METHODS FOR DETECTING AAV

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a National Phase application under 35 U.S.C. § 371 of International Application No. PCT/US2017/046814, filed Aug. 14, 2017, which claims the priority benefit of U.S. Provisional Application No. 62/375,314, filed Aug. 15, 2016, the disclosure of each of which is hereby incorporated by reference in its entirety.

SEQUENCE LISTING

The content of the following submission on ASCII text file is incorporated herein by reference in its entirety: a computer readable form (CRF) of the Sequence Listing (file name: 1597920141005EQLIST.TXT, date recorded: Feb. 13, 2019, size: 52 KB).

FIELD OF THE INVENTION

The present invention relates to methods for serotyping and/or determining the heterogeneity of a viral particle (e.g., an adeno-associated virus (AAV) particle) using mass determination, e.g., by employing liquid chromatography/mass spectrometry (LC/MS) or liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS). In some aspects, the present invention relates to methods to improve the stability of AAV particles.

BACKGROUND OF THE INVENTION

Complete characterization of the viral capsid proteins of viral vectors (e.g., AAV vectors), including their sequence and post-translation modifications, is desired in gene therapy research and development since viral capsid proteins (VPs) are critical for viral infectivity.

Viral vector products such as recombinant Adeno-Associated Virus (rAAV) products are typically identified using molecular tools targeting the nucleic acid transgene. These methods may include polymerase chain reaction (PCR) targeting transgene-specific sequences and Restriction Fragment Length Polymorphism (RFLP) techniques. As rAAV technologies evolve, many facilities are beginning to investigate multiple AAV capsid serotypes encoding their therapeutic transgene in an effort to improve targeted tissue tropism.

Traditional molecular identification methods identify products containing unique transgenes but are unable to discern those that have differing AAV capsid serotypes. Currently, most AAV serotype identity tests are based on SDS-PAGE banding patterns, an antibody-based ELISA, or a Western blot assay. However, the banding patterns and antibodies are not specific enough to differentiate different AAV serotypes. Gel-LC/MS/MS has been reported as a capsid serotype identification method. However, this method involves multiple steps including SDS-PAGE, in-gel digestion, and LC/MS/MS and thus requires multiple days for the analysis while providing limited sequence coverage. Methods for identifying vectors such as rAAV vectors are of interest to gene therapy vectors (see, e.g., U.S. PG Pub. No. US20110275529). Thus, it would be useful to have improved methods of characterizing viral particles.

All references cited herein, including patent applications and publications, are incorporated by reference in their entirety.

BRIEF SUMMARY OF THE INVENTION

Using rAAV as an example, described herein is the use of LC/MS as an analytical tool to specifically identify different viral capsid serotypes (e.g., rAAV capsid serotypes). As part of viral characterization, LC/MS can be used to augment the molecular identification methods. This analytical combination can satisfy regulatory requirements by discerning both the identity of the product's therapeutic transgene and the identity of the capsid serotype. This method can be used e.g., as an AAV serotype identity test or to monitor viral capsid protein heterogeneity in recombinant AAV gene therapy development. It can also be used to confirm VP sequences in capsid engineering research. In addition, this technique can be used to study the impact of post translation modifications, such as N terminal acetylation of viral capsid proteins, on transfection potency and intracellular protein trafficking.

The methods described herein can also be used to design AAV particles for greater stability and/or improved transduction efficiency; for example by altering the amino acid residue at position 2 of VP1 and/or VP3 of the AAV capsid such that the amino acid at position 2 is acetylated to a higher extent compared to a wild type AAV capsid. In some embodiments, the methods can be used to design AAV particles with reduced transduction efficiency; for example by altering the amino acid residue at position 2 of VP1 and/or VP3 of the AAV capsid such that the amino acid at position 2 is deacetylated to a higher or lower extent compared to a wild type AAV capsid.

In some aspects, the invention provides a method to determine the serotype of a viral particle comprising a) denaturing the viral particle, b) subjecting the denatured viral particle to liquid chromatography/mass spectrometry (LC/MS), and c) determining the masses of one or more capsid proteins of the viral particle; wherein the specific combination of masses of the one or more capsid proteins are indicative of the virus serotype. In some embodiments, the calculated masses of the one or more capsid proteins are compared to the theoretical masses of the one or more capsid proteins of one or more virus serotypes.

In some aspects, the invention provides a method of determining the heterogeneity of a viral particle comprising a) denaturing the viral particle, b) subjecting the denatured viral particle to liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS), c) determining the masses of one or more capsid proteins of the viral particle, and d) comparing the masses of step c) with the theoretical masses of the one or more capsid proteins of the virus serotype; wherein a deviation of one or more of the masses of the one or more capsid proteins are indicative of the viral capsid heterogeneity. In some embodiments, the heterogeneity comprises one or more of mixed serotypes, variant capsids, capsid amino acid substitutions, truncated capsids, or modified capsids.

In some embodiments of the above aspects, the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography. In some embodiments, the viral particle comprises a viral vector encoding a heterologous transgene.

In some aspects, the invention provide a method to determine the serotype of a viral particle comprising a) denaturing the viral particle, b) subjecting the denatured viral particle to reduction and/or alkylation, c) subjecting the denatured viral particle to digestion to generate fragments of one or more capsid proteins of the viral particle, d) subjecting the fragments of the one or more capsid proteins to liquid

chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), and e) determining the masses of fragments of the one or more capsid proteins of the viral particle; wherein the specific combination of masses of fragments of the one or more capsid proteins are indicative of the viral serotype. In some embodiments, the calculated masses of the fragments of the one or more capsid proteins are compared to the theoretical masses of fragments of the one or more capsid proteins of one or more viral serotypes.

In some aspects, the invention provides a method of determining the heterogeneity of a serotype of a viral particle comprising a) denaturing the viral particle, b) subjecting the denatured viral particle to reduction and/or alkylation, c) subjecting the denatured viral particle to digestion to generate fragments of one or more capsid proteins of the viral particle, d) subjecting the fragments of the one or more capsid proteins to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), e) determining the masses of fragments of the one or more capsid proteins of the viral particle, and f) comparing the masses of step e) with the theoretical masses of fragments of the one or more capsid proteins of the viral serotype; wherein a deviation of one or more of the masses of the one or more capsid proteins are indicative of the viral capsid heterogeneity. In some embodiments, the heterogeneity comprises one or more of mixed serotypes, variant capsids, capsid amino acid substitutions, truncated capsids, or modified capsids. In some embodiments, the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography.

As shown herein the methods can be performed in the absence of a gel separation step (e.g., sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)).

In some embodiments of the above aspects and embodiments, the viral particle comprises a viral vector encoding a heterologous transgene. In some embodiments, the viral particle belongs to a viral family selected from the group consisting of Adenoviridae, Parvoviridae, Retroviridae, Baculoviridae, and Herpesviridae. In some embodiments, the viral particle belongs to a viral genus selected from the group consisting of Atadenovirus, Aviadenovirus, Ichtadenovirus, Mastadenovirus, Siadenovirus, Ambidensovirus, Brevidensovirus, Hepandensovirus, Iteradensovirus, Penstyldensovirus, Amdoparvovirus, Aveparvovirus, Bocaparvovirus, Copiparvovirus, Dependoparvovirus, Erythroparvovirus, Protoparvovirus, Tetraparvovirus, Alpharetrovirus, Betaretrovirus, Deltaretrovirus, Epsilonretrovirus, Gammaretrovirus, Lentivirus, Spumavirus, Alphabaculovirus, Betabaculovirus, Deltabaculovirus, Gammabaculovirus, Iltovirus, Mardivirus, Simplexvirus, Varicellovirus, Cytomegalovirus, Muromegalovirus, Proboscivirus, Roseolovirus, Lymphocryptovirus, Macavirus, Percavirus, and Rhadinovirus.

In some aspects, the invention provides a method to determine the serotype of an adeno-associated virus (AAV) particle comprising a) denaturing the AAV particle, b) subjecting the denatured AAV particle to liquid chromatography/mass spectrometry (LC/MS), and c) determining the masses of VP1, VP2 and VP3 of the AAV particle; wherein the specific combination of masses of VP1, VP2 and VP3 are indicative of the AAV serotype. In some embodiments, the calculated masses of VP1, VP2 and VP3 are compared to the theoretical masses of VP1, VP2 and VP3 of one or more AAV serotypes.

In some aspects, the invention provides a method of determining the heterogeneity of an AAV particle comprising a) denaturing the AAV particle, b) subjecting the denatured AAV particle to liquid chromatography/mass spectrometry (LC/MS/MS), c) determining the masses of VP1, VP2 and VP3 of the AAV particle, and d) comparing the masses of step c) with the theoretical masses of VP1, VP2 and VP3 of the AAV serotype; wherein a deviation of one or more of the masses of VP1, VP2 or VP3 are indicative of the AAV capsid heterogeneity. In some embodiments, the heterogeneity comprises one or more of mixed serotypes, variant capsids, capsid amino acid substitutions, truncated capsids, or modified capsids.

In some embodiments of the above aspects and embodiments, the AAV particle is denatured with acetic acid, guanidine hydrochloride and/or an organic solvent. In some embodiments, the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography. In some embodiments, the liquid chromatography is reverse phase liquid chromatography. In some embodiments, the reverse phase chromatography is a C4 or C8 reverse chromatography. In some embodiments, the chromatography uses a mobile phase A comprising formic acid in water. In some embodiments, the mobile phase A comprises about 0.1% formic acid. In some embodiments, the chromatography comprises a mobile phase B comprising formic acid in acetonitrile. In some embodiments, the mobile phase B comprises about 0.1% formic acid. In some embodiments, the proportion of mobile phase B in the chromatography increases over time. In some embodiments, the proportion of mobile phase B in the chromatography increases in a stepwise manner. In some embodiments, mobile phase B increases from about 10% to about 20%, from about 20% to about 30%, and from about 30% to about 38%. In some embodiments, mobile phase B increases from about 10% to about 20% in about 6 minutes, from about 20% to about 30% in about 10 minutes, and from about 30% to about 38% in about 40 minutes. In some embodiments, the liquid chromatography is ultra-performance liquid chromatography (UPLC).

In some embodiments of the above aspects and embodiments, the mass spectrometry comprises a capillary voltage of about 3.5 kV. In some embodiments, the mass spectrometry comprises a sampling cone voltage of about 45 V. In some embodiments, the mass spectrometry comprises assisted calibration. In some embodiments, sodium iodide is used as a calibrant.

In some embodiments of the above aspects and embodiments, the N-terminus of VP1 and/or VP3 is acetylated. In some embodiments, the AAV particle is a recombinant AAV (rAAV) particle. In some embodiments, the AAV particle comprises an AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAV5 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an AAV LK03 capsid, an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid, an AAV DJ8 capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, or a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV/human bocavirus virus 1). In some embodiments, the AAV capsid comprises a tyrosine mutation or a heparin binding mutation. In some embodiments, the masses of VP1, VP2, and VP3 are compared to the theoretical masses of one or more of AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid,

an AAVrh8 capsid, an AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an AAV LK03 capsid, an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid, an AAV DJ8 capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, or a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV/human bocavirus virus 1), an AAV2HBKO capsid, an AAVPHP.B capsid or an AAVPHP.eB capsid.

In some embodiments of the above aspects and embodiments, the viral particle comprises an AAV1 ITR, an AAV2 ITR, an AAV3 ITR, an AAV4 ITR, an AAV5 ITR, an AAV6 ITR, an AAV7 ITR, an AAV8 ITR, an AAVrh8 ITR, an AAV9 ITR, an AAV10 ITR, an AAVrh10 ITR, an AAV11 ITR, or an AAV12 ITR. In some embodiments, the AAV particle comprises an AAV vector encoding a heterologous transgene.

In some aspects, the invention provides a method to determine the serotype of an adeno-associated virus (AAV) particle comprising a) denaturing the AAV particle, b) subjecting the denatured AAV particle to reduction and/or alkylation, c) subjecting the denatured AAV particle to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle, d) subjecting the fragments of VP1, VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), and e) determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle; wherein the specific combination of masses of fragments of VP1, VP2 and VP3 are indicative of the AAV serotype. In some embodiments, the calculated masses of the fragments of VP1, VP2 and/or VP3 are compared to the theoretical masses of fragments of VP1, VP2 and/or VP3 of one or more AAV serotypes.

In some aspects, the invention provides a method of determining the heterogeneity of a serotype of an AAV particle comprising a) denaturing the AAV particle, b) subjecting the denatured AAV particle to reduction and/or alkylation, c) subjecting the denatured AAV particle to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle, d) subjecting the fragments of VP1, VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), e) determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle, and f) comparing the masses of step e) with the theoretical masses of fragments of VP1, VP2 and VP3 of the AAV serotype; wherein a deviation of one or more of the masses of VP1, VP2 or VP3 are indicative of the AAV capsid heterogeneity. In some embodiments, the heterogeneity comprises one or more of mixed serotypes, variant capsids, capsid amino acid substitutions, truncated capsids, or modified capsids. In some embodiments, the reduction is by subjecting the AAV particle to dithiothreitol, beta-mercaptoethanol, or tris(2-carboxyethyl)phosphine (TCEP). In some embodiments, the alkylation is by subjecting the AAV particle to iodoacetic acid, iodoacetamide, or 4-vinylpyridine. In some embodiments, the digestion is an enzymatic digestion or a chemical digestion. In some embodiments, the enzymatic digestion is an endopeptidase digestion. In some embodiments, the enzymatic digestion is a trypsin digestion, a LysC digestion, an Asp-N digestion or a Glu-C digestion. In some embodiments, the chemical digestion is cyanogen bromide digestion or an acid digestion. In some embodiments, the AAV particle is denatured with acetic acid, guanidine hydrochloride and/or an organic solvent.

In some embodiments of the above aspects and embodiments, the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography. In some embodiments, the liquid chromatography is reverse phase liquid chromatography. In some embodiments, the reverse phase chromatography is a C18 reverse chromatography. In some embodiments, the chromatography uses a mobile phase A comprising formic acid in water. In some embodiments, the mobile phase A comprises about 0.1% formic acid. In some embodiments, the chromatography comprises a mobile phase B comprising formic acid in acetonitrile. In some embodiments, the mobile phase B comprises about 0.1% formic acid. In some embodiments, the proportion of mobile phase B in the chromatography increases over time. In some embodiments, mobile phase B increases from about 2% to about 60%. In some embodiments, mobile phase B increases from about 2% to about 60% in about 121 minutes. In some embodiments, the liquid chromatography is high-performance liquid chromatography (HPLC).

In some embodiments of the above aspects and embodiments, the mass spectrometry comprises a capillary voltage of about 3.5 kV. In some embodiments, the mass spectrometry comprises a sampling cone voltage of about 45 V. In some embodiments, the mass spectrometry comprises assisted calibration. In some embodiments, sodium iodide is used as a calibrant.

In some embodiments of the above aspects and embodiments, the N-terminus of VP1 and/or VP3 is acetylated. In some embodiments, the AAV particle is a recombinant AAV (rAAV) particle. In some embodiments, the AAV particle comprises an AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an AAV LK03 capsid, an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid, an AAV DJ8 capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, or a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV/human bocavirus virus 1). In some embodiments, the AAV capsid comprises a tyrosine mutation or a heparin binding mutation. In some embodiments, the masses of VP1, VP2, and VP3 are compared to the theoretical masses of one or more of AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an AAV LK03 capsid, an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid, an AAV DJ8 capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, or a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV/human bocavirus virus 1).

In some embodiments of the above aspects and embodiments, the viral particle comprises an AAV1 ITR, an AAV2 ITR, an AAV3 ITR, an AAV4 ITR, an AAV5 ITR, an AAV6 ITR, an AAV7 ITR, an AAV8 ITR, an AAVrh8 ITR, an AAV9 ITR, an AAV10 ITR, an AAVrh10 ITR, an AAV11 ITR, or an AAV12 ITR. In some embodiments, the AAV particle comprises an AAV vector encoding a heterologous transgene.

In some embodiments, the invention provides a recombinant AAV (rAAV) particle comprising an amino acid substitution at amino acid residue 2 of VP1 and/or VP3;

wherein the amino acid substitution at amino acid residue 2 of VP1 and/or VP3 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of VP1 and/or VP3 of the parent AAV particle. In some embodiments, the substitution results in a higher frequency of N-terminal acetylation or a lower frequency of N-terminal acetylation. In some embodiments, the rAAV particle comprises an amino acid substitution at amino acid residue 2 of VP1; wherein the amino acid substitution at amino acid residue 2 of VP1 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of VP1 of the parent AAV particle. In some embodiments, the rAAV particle comprises an amino acid substitution at amino acid residue 2 of VP3; wherein the amino acid substitution at amino acid residue 2 of VP3 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of VP3 of the parent AAV particle. In some embodiments, amino acid residue 2 is substituted with Cys, Ser, Thr, Val, Gly, Asn, Asp, Glu, Ile, Leu, Phe, Gln, Lys, Met, Pro or Tyr. In some embodiments, amino acid residue 2 is substituted with Ser, Asp or Glu.

In some embodiments of the above aspects and embodiments, the AAV particle comprises an AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV2/2-7m8, AAV DJ, an AAV DJ8 capsid, AAV2 N587A, AAV2 E548A, AAV2 N708A, AAV V708K, goat AAV, AAV1/AAV2 chimeric, bovine AAV, mouse AAV, rAAV2/HBoV1, AAV2HBKO, AAVPHP.B, or AAVPHP.eB serotype capsid. In some embodiments, the AAV capsid further comprises a tyrosine mutation or a heparin binding mutation. In some embodiments, the rAAV particle comprises a rAAV vector. In some embodiments, the rAAV vector comprises one or more AAV ITRs. In some embodiments, the rAAV vector comprises an AAV1 ITR, an AAV2 ITR, an AAV3 ITR, an AAV4 ITR, an AAV5 ITR, an AAV6 ITR, an AAV7 ITR, an AAV8 ITR, an AAVrh8 ITR, an AAV9 ITR, an AAV10 ITR, an AAVrh10 ITR, an AAV11 ITR, or an AAV12 ITR. In some embodiments, the AAV capsid and the AAV ITRs are derived from the same serotype. In some embodiments, the AAV capsid and the AAV ITRs are derived from different serotypes. In some embodiments, the AAV particle comprises an AAV vector encoding a heterologous transgene flanked by one or more AAV ITRs.

In some embodiments of the above aspects and embodiments, the rAAV vector is a self-complementary vector. In some embodiments, the rAAV vector comprises first nucleic acid sequence encoding the transgene and a second nucleic acid sequence encoding a complement of the transgene, wherein the first nucleic acid sequence can form intrastrand base pairs with the second nucleic acid sequence along most or all of its length. In some embodiments, the first nucleic acid sequence and the second nucleic acid sequence are linked by a mutated AAV ITR, wherein the mutated AAV ITR comprises a deletion of the D region and comprises a mutation of the terminal resolution sequence.

In some embodiments of the above aspects and embodiments, the rAAV particle is produced by transfecting a host cell with nucleic acid encoding the rAAV vector and nucleic acid encoding AAV rep and cap functions, and providing nucleic acid encoding AAV helper functions. In some embodiments, the AAV helper functions are provided by transfecting the host cell with nucleic acid encoding the AAV helper functions. In some embodiments, the AAV helper functions are provided by infecting the host cell with an AAV helper virus that provides the AAV helper functions. In some embodiments, the AAV helper virus is an adenovirus, a herpes simplex virus or a baculovirus. In some embodiments, the rAAV particle is produced by an AAV producer cell comprising nucleic acid encoding the rAAV vector and nucleic acid encoding AAV rep and cap functions, and providing nucleic acid encoding AAV helper functions. In some embodiments, the AAV producer cell comprises nucleic acid encoding AAV helper functions. In some embodiments, the AAV helper functions are provided by infecting the AAV producer cells with an AAV helper virus that provides the AAV helper functions. In some embodiments, the AAV helper virus is an adenovirus, a herpes simplex virus, or a baculovirus. In some embodiments, the AAV cap functions provide an amino acid substitution at amino acid residue 2 of VP1 and/or VP3, wherein the amino acid substitution at amino acid residue 2 of VP1 and/or VP3 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of VP1 and/or VP3 of the parent AAV particle.

In some aspects, the invention provides a pharmaceutical composition comprising the rAAV particle as described herein. In some aspects, the invention provides a kit comprising the rAAV particle or the pharmaceutical composition as described herein. In some aspects, the invention provides an article of manufacture comprising the rAAV particle or the pharmaceutical composition as described herein.

In some aspects, the invention provides as AAV capsid protein comprising an amino acid substitution at amino acid residue 2 of a parent AAV capsid protein; wherein the amino acid substitution at amino acid residue 2 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of the parent AAV capsid protein. In some embodiments, the substitution results in a higher frequency of N-terminal acetylation or a lower frequency of N-terminal acetylation. In some embodiments, the AAV capsid protein is VP1 or VP3. In some embodiments, amino acid residue 2 of the AAV capsid protein is substituted with Cys, Ser, Thr, Val, Gly, Asn, Asp, Glu, Ile, Leu, Phe, Gln, Lys, Met, Pro or Tyr. In some embodiments, amino acid residue 2 of the AAV capsid protein is substituted with Ser, Asp or Glu. In some embodiments, the amino acid substitution results in less deamidation of the AAV capsid.

In some embodiments of the above aspects and embodiments, the AAV particle comprises an AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV2/2-7m8, AAV DJ, an AAV DJ8 capsid, AAV2 N587A, AAV2 E548A, AAV2 N708A, AAV V708K, goat AAV, AAV1/AAV2 chimeric, bovine AAV, mouse AAV, rAAV2/HBoV1, AAV2HBKO, AAVPHP.B, or AAVPHP.eB serotype capsid. In some embodiments, the AAV capsid further comprises a tyrosine mutation or a heparin binding mutation.

In some aspects, the invention provides a method of improving stability of a rAAV particle comprising substituting amino acid residue 2 of VP1 and/or VP3 of a parent VP1 and/or VP3; wherein the substituting amino acid residue 2 alters N-terminal acetylation of VP1 and/or VP3, as compared to amino acid residue 2 of the parent VP1 and/or VP3. In some aspects, the invention provides a method of improving assembly of rAAV particles in a cell comprising substituting amino acid residue 2 of VP1 and/or VP3 or a parental VP1 and/or VP3; wherein substituting amino acid at position 2 alters N-terminal acetylation of VP1 and/or VP3, as compared to amino acid residue 2 of the parent VP1 and/or VP3. In some aspects, the invention provides a method of improving the transduction of rAAV particles in

a cell comprising substituting amino acid residue 2 of VP1 and/or VP3 or a parental VP1 and/or VP3; wherein substituting amino acid residue 2 alters N-terminal acetylation of VP1 and/or VP3, as compared to amino acid residue 2 of the parent VP1 and/or VP3. In some embodiments, the substituted amino acid results in a higher frequency of N-terminal acetylation or a lower frequency of N-terminal acetylation. In some embodiments, the amino acid substitution at amino acid residue 2 of VP1 is substituted. In some embodiments, the amino acid substitution at amino acid residue 2 of VP3 is substituted. In some embodiments, amino acid residue 2 is substituted with Cys, Ser, Thr, Val, Gly, Asn, Asp, Glu, Ile, Leu, Phe, Gln, Lys, Met, Pro or Tyr. In some embodiments, amino acid residue 2 is substituted with Ser, Asp or Glu. In some aspects, the invention provides a method of reducing the transduction of rAAV particles in a cell comprising substituting amino acid residue 2 of VP1 and/or VP3; wherein the substituted amino acid at position 2 alters N-terminal acetylation of VP1 and/or VP3, as compared to amino acid residue 2 of the parent VP1 and/or VP3.

In some embodiments of the above aspects and embodiments, the AAV particle comprises an AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV2/2-7m8, AAV DJ, an AAV DJ8 capsid, AAV2 N587A, AAV2 E548A, AAV2 N708A, AAV V708K, goat AAV, AAV1/AAV2 chimeric, bovine AAV, mouse AAV, rAAV2/HBoV1, AAV2HBKO, AAVPHP.B, or AAVPHP.eB serotype capsid. In some embodiments, the AAV capsid further comprises a tyrosine mutation or a heparin binding mutation. In some embodiments, the rAAV particle comprises a rAAV vector. In some embodiments, the rAAV vector comprises one or more AAV ITRs. In some embodiments, the rAAV vector comprises an AAV1 ITR, an AAV2 ITR, an AAV3 ITR, an AAV4 ITR, an AAV5 ITR, an AAV6 ITR, an AAV7 ITR, an AAV8 ITR, an AAVrh8 ITR, an AAV9 ITR, an AAV10 ITR, an AAVrh10 ITR, an AAV11 ITR, or an AAV12 ITR. In some embodiments, the AAV capsid and the AAV ITRs are derived from the same serotype. In some embodiments, the AAV capsid and the AAV ITRs are derived from different serotypes. In some embodiments, the AAV particle comprises an AAV vector encoding a heterologous transgene flanked by one or more AAV ITRs.

In some embodiments of the above aspects and embodiments, the rAAV vector is a self-complementary vector. In some embodiments, the rAAV vector comprises first nucleic acid sequence encoding the transgene and a second nucleic acid sequence encoding a complement of the transgene, wherein the first nucleic acid sequence can form intrastrand base pairs with the second nucleic acid sequence along most or all of its length. In some embodiments, the first nucleic acid sequence and the second nucleic acid sequence are linked by a mutated AAV ITR, wherein the mutated AAV ITR comprises a deletion of the D region and comprises a mutation of the terminal resolution sequence.

In some embodiments of the above aspects and embodiments, the rAAV particle is produced by transfecting a host cell with nucleic acid encoding the rAAV vector and nucleic acid encoding AAV rep and cap functions, and providing nucleic acid encoding AAV helper functions. In some embodiments, the AAV helper functions are provided by transfecting the host cell with nucleic acid encoding the AAV helper functions. In some embodiments, the AAV helper functions are provided by infecting the host cell with an AAV helper virus that provides the AAV helper functions. In some embodiments, the AAV helper virus is an adenovirus, a herpes simplex virus or a baculovirus. In some embodiments, the rAAV particle is produced by an AAV producer cell comprising nucleic acid encoding the rAAV vector and nucleic acid encoding AAV rep and cap functions, and providing nucleic acid encoding AAV helper functions. In some embodiments, the AAV producer cell comprises nucleic acid encoding AAV helper functions. In some embodiments, the AAV helper functions are provided by infecting the AAV producer cells with an AAV helper virus that provides the AAV helper functions. In some embodiments, the AAV helper virus is an adenovirus, a herpes simplex virus, or a baculovirus. In some embodiments, the AAV cap functions provide an amino acid substitution at amino acid residue 2 of VP1 and/or VP3, wherein the amino acid substitution at amino acid residue 2 of VP1 and/or VP3 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of VP1 and/or VP3 of the parent AAV particle.

In some aspects, the invention provides a recombinant AAV (rAAV) particle comprising one or more amino acid substitutions at amino acid residue A35, N57, G58, N382, G383, N511, G512, N715, or G716 of VP1 or VP3 of a parent particle, residue numbering based on VP1 of AAV2; wherein the one or more amino acid substitutions alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some embodiments, the one or more amino acid substitution is at amino acid residue A35, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3 and alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some embodiments, the one or more amino acid substitutions comprises a substitution with Asp at N57 of VP1, N382 of VP3, N511 of VP3, or N715 of VP3; and results in a higher frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some embodiments, the one or more amino acid substitutions comprise a N57K or a N57Q substitution and results in a lower frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some embodiments, the one or more amino acid substitution comprise a substitution with Asp at A35 of VP1 and results in a higher frequency of deamidation as compared to deamidation of VP1 of the parent AAV particle. In some embodiments, the one or more amino acid substitutions is at G58 of VP1, G383 of VP3, G512 of VP3, or G716 of VP3 and results in a lower frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some embodiments, the G58 of VP1 is substituted with Asp. In some embodiments, the rAAV particle is an AAV1 particle or an AAV2 particle.

In some aspects, the invention provides pharmaceutical compositions comprising AAV particles comprising one or more amino acid substitutions at amino acid residue A35, N57, G58, N382, G383, N511, G512, N715, or G716 of VP1 or VP3, residue numbering based on VP1 of AAV2; wherein the amino acid substitution alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some aspects, the invention provides kits comprising AAV particles or compositions comprising AAV particles wherein the AAV particles comprise one or more amino acid substitutions at amino acid residue A35, N57, G58, N382, G383, N511, G512, N715, or G716 of VP1 or VP3, residue numbering based on VP1 of AAV2; wherein the amino acid substitution alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some aspects, the invention provides articles of

manufacture comprising AAV particles or compositions comprising AAV particles wherein the AAV particles comprise one or more amino acid substitutions at amino acid residue A35, N57, G58, N382, G383, N511, G512, N715, or G716 of VP1 or VP3, residue numbering based on VP1 of AAV2; wherein the amino acid substitution alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some aspects, the invention provides an AAV capsid protein comprising an amino acid substitution of a parent AAV capsid protein; wherein the amino acid substitution alters deamidation of the capsid compared to the parent AAV capsid protein.

In some aspects, the invention provides a method of improving the stability of a rAAV particle comprising substituting one or more amino acid residues, wherein the one or more amino acid residues is A35, N57, G58, N382, G383, N511, G512, N715, or G716, residue numbering based on VP1 of AAV2; wherein the amino acid substitution alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some aspects, the invention provides a method of improving the assembly of rAAV particles in a cell comprising substituting one or more amino acid residues, wherein the one or more amino acid residues is A35, N57, G58, N382, G383, N511, G512, N715, or G716, residue numbering based on VP1 of AAV2; wherein the amino acid substitution alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some aspects, the invention provides a method of improving the transduction of rAAV particles in a cell comprising substituting one or more amino acid residues, wherein the one or more amino acid residues is A35, N57, G58, N382, G383, N511, G512, N715, or G716, residue numbering based on VP1 of AAV2; wherein the amino acid substitution alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some embodiments, the one or more amino acid substitutions is at A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3; wherein the amino acid substitution alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some embodiments, the parental Ala residue at position 35 of VP1 is substituted with Asn. In some embodiments, the parental Gly residue at position 58 of VP1 is substituted with Asp. In some embodiments, the rAAV particle is an AAV1 particle or an AAV2 particle.

In some embodiments, the invention provides a method of improving the stability, assembly and/or transduction efficiency of a rAAV particle comprising substituting one or more amino acid residues, wherein the one or more amino acid residues is A35, N57, G58, N382, G383, N511, G512, N715, or G716, residue numbering based on VP1 of AAV2; wherein the amino acid substitution alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle as described above, wherein the AAV particle comprises an AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV2/2-7m8, AAV DJ, an AAV DJ8 capsid, AAV2 N587A, AAV2 E548A, AAV2 N708A, AAV V708K, goat AAV, AAV1/AAV2 chimeric, bovine AAV, mouse AAV, or rAAV2/HBoV1 serotype capsid. In some embodiments, the AAV capsid further comprises a tyrosine mutation or a heparin binding mutation. In some embodiments, the rAAV particle comprises a rAAV vector. In some embodiments, the rAAV vector comprises one or more AAV ITRs. In some embodiments, the rAAV vector comprises an AAV1 ITR, an AAV2 ITR, an AAV3 ITR, an AAV4 ITR, an AAV5 ITR, an AAV6 ITR, an AAV7 ITR, an AAV8 ITR, an AAVrh8 ITR, an AAV9 ITR, an AAV10 ITR, an AAVrh10 ITR, an AAV11 ITR, or an AAV12 ITR. In some embodiments, the AAV capsid and the AAV ITRs are derived from the same serotype. In some embodiments, the AAV capsid and the AAV ITRs are derived from different serotypes. In some embodiments, the AAV particle comprises an AAV vector encoding a heterologous transgene flanked by one or more AAV ITRs.

In some embodiments of the above aspects and embodiments, the rAAV vector is a self-complementary vector. In some embodiments, the rAAV vector comprises first nucleic acid sequence encoding the transgene and a second nucleic acid sequence encoding a complement of the transgene, wherein the first nucleic acid sequence can form intrastrand base pairs with the second nucleic acid sequence along most or all of its length. In some embodiments, the first nucleic acid sequence and the second nucleic acid sequence are linked by a mutated AAV ITR, wherein the mutated AAV ITR comprises a deletion of the D region and comprises a mutation of the terminal resolution sequence.

In some embodiments of the above aspects and embodiments, the rAAV particle is produced by transfecting a host cell with nucleic acid encoding the rAAV vector and nucleic acid encoding AAV rep and cap functions, and providing nucleic acid encoding AAV helper functions. In some embodiments, the AAV helper functions are provided by transfecting the host cell with nucleic acid encoding the AAV helper functions. In some embodiments, the AAV helper functions are provided by infecting the host cell with an AAV helper virus that provides the AAV helper functions. In some embodiments, the AAV helper virus is an adenovirus, a herpes simplex virus or a baculovirus. In some embodiments, the rAAV particle is produced by an AAV producer cell comprising nucleic acid encoding the rAAV vector and nucleic acid encoding AAV rep and cap functions, and providing nucleic acid encoding AAV helper functions. In some embodiments, the AAV producer cell comprises nucleic acid encoding AAV helper functions. In some embodiments, the AAV helper functions are provided by infecting the AAV producer cells with an AAV helper virus that provides the AAV helper functions. In some embodiments, the AAV helper virus is an adenovirus, a herpes simplex virus, or a baculovirus. In some embodiments, the AAV cap functions provide an amino acid substitution of VP1 and/or VP3, wherein the amino acid substitution modulated deamidation of the capsid compared to the parent AAV particle.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. **1**A-D provide total ion Chromatograms of LC/MS of AAV2 VPs. FIG. **1**A: 10 cm long BEH C4 column with 1.7%/min gradient, FIG. **1**B: 10 cm long BEH C4 column with 0.5%/min gradient; FIG. **1**C: 15 cm long BEH C4 column with 0.5%/min gradient. FIG. **1**D: 15 cm long BEH C8 column with 0.5%/min gradient.

FIGS. **2**A&B provide deconvoluted mass spectra from FIG. **1**D peak 1 (FIG. **2**A) and FIG. **1**D peak 2 (FIG. **2**B).

FIG. **3** provides the sequence coverage of AAV2 VP1 (SEQ ID NO:3): green, tryptic peptides, blue, Lys-C peptides, pink, Asp-N peptides.

FIGS. **4**A-**4**C provide MS/MS spectra of AAV2 VP N-terminal peptides. FIG. **4**A: VP1 N-terminal tryptic peptide A(Ac)ADGYLPDWLEDTLSEGIR (SEQ ID NO: 4), FIG. **4**B VP2 N-terminal Asp-N peptide APGKKRPVEHSPVEP

(SEQ ID NO: 15). FIG. **4**C: VP-3 N-terminal Asp-N derived peptide A(Ac)TGSGAPM (SEQ ID NO: 5).

FIG. **5** provides the sequence alignment of 13 AAV serotypes black letter/white background: non-similar; blue letter/blue background: conservative; black letter/green background: block of similar; red letter/yellow background: identical; green letter/white background: weakly similar. AAVRh10 (SEQ ID NO: 17); AAV10 (SEQ ID NO: 18); AAV8 (SEQ ID NO: 19); AAV7 (SEQ ID NO: 20); AAV1 (SEQ ID NO: 21); AAV6 (SEQ ID NO: 22); AAV2 (SEQ ID NO: 23); AAV3 (SEQ ID NO: 24); AAV11 (SEQ ID NO: 25); AAV12 (SEQ ID NO: 26); AAV4 (SEQ ID NO: 27); AAV5 (SEQ ID NO: 28); AAV9 (SEQ ID NO: 29); Consensus (SEQ ID NO: 30).

FIGS. **6**A & **6**B show the results of LC/MS/MS analysis comparing the percentage of deamidation in AAV1 and AAV2 particles produced by the TTx and PCL methods. The T9 peptide YLGPFNGLDK (SEQ ID NO: 9) was used to monitor potential deamidation site N57 in both AAV1 and AAV2.

FIGS. **7**A & **7**B show the results of LC/MS/MS analysis comparing the percentage of deamidation in AAV1 and AAV2 particles produced by the TTx and PCL methods. The T49 peptides YNLNGR (SEQ ID NO: 11) and YHLNGR (SEQ ID NO: 12) were used to monitor potential deamidation site N511 in AAV1 and AAV2, respectively.

FIGS. **8**A & **8**B show the results of LC/MS/MS analysis comparing the percentage of deamidation in AAV1 and AAV2 particles produced by the TTx and PCL methods. The T67 peptides SANVDFTVDNNGLYTEPR (SEQ ID NO: 13) and SVNVDFTVDTNGVYSEPR (SEQ ID NO: 14) were used to monitor potential deamidation site N715 in AAV1 and AAV2, respectively.

FIG. **9** shows the results of SYPRO protein gel analysis of production and VP1:VP2:VP3 ratio of AAV5 deacetylated mutant variants.

FIG. **10** illustrates an in vitro transduction assay for testing transduction efficiency of AAV5 deacetylated variants.

FIG. **11** shows the efficiency of cell entry by the indicated AAV5 deacetylated variants or parental unmodified AAV5, as measured by vector genome copies/μg protein. Three cell lines were used: 293, HeLa, and HuH7.

FIG. **12** shows eGFP expression (as measured by ELISA) by cells transduced with the indicated AAV5 deacetylated variants as compared to transduction with parental unmodified AAV5. Three cell lines were used: 293, HeLa, and HuH7.

FIG. **13** provides the sequence alignment of 13 AAV serotypes, highlighting the conserved N57G58 deamidation site and the A35 residue in AAV2. AAVRh10 (SEQ ID NO: 31); AAV10 (SEQ ID NO: 31); AAV8 (SEQ ID NO: 32); AAV7 (SEQ ID NO: 33); AAV1 (SEQ ID NO: 31); AAV6 (SEQ ID NO: 31); AAV2 (SEQ ID NO: 34); AAV3 (SEQ ID NO: 35); AAV11 (SEQ ID NO: 31); AAV12 (SEQ ID NO: 36); AAV4 (SEQ ID NO: 37); AAV5 (SEQ ID NO: 38); AAV9 (SEQ ID NO: 39); Consensus (SEQ ID NO: 40).

FIG. **14** shows a protein gel of VP1, VP2, and VP3 capsid proteins from AAV1 or AAV2 particles produced by the PCL or TTx method. *highlights the truncated VP1 (tVP1) protein.

FIG. **15** shows the results of LC/MS analysis of deamidation of the indicated AAV2 mutants, as compared to control AAV2 capsids.

FIG. **16** shows the results of SYPRO protein gel analysis of production and VP1:VP2:VP3 ratio of AAV2 deamidation mutant variants.

FIG. **17** illustrates an in vitro transduction assay for testing transduction efficiency of AAV2 deamidation variants.

FIG. **18** shows the efficiency of cell entry by the indicated AAV2 deamidation variants or parental unmodified AAV2, as measured by vector genome copies/μg protein. Three cell lines were used: 293, HeLa, and HuH7.

FIG. **19** shows eGFP expression (as measured by ELISA) by cells transduced with the indicated AAV2 deamidation variants as compared to transduction with parental unmodified AAV2. Three cell lines were used: 293, HeLa, and HuH7.

DETAILED DESCRIPTION

In some aspects, the invention provides a method to determine the serotype of an adeno-associated virus (AAV) particle(s) comprising: a) denaturing the AAV particle, b) injecting the denatured AAV particle to liquid chromatography/mass spectrometry (LC/MS), and c) determining the masses of VP1, VP2 and VP3 of the AAV particle; wherein the specific combination of masses of VP1, VP2 and VP3 are indicative of the AAV serotype.

In other aspects, the invention provides a method of determining the heterogeneity of an AAV particle comprising: a) denaturing the AAV particle, b) injecting the denatured AAV particle to liquid chromatography/mass spectrometry (LC/MS), and c) determining the masses of VP1, VP2 and VP3 of the AAV particle, and comparing the masses of step c) with the theoretical masses of VP1, VP2 and VP3 of the AAV serotype; wherein a deviation of one or more of the masses of VP1, VP2 or VP3 are indicative of the AAV capsid heterogeneity.

In other aspects, the invention provides a method to determine the serotype of an adeno-associated virus (AAV) particle comprising a) denaturing the AAV particle, b) subjecting the denatured AAV particle to reduction and/or alkylation, c) injecting the denatured AAV particle to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle, d) subjecting the fragments of VP1, VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), and e) determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle; wherein the specific combination of masses of fragments of VP1, VP2 and VP3 are indicative of the AAV serotype.

In other aspects, the invention provides a method of determining the heterogeneity of an AAV particle of a serotype comprising: a) denaturing the AAV particle, b) subjecting the denatured AAV particle to reduction and/or alkylation, c) injecting the denatured AAV particle to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle, d) subjecting the fragments of VP1, VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), e) determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle, and f) comparing the masses of step e) with the theoretical masses of fragments of VP1, VP2 and VP3 of the AAV serotype; wherein a deviation of one or more of the masses of VP1, VP2 or VP3 are indicative of the AAV capsid heterogeneity.

In some aspects, the invention provides a recombinant AAV (rAAV) particle comprising an amino acid substitution at amino acid residue 2 of VP1 and/or VP3; wherein the amino acid substitution at amino acid residue 2 of VP1

and/or VP3 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of VP1 and/or VP3 of the parent AAV particle.

In some aspects, the invention provides a method of improving the assembly of rAAV particles in a cell comprising substituting amino acid residue 2 of VP1 and/or VP3; wherein the substituted amino acid at position 2 is N-acetylated at a higher frequency than amino acid residue 2 of the parent VP1 and/or VP3. In some aspects, the invention provides a method of improving the transduction of rAAV particles in a cell comprising substituting amino acid residue 2 of VP1 and/or VP3; wherein the substituted amino acid at position 2 is N-acetylated at a higher frequency than amino acid residue 2 of the parent VP1 and/or VP3.

I. General Techniques

The techniques and procedures described or referenced herein are generally well understood and commonly employed using conventional methodology by those skilled in the art, such as, for example, the widely utilized methodologies described in *Molecular Cloning: A Laboratory Manual* (Sambrook et al., 4th ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2012); *Current Protocols in Molecular Biology* (F. M. Ausubel, et al. eds., 2003); the series Methods in Enzymology (Academic Press, Inc.); *PCR 2: A Practical Approach* (M. J. MacPherson, B. D. Hames and G. R. Taylor eds., 1995); *Antibodies, A Laboratory Manual* (Harlow and Lane, eds., 1988); *Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications* (R. I. Freshney, 6th ed., J. Wiley and Sons, 2010); *Oligonucleotide Synthesis* (M. J. Gait, ed., 1984); *Methods in Molecular Biology*, Humana Press; *Cell Biology: A Laboratory Notebook* (J. E. Cellis, ed., Academic Press, 1998); *Introduction to Cell and Tissue Culture* (J. P. Mather and P. E. Roberts, Plenum Press, 1998); *Cell and Tissue Culture: Laboratory Procedures* (A. Doyle, J. B. Griffiths, and D. G. Newell, eds., J. Wiley and Sons, 1993-8); *Handbook of Experimental Immunology* (D. M. Weir and C. C. Blackwell, eds., 1996); *Gene Transfer Vectors for Mammalian Cells* (J. M. Miller and M. P. Calos, eds., 1987); *PCR: The Polymerase Chain Reaction*, (Mullis et al., eds., 1994); *Current Protocols in Immunology* (J. E. Coligan et al., eds., 1991); *Short Protocols in Molecular Biology* (Ausubel et al., eds., J. Wiley and Sons, 2002); *Immunobiology* (C. A. Janeway et al., 2004); *Antibodies* (P. Finch, 1997); *Antibodies: A Practical Approach* (D. Catty., ed., IRL Press, 1988-1989); *Monoclonal Antibodies: A Practical Approach* (P. Shepherd and C. Dean, eds., Oxford University Press, 2000); *Using Antibodies: A Laboratory Manual* (E. Harlow and D. Lane, Cold Spring Harbor Laboratory Press, 1999); *The Antibodies* (M. Zanetti and J. D. Capra, eds., Harwood Academic Publishers, 1995); and *Cancer: Principles and Practice of Oncology* (V. T. DeVita et al., eds., J.B. Lippincott Company, 2011).

II. Definitions

A "vector," as used herein, refers to a recombinant plasmid or virus that comprises a nucleic acid to be delivered into a host cell, either in vitro or in vivo.

The term "polynucleotide" or "nucleic acid" as used herein refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. Thus, this term includes, but is not limited to, single-, double- or multi-stranded DNA or RNA, genomic DNA, cDNA, DNA-RNA hybrids, or a polymer comprising purine and pyrimidine bases, or other natural, chemically or biochemically modified, non-natural, or derivatized nucleotide bases. The backbone of the nucleic acid can comprise sugars and phosphate groups (as may typically be found in RNA or DNA), or modified or substituted sugar or phosphate groups. Alternatively, the backbone of the nucleic acid can comprise a polymer of synthetic subunits such as phosphoramidates and thus can be an oligodeoxynucleoside phosphoramidate ($P—NH_2$) or a mixed phosphoramidate-phosphodiester oligomer. In addition, a double-stranded nucleic acid can be obtained from the single stranded polynucleotide product of chemical synthesis either by synthesizing the complementary strand and annealing the strands under appropriate conditions, or by synthesizing the complementary strand de novo using a DNA polymerase with an appropriate primer.

The terms "polypeptide" and "protein" are used interchangeably to refer to a polymer of amino acid residues, and are not limited to a minimum length. Such polymers of amino acid residues may contain natural or non-natural amino acid residues, and include, but are not limited to, peptides, oligopeptides, dimers, trimers, and multimers of amino acid residues. Both full-length proteins and fragments thereof are encompassed by the definition. The terms also include post-translational modifications of the polypeptide, for example, glycosylation, sialylation, acetylation, phosphorylation, and the like. Furthermore, for purposes of the present invention, a "polypeptide" refers to a protein which includes modifications, such as deletions, additions, and substitutions (generally conservative in nature), to the native sequence, as long as the protein maintains the desired activity. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutations of hosts which produce the proteins or errors due to PCR amplification.

A "recombinant viral vector" refers to a recombinant polynucleotide vector comprising one or more heterologous sequences (i.e., nucleic acid sequence not of viral origin). In the case of recombinant AAV vectors, the recombinant nucleic acid is flanked by at least one, e.g., two, inverted terminal repeat sequences (ITRs).

A "recombinant AAV vector (rAAV vector)" refers to a polynucleotide vector comprising one or more heterologous sequences (i.e., nucleic acid sequence not of AAV origin) that are flanked by at least one, e.g., two, AAV inverted terminal repeat sequences (ITRs). Such rAAV vectors can be replicated and packaged into infectious viral particles when present in a host cell that has been infected with a suitable helper virus (or that is expressing suitable helper functions) and that is expressing AAV rep and cap gene products (i.e. AAV Rep and Cap proteins). When a rAAV vector is incorporated into a larger polynucleotide (e.g., in a chromosome or in another vector such as a plasmid used for cloning or transfection), then the rAAV vector may be referred to as a "pro-vector" which can be "rescued" by replication and encapsidation in the presence of AAV packaging functions and suitable helper functions. A rAAV vector can be in any of a number of forms, including, but not limited to, plasmids, linear artificial chromosomes, complexed with lipids, encapsulated within liposomes, and, in embodiments, encapsidated in a viral particle, particularly an AAV particle. A rAAV vector can be packaged into an AAV virus capsid to generate a "recombinant adeno-associated viral particle (rAAV particle)".

An "rAAV virus" or "rAAV viral particle" refers to a viral particle composed of at least one AAV capsid protein and an encapsidated rAAV vector genome.

A "parent AAV particle" and "parent AAV capsid protein" as used herein in the context of comparing N-acetylation and/or deamidation refers to an AAV particle or capsid protein into which amino acid modifications are introduced to modulate N-acetylation and/or deamidation (e.g., an AAV particle/capsid protein that is the same as or similar to the AAV particle/capsid of the subject invention but does not comprise the mutations that modulate/alter N-acetylation and/or deamidation as described herein). In some embodiments, the parent AAV particle is a recombinant AAV particle comprising a recombinant AAV genome. In some embodiments, the parent AAV capsid particle or parent AAV capsid protein comprises amino acid substitutions that affect other aspects of the AAV particle. For example, the parent AAV particle may comprise amino acid substitutions that affect the binding of AAV to its receptor, such as affecting binding of AAV2 to heparin sulfate proteoglycan (e.g. an AAV2 HBKO particle). An AAV2 HBKO particle can be mutated to introduce amino acid substitutions that modulate N-acetylation and/or deamidation. Such a mutated AAV particle may then be compared to the parent AAV2 HBKO particle in aspects of the invention as described herein. A parent AAV capsid protein may include a parent VP1 capsid protein, a parent VP2 capsid protein, or a VP3 capsid protein.

As used herein, the term "modulate" or "alter" in reference to a parent molecule means to change a feature of the parent molecule. For example, an AAV particle with altered N-acetylation may show increased or decreased N-acetylation compared to the parent AAV particle and an AAV particle with altered deamidation may show increased or decreased deamidation compared to the parent AAV particle.

"Heterologous" means derived from a genotypically distinct entity from that of the rest of the entity to which it is compared or into which it is introduced or incorporated. For example, a nucleic acid introduced by genetic engineering techniques into a different cell type is a heterologous nucleic acid (and, when expressed, can encode a heterologous polypeptide). Similarly, a cellular sequence (e.g., a gene or portion thereof) that is incorporated into a viral vector is a heterologous nucleotide sequence with respect to the vector.

The term "transgene" refers to a nucleic acid that is introduced into a cell and is capable of being transcribed into RNA and optionally, translated and/or expressed under appropriate conditions. In aspects, it confers a desired property to a cell into which it was introduced, or otherwise leads to a desired therapeutic or diagnostic outcome. In another aspect, it may be transcribed into a molecule that mediates RNA interference, such as siRNA.

The terms "genome particles (gp)," "genome equivalents," or "genome copies" as used in reference to a viral titer, refer to the number of virions containing the recombinant AAV DNA genome, regardless of infectivity or functionality. The number of genome particles in a particular vector preparation can be measured by procedures such as described in the Examples herein, or for example, in Clark et al. (1999) *Hum. Gene Ther.*, 10:1031-1039; Veldwijk et al. (2002) *Mol. Ther.*, 6:272-278.

The terms "infection unit (iu)," "infectious particle," or "replication unit," as used in reference to a viral titer, refer to the number of infectious and replication-competent recombinant AAV vector particles as measured by the infectious center assay, also known as replication center assay, as described, for example, in McLaughlin et al. (1988) *J. Virol.*, 62:1963-1973.

The term "transducing unit (tu)" as used in reference to a viral titer, refers to the number of infectious recombinant AAV vector particles that result in the production of a functional transgene product as measured in functional assays such as described in Examples herein, or for example, in Xiao et al. (1997) *Exp. Neurobiol.*, 144:113-124; or in Fisher et al. (1996) *J. Virol.*, 70:520-532 (LFU assay).

An "inverted terminal repeat" or "ITR" sequence is a term well understood in the art and refers to relatively short sequences found at the termini of viral genomes which are in opposite orientation.

An "AAV inverted terminal repeat (ITR)" sequence, a term well-understood in the art, is an approximately 145-nucleotide sequence that is present at both termini of the native single-stranded AAV genome. The outermost 125 nucleotides of the ITR can be present in either of two alternative orientations, leading to heterogeneity between different AAV genomes and between the two ends of a single AAV genome. The outermost 125 nucleotides also contains several shorter regions of self-complementarity (designated A, A', B, B', C, C' and D regions), allowing intrastrand base-pairing to occur within this portion of the ITR.

A "terminal resolution sequence" or "trs" is a sequence in the D region of the AAV ITR that is cleaved by AAV rep proteins during viral DNA replication. A mutant terminal resolution sequence is refractory to cleavage by AAV rep proteins. "AAV helper functions" refer to functions that allow AAV to be replicated and packaged by a host cell. AAV helper functions can be provided in any of a number of forms, including, but not limited to, helper virus or helper virus genes which aid in AAV replication and packaging. Other AAV helper functions are known in the art such as genotoxic agents.

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A "helper virus" for AAV refers to a virus that allows AAV (which is a defective parvovirus) to be replicated and packaged by a host cell. A number of such helper viruses have been identified, including adenoviruses, herpesviruses, poxviruses such as vaccinia, and baculovirus. The adenoviruses encompass a number of different subgroups, although Adenovirus type 5 of subgroup C (Ad5) is most commonly used. Numerous adenoviruses of human, non-human mammalian and avian origin are known and are available from depositories such as the ATCC. Viruses of the herpes family, which are also available from depositories such as ATCC, include, for example, herpes simplex viruses (HSV), Epstein-Barr viruses (EBV), cytomegaloviruses (CMV) and pseudorabies viruses (PRV). Baculoviruses available from depositories include *Autographa californica* nuclear polyhedrosis virus.

"Percent (%) sequence identity" with respect to a reference polypeptide or nucleic acid sequence is defined as the percentage of amino acid residues or nucleotides in a candidate sequence that are identical with the amino acid residues or nucleotides in the reference polypeptide or nucleic acid sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid or nucleic acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software programs, for

example, those described in Current Protocols in Molecular Biology (Ausubel et al., eds., 1987), Supp. 30, section 7.7.18, Table 7.7.1, and including BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. A potential alignment program is ALIGN Plus (Scientific and Educational Software, Pennsylvania). Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For purposes herein, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows: 100 times the fraction X/Y, where X is the number of amino acid residues scored as identical matches by the sequence alignment program in that program's alignment of A and B, and where Y is the total number of amino acid residues in B. It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A. For purposes herein, the % nucleic acid sequence identity of a given nucleic acid sequence C to, with, or against a given nucleic acid sequence D (which can alternatively be phrased as a given nucleic acid sequence C that has or comprises a certain % nucleic acid sequence identity to, with, or against a given nucleic acid sequence D) is calculated as follows: 100 times the fraction W/Z, where W is the number of nucleotides scored as identical matches by the sequence alignment program in that program's alignment of C and D, and where Z is the total number of nucleotides in D. It will be appreciated that where the length of nucleic acid sequence C is not equal to the length of nucleic acid sequence D, the % nucleic acid sequence identity of C to D will not equal the % nucleic acid sequence identity of D to C.

An "isolated" molecule (e.g., nucleic acid or protein) or cell means it has been identified and separated and/or recovered from a component of its natural environment.

"Mass spectrometry" refers to the analytical chemistry technique of identifying an amount and/or type of a compound (e.g., a polypeptide) by measuring the mass-to-charge ratio and abundance of gas-phase ions. The term "mass spectrometry" may be used interchangeably herein.

"Heterogeneity" when used in reference to an AAV capsid refers to an AAV capsid characterized by one or more capsid polypeptides observed to deviate from a reference mass of a VP1, VP2, and/or VP3 polypeptide, or fragment thereof. A reference mass may include, without limitation, a theoretical, predicted, or expected mass of a VP1, VP2, and/or VP3 polypeptide, e.g., of a known AAV serotype. For example, an AAV capsid may be said to display heterogeneity if it demonstrates one or more of the following properties (without limitation): a mixed serotype, a variant capsid, a capsid amino acid substitution, a truncated capsid, or a modified capsid.

Reference to "about" a value or parameter herein includes (and describes) embodiments that are directed to that value or parameter per se. For example, description referring to "about X" includes description of "X."

As used herein, the singular form of the articles "a," "an," and "the" includes plural references unless indicated otherwise.

It is understood that aspects and embodiments of the invention described herein include "comprising," "consisting," and/or "consisting essentially of" aspects and embodiments.

III. Methods

Certain aspects of the present disclosure relate to methods of determining the serotype of a viral particle. Other aspects of the present disclosure relate to methods of determining the heterogeneity of a viral particle. As described below, the accurate masses of VP1, VP2 and VP3 of each AAV serotype are unique and can be used to identify or differentiate AAV capsid serotypes. These methods are based in part on the discovery described herein that direct LC/MS of different types of AAVs after denaturation may be used to monitor the protein sequence and post-translational modifications with accurate mass measurement in the intact protein level. Further, acetylations of N-termini of VP1 and VP3 may also be identified and/or monitored in different AAV serotypes. Based on these AAV results and the guidance provided herein, it is contemplated that such methods may readily be applied to profile a variety of viruses, e.g., the viral families, subfamilies, and genera of the present disclosure. The methods of the present disclosure may find use, e.g., in profile VPs to monitor VP expressions, posttranslational modifications, and truncations and to ensure product consistency during VLP production, to confirm site-direct mutagenesis or structural characterization for capsid protein engineering applications, and/or to monitor or detect heterogeneity of a viral particle or preparation.

In some embodiments, the methods include denaturing a viral particle. In some embodiments, a viral particle such as an AAV particle may be denatured using detergent, heat, high salt, or buffering with a low or high pH. In certain embodiments, an AAV particle may be denatured using acetic acid or guanidine hydrochloride. The skilled artisan will recognize that a variety of methods useful for promoting and/or monitoring protein denaturation are available in the art and may suitably select a denaturation method compatible with liquid chromatography/mass spectrometry. For example, if heat denaturation is used, care may be applied to avoid protein precipitation and reverse phase column clogging. Similarly, high salt denaturation may be coupled with a desalting step prior to LC/MS or LC/MS/MS. In other embodiments, high pH denaturation, low pH denaturation, or denaturation using organic solvents is used.

In some embodiments, the methods include subjecting a denatured viral particle of the present disclosure to liquid chromatography/mass spectrometry (LC/MS). As is known in the art, LC/MS utilizes liquid chromatography for physical separation of ions and mass spectrometry for generation of mass spectral data from the ions. Such mass spectral data may be used to determine, e.g., molecular weight or structure, identification of particles by mass, quantity, purity, and so forth. These data may represent properties of the detected ions such as signal strength (e.g., abundance) over time (e.g., retention time), or relative abundance over mass-to-charge ratio.

In some embodiments, liquid chromatography (e.g., used in LC/MS as described herein) is ultra-performance liquid chromatography (UPLC: the term "ultra high performance liquid chromatography" or UHPLC may be used interchangeably herein). UPLC is known in the art as an LC technique that relies upon a column with reduced particle size (e.g., less than 2 μm) and increased flow velocity to improve chromatographic resolution, efficiency, peak capac-

ity, and sensitivity (see, e.g., Plumb, R. et al. (2004) *Rapid Commun. Mass Spectrom.* 18:2331-2337). In some embodiments, UPLC refers to the use of a column with a particle size less than 2 μm in liquid chromatography. In some embodiments, UPLC refers to the use of a high linear solvent velocity (e.g., as observed when operating at 6000 psi or higher) in liquid chromatography. Exemplary UPLC machines are commercially available (e.g., the ACQUITY UPLC® from Waters; Milford, Mass.).

In some embodiments, mass spectrometry (e.g., used in LC/MS as described herein) may refer to electrospray ionization mass spectrometry (ESI-MS). ESI-MS is known in the art as a technique that uses electrical energy to analyze ions derived from a solution using mass spectrometry (see, e.g., Yamashita, M. and Fenn, J. B. (1984) *J. Phys. Chem.* 88:4451-4459). Ionic species (or neutral species that are ionized in solution or in gaseous phase) are transferred from a solution to a gaseous phase by dispersal in an aerosol of charged droplets, followed by solvent evaporation that reduces the size of the charged droplets and sample ion ejection from the charge droplets as the solution is passed through a small capillary with a voltage relative to ground (e.g., the wall of the surrounding chamber). In some embodiments, the capillary voltage is from about 2 kV to about 10 kV, or about 2.5 kV to about 6.0 kV. In certain embodiments, liquid chromatography (e.g., used in LC/MS as described herein) uses a capillary voltage of about 3.5 kV. In some embodiments the capillary voltage ranges from about 1 kV to about 10 kV. In other embodiments, mass spectrometry (e.g., used in LC/MS as described herein) may refer to matrix-assisted laser desorption/ionization (MALDI).

In some embodiments, mass spectrometry (e.g., used in LC/MS as described herein) uses a sampling cone and/or skimmer, through which ions may pass before entering the analyzer. In some embodiments, e.g., when applying voltage to the capillary as described above, the sample cone is held at a lower voltage than the capillary voltage. In certain embodiments, liquid chromatography (e.g., used in LC/MS as described herein) uses a sampling cone voltage of about 45 V. In some embodiments the sampling cone voltage ranges from about 0 V to about 200 V.

In some embodiments, mass spectrometry (e.g., used in LC/MS as described herein) uses assisted calibration. Calibration, when used in reference to mass spectrometry, may include the introduction of one or more compounds having a known mass (e.g., a standard) for the purpose of calibrating the instrument with respect to mass detection (e.g., m/z measurements). In some embodiments, assisted calibration may refer to using software to correlate a peak and/or position of a known standard (e.g., a calibrant) to a specific mass-to-charge (m/z) ratio. Once calibrated, the user may then perform mass spectrometry on a sample having one or more unknown compounds, or compounds present at an unknown concentration, within a certain degree of accuracy or error, and/or a desired level of reproducibility, e.g., as compared to a previous or known experimental condition. Various calibrants are known in the art, including without limitation sodium iodide, sodium cesium iodide, polyethylene glycol, and perfluorotributylamine. In certain embodiments, sodium iodide is used as a calibrant. In some embodiments the calibrants are Glu-1-fibrinopeptide B and leucine encephalin peptide to lock mass during LC/MS operation.

In some embodiments, the methods include subjecting a denatured viral particle of the present disclosure, or subjecting digested fragments of a denatured viral particle of the present disclosure, to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS). As is known in the art, LC/MS/MS (the term "liquid chromatography-tandem mass spectrometry" may be used interchangeably herein) utilizes liquid chromatography for physical separation of ions and mass spectrometry for generation of mass spectral data from the ions, where the mass spectrometry uses multiple stages of mass (e.g., m/z) separation, typically separated by a fragmentation step. For example, ions of interest within a range of m/z may be separated out in a first round of MS, fragmented, and then further separated based on individual m/z in a second round of MS. Ion fragmentation may include without limitation a technique such as collision-induced dissociation (CID), higher energy collision dissociation (HCD), electron-capture dissociation (ECD), or electron-transfer dissociation (ETD).

In some embodiments, the methods include subjecting a denatured viral particle of the present disclosure to reduction and/or alkylation. Means to reduce the viral particle include but are not limited to treatment with dithiothreitol, β-mercaptoethanol, or tris(2-carboxyethyl)phosphine (TCEP). Means to alkylate the viral particle include but are not limited to treating the AAV particle with iodoacetic acid, iodoacetamide, or 4-vinylpyridine.

In some embodiments, the methods include subjecting a denatured viral particle of the present disclosure to digestion, e.g., to generate fragments of VP1, VP2 and/or VP3 of an AAV particle. For example, a denatured AAV particle may be subjected to digestion to generate peptide fragments that may be analyzed, e.g., using LC for separation and MS/MS for analysis (see below for greater description). In some embodiments, the digestion is an enzymatic digestion. In some embodiments, the digestion uses chemical digestion such as CNBr treatment of instrument fragmentation (e.g., top down). In some embodiments, the digestion uses chemical digestion such as acid digestion.

In some embodiments, the enzymatic digestion is an endopeptidase digestion. An endopeptidase may include any peptidase that catalyzes the proteolysis of peptide bonds of non-terminal amino acids of a polypeptide. Known endopeptidases may include, without limitation, trypsin, chymotrypsin, AspN, Glu-C, LysC, pepsin, thermolysin, glutamyl endopeptidase, elastase, and neprilysin. In certain embodiments, the enzymatic digestion is a trypsin digestion or a LysC digestion.

In some embodiments, the liquid chromatography (e.g., used in LC/MS or LC/MS/MS as described herein) is reverse phase liquid chromatography (the terms "reversed phase liquid chromatography" or RPLC may be used interchangeably herein with reverse phase liquid chromatography). As is known in the art, reverse phase liquid chromatography may refer to a chromatographic separation using a hydrophobic stationary phase (e.g., a support or substrate such as a column) to adsorb hydrophobic molecules in a polar mobile phase. By decreasing the polarity of the mobile phase (e.g., by adding an organic solvent), one may achieve gradient separation of molecules by hydrophobicity, since more hydrophobic molecules will stay on the column in higher concentrations of organic solvent due to stronger hydrophobic interactions with the column. In some embodiment, separation is by capillary electrophoresis (CE), size exclusion chromatography (SEC), ion exchange chromatography (IEC) such as cation exchange chromatography, hydrophobic interaction chromatography (HIC), hydrophilic interaction liquid chromatography (HILIC), but not limited to on-line LC/MS such as offline separation before MS; e.g., tips, columns; plates or cartridges.

Generally, a stationary phase suitable for reverse phase liquid chromatography (e.g., a hydrophobic moiety) may be

coupled to a support including without limitation a column or resin packed with particles or beads (e.g., porous silica particles or polystyrene). A variety of hydrophobic stationary phases are known in the art, including without limitation hydrophobic alkyl chains, octyl or octadecyl silyl moieties, cyano moieties, and amino moieties. In some embodiments, the stationary phase may include a hydrophobic alkyl chain of a particular length, such as C4, C8, or C18. In certain embodiments, the reverse phase chromatography is a C4 or C8 reverse chromatography (e.g., reverse phase chromatography utilizing a C4 or C8 stationary phase). One of skill in the art may suitably select a stationary phase based on the molecule of interest (e.g., a denatured AAV particle or fragment thereof).

A variety of mobile phases suitable for reverse phase liquid chromatography are known in the art. As described above, a reverse phase liquid chromatography mobile phase may include a mixture of organic (e.g., hydrophobic) and aqueous (e.g., polar) solvents. Increasing the proportion of organic solvent increases its power to elute hydrophobic compounds from the stationary phase. Compound retention and/or selectivity may be altered, e.g., by changing the type or exposure of the stationary phase, adding polar reagents such as end capping reagents, altering the temperature, and/or altering mobile phase characteristics such as the proportion of organic solvent, pH, buffers, and the type of organic solvent used. In some embodiments, the polar component of the mobile phase may include without limitation water or an aqueous buffer. In some embodiments, the polar component of the mobile phase may include without limitation acetonitrile, methanol, ethanol, isopropyl alcohol, tetrahydrofuran (THF), and formic acid.

In some embodiments, two or more mobile phases may be used (e.g., mobile phase A, mobile phase B, etc.) in a gradient or proportion of interest. In certain embodiments, the chromatography uses a mobile phase A comprising formic acid in water. In certain embodiments, the mobile phase A comprises about 0.1% formic acid. In certain embodiments, the mobile phase A comprises about 0.1% to about 5% formic acid. In certain embodiments, the chromatography uses a mobile phase B comprising formic acid in acetonitrile. In certain embodiments, the mobile phase B comprises about 0.1% formic acid.

In some embodiments, the proportion of mobile phase B in the chromatography increases over time. For example, the proportion of mobile phase B in the chromatography may be increased in a stepwise manner. In certain embodiments, mobile phase B increases from about 10% to about 20%, from about 20% to about 30%, and from about 30% to about 38%. In other embodiments, mobile phase B increases from about 2% to about 60%. In other embodiments, mobile phase B increases from about 2% to about 100% from about 1 min to about 200 min In some embodiments, the remainder of the mobile phase is a second mobile phase of the present disclosure, e.g., mobile phase A. In certain embodiments, mobile phase B increases from about 10% to about 20% in about 6 minutes, from about 20% to about 30% in about 10 minutes, and from about 30% to about 38% in about 40 minutes. In other embodiments, mobile phase B increases from about 2% to about 60% in about 121 minutes. One of skill in the art may suitably adjust the mobile phase of interest and the gradient timing used based on the desired chromatographic separation and/or analyte of interest.

In some embodiments, the liquid chromatography is high-performance liquid chromatography (HPLC). HPLC is known in the art as a form of liquid chromatography in which a liquid solvent containing a sample is pressurized as it passes through a column containing solid phase. While traditional or low pressure LC may use gravity to pass a mobile phase through the solid phase, HPLC uses pumps to apply a pressure to the mobile phase and typically uses a solid phase with smaller particles to increase resolution. In some embodiments, the HPLC uses a pressure of between about 50 bar and about 350 bar. In some embodiments, reversed phase HPLC may be used to concentrate and/or desalt proteins (e.g., AAV capsid proteins) for MS analysis.

In some embodiments, one or more parameters including without limitation source voltage, capillary temperature, ESI voltage (if using ESI-MS), CID energy, and the number of MS/MS events may be adjusted, e.g., in LC/MS/MS as used herein, based on the findings described herein. In some embodiments, mass spectrometry (e.g., used in LC/MS/MS as described herein) uses a source voltage (e.g., capillary voltage) of about 2.5 kV. In some embodiments, mass spectrometry (e.g., used in LC/MS/MS as described herein) uses a capillary temperature of about 275° C. In some embodiments, the capillary temperature ranges from about 20° C. to about 400° C.

A variety of mass analyzers suitable for LC/MS and/or LC/MS/MS are known in the art, including without limitation time-of-flight (TOF) analyzers, quadrupole mass filters, quadrupole TOF (QTOF), and ion traps (e.g., a Fourier transform-based mass spectrometer or an Orbitrap). In Orbitrap, a barrel-like outer electrode at ground potential and a spindle-like central electrode are used to trap ions in trajectories rotating elliptically around the central electrode with oscillations along the central axis, confined by the balance of centrifugal and electrostatic forces. The use of such instruments employs a Fourier transform operation to convert a time domain signal (e.g., frequency) from detection of image current into a high resolution mass measurement (e.g., nano LC/MS/MS). Further descriptions and details may be found, e.g., in Scheltema, R. A. et al. (2014) *Mol. Cell Proteomics* 13:3698-3708; Perry, R. H. et al. (2008) *Mass. Spectrom. Rev.* 27:661-699; and Scigelova, M. et al. (2011) *Mol. Cell Proteomics* 10:M111.009431.

As described above, in some embodiments, the MS includes nano LC/MS/MS, e.g., using an Orbitrap mass analyzer. In some embodiments, the ion source may include a stacked-ring ion guide or S-lens. As is known in the art, an S-lens may be employed to focus the ion beam using radio frequency (RF), thereby increasing transmission of ions into the instrument. This may improve sensitivity (e.g., for low-intensity ions) and/or improve the scan rate. In certain embodiments, the S-lens RF level of the mass spectrometry is about 55%. In certain embodiments, the S-lens RF level of the mass spectrometry is about 20% to about 100%.

In some embodiments, masses of viral capsid proteins may be determined, e.g., based on LC/MS and/or LC/MS/MS data. In some embodiments, masses of VP1, VP2 and VP3 of an AAV particle, or of fragments of VP1, VP2 and VP3 of the AAV particle, may be determined, e.g., based on LC/MS and/or LC/MS/MS data. Various methods to determine protein mass and/or identity from MS data are known in the art. For example, peptide mass fingerprinting may be used to determine protein sequence based on MS data, or proteins may be identified based on MS/MS data related to one or more constituent peptides. When using tandem MS, product ion scanning may be used to analyze m/z data related to one or more peptides of a protein of interest. Software known in the art may then be used, e.g., to match identified peaks to reference or known peaks, to group peaks into isotopomer envelopes, and so forth. Peptide mass values may be compared to a database of known peptide sequences.

For example, Mascot may be used to match observed peptides with theoretical database peptides, e.g., resulting from application of a particular digest pattern to an in silico protein database. Other suitable software may include without limitation Proteome Discoverer, ProteinProspector, X!Tandem, Pepfinder, Bonics, or MassLynx™ (Waters). Other software suitable for various steps of MS data analysis may be found, e.g., at www.ms-utils.org/wiki/pmwiki.php/Main/SoftwareList.

In some embodiments, a determined or calculated mass of the present disclosure (e.g., the determined or calculated mass of VP1, VP2 and/or VP3 of the AAV particle) may be compared with a reference, e.g., a theoretical mass of a VP1, VP2, and/or VP3 of one or more AAV serotypes. A reference of the present disclosure may include a theoretical mass of a VP1, VP2, and/or VP3 of one or more of any of the AAV serotypes described herein. For example, in some embodiments, the masses of VP1, VP2, and/or VP3 are compared to the theoretical masses of one or more of an AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an AAV LK03 capsid (see U.S. Pat. No. 9,169,299), an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid (see U.S. Pat. No. 7,588,772), an AAV DJ8 capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, or a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV/human bocavirus virus 1), an AAV2HBKO capsid, an AAVPHP.B capsid or an AAVPHP.eB capsid. In some embodiments, a determined or calculated mass of the present disclosure (e.g., the determined or calculated mass of VP1, VP2 and/or VP3 of the AAV particle) may be compared with a theoretical mass of a VP1, VP2, and/or VP3 of the corresponding AAV serotype.

In some embodiments, the methods of the present disclosure may include determining the heterogeneity of an AAV particle. In some embodiments, a deviation of one or more of the masses of VP1, VP2 and/or VP3 (e.g., from a reference mass, such as a theoretical, predicted, or expected mass) is indicative of the AAV capsid heterogeneity. In some embodiments, heterogeneity may include one or more of the following, without limitation: mixed serotypes, variant capsids, capsid amino acid substitutions, truncated capsids, or modified capsids.

In some embodiments, the use of LC/MS and LC/MS/MS as described herein may be combined. In some embodiments, a method of determining the serotype of an AAV particle may include subjecting a denatured AAV particle to LC/MS (e.g., as described herein) and determining the masses of VP1, VP2 and VP3 of the AAV particle; as well as subjecting fragments of VP1, VP2 and/or VP3 to LC/MS/MS and determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle (the specific combination of masses of fragments of VP1, VP2 and VP3 are indicative of the AAV serotype). In some embodiments, a method of determining the heterogeneity of an AAV particle may include subjecting a denatured AAV particle to LC/MS (e.g., as described herein), determining the masses of VP1, VP2 and VP3 of the AAV particle, and comparing these masses with the theoretical masses of VP1, VP2 and VP3 of the AAV serotype; as well as subjecting fragments of VP1, VP2 and/or VP3 to LC/MS/MS, determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle, and comparing these masses with the theoretical masses of VP1, VP2 and VP3 of the AAV serotype (a deviation of one or more of the masses of VP1, VP2 or VP3 are indicative of the AAV capsid heterogeneity).

In some embodiments, an AAV particle of the present disclosure may be acetylated. For example, in some embodiments, the N-terminus of VP1 and/or VP3 is acetylated. As described in greater detail below, the amino acid at the 2nd position to the initiating methionine (iMet X) of an AAV capsid protein may be mutated in order to determine its effect on N-terminal (Nt-) acetylation, as well as the functional consequences of affecting Nt-acetylation on AAV particle trafficking, transduction, and/or post-translational modification (e.g., glycosylation, ubiquitination, and so forth). In some embodiments, the N-terminus of an AAV capsid protein (e.g., VP1 or VP3) may refer to the first amino acid after the initiating methionine, which in some cases may be removed by, e.g., a Met-aminopeptidase.

In some embodiments, an AAV particle of the present disclosure (e.g., a recombinant AAV or rAAV particle) comprises an amino acid substitution at amino acid residue 2 of VP1 and/or VP3. In some embodiments, the amino acid substitution at amino acid residue 2 of VP1 and/or VP3 leads to a VP1 and/or VP3 with a different frequency or proportion of N-terminal acetylation as compared to a reference (e.g., the parent AAV particle before the amino acid substitution, or an AAV particle with a different amino acid residue 2 of VP1 and/or VP3). In some embodiments, the amino acid substitution at amino acid residue 2 of VP1 and/or VP3 alters N-terminal acetylation as compared to N-terminal acetylation at amino acid residue 2 of VP1 and/or VP3 of the parent AAV particle. For example, in certain embodiments, the amino acid substitution at amino acid residue 2 of VP1 alters N-terminal acetylation as compared to N-terminal acetylation at amino acid residue 2 of VP1 of the parent AAV particle. In certain embodiments, the amino acid substitution at amino acid residue 2 of VP3 alters N-terminal acetylation as compared to N-terminal acetylation at amino acid residue 2 of VP3 of the parent AAV particle. In some embodiments, an amino acid substitution (e.g., an amino acid substitution at amino acid residue 2 of VP1 or VP3) that "alters" N-terminal acetylation results in a higher frequency of N-terminal acetylation or a lower frequency of N-terminal acetylation, e.g., as compared to a VP1 or VP3 without the substitution, such as the parental VP1 or VP3. The VP1 and/or VP3 may belong to any of the exemplary AAV serotypes described herein, including variants or hybrids thereof (e.g., bearing tyrosine mutation or heparin binding mutations). Exemplary assays for N-terminal acetylation include without limitation mass spectrometry, isotope labeling (e.g., with an isotope-labeled acetyl group or precursor thereof), Western blotting with an acetylation-specific antibody, and so forth. In some embodiments, amino acid residue 2 of the AAV capsid protein (e.g., VP1 or VP3) is substituted with Cys, Ser, Thr, Val, Gly, Asn, Asp, Glu, Ile, Leu, Phe, Gln, Lys, Met, Pro or Tyr. In some embodiments, the amino acid substitution results in less deamidation of the AAV capsid.

In some embodiments, an AAV particle of the present disclosure may be deamidated. For example, in some embodiments, N57 of VP1 and/or N382, N511, and/or N715 VP3 is deamidated. As described in greater detail below, an amino acid selected from A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3 of an AAV capsid protein (e.g., VP1 or VP3) may be mutated in order to determine its effect on deamidation, as well as the functional consequences of affecting deamidation on AAV particle

trafficking, transduction, and/or post-translational modification (e.g., glycosylation, ubiquitination, and so forth).

In some embodiments, an AAV particle of the present disclosure (e.g., a recombinant AAV or rAAV particle) comprises an amino acid substitution at one or more amino acid residues selected from A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, and G716 of VP3. In some embodiments, the amino acid substitution at A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, and/or G716 of VP3 leads to a VP1 and/or VP3 with a different frequency or proportion of deamidation as compared to a reference (e.g., the parent AAV particle before the amino acid substitution, or an AAV particle with a different corresponding amino acid residue 2). In some embodiments, an amino acid substitution (e.g., an amino acid substitution at A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3) that "alters" deamidation results in a higher frequency of deamidation or a lower frequency of deamidation, e.g., as compared to a VP1 or VP3 without the substitution, such as the parental VP1 or VP3. The VP1 and/or VP3 may belong to any of the exemplary AAV serotypes described herein, including variants or hybrids thereof (e.g., bearing tyrosine mutation or heparin binding mutations). Exemplary assays for deamidation include without limitation mass spectrometry, HPLC (see, e.g., the ISOQUANT® isoaspartate detection kit from Promega), and so forth. In some embodiments, N57 of VP1, N382 of VP3, N511 of VP3, and/or N715 of VP3 is substituted with Asp, and the amino acid substitution results in a higher frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In other embodiments, the amino acid substitution is N57K or N57Q, and the amino acid substitution results in a lower frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In yet other embodiments, G58 of VP1, G383 of VP3, G512 of VP3, and/or G716 of VP3 is substituted with an amino acid that is not Gly (e.g., Ala, Arg, Asn, Asp, Cys, Glu, Gln, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val), and the amino acid substitution results in a lower frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In yet other embodiments, A35 of VP1 is substituted with Asn and results in a higher frequency of deamidation as compared to deamidation of VP1 of a parent particle.

As used herein "N-acetylation" refers to a process whereby an acetyl group is covalently added to the amino group of the N-terminal amino acid of a protein. Typically, N-terminal acetyltransferases (NATs) transfer an acetyl group from acetyl-coenzyme A (Ac-CoA) to the α-amino group of the first amino acid residue of the protein.

As used here in, "deamidation" refers to a chemical reaction in which an amide functional group in the side chain of asparagine or glutamine is removed or converted to another functional group. For example, asparagine may be converted to aspartic acid or isoaspartic acid. In other examples, glutamine is converted to glutamic acid or pyroglutamic acid (5-oxoproline).

In some embodiments, the AAV particle is N-acetylated to a higher extent compared to a parental AAV capsid protein. In some embodiments, the AAV particle comprises more than about any of 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% more N-acetyl groups compared to a parent AAV particle. In some embodiments, the AAV particle comprises between about any of 5%-10%, 10%-15%, 15%-20%, 20%-25%, 25%-30%, 30%-35%, 35%-40%, 40%-55%, 45%-50%, 50%-55%, 55%-60%, 60%-65%, 65%-70%, 70%-75%, 75%-80%, 80%-85%, 85%-90%, 90%-95%, 95%-100%, 5-25%, 25-50%, 50-75%, 75%-100%, 5-50% or 50%-100% more N-acetyl groups compared to a parent AAV particle. In some embodiments, the AAV particle comprises more than about any of 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 25-fold, 50-fold, 100-fold, 500-fold, or 1000-fold more N-acetyl groups compared to a parent AAV particle. In some embodiments, the AAV particle comprises between about any of 2-fold to 3-fold, 3-fold to 4-fold, 4-fold to 5-fold, 5-fold to 10-fold, 10-fold to 25-fold, 25-fold to 50-fold, 50-fold to 100-fold, 100-fold to 500-fold, 500-fold to 1000-fold, 2-fold to 10-fold, 10-fold to 100-fold, or 100-fold to 1000-fold more N-acetyl groups compared to a parent AAV particle.

In some embodiments, the AAV particle N-acetlyated to a lower extent compared to a parental AAV capsid protein. In some embodiments, the AAV particle comprises more than about any of 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% less N-acetyl groups compared to a parent AAV particle. In some embodiments, the AAV particle comprises between about any of 5%-10%, 10%-15%, 15%-20%, 20%-25%, 25%-30%, 30%-35%, 35%-40%, 40%-55%, 45%-50%, 50%-55%, 55%-60%, 60%-65%, 65%-70%, 70%-75%, 75%-80%, 80%-85%, 85%-90%, 90%-95%, 95%-100%, 5-25%, 25-50%, 50-75%, 75%-100%, 5-50% or 50%-100% less N-acetyl groups compared to a parent AAV particle. In some embodiments, the AAV particle comprises more than about any of 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 25-fold, 50-fold, 100-fold, 500-fold, or 1000-fold less N-acetyl groups compared to a parent AAV particle. In some embodiments, the AAV particle comprises between about any of 2-fold to 3-fold, 3-fold to 4-fold, 4-fold to 5-fold, 5-fold to 10-fold, 10-fold to 25-fold, 25-fold to 50-fold, 50-fold to 100-fold, 100-fold to 500-fold, 500-fold to 1000-fold, 2-fold to 10-fold, 10-fold to 100-fold, or 100-fold to 1000-fold less N-acetyl groups compared to a parent AAV particle.

In some embodiments, the AAV particle is deamidated to a higher extent compared to a parental AAV particle. In some embodiments, the AAV particle is more than about any of 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% more deamidated compared to a parent AAV particle. In some embodiments, the AAV particle is deamidated between about any of 5%-10%, 10%-15%, 15%-20%, 20%-25%, 25%-30%, 30%-35%, 35%-40%, 40%-55%, 45%-50%, 50%-55%, 55%-60%, 60%-65%, 65%-70%, 70%-75%, 75%-80%, 80%-85%, 85%-90%, 90%-95%, 95%-100%, 5-25%, 25-50%, 50-75%, 75%-100%, 5-50% or 50%-100% more than a parent AAV particle. In some embodiments, the AAV particle is deamidated more than about any of 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 25-fold, 50-fold, 100-fold, 500-fold, or 1000-fold compared to a parent AAV particle. In some embodiments, the AAV particle is deamidated between about any of 2-fold to 3-fold, 3-fold to 4-fold, 4-fold to 5-fold, 5-fold to 10-fold, 10-fold to 25-fold, 25-fold to 50-fold, 50-fold to 100-fold, 100-fold to 500-fold, 500-fold to 1000-fold, 2-fold to 10-fold, 10-fold to 100-fold, or 100-fold to 1000-fold more than a parent AAV particle.

In some embodiments, a capsid protein of AAV is deamidated to a lower extent compared to a parental AAV capsid protein. In some embodiments, the AAV particle is more than about any of 5%, 10%, 15%, 20%, 25%, 30%, 35%,

40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% less deamidated compared to a parent AAV particle. In some embodiments, the AAV particle is deamidated between about any of 5%-10%, 10%-15%, 15%-20%, 20%-25%, 25%-30%, 30%-35%, 35%-40%, 40%-55%, 45%-50%, 50%-55%, 55%-60%, 60%-65%, 65%-70%, 70%-75%, 75%-80%, 80%-85%, 85%-90%, 90%-95%, 95%-100%, 5-25%, 25-50%, 50-75%, 75%-100%, 5-50% or 50%-100% less than a parent AAV particle. In some embodiments, the AAV particle is deamidated more than about any of 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 25-fold, 50-fold, 100-fold, 500-fold, or 1000-fold less than a parent AAV particle. In some embodiments, the AAV particle is deamidated between about any of 2-fold to 3-fold, 3-fold to 4-fold, 4-fold to 5-fold, 5-fold to 10-fold, 10-fold to 25-fold, 25-fold to 50-fold, 50-fold to 100-fold, 100-fold to 500-fold, 500-fold to 1000-fold, 2-fold to 10-fold, 10-fold to 100-fold, or 100-fold to 1000-fold less than a parent AAV particle.

The invention provides any combination of N-acetylation and deamidation. For example, the AAV capsid protein may be N-acetylated to a higher extent than a parent AAV capsid protein and deamidated to a higher extent than a parent AAV capsid protein, the AAV capsid protein may be N-acetylated to a higher extent than a parent AAV capsid protein and deamidated to the same extent than a parent AAV capsid protein, the AAV capsid protein may be N-acetylated to a higher extent than a parent AAV capsid protein and deamidated to a lower extent than a parent AAV capsid protein, the AAV capsid protein may be N-acetylated to the same extent than a parent AAV capsid protein and deamidated to a higher extent than a parent AAV capsid protein, the AAV capsid protein may be N-acetylated to the same extent than a parent AAV capsid protein and deamidated to the same extent than a parent AAV capsid protein, the AAV capsid protein may be N-acetylated to the same extent than a parent AAV capsid protein and deamidated to a lower extent than a parent AAV capsid protein, the AAV capsid protein may be N-acetylated to a lower extent than a parent AAV capsid protein and deamidated to a higher extent than a parent AAV capsid protein, the AAV capsid protein may be N-acetylated to a lower extent than a parent AAV capsid protein and deamidated to the same extent than a parent AAV capsid protein, or the AAV capsid protein may be N-acetylated to a lower extent than a parent AAV capsid protein and deamidated to a lower extent than a parent AAV capsid protein.

IV. Vectors

In certain aspects, the invention relates to viral particles, suitable for use in any of the methods described herein, which may comprise AAV vectors (e.g., rAAV vectors) or vectors derived from another virus. In some embodiments, the viral particle comprises a vector encoding a heterologous nucleic acid, e.g., a heterologous transgene. In some embodiments, the AAV particle comprises an AAV vector genome encoding a heterologous nucleic acid, e.g., a heterologous transgene.

The present invention contemplates the use of a recombinant viral genome for introduction of one or more nucleic acid sequences encoding a therapeutic polypeptide and/or nucleic acid for packaging into a rAAV viral particle. The recombinant viral genome may include any element to establish the expression of the therapeutic polypeptide and/or nucleic acid, for example, a promoter, an ITR of the present disclosure, a ribosome binding element, terminator, enhancer, selection marker, intron, polyA signal, and/or origin of replication.

In some embodiments, the heterologous nucleic acid encodes a therapeutic polypeptide. A therapeutic polypeptide may, e.g., supply a polypeptide and/or enzymatic activity that is absent or present at a reduced level in a cell or organism. Alternatively, a therapeutic polypeptide may supply a polypeptide and/or enzymatic activity that indirectly counteracts an imbalance in a cell or organism. For example, a therapeutic polypeptide for a disorder related to buildup of a metabolite caused by a deficiency in a metabolic enzyme or activity may supply a missing metabolic enzyme or activity, or it may supply an alternate metabolic enzyme or activity that leads to reduction of the metabolite. A therapeutic polypeptide may also be used to reduce the activity of a polypeptide (e.g., one that is overexpressed, activated by a gain-of-function mutation, or whose activity is otherwise misregulated) by acting, e.g., as a dominant-negative polypeptide.

The nucleic acids of the invention may encode polypeptides that are intracellular proteins, anchored in the cell membrane, remain within the cell, or are secreted by the cell transduced with the vectors of the invention. For polypeptides secreted by the cell that receives the vector; the polypeptide can be soluble (i.e., not attached to the cell). For example, soluble polypeptides are devoid of a transmembrane region and are secreted from the cell. Techniques to identify and remove nucleic acid sequences which encode transmembrane domains are known in the art.

The nucleic acids if the invention (e.g. the AAV vector genome) may comprise as a transgene, a nucleic acid encoding a protein or functional RNA that modulates or treats a CNS-associated disorder. The following is a non-limiting list of genes associated with CNS-associated disorders: neuronal apoptosis inhibitory protein (NAIP), nerve growth factor (NGF), glial-derived growth factor (GDNF), brain-derived growth factor (BDNF), ciliary neurotrophic factor (CNTF), tyrosine hydroxylase (TM, GTP-cyclohydrolase (GTPCH), aspartoacylase (ASPA), superoxide dismutase (SOD1), an anti-oxidant, an anti-angiogenic polypeptide, an anti-inflammatory polypeptide, and amino acid decorboxylase (AADC). For example, a useful transgene in the treatment of Parkinson's disease encodes TH, which is a rate limiting enzyme in the synthesis of dopamine. A transgene encoding GTPCII, which generates the TII cofactor tetrahydrobiopterin, may also be used in the treatment of Parkinson's disease. A transgene encoding GDNF or BDNF, or AADC, which facilitates conversion of L-Dopa to DA, may also be used for the treatment of Parkinson's disease. For the treatment of ALS, a useful transgene may encode: GDNF, BDNF or CNTF. Also for the treatment of ALS, a useful transgene may encode a functional RNA, e.g., shRNA, miRNA, that inhibits the expression of SOD1. For the treatment of ischemia a useful transgene may encode NAIP or NGF. A transgene encoding Beta-glucuronidase (GUS) may be useful for the treatment of certain lysosomal storage diseases (e.g., Mucopolysacharidosis type VII (MPS VII)). A transgene encoding a prodrug activation gene, e.g., HSV-Thymidine kinase which converts ganciclovir to a toxic nucleotide which disrupts DNA synthesis and leads to cell death, may be useful for treating certain cancers, e.g., when administered in combination with the prodrug. A transgene encoding an endogenous opioid, such a β-endorphin may be useful for treating pain. Examples of anti-oxidants include without limitation SOD1; SOD2; Catalase; Sirtuins 1, 3, 4, or 5; NRF2; PGC1a; GCL (catalytic sub-

unit); GCL (modifier subunit); adiponectin; glutathione peroxidase 1; and neuroglobin. Examples of anti-angiogenic polypeptides include without limitation angiostatin, endostatin, PEDF, a soluble VEGF receptor, and a soluble PDGF receptor. Examples of anti-inflammatory polypeptides include without limitation IL-10, soluble IL17R, soluble TNF-R, TNF-R-Ig, an IL-1 inhibitor, and an IL18 inhibitor. Other examples of transgenes that may be used in the rAAV vectors of the invention will be apparent to the skilled artisan (See, e.g., Costantini L C, et al., *Gene Therapy* (2000) 7, 93-109).

In some embodiments, the heterologous nucleic acid encodes a therapeutic nucleic acid. In some embodiments, a therapeutic nucleic acid may include without limitation an siRNA, an shRNA, an RNAi, a miRNA, an antisense RNA, a ribozyme or a DNAzyme. As such, a therapeutic nucleic acid may encode an RNA that when transcribed from the nucleic acids of the vector can treat a disorder by interfering with translation or transcription of an abnormal or excess protein associated with a disorder of the invention. For example, the nucleic acids of the invention may encode for an RNA which treats a disorder by highly specific elimination or reduction of mRNA encoding the abnormal and/or excess proteins. Therapeutic RNA sequences include RNAi, small inhibitory RNA (siRNA), micro RNA (miRNA), and/or ribozymes (such as hammerhead and hairpin ribozymes) that can treat disorders by highly specific elimination or reduction of mRNA encoding the abnormal and/or excess proteins.

In some embodiments, the therapeutic polypeptide or therapeutic nucleic acid is used to treat a disorder of the CNS. Without wishing to be bound to theory, it is thought that a therapeutic polypeptide or therapeutic nucleic acid may be used to reduce or eliminate the expression and/or activity of a polypeptide whose gain-of-function has been associated with a disorder, or to enhance the expression and/or activity of a polypeptide to complement a deficiency that has been associated with a disorder (e.g., a mutation in a gene whose expression shows similar or related activity). Non-limiting examples of disorders of the invention that may be treated by a therapeutic polypeptide or therapeutic nucleic acid of the invention (exemplary genes that may be targeted or supplied are provided in parenthesis for each disorder) include stroke (e.g., caspase-3, Beclin1, Ask1, PAR1, HIF1α, PUMA, and/or any of the genes described in Fukuda, A. M. and Badaut, J. (2013) *Genes* (*Basel*) 4:435-456). Huntington's disease (mutant HTT), epilepsy (e.g., SCN1A, NMDAR, ADK, and/or any of the genes described in Boison, D. (2010) *Epilepsia* 51:1659-1668), Parkinson's disease (alpha-synuclein), Lou Gehrig's disease (also known as amyotrophic lateral sclerosis; SOD1), Alzheimer's disease (tau, amyloid precursor protein), corticobasal degeneration or CBD (tau), corticogasal ganglionic degeneration or CBGD (tau), frontotemporal dementia or FTD (tau), progressive supranuclear palsy or PSP (tau), multiple system atrophy or MSA (alpha-synuclein), cancer of the brain (e.g., a mutant or overexpressed oncogene implicated in brain cancer), and lysosomal storage diseases (LSD). Disorders of the invention may include those that involve large areas of the cortex, e.g., more than one functional area of the cortex, more than one lobe of the cortex, and/or the entire cortex. Other non-limiting examples of disorders of the invention that may be treated by a therapeutic polypeptide or therapeutic nucleic acid of the invention include traumatic brain injury, enzymatic dysfunction disorders, psychiatric disorders (including post-traumatic stress syndrome), neurodegenerative diseases, and cognitive disorders (including dementias, autism, and depression). Enzymatic dysfunction disorders include without limitation leukodystrophies (including Canavan's disease) and any of the lysosomal storage diseases described below.

In some embodiments, the therapeutic polypeptide or therapeutic nucleic acid is used to treat a lysosomal storage disease. As is commonly known in the art, lysosomal storage disease are rare, inherited metabolic disorders characterized by defects in lysosomal function. Such disorders are often caused by a deficiency in an enzyme required for proper mucopolysaccharide, glycoprotein, and/or lipid metabolism, leading to a pathological accumulation of lysosomally stored cellular materials. Non-limiting examples of lysosomal storage diseases of the invention that may be treated by a therapeutic polypeptide or therapeutic nucleic acid of the invention (exemplary genes that may be targeted or supplied are provided in parenthesis for each disorder) include Gaucher disease type 2 or type 3 (acid beta-glucosidase, GBA), GM1 gangliosidosis (beta-galactosidase-1, GLB1), Hunter disease (iduronate 2-sulfatase, IDS), Krabbe disease (galactosylceramidase, GALC), a mannosidosis disease (a mannosidase, such as alpha-D-mannosidase, MAN2B1), β mannosidosis disease (beta-mannosidase, MANBA), metachromatic leukodystrophy disease (pseudoarylsulfatase A, ARSA), mucolipidosisII/III disease (N-acetylglucosamine-1-phosphotransferase, GNP TAB), Niemann-Pick A disease (acid sphingomyelinase, ASM), Niemann-Pick C disease (Niemann-Pick C protein, NPC1), Pompe disease (acid alpha-1,4-glucosidase, GAA), Sandhoff disease (hexosaminidase beta subunit, HEXB), Sanfilippo A disease (N-sulfoglucosamine sulfohydrolase, MPS3A), Sanfilippo B disease (N-alpha-acetylglucosaminidase, NAGLU), Sanfilippo C disease (heparin acetyl-CoA:alpha-glucosaminidase N-acetyltransferase, MP S3C), Sanfilippo D disease (N-acetylglucosamine-6-sulfatase, GNS), Schindler disease (alpha-N-acetylgalactosaminidase, NAGA), Sly disease (beta-glucuronidase, GUSB), Tay-Sachs disease (hexosaminidase alpha subunit, HEXA), and Wolman disease (lysosomal acid lipase, LIPA).

Additional lysosomal storage diseases, as well as the defective enzyme associated with each disease, are listed in Table 1 below. In some embodiments, a disease listed in the table below is treated by a therapeutic polypeptide or therapeutic nucleic acid of the invention that complements or otherwise compensates for the corresponding enzymatic defect.

TABLE 1

Lysosomal storage disorders and associated defective enzymes.

Lysosomal storage disease	Defective enzyme
Aspartylglusaminuria	Aspartylglucosaminidase
Fabry	Alpha-galactosidase A
Infantile Batten Disease (CNL1)	Palmitoyl protein thioesterase
Classic Late Infantile Batten Disease (CNL2)	Tripeptidyl peptidase
Juvenile Batten Disease (CNL3)	Lysosomal transmembrane protein
Batten, other forms (CNL4-CNL8)	multiple gene products
Cystinosis	Cysteine transporter
Farber	Acid ceramidase
Fucosidosis	Acid alpha-L-fucosidase
Galactosidosialidosis	Protective protein/cathepsin A
Gaucher types 1, 2, and 3	Acid beta-glucosidase
GM1 gangliosidosis	Acid beta-galactosidase
Hunter	Iduronate-2-sulfatase
Hurler-Scheie	Alpha-L-iduronidase
Krabbe	Galactocerebrosidase

TABLE 1-continued

Lysosomal storage disorders and associated defective enzymes.

Lysosomal storage disease	Defective enzyme
alpha-mannosidosis	Acid alpha-mannosidase
beta-mannosidosis	Acid beta-mannosidase
Maroteaux-Lamy	Arylsulfatase B
Metachromatic leukodystrophy	Aryl sulfatase A
Morquio A	N-acetylgalactosamine-6-sulfate
Morquio B	Acid beta-galactosidase
Mucolipidosis II/III	N-acetylglucosamine-1-phosphotransferase
Niemann-Pick A, B	Acid sphingomyelinase
Niemann-Pick C	NPC-1
Pompe acid	alpha-glucosidase
Sandhoff	beta-hexosaminidase B
Sanfilippo A	Heparan N-sulfatase
Sanfilippo B	alpha-N-acetylglucosaminidase
Sanfilippo C	Acetyl-CoA:alpha-glucosaminide N-acetyltransferase
Sanfilippo D	N-acetylglucosamine-6-sulfate
Schindler disease	alpha-N-acetylgalactosaminidase
Schindler-Kanzaki	alpha-N-acetylgalactosaminidase
Sialidosis	alpha-neuramidase
Sly	beta-glucuronidase
Tay-Sachs	beta-hexosaminidase A
Wolman	Acid lipase

In some embodiments, the heterologous nucleic acid is operably linked to a promoter. Exemplary promoters include, but are not limited to, the cytomegalovirus (CMV) immediate early promoter, the RSV LTR, the MoMLV LTR, the phosphoglycerate kinase-1 (PGK) promoter, a simian virus 40 (SV40) promoter and a CK6 promoter, a transthyretin promoter (TTR), a TK promoter, a tetracycline responsive promoter (TRE), an HBV promoter, an hAAT promoter, a LSP promoter, chimeric liver-specific promoters (LSPs), the E2F promoter, the telomerase (hTERT) promoter; the cytomegalovirus enhancer/chicken beta-actin/Rabbit 3-globin promoter (CAG promoter; Niwa et al., *Gene*, 1991, 108(2):193-9) and the elongation factor 1-alpha promoter (EF1-alpha) promoter (Kim et al., *Gene*, 1990, 91(2):217-23 and Guo et al., *Gene Ther.*, 1996, 3(9):802-10). In some embodiments, the promoter comprises a human β-glucuronidase promoter or a cytomegalovirus enhancer linked to a chicken β-actin (CBA) promoter. The promoter can be a constitutive, inducible or repressible promoter. In some embodiments, the invention provides a recombinant vector comprising nucleic acid encoding a heterologous transgene of the present disclosure operably linked to a CBA promoter. Exemplary promoters and descriptions may be found, e.g., in U.S. PG Pub. 20140335054.

Examples of constitutive promoters include, without limitation, the retroviral Rous sarcoma virus (RSV) LTR promoter (optionally with the RSV enhancer), the cytomegalovirus (CMV) promoter (optionally with the CMV enhancer) [see, e.g., Boshart et al., Cell, 41:521-530 (1985)], the SV40 promoter, the dihydrofolate reductase promoter, the 13-actin promoter, the phosphoglycerol kinase (PGK) promoter, and the EF1a promoter [Invitrogen].

Inducible promoters allow regulation of gene expression and can be regulated by exogenously supplied compounds, environmental factors such as temperature, or the presence of a specific physiological state, e.g., acute phase, a particular differentiation state of the cell, or in replicating cells only. Inducible promoters and inducible systems are available from a variety of commercial sources, including, without limitation, Invitrogen, Clontech and Ariad. Many other systems have been described and can be readily selected by one of skill in the art. Examples of inducible promoters regulated by exogenously supplied promoters include the zinc-inducible sheep metallothionine (MT) promoter, the dexamethasone (Dex)-inducible mouse mammary tumor virus (MMTV) promoter, the T7 polymerase promoter system (WO 98/10088); the ecdysone insect promoter (No et al., *Proc. Natl. Acad. Sci. USA*, 93:3346-3351 (1996)), the tetracycline-repressible system (Gossen et al., *Proc. Natl. Acad. Sci. USA*, 89:5547-5551 (1992)), the tetracycline-inducible system (Gossen et al., *Science*, 268:1766-1769 (1995), see also Harvey et al., *Curr. Opin. Chem. Biol.*, 2:512-518 (1998)), the RU486-inducible system (Wang et al., *Nat. Biotech.*, 15:239-243 (1997) and Wang et al., *Gene Ther.*, 4:432-441 (1997)) and the rapamycin-inducible system (Magari et al., *J. Clin. Invest.*, 100:2865-2872 (1997)). Still other types of inducible promoters which may be useful in this context are those which are regulated by a specific physiological state, e.g., temperature, acute phase, a particular differentiation state of the cell, or in replicating cells only.

In another embodiment, the native promoter, or fragment thereof, for the transgene will be used. The native promoter can be used when it is desired that expression of the transgene should mimic the native expression. The native promoter may be used when expression of the transgene must be regulated temporally or developmentally, or in a tissue-specific manner, or in response to specific transcriptional stimuli. In a further embodiment, other native expression control elements, such as enhancer elements, polyadenylation sites or Kozak consensus sequences may also be used to mimic the native expression

In some embodiments, the regulatory sequences impart tissue-specific gene expression capabilities. In some cases, the tissue-specific regulatory sequences bind tissue-specific transcription factors that induce transcription in a tissue specific manner. Such tissue-specific regulatory sequences (e.g., promoters, enhancers, etc.) are well known in the art.

In some embodiments, the vector comprises an intron. For example, in some embodiments, the intron is a chimeric intron derived from chicken beta-actin and rabbit beta-globin. In some embodiments, the intron is a minute virus of mice (MVM) intron.

In some embodiments, the vector comprises a polyadenylation (polyA) sequence. Numerous examples of polyadenylation sequences are known in the art, such as a bovine growth hormone (BGH) Poly(A) sequence (see, e.g., accession number EF592533), an SV40 polyadenylation sequence, and an HSV TK pA polyadenylation sequence.

V. Viral Particles and Methods of Producing Viral Particles

Certain aspects of the present disclosure relate to recombinant viral particles (e.g., rAAV particles).

Based on the guidance provided herein, the techniques of the present disclosure may suitably be adapted by one of skill in the art for use with a variety of different viruses.

In some embodiments, the virus is of the family Adenoviridae, which includes nonenveloped viruses typically known as Adenoviruses. In some embodiments, the virus is of the genus *Atadenovirus*, *Aviadenovirus*, *Ichtadenovirus*, *Mastadenovirus*, or *Siadenovirus*.

In some embodiments, the virus is of the family Parvoviridae, which includes nonenveloped viruses such as AAV and Bocaparvovirus. In some embodiments, the virus is of the subfamily Densovirinae. In some embodiments, the virus is of the genus *Ambidensovirus*, *Brevidensovirus*, *Hepandensovirus*, *Iteradensovirus*, or *Penstyldensovirus*. In some

embodiments, the virus is of the subfamily Parvovirinae. In some embodiments, the virus is of the genus *Amdoparvovirus, Aveparvovirus, Bocaparvovirus, Copiparvovirus, Dependoparvovirus, Erythroparvovirus, Protoparvovirus*, or *Tetraparvovirus*.

In some embodiments, the virus is of the family Retroviridae, which includes enveloped viruses including lentivirus. In some embodiments, the virus is of the subfamily Orthoretrovirinae. In some embodiments, the virus is of the genus *Alpharetrovirus, Betaretrovirus, Deltaretrovirus, Epsilonretrovirus, Gammaretrovirus*, or *Lentivirus*. In some embodiments, the virus is of the subfamily Spumaretrovirinae. In some embodiments, the virus is of the genus *Spumavirus*.

In some embodiments, the virus is of the family Baculoviridae, which includes enveloped viruses including alphabaculovirus. In some embodiments, the virus is of the genus *Alphabaculovirus, Betabaculovirus, Deltabaculovirus*, or *Gammabaculovirus*.

In some embodiments, the virus is of the family Herpesviridae, which includes enveloped viruses such as the simplex viruses HSV-1 and HSV-2. In some embodiments, the virus is of the subfamily Alphaherpesvirinae. In some embodiments, the virus is of the genus *Iltovirus, Mardivirus, Simplexvirus*, or *Varicellovirus*. In some embodiments, the virus is of the subfamily Betaherpesvirinae. In some embodiments, the virus is of the genus *Cytomegalovirus, Muromegalovirus, Proboscivirus*, or *Roseolovirus*. In some embodiments, the virus is of the subfamily Gammaherpesvirinae. In some embodiments, the virus is of the genus *Lymphocryptovirus, Macavirus, Percavirus*, or *Rhadinovirus*.

In some embodiments, the virus is an AAV virus. In an AAV particle, a nucleic acid is encapsidated in the AAV particle. The AAV particle also comprises capsid proteins. In some embodiments, the nucleic acid comprises a heterologous nucleic acid and/or one or more of the following components, operatively linked in the direction of transcription, control sequences including transcription initiation and termination sequences, thereby forming an expression cassette.

In some embodiments, the viral particle comprises an AAV ITR sequence. For example, an expression cassette may be flanked on the 5' and 3' end by at least one functional AAV ITR sequence. By "functional AAV ITR sequences" it is meant that the ITR sequences function as intended for the rescue, replication and packaging of the AAV virion. See Davidson et al., *PNAS*, 2000, 97(7)3428-32; Passini et al., *J. Virol.*, 2003, 77(12):7034-40; and Pechan et al., *Gene Ther.*, 2009, 16:10-16, all of which are incorporated herein in their entirety by reference. For practicing some aspects of the invention, the recombinant vectors comprise at least all of the sequences of AAV essential for encapsidation and the physical structures for infection by the rAAV. AAV ITRs for use in the vectors of the invention need not have a wild-type nucleotide sequence (e.g., as described in Kotin, *Hum. Gene Ther.*, 1994, 5:793-801), and may be altered by the insertion, deletion or substitution of nucleotides or the AAV ITRs may be derived from any of several AAV serotypes. More than 40 serotypes of AAV are currently known, and new serotypes and variants of existing serotypes continue to be identified. See Gao et al., *PNAS*, 2002, 99(18): 11854-6; Gao et al., *PNAS*, 2003, 100(10):6081-6; and Bossis et al., *J. Virol.*, 2003, 77(12):6799-810. Use of any AAV serotype is considered within the scope of the present invention. In some embodiments, a rAAV vector is a vector derived from an AAV serotype, including without limitation, AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV DJ, AAV DJ8, a goat AAV, bovine AAV, or mouse AAV ITRs or the like. In some embodiments, the nucleic acid in the AAV (e.g., an rAAV vector) comprises an ITR of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV DJ, AAV DJ8, a goat AAV, bovine AAV, or mouse AAV ITRs or the like. In some embodiments, the AAV particle comprises an AAV vector encoding a heterologous transgene flanked by one or more AAV ITRs.

In some embodiments, a rAAV particle comprises an encapsulation protein selected from AAV1, AAV2, AAV3, AAV4, AAV5, AAV6 (e.g., a wild-type AAV6 capsid, or a variant AAV6 capsid such as ShH10, as described in U.S. PG Pub. 2012/0164106), AAV7, AAV8, AAVrh8, AAVrh8R, AAV9 (e.g., a wild-type AAV9 capsid, or a modified AAV9 capsid as described in U.S. PG Pub. 2013/0323226), AAV10, AAVrh10, AAV11, AAV12, a tyrosine capsid mutant, a heparin binding capsid mutant, an AAV2R471A capsid, an AAVAAV2/2-7m8 capsid, an AAV LK03 capsid, an AAV DJ capsid (e.g., an AAV-DJ/8 capsid, an AAV-DJ/9 capsid, or any other of the capsids described in U.S. PG Pub. 2012/0066783), AAV2 N587A capsid, AAV2 E548A capsid, AAV2 N708A capsid, AAV V708K capsid, goat AAV capsid, AAV1/AAV2 chimeric capsid, bovine AAV capsid, mouse AAV capsid, rAAV2/HBoV1 capsid, an AAV2HBKO capsid, an AAVPHP.B capsid or an AAVPHP.eB capsid, or an AAV capsid described in U.S. Pat. No. 8,283,151 or International Publication No. WO/2003/042397. In further embodiments, a rAAV particle comprises capsid proteins of an AAV serotype from Clades A-F.

Certain aspects of the present disclosure relate to an AAV (e.g., a rAAV) capsid protein comprising an amino acid substitution at amino acid residue 2. In some embodiments, the amino acid substitution at amino acid residue 2 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of the parent AAV capsid protein. As described herein, the amino acid at the 2nd position to the initiating methionine (iMet X) of an AAV capsid protein may be examined for effects on N-terminal acetylation, trafficking, transduction, and/or other post-translational modification(s) (e.g., glycosylation, ubiquitination, and so forth). Any assay described herein for examining acetylation, or a functional consequence thereof related to AAV particles, may be used to assess N-terminal acetylation. In some embodiments, amino acid residue 2 of the AAV capsid protein (e.g., VP1 or VP3) is substituted with Cys, Ser, Thr, Val, Gly, Asn, Asp, Glu, Ile, Leu, Phe, Gln, Lys, Met, Pro or Tyr. In some embodiments, the amino acid substitution results in less deamidation of the AAV capsid.

Other aspects of the present disclosure relate to an AAV (e.g., a rAAV) capsid protein comprising an amino acid substitution that alters deamidation. In some embodiments, an amino acid substitution (e.g., an amino acid substitution at A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3) that "alters" deamidation results in a higher frequency of deamidation or a lower frequency of deamidation, e.g., as compared to a VP1 or VP3 without the substitution, such as the parental VP1 or VP3. As described herein, a potential deamidation site of an AAV capsid protein (e.g., VP1 or VP3) may be examined for effects on deamidation, trafficking, transduction, and/or other post-translational modification(s) (e.g., glycosylation, ubiquitination, and so forth). Any assay described herein for examining

deamidation, or a functional consequence thereof related to AAV particles, may be used to assess deamidation.

Several potential deamidation sites are described herein. In some embodiments, an amino acid substitution that alters deamidation is selected from A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3. For example, in some embodiments, N57 of VP1, N382 of VP3, N511 of VP3, and/or N715 of VP3 is substituted with Asp, and the amino acid substitution results in a higher frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In other embodiments, the amino acid substitution is N57K or N57Q, and the amino acid substitution results in a lower frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In yet other embodiments, G58 of VP1, G383 of VP3, G512 of VP3, and/or G716 of VP3 is substituted with an amino acid that is not Gly (e.g., Ala, Arg, Asn, Asp, Cys, Glu, Gln, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val), and the amino acid substitution results in a lower frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle.

In some embodiments, the AAV capsid protein is VP1, VP2, or VP3. The AAV particle may comprise any of the exemplary AAV capsid serotypes described herein, such as AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV2/2-7m8, AAV DJ, AAV DJ8, AAV2 N587A, AAV2 E548A, AAV2 N708A, AAV V708K, goat AAV, AAV1/AAV2 chimeric, bovine AAV, mouse AAV, or rAAV2/HBoV1. The AAV capsid protein may further comprise any of the capsid protein mutations described herein, such as tyrosine and/or heparin binding mutations.

Other aspects of the present disclosure relate to methods of improving the stability of a rAAV particle. In some embodiments, the methods include substituting amino acid residue 2 of VP1 and/or VP3, e.g., as described herein. For example, in some embodiments, amino acid residue 2 of VP1 is substituted. In other embodiments, amino acid residue 2 of VP3 is substituted. In some embodiments, the substituted amino acid at position 2 is N-acetylated at a higher frequency than amino acid residue 2 of the parent VP1 and/or VP3, e.g., as described herein. In some embodiments, substituting amino acid residue 2 of VP1 and/or VP3 improves the stability of a rAAV particle by at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 100%. In some embodiments, the stability of a rAAV particle with a substituted amino acid at position 2 may be compared to a wild-type or parental AAV capsid, e.g., of the same serotype. For example, in some embodiments, substituting amino acid residue 2 of VP1 and/or VP3 improves the stability of a rAAV particle by any one of about 10% to about 100%, about 20% to about 100%, about 30% to about 100%, about 40% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 10% to about 90%, about 20% to about 90%, about 30% to about 90%, about 40% to about 90%, about 50% to about 90%, about 60% to about 90%, about 70% to about 90%, about 80% to about 90%, about 10% to about 80%, about 20% to about 80%, about 30% to about 80%, about 40% to about 80%, about 50% to about 80%, about 60% to about 80%, about 70% to about 80%, about 10% to about 70%, about 20% to about 70%, about 30% to about 70%, about 40% to about 70%, about 50% to about 70%, about 60% to about 70%, about 10% to about 60%, about 20% to about 60%, about 30% to about 60%, about 40% to about 60%, about 50% to about 60%, about 10% to about 50%, about 20% to about 50%, about 30% to about 50%, about 40% to about 50%, about 10% to about 40%, about 20% to about 40%, about 30% to about 40%, about 10% to about 30%, about 20% to about 30%, or about 10% to about 20%, e.g., as compared to stability of a rAAV particle comprising a wild-type capsid. AAV particle stability may be measured using various assays known in the art, including without limitation differential scanning fluorescence (DSF), differential scanning calorimetry (DSC), other thermal denaturation assays, susceptibility to proteolysis, imaging or structural analysis to observe denaturation (e.g., using electron microscopy), transduction efficiency or another functional assay on AAV particle compositions kept for a designated time interval at a particular temperature (e.g., room temperature, or 4° C., for thermal stability) or treated at a particular pH (e.g., pH stability), and the like.

Other aspects of the present disclosure relate to methods of improving the assembly of a rAAV particle. In some embodiments, the methods include substituting amino acid residue 2 of VP1 and/or VP3, e.g., as described herein. For example, in some embodiments, amino acid residue 2 of VP1 is substituted. In other embodiments, amino acid residue 2 of VP3 is substituted. In some embodiments, the substituted amino acid at position 2 is N-acetylated at a higher frequency than amino acid residue 2 of the parent VP1 and/or VP3, e.g., as described herein. In some embodiments, substituting amino acid residue 2 of VP1 and/or VP3 improves the assembly of a rAAV particle by at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 100%. In some embodiments, the assembly of a rAAV particle with a substituted amino acid at position 2 may be compared to a wild-type or parental AAV capsid, e.g., of the same serotype. For example, in some embodiments, substituting amino acid residue 2 of VP1 and/or VP3 improves the assembly of a rAAV particle by any one of about 10% to about 100%, about 20% to about 100%, about 30% to about 100%, about 40% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 10% to about 90%, about 20% to about 90%, about 30% to about 90%, about 40% to about 90%, about 50% to about 90%, about 60% to about 90%, about 70% to about 90%, about 80% to about 90%, about 10% to about 80%, about 20% to about 80%, about 30% to about 80%, about 40% to about 80%, about 50% to about 80%, about 60% to about 80%, about 70% to about 80%, about 10% to about 70%, about 20% to about 70%, about 30% to about 70%, about 40% to about 70%, about 50% to about 70%, about 60% to about 70%, about 10% to about 60%, about 20% to about 60%, about 30% to about 60%, about 40% to about 60%, about 50% to about 60%, about 10% to about 50%, about 20% to about 50%, about 30% to about 50%, about 40% to about 50%, about 10% to about 40%, about 20% to about 40%, about 30% to about 40%, about 10% to about 30%, about 20% to about 30%, or about 10%

to about 20%, e.g., as compared to assembly of a rAAV particle comprising a wild-type capsid. AAV particle assembly may be measured using various assays known in the art, including without limitation, measuring particle production amount and/or rate, quantifying capsid production (e.g., after purification using any of the methods described herein), assaying production of complete vectors vs. empty capsids, measuring transduction efficiency, imaging or structural analysis to observe particle formation (e.g., using electron microscopy), production of AAV capsid proteins (e.g., as assayed by Western blotting), and the like.

Other aspects of the present disclosure relate to methods of improving the transduction of a rAAV particle. In some embodiments, the methods include substituting amino acid residue 2 of VP1 and/or VP3, e.g., as described herein. For example, in some embodiments, amino acid residue 2 of VP1 is substituted. In other embodiments, amino acid residue 2 of VP3 is substituted. In some embodiments, the substituted amino acid at position 2 is N-acetylated at a higher frequency than amino acid residue 2 of the parent VP1 and/or VP3, e.g., as described herein. In some embodiments, substituting amino acid residue 2 of VP1 and/or VP3 improves the transduction of a rAAV particle by at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 100%. In some embodiments, the transduction of a rAAV particle with a substituted amino acid at position 2 may be compared to a wild-type or parental AAV capsid, e.g., of the same serotype. For example, in some embodiments, substituting amino acid residue 2 of VP1 and/or VP3 improves the transduction of a rAAV particle by any one of about 10% to about 100%, about 20% to about 100%, about 30% to about 100%, about 40% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 10% to about 90%, about 20% to about 90%, about 30% to about 90%, about 40% to about 90%, about 50% to about 90%, about 60% to about 90%, about 70% to about 90%, about 80% to about 90%, about 10% to about 80%, about 20% to about 80%, about 30% to about 80%, about 40% to about 80%, about 50% to about 80%, about 60% to about 80%, about 70% to about 80%, about 10% to about 70%, about 20% to about 70%, about 30% to about 70%, about 40% to about 70%, about 50% to about 70%, about 60% to about 70%, about 10% to about 60%, about 20% to about 60%, about 30% to about 60%, about 40% to about 60%, about 50% to about 60%, about 10% to about 50%, about 20% to about 50%, about 30% to about 50%, about 40% to about 50%, about 10% to about 40%, about 20% to about 40%, about 30% to about 40%, about 10% to about 30%, about 20% to about 30%, or about 10% to about 20%, e.g., as compared to transduction of a rAAV particle comprising a wild-type capsid. AAV particle transduction may be measured using various assays known in the art, including without limitation, the transduction efficiency assays described herein. In some embodiments, the invention provide methods of reducing the transduction of a rAAV particle; for example, by substituting amino acid residue 2 of VP1 and/or VP3.

Other aspects of the present disclosure relate to methods of improving the stability of a rAAV particle. In some embodiments, the methods include substituting an amino acid of VP1 and/or VP3 that alters deamidation, e.g., as described herein. For example, in some embodiments, A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3 is substituted. In some embodiments, the substituted amino acid is deamidated at a higher frequency than the amino acid residue of the parent VP1 and/or VP3, e.g., as described herein. In some embodiments, substituting A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, and/or G716 of VP3 improves the stability of a rAAV particle by at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 100%. In some embodiments, the stability of a rAAV particle with a substituted A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3 may be compared to a wild-type or parental AAV capsid, e.g., of the same serotype. For example, in some embodiments, substituting A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, and/or G716 of VP3 improves the stability of a rAAV particle by any one of about 10% to about 100%, about 20% to about 100%, about 30% to about 100%, about 40% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 10% to about 90%, about 20% to about 90%, about 30% to about 90%, about 40% to about 90%, about 50% to about 90%, about 60% to about 90%, about 70% to about 90%, about 80% to about 90%, about 10% to about 80%, about 20% to about 80%, about 30% to about 80%, about 40% to about 80%, about 50% to about 80%, about 60% to about 80%, about 70% to about 80%, about 10% to about 70%, about 20% to about 70%, about 30% to about 70%, about 40% to about 70%, about 50% to about 70%, about 60% to about 70%, about 10% to about 60%, about 20% to about 60%, about 30% to about 60%, about 40% to about 60%, about 50% to about 60%, about 10% to about 50%, about 20% to about 50%, about 30% to about 50%, about 40% to about 50%, about 10% to about 40%, about 20% to about 40%, about 30% to about 40%, about 10% to about 30%, about 20% to about 30%, or about 10% to about 20%, e.g., as compared to stability of a rAAV particle comprising a wild-type capsid. AAV particle stability may be measured using various assays known in the art, including without limitation differential scanning fluorescence (DSF), differential scanning calorimetry (DSC), other thermal denaturation assays, susceptibility to proteolysis, imaging or structural analysis to observe denaturation (e.g., using electron microscopy), transduction efficiency or another functional assay on AAV particle compositions kept for a designated time interval at a particular temperature (e.g., room temperature, or 4° C., for thermal stability) or treated at a particular pH (e.g., pH stability), and the like.

Other aspects of the present disclosure relate to methods of improving the assembly of a rAAV particle. In some embodiments, the methods include substituting an amino acid of VP1 and/or VP3 that alters deamidation, e.g., as described herein. For example, in some embodiments, A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3 is substituted. In some embodiments, the substituted amino acid is deamidated at a higher frequency than the

amino acid residue of the parent VP1 and/or VP3, e.g., as described herein. In some embodiments, substituting A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, and/or G716 of VP3 improves the assembly of a rAAV particle by at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 100%. In some embodiments, the stability of a rAAV particle with a substituted A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3 may be compared to a wild-type or parental AAV capsid, e.g., of the same serotype. For example, in some embodiments, substituting A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, and/or G716 of VP3 improves the assembly of a rAAV particle by any one of about 10% to about 100%, about 20% to about 100%, about 30% to about 100%, about 40% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 10% to about 90%, about 20% to about 90%, about 30% to about 90%, about 40% to about 90%, about 50% to about 90%, about 60% to about 90%, about 70% to about 90%, about 80% to about 90%, about 10% to about 80%, about 20% to about 80%, about 30% to about 80%, about 40% to about 80%, about 50% to about 80%, about 60% to about 80%, about 70% to about 80%, about 10% to about 70%, about 20% to about 70%, about 30% to about 70%, about 40% to about 70%, about 50% to about 70%, about 60% to about 70%, about 10% to about 60%, about 20% to about 60%, about 30% to about 60%, about 40% to about 60%, about 50% to about 60%, about 10% to about 50%, about 20% to about 50%, about 30% to about 50%, about 40% to about 50%, about 10% to about 40%, about 20% to about 40%, about 30% to about 40%, about 10% to about 30%, about 20% to about 30%, or about 10% to about 20%, e.g., as compared to assembly of a rAAV particle comprising a wild-type capsid. AAV particle assembly may be measured using various assays known in the art, including without limitation, measuring particle production amount and/or rate, quantifying capsid production (e.g., after purification using any of the methods described herein), assaying production of complete vectors vs. empty capsids, measuring transduction efficiency, imaging or structural analysis to observe particle formation (e.g., using electron microscopy), production of AAV capsid proteins (e.g., as assayed by Western blotting), and the like.

Other aspects of the present disclosure relate to methods of improving the transduction of a rAAV particle. In some embodiments, the methods include substituting an amino acid of VP1 and/or VP3 that alters deamidation, e.g., as described herein. For example, in some embodiments, A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3 is substituted. In some embodiments, the substituted amino acid is deamidated at a higher frequency than the amino acid residue of the parent VP1 and/or VP3, e.g., as described herein. In some embodiments, substituting A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, and/or G716 of VP3 improves the transduction of a rAAV particle by at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 100%. In some embodiments, the stability of a rAAV particle with a substituted A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3 may be compared to a wild-type or parental AAV capsid, e.g., of the same serotype. For example, in some embodiments, substituting A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, and/or G716 of VP3 improves the transduction of a rAAV particle by any one of about 10% to about 100%, about 20% to about 100%, about 30% to about 100%, about 40% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 10% to about 90%, about 20% to about 90%, about 30% to about 90%, about 40% to about 90%, about 50% to about 90%, about 60% to about 90%, about 70% to about 90%, about 80% to about 90%, about 10% to about 80%, about 20% to about 80%, about 30% to about 80%, about 40% to about 80%, about 50% to about 80%, about 60% to about 80%, about 70% to about 80%, about 10% to about 70%, about 20% to about 70%, about 30% to about 70%, about 40% to about 70%, about 50% to about 70%, about 60% to about 70%, about 10% to about 60%, about 20% to about 60%, about 30% to about 60%, about 40% to about 60%, about 50% to about 60%, about 10% to about 50%, about 20% to about 50%, about 30% to about 50%, about 40% to about 50%, about 10% to about 40%, about 20% to about 40%, about 30% to about 40%, about 10% to about 30%, about 20% to about 30%, or about 10% to about 20%, e.g., as compared to transduction of a rAAV particle comprising a wild-type capsid. AAV particle transduction may be measured using various assays known in the art, including without limitation, the transduction efficiency assays described herein.

In some aspects, the invention provides viral particles comprising a recombinant self-complementing genome (e.g., a self-complementary or self-complimenting rAAV vector). AAV viral particles with self-complementing vector genomes and methods of use of self-complementing AAV genomes are described in U.S. Pat. Nos. 6,596,535; 7,125,717; 7,465,583; 7,785,888; 7,790,154; 7,846,729; 8,093,054; and 8,361,457; and Wang Z., et al., (2003) *Gene Ther* 10:2105-2111, each of which are incorporated herein by reference in its entirety. A rAAV comprising a self-complementing genome will quickly form a double stranded DNA molecule by virtue of its partially complementing sequences (e.g., complementing coding and non-coding strands of a heterologous nucleic acid). In some embodiments, the vector comprises a first nucleic acid sequence encoding a heterologous nucleic acid and a second nucleic acid sequence encoding a complement of the nucleic acid, where the first nucleic acid sequence can form intrastrand base pairs with the second nucleic acid sequence along most or all of its length.

In some embodiments, the first heterologous nucleic acid sequence and a second heterologous nucleic acid sequence are linked by a mutated ITR (e.g., the right ITR). In some embodiments, the ITR comprises the polynucleotide sequence 5'-CACTCCCTCTCTGCGCGCTCGCTCGCTCACTGAGGCC GGGCGACCAAAGGTCGCCCACGCCCGGGCTTTGCCCGGGCG-3' (SEQ ID NO:8).

The mutated ITR comprises a deletion of the D region comprising the terminal resolution sequence. As a result, on replicating an AAV viral genome, the rep proteins will not cleave the viral genome at the mutated ITR and as such, a recombinant viral genome comprising the following in 5' to 3' order will be packaged in a viral capsid: an AAV ITR, the first heterologous polynucleotide sequence including regulatory sequences, the mutated AAV ITR, the second heterologous polynucleotide in reverse orientation to the first heterologous polynucleotide and a third AAV ITR.

Different AAV serotypes are used to optimize transduction of particular target cells or to target specific cell types within a particular target tissue (e.g., a diseased tissue). A rAAV particle can comprise viral proteins and viral nucleic acids of the same serotype or a mixed serotype. For example, a rAAV particle may contain one or more ITRs and capsid derived from the same AAV serotype, or a rAAV particle may contain one or more ITRs derived from a different AAV serotype than capsid of the rAAV particle.

In some embodiments, the AAV capsid comprises a mutation, e.g., the capsid comprises a mutant capsid protein. In some embodiments, the mutation is a tyrosine mutation or a heparin binding mutation. In some embodiments, a mutant capsid protein maintains the ability to form an AAV capsid. In some embodiments, the rAAV particle comprises an AAV2 or AAV5 tyrosine mutant capsid (see, e.g., Zhong L. et al., (2008) *Proc Natl Acad Sci USA* 105(22):7827-7832), such as a mutation in Y444 or Y730 (numbering according to AAV2). In further embodiments, the rAAV particle comprises capsid proteins of an AAV serotype from Clades A-F (Gao, et al., *J. Virol.* 2004, 78(12):6381).

In some embodiments, a capsid protein comprises one or more amino acid substitutions at one or more positions that interact with a heparin sulfate proteoglycan or at one or more positions corresponding to amino acids 484, 487, 527, 532, 585 or 588, numbering based on VP1 numbering of AAV2. Heparan sulfate proteoglycan (HSPG) is known in the art to act as the cellular receptor for AAV2 particles (Summerford, C. and Samulski, R. J. (1998) *J. Virol.* 72(2):1438-45). Binding between an AAV2 particle and HSPG at the cell membrane serves to attach the particle to the cell. Other cell surface proteins such as fibroblast growth factor receptor and αvβ5 integrin may also facilitate cellular infection. After binding, an AAV2 particle may enter the cell through mechanisms including receptor mediated endocytosis via clathrin-coated pits. An AAV2 particle may be released from an endocytic vesicle upon endosomal acidification. This allows the AAV2 particle to travel to the perinuclear region and then the cell nucleus. AAV3 particles are also known to bind heparin (Rabinowitz, J. E., et al. (2002) *J. Virol.* 76(2):791-801).

The binding between AAV2 capsid proteins and HSPG is known to occur via electrostatic interactions between basic AAV2 capsid protein residues and negatively charged glycosaminoglycan residues (Opie, S R et al., (2003) *J. Virol.* 77:6995-7006; Kern, A et al., (2003) *J. Virol.* 77:11072-11081). Specific capsid residues implicated in these interactions include R484, R487, K527, K532, R585, and R588. Mutations in these residues have been shown to reduce AAV2 binding to Hela cells and heparin itself (Opie, S R et al., (2003) *J. Virol.* 77:6995-7006; Kern, A et al., (2003) *J. Virol.* 77:11072-11081; WO 2004/027019 A2, U.S. Pat. No. 7,629,322). Further, without wishing to be bound to theory, it is thought that amino acid substitution(s) at one or more of the residues corresponding to amino acids 484, 487, 527, 532, 585 or 588, numbering based on VP1 numbering of AAV2 may modulate the transduction properties of AAV capsid types that do not bind to HSPG, or may modulate the transduction properties of AAV capsid types independent from their ability to bind HSPG. In some embodiments, the one or more amino acid substitutions comprises a substitution at position R484, R487, K527, K532, R585 and/or R588 of VP1, VP2 and/or VP3, numbering based on VP1 of AAV2.

In some embodiments, the one or more amino acid substitutions reduce binding of the rAAV particle to the heparin sulfate proteoglycan by about at least 10%, about at least 25%, about at least 50%, about at least 75%, or about at least 100%. In some embodiments, the one or more amino acid substitutions reduce binding of the rAAV particle to the heparin sulfate proteoglycan by about at least 10%, about at least 15%, about at least 20%, about at least 25%, about at least 30%, about at least 35%, about at least 40%, about at least 45%, about at least 50%, about at least 55%, about at least 60%, about at least 65%, about at least 70%, about at least 75%, about at least 80%, about at least 85%, about at least 90%, about at least 95%, or about at least 100% (as compared to binding of a rAAV particle comprising a wild-type capsid). In some embodiments, the one or more amino acid substitutions reduce binding of the rAAV particle to the heparin sulfate proteoglycan by any one of about 10% to about 100%, about 20% to about 100%, about 30% to about 100%, about 40% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 10% to about 90%, about 20% to about 90%, about 30% to about 90%, about 40% to about 90%, about 50% to about 90%, about 60% to about 90%, about 70% to about 90%, about 80% to about 90%, about 10% to about 80%, about 20% to about 80%, about 30% to about 80%, about 40% to about 80%, about 50% to about 80%, about 60% to about 80%, about 70% to about 80%, about 10% to about 70%, about 20% to about 70%, about 30% to about 70%, about 40% to about 70%, about 50% to about 70%, about 60% to about 70%, about 10% to about 60%, about 20% to about 60%, about 30% to about 60%, about 40% to about 60%, about 50% to about 60%, about 10% to about 50%, about 20% to about 50%, about 30% to about 50%, about 40% to about 50%, about 10% to about 40%, about 20% to about 40%, about 30% to about 40%, about 10% to about 30%, about 20% to about 30%, or about 10% to about 20%, (as compared to binding of a rAAV particle comprising a wild-type capsid). In some embodiments, the one or more amino acid substitutions results in no detectable binding of the rAAV particle to the heparin sulfate proteoglycan compared to binding of a wild-type rAAV particle. Means to measure binding of AAV particles to HSPG are known in the art; e.g., binding to a heparin sulfate chromatography media or binding to a cell known to express HSPG on its surface. For example, see Opie, S R et al., (2003) *J. Virol.* 77:6995-7006 and Kern, A et al., (2003) *J. Virol.* 77:11072-11081. In some embodiments, the one or more amino acid substitutions improve the transduction efficiency of the rAAV particle to a cell (e.g., a cell in the eye or CNS) by about at least 10%, about at least 15%, about at least 20%, about at least 25%, about at least 30%, about at least 35%, about at least 40%, about at least 45%, about at least 50%, about at least 55%, about at least 60%, about at least 65%, about at least 70%, about at least 75%, about at least 80%, about at least 85%, about at least 90%, about at least 95%, or about at least 100% (as compared to transduction efficiency of a rAAV particle comprising a wild-type capsid). In some embodiments, the one or more amino acid substitutions improve the transduction efficiency of the rAAV particle to a cell (e.g., a

cell in the eye or CNS) by any one of about 10% to about 100%, about 20% to about 100%, about 30% to about 100%, about 40% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 10% to about 90%, about 20% to about 90%, about 30% to about 90%, about 40% to about 90%, about 50% to about 90%, about 60% to about 90%, about 70% to about 90%, about 80% to about 90%, about 10% to about 80%, about 20% to about 80%, about 30% to about 80%, about 40% to about 80%, about 50% to about 80%, about 60% to about 80%, about 70% to about 80%, about 10% to about 70%, about 20% to about 70%, about 30% to about 70%, about 40% to about 70%, about 50% to about 70%, about 60% to about 70%, about 10% to about 60%, about 20% to about 60%, about 30% to about 60%, about 40% to about 60%, about 50% to about 60%, about 10% to about 50%, about 20% to about 50%, about 30% to about 50%, about 40% to about 50%, about 10% to about 40%, about 20% to about 40%, about 30% to about 40%, about 10% to about 30%, about 20% to about 30%, or about 10% to about 20%, (as compared to transduction efficiency of a rAAV particle comprising a wild-type capsid). Means to measure transduction efficiency of AAV particles to a cell (e.g., a cell in culture or part of a tissue) are known in the art. For example, a population of cells (e.g., in culture or part of a tissue) may be infected with a concentration of rAAV particles containing a vector that, when expressed in the cells, produces an assayable reporter (e.g., GFP fluorescence, sFLT production, etc.).

AAV Capsid Proteins

In some aspects, the invention provides an AAV capsid protein comprising an amino acid substitution at amino acid residue 2; wherein the amino acid substitution at amino acid residue 2 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of the parent AAV capsid protein. In some embodiments, the AAV capsid protein is VP1 or VP3. In some embodiments, amino acid residue 2 of the AAV capsid protein (e.g., VP1 or VP3) is substituted with Cys, Ser, Thr, Val, Gly, Asn, Asp, Glu, Ile, Leu, Phe, Gln, Lys, Met, Pro or Tyr. In some embodiments, the amino acid substitution results in less deamidation of the AAV capsid protein. Non-limiting examples of AAV capsid proteins of the invention include VP1 and/or VP3 of any of the following AAV serotypes: AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV2/2-7m8, AAV DJ, AAV DJ8, AAV2 N587A, AAV2 E548A, AAV2 N708A, AAV V708K, goat AAV, AAV1/AAV2 chimeric, bovine AAV, mouse AAV, or rAAV2/HBoV1 serotype capsid. In some embodiments, the AAV capsid further comprises a tyrosine mutation or a heparin binding mutation.

Production of AAV Particles

Numerous methods are known in the art for production of rAAV vectors, including transfection, stable cell line production, and infectious hybrid virus production systems which include adenovirus-AAV hybrids, herpesvirus-AAV hybrids (Conway, J E et al., (1997) *J. Virology* 71(11):8780-8789) and baculovirus-AAV hybrids (Urabe, M. et al., (2002) *Human Gene Therapy* 13(16):1935-1943; Kotin, R. (2011) *Hum Mol Genet.* 20(R1): R2-R6). rAAV production cultures for the production of rAAV viral particles all require; 1) suitable host cells, 2) suitable helper virus function, 3) AAV rep and cap genes and gene products; 4) a nucleic acid (such as a therapeutic nucleic acid) flanked by at least one AAV ITR sequences (e.g., an AAV genome encoding GNPTAB); and 5) suitable media and media components to support rAAV production. In some embodiments, the suitable host cell is a primate host cell. In some embodiments, the suitable host cell is a human-derived cell lines such as HeLa, A549, 293, or Perc.6 cells. In some embodiments, the suitable helper virus function is provided by wild-type or mutant adenovirus (such as temperature sensitive adenovirus), herpes virus (HSV), baculovirus, or a plasmid construct providing helper functions. In some embodiments, the AAV rep and cap gene products may be from any AAV serotype. In general, but not obligatory, the AAV rep gene product is of the same serotype as the ITRs of the rAAV vector genome as long as the rep gene products may function to replicated and package the rAAV genome. Suitable media known in the art may be used for the production of rAAV vectors. These media include, without limitation, media produced by Hyclone Laboratories and JRH including Modified Eagle Medium (MEM), Dulbecco's Modified Eagle Medium (DMEM), custom formulations such as those described in U.S. Pat. No. 6,566,118, and Sf-900 II SFM media as described in U.S. Pat. No. 6,723,551, each of which is incorporated herein by reference in its entirety, particularly with respect to custom media formulations for use in production of recombinant AAV vectors. In some embodiments, the AAV helper functions are provided by adenovirus or HSV. In some embodiments, the AAV helper functions are provided by baculovirus and the host cell is an insect cell (e.g., *Spodoptera frugiperda* (Sf9) cells). In some embodiments, the AAV cap functions provide an amino acid substitution at amino acid residue 2 of VP1 and/or VP3, wherein the amino acid substitution at amino acid residue 2 of VP1 and/or VP3 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of VP1 and/or VP3 of the parent AAV particle. In some embodiments, amino acid residue 2 of the AAV capsid protein (e.g., VP1 or VP3) is substituted with Cys, Ser, Thr, Val, Gly, Asn, Asp, Glu, Ile, Leu, Phe, Gln, Lys, Met, Pro or Tyr. In some embodiments, the amino acid substitution results in less deamidation of the AAV capsid.

One method for producing rAAV particles is the triple transfection method. Briefly, a plasmid containing a rep gene and a capsid gene, along with a helper adenoviral plasmid, may be transfected (e.g., using the calcium phosphate method) into a cell line (e.g., HEK-293 cells), and virus may be collected and optionally purified. As such, in some embodiments, the rAAV particle was produced by triple transfection of a nucleic acid encoding the rAAV vector, a nucleic acid encoding AAV rep and cap, and a nucleic acid encoding AAV helper virus functions into a host cell, wherein the transfection of the nucleic acids to the host cells generates a host cell capable of producing rAAV particles.

In some embodiments, rAAV particles may be produced by a producer cell line method (see Martin et al., (2013) *Human Gene Therapy Methods* 24:253-269; U.S. PG Pub. No. US2004/0224411; and Liu, X. L. et al. (1999) *Gene Ther.* 6:293-299). Briefly, a cell line (e.g., a HeLa, 293, A549, or Perc.6 cell line) may be stably transfected with a plasmid containing a rep gene, a capsid gene, and a vector genome comprising a promoter-heterologous nucleic acid sequence (e.g., GNPTAB). Cell lines may be screened to select a lead clone for rAAV production, which may then be expanded to a production bioreactor and infected with a helper virus (e.g., an adenovirus or HSV) to initiate rAAV production. Virus may subsequently be harvested, adenovirus may be inactivated (e.g., by heat) and/or removed, and the rAAV particles may be purified. As such, in some embodiments, the rAAV particle was produced by a pro-

ducer cell line comprising one or more of nucleic acid encoding the rAAV vector, a nucleic acid encoding AAV rep and cap, and a nucleic acid encoding AAV helper virus functions. As described herein, the producer cell line method may be advantageous for the production of rAAV particles with an oversized genome, as compared to the triple transfection method.

In some embodiments, the nucleic acid encoding AAV rep and cap genes and/or the rAAV genome are stably maintained in the producer cell line. In some embodiments, nucleic acid encoding AAV rep and cap genes and/or the rAAV genome is introduced on one or more plasmids into a cell line to generate a producer cell line. In some embodiments, the AAV rep, AAV cap, and rAAV genome are introduced into a cell on the same plasmid. In other embodiments, the AAV rep, AAV cap, and rAAV genome are introduced into a cell on different plasmids. In some embodiments, a cell line stably transfected with a plasmid maintains the plasmid for multiple passages of the cell line (e.g., 5, 10, 20, 30, 40, 50 or more than 50 passages of the cell). For example, the plasmid(s) may replicate as the cell replicates, or the plasmid(s) may integrate into the cell genome. A variety of sequences that enable a plasmid to replicate autonomously in a cell (e.g., a human cell) have been identified (see, e.g., Krysan, P. J. et al. (1989) *Mol. Cell Biol.* 9:1026-1033). In some embodiments, the plasmid(s) may contain a selectable marker (e.g., an antibiotic resistance marker) that allows for selection of cells maintaining the plasmid. Selectable markers commonly used in mammalian cells include without limitation blasticidin, G418, hygromycin B, zeocin, puromycin, and derivatives thereof. Methods for introducing nucleic acids into a cell are known in the art and include without limitation viral transduction, cationic transfection (e.g., using a cationic polymer such as DEAE-dextran or a cationic lipid such as lipofectamine), calcium phosphate transfection, microinjection, particle bombardment, electroporation, and nanoparticle transfection (for more details, see e.g., Kim, T. K. and Eberwine, J. H. (2010) *Anal. Bioanal. Chem.* 397:3173-3178).

In some embodiments, the nucleic acid encoding AAV rep and cap genes and/or the rAAV genome are stably integrated into the genome of the producer cell line. In some embodiments, nucleic acid encoding AAV rep and cap genes and/or the rAAV genome is introduced on one or more plasmids into a cell line to generate a producer cell line. In some embodiments, the AAV rep, AAV cap, and rAAV genome are introduced into a cell on the same plasmid. In other embodiments, the AAV rep, AAV cap, and rAAV genome are introduced into a cell on different plasmids. In some embodiments, the plasmid(s) may contain a selectable marker (e.g., an antibiotic resistance marker) that allows for selection of cells maintaining the plasmid. Methods for stable integration of nucleic acids into a variety of host cell lines are known in the art. For example, repeated selection (e.g., through use of a selectable marker) may be used to select for cells that have integrated a nucleic acid containing a selectable marker (and AAV cap and rep genes and/or a rAAV genome). In other embodiments, nucleic acids may be integrated in a site-specific manner into a cell line to generate a producer cell line. Several site-specific recombination systems are known in the art, such as FLP/FRT (see, e.g., O'Gorman, S. et al. (1991) *Science* 251:1351-1355), Cre/loxP (see, e.g., Sauer, B. and Henderson, N. (1988) *Proc. Natl. Acad. Sci.* 85:5166-5170), and phi C31-att (see, e.g., Groth, A. C. et al. (2000) Proc. *Natl. Acad. Sci.* 97:5995-6000).

In some embodiments, the producer cell line is derived from a primate cell line (e.g., a non-human primate cell line, such as a Vero or FRhL-2 cell line). In some embodiments, the cell line is derived from a human cell line. In some embodiments, the producer cell line is derived from HeLa, 293, A549, or PERC.6® (Crucell) cells. For example, prior to introduction and/or stable maintenance/integration of nucleic acid encoding AAV rep and cap genes and/or the oversized rAAV genome into a cell line to generate a producer cell line, the cell line is a HeLa, 293, A549, or PERC.6® (Crucell) cell line, or a derivative thereof.

In some embodiments, the producer cell line is adapted for growth in suspension. As is known in the art, anchorage-dependent cells are typically not able to grow in suspension without a substrate, such as microcarrier beads. Adapting a cell line to grow in suspension may include, for example, growing the cell line in a spinner culture with a stirring paddle, using a culture medium that lacks calcium and magnesium ions to prevent clumping (and optionally an antifoaming agent), using a culture vessel coated with a siliconizing compound, and selecting cells in the culture (rather than in large clumps or on the sides of the vessel) at each passage. For further description, see, e.g., ATCC frequently asked questions document (available on the world wide web at atcc.org/Global/FAQs/9/1/Adapting%20a%20monolayer%20cell%20line%20to%20-suspension-40.aspx) and references cited therein.

Suitable AAV production culture media of the present invention may be supplemented with serum or serum-derived recombinant proteins at a level of 0.5%-20% (v/v or w/v). Alternatively, as is known in the art, AAV vectors may be produced in serum-free conditions which may also be referred to as media with no animal-derived products. One of ordinary skill in the art may appreciate that commercial or custom media designed to support production of AAV vectors may also be supplemented with one or more cell culture components know in the art, including without limitation glucose, vitamins, amino acids, and or growth factors, in order to increase the titer of AAV in production cultures.

AAV production cultures can be grown under a variety of conditions (over a wide temperature range, for varying lengths of time, and the like) suitable to the particular host cell being utilized. As is known in the art, AAV production cultures include attachment-dependent cultures which can be cultured in suitable attachment-dependent vessels such as, for example, roller bottles, hollow fiber filters, microcarriers, and packed-bed or fluidized-bed bioreactors. AAV vector production cultures may also include suspension-adapted host cells such as HeLa, 293, and SF-9 cells which can be cultured in a variety of ways including, for example, spinner flasks, stirred tank bioreactors, and disposable systems such as the Wave bag system.

AAV vector particles of the invention may be harvested from AAV production cultures by lysis of the host cells of the production culture or by harvest of the spent media from the production culture, provided the cells are cultured under conditions known in the art to cause release of AAV particles into the media from intact cells, as described more fully in U.S. Pat. No. 6,566,118). Suitable methods of lysing cells are also known in the art and include for example multiple freeze/thaw cycles, sonication, microfluidization, and treatment with chemicals, such as detergents and/or proteases.

In a further embodiment, the AAV particles are purified. The term "purified" as used herein includes a preparation of AAV particles devoid of at least some of the other components that may also be present where the AAV particles naturally occur or are initially prepared from. Thus, for example, isolated AAV particles may be prepared using a

purification technique to enrich it from a source mixture, such as a culture lysate or production culture supernatant. Enrichment can be measured in a variety of ways, such as, for example, by the proportion of DNase-resistant particles (DRPs) or genome copies (gc) present in a solution, or by infectivity, or it can be measured in relation to a second, potentially interfering substance present in the source mixture, such as contaminants, including production culture contaminants or in-process contaminants, including helper virus, media components, and the like.

In some embodiments, the AAV production culture harvest is clarified to remove host cell debris. In some embodiments, the production culture harvest is clarified by filtration through a series of depth filters including, for example, a grade DOHC Millipore Millistak+HC Pod Filter, a grade A1HC Millipore Millistak+HC Pod Filter, and a 0.2 μm Filter Opticap XL1O Millipore Express SHC Hydrophilic Membrane filter. Clarification can also be achieved by a variety of other standard techniques known in the art, such as, centrifugation or filtration through any cellulose acetate filter of 0.2 μm or greater pore size known in the art.

In some embodiments, the AAV production culture harvest is further treated with Benzonase® to digest any high molecular weight DNA present in the production culture. In some embodiments, the Benzonase® digestion is performed under standard conditions known in the art including, for example, a final concentration of 1-2.5 units/ml of Benzonase® at a temperature ranging from ambient to 37° C. for a period of 30 minutes to several hours.

AAV particles may be isolated or purified using one or more of the following purification steps: equilibrium centrifugation; flow-through anionic exchange filtration; tangential flow filtration (TFF) for concentrating the AAV particles; AAV capture by apatite chromatography; heat inactivation of helper virus; AAV capture by hydrophobic interaction chromatography; buffer exchange by size exclusion chromatography (SEC); nanofiltration; and AAV capture by anionic exchange chromatography, cationic exchange chromatography, or affinity chromatography. These steps may be used alone, in various combinations, or in different orders. In some embodiments, the method comprises all the steps in the order as described below. Methods to purify AAV particles are found, for example, in Xiao et al., (1998) *Journal of Virology* 72:2224-2232; U.S. Pat. Nos. 6,989,264 and 8,137,948; and WO 2010/148143.

Pharmaceutical Compositions

In some embodiments, an AAV particle of the present disclosure (e.g., a rAAV particle) is in a pharmaceutical composition. The pharmaceutical compositions may be suitable for any mode of administration described herein or known in the art. In some embodiments, the pharmaceutical composition comprises rAAV particles modified to improve the stability and/or improve the transduction efficiency of rAAV particles; for example, for use in substituting the amino acid residue at position 2 of VP1 and/or VP3 to improve acetylation of rAAV capsid proteins. In some embodiments, the pharmaceutical composition comprises rAAV particles modified to modulate the stability and/or the transduction efficiency of rAAV particles (e.g., increase stability and/or transduction efficiency or decrease stability and/or transduction efficiency); for example, for use in substituting the amino acid residues that modulate deamidation (e.g., increase deamidation or decrease deamidation).

In some embodiments, the rAAV particle is in a pharmaceutical composition comprising a pharmaceutically acceptable excipient. As is well known in the art, pharmaceutically acceptable excipients are relatively inert substances that facilitate administration of a pharmacologically effective substance and can be supplied as liquid solutions or suspensions, as emulsions, or as solid forms suitable for dissolution or suspension in liquid prior to use. For example, an excipient can give form or consistency, or act as a diluent. Suitable excipients include but are not limited to stabilizing agents, wetting and emulsifying agents, salts for varying osmolarity, encapsulating agents, pH buffering substances, and buffers. Such excipients include any pharmaceutical agent suitable for direct delivery to the eye which may be administered without undue toxicity. Pharmaceutically acceptable excipients include, but are not limited to, sorbitol, any of the various TWEEN compounds, and liquids such as water, saline, glycerol and ethanol. Pharmaceutically acceptable salts can be included therein, for example, mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. A thorough discussion of pharmaceutically acceptable excipients is available in REMINGTON'S PHARMACEUTICAL SCIENCES (Mack Pub. Co., N.J. 1991). In some embodiments, the pharmaceutical composition comprising a rAAV particle described herein and a pharmaceutically acceptable carrier is suitable for administration to human. Such carriers are well known in the art (see, e.g., Remington's Pharmaceutical Sciences, 15th Edition, pp. 1035-1038 and 1570-1580).

Such pharmaceutically acceptable carriers can be sterile liquids, such as water and oil, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, and the like. Saline solutions and aqueous dextrose, polyethylene glycol (PEG) and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. The pharmaceutical composition may further comprise additional ingredients, for example preservatives, buffers, tonicity agents, antioxidants and stabilizers, nonionic wetting or clarifying agents, viscosity-increasing agents, and the like. The pharmaceutical compositions described herein can be packaged in single unit dosages or in multidosage forms. The compositions are generally formulated as sterile and substantially isotonic solution.

Kits and Articles of Manufacture

The present invention also provides kits or articles of manufacture comprising any of the rAAV particles and/or pharmaceutical compositions of the present disclosure. The kits or articles of manufacture may comprise any of the rAAV particles or rAAV particle compositions of the invention. In some embodiments the kits are used to improve the stability and/or improve the transduction efficiency of rAAV particles; for example, for use in substituting the amino acid residue at position 2 of VP1 and/or VP3 to improve acetylation of rAAV capsid proteins. In some embodiments the kits are used to modulate the stability and/or the transduction efficiency of rAAV particles (e.g., increase stability and/or transduction efficiency or decrease stability and/or transduction efficiency); for example, for use in substituting the amino acid residues that modulate deamidation (e.g., increase deamidation or decrease deamidation).

In some embodiments, the kits or articles of manufacture further include instructions for administration of a composition of rAAV particles. The kits or articles of manufacture described herein may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for performing any methods described herein. Suitable packaging materials may also be

included and may be any packaging materials known in the art, including, for example, vials (such as sealed vials), vessels, ampules, bottles, jars, flexible packaging (e.g., sealed Mylar or plastic bags), and the like. These articles of manufacture may further be sterilized and/or sealed.

In some embodiments, the kits or articles of manufacture further contain one or more of the buffers and/or pharmaceutically acceptable excipients described herein (e.g., as described in REMINGTON'S PHARMACEUTICAL SCIENCES (Mack Pub. Co., N.J. 1991). In some embodiments, the kits or articles of manufacture include one or more pharmaceutically acceptable excipients, carriers, solutions, and/or additional ingredients described herein. The kits or articles of manufacture described herein can be packaged in single unit dosages or in multidosage forms. The contents of the kits or articles of manufacture are generally formulated as sterile and can be lyophilized or provided as a substantially isotonic solution.

EXAMPLES

The invention will be more fully understood by reference to the following examples. They should not, however, be construed as limiting the scope of the invention. It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

Example 1: Direct LC/NIS and LC/NIS/NIS for Complete Characterization of Recombinant AAV Viral Capsid Protein

Recombinant adeno-associated viruses (rAAVs) have become popular gene therapy vectors due to their nonpathogenic nature, ability to infect both dividing and non-dividing cells and long term gene expression. Currently, AAV-based gene therapies are used in clinical trials for numerous disease targets, such as muscular dystrophy, hemophilia, Parkinson's disease, Leber's congenital amaurosis and macular degeneration.

AAV is a small and nonenveloped parvovirus with a single stranded DNA genome encapsulated in an icosahedra shell. Each capsid includes sixty copies of three viral capsid proteins VP1 (87 kDa), VP2 (73 kDa) and VP3 (62 kDa) in an approximately 1:1:10 ratio. The three viral capsid proteins are expressed from the same open reading frame by using alternative splicing and an atypical start codon and thus have overlapping sequences. VP1 has ~137 additional N-terminal amino acid residues compared to VP3 while VP2 has ~65 additional N-terminal amino acid residues compared to VP3. At least 13 AAV serotypes and ~150 gene sequences have been isolated from human and non-human primate tissues; AAV serotypes differ in the amino acid sequence of viral capsid proteins and their corresponding cellular receptors and co-receptors for targeting.

The AAV capsid, in addition to protecting the genome inside, plays an important role in mediating receptor binding, escape of virus from endosome, and transport of viral DNA into nucleus in the viral infection cycle, thus directly impacting viral infectivity. It has been shown that the VP1 N-terminus contains a phospholipase PLA2 domain (a.a. 52-97) which is critical in endosomal escaping of virus [1-3]. N-termini of VP1 and VP2 also contain three basic amino acid clusters as nuclear localization signals. These sequences are highly conserved among different AAV serotypes. Mutations of these amino acids have been shown to reduce or abolish infectivity completely [4]. In addition, each AAV serotype has corresponding sequence-specific receptors and co-receptors. For example, heparin sulfate proteoglycan was identified as a major receptor of AAV2 and several other co-receptors, including $\alpha V\beta 5$ integrin, fibroblast growth factor receptor 1, and hepatocyte growth receptor have been identified [5-8]. Mutation analysis of AAV2 capsid proteins has identified a group of basic amino acids (Arginine484, 487, 585, and Lysine532) as a heparin-binding motif which contributes to the heparin and HeLa cell binding [9]. NGR domain in AAV2 was identified as an integrin $\alpha 5\beta 1$ binding domain which is essential for viral cell entry [10]. In summary, viral capsid protein sequences are important in cellular targeting and trafficking in the viral infection cycle. Since different production conditions may cause different expression levels of viral capsid proteins, post-translational modifications, and truncations, the viral capsid proteins need to be characterized and monitored to ensure the product consistency in gene therapy development programs.

Traditionally, SDS-PAGE has been used to characterize the AAV viral capsid proteins, providing rough molecular weight information such as 87 kDa, 73 kDa and 62 kDa. No sequence information was obtained from Edman sequencing, possibly due to the blocked N-termini of viral capsid proteins, except VP2. Although X-ray structures of multiple AAVs have been solved, only the VP3 region sequence was observed in the crystal structures. Fifteen N-terminal amino acid residues of VP3 were still missing in the X-ray structure, possibly due to its intrinsic disorder [11-13]. It is possible that the lack of information of VP1 and VP2 N-terminal regions in the atomic structure might be due to low stoichiometry of VP1 and VP2 in the capsid. In addition, N-termini of VP1 and VP2 are buried inside the capsid and are not accessible to antibodies in the native state as reported in the some literature [3, 14, 15]. Conventionally, a Gel-LC/MS method (SDS-PAGE, in-gel tryptic digestion and LC/MS/MS) was used in characterization of VPs [16-18]. However, N-termini of VP1, VP2 and VP3 have not been confirmed using this approach, since this method failed to obtain 100% sequence coverage of VPs due to the limited recovery of peptide from gel.

Direct analysis using MALDI-TOF MS was reported for several virus capsid proteins including tobacco mosaic virus U2 after dissociation with organic acid [19]. Direct peptide mapping after amide hydrogen exchange and mass spectrometry have been used to study the pH-induced structural changes in the capsid of brome mosaic virus (BMV) [20]. Since AAVs are nonenveloped viruses containing only capsid proteins and genome, AAVs capsids could be directly analyzed by RP-LC/MS of proteins and LC/MS/MS of peptide mapping to achieve 100% sequence coverage after capsid dissociation without SDS-PAGE separation. The DNA fragments could elute in the void volume and thus have no interference on protein/peptide detection by LC/MS. In order to investigate these methods, direct LC/MS of different types of AAVs after denaturation was used to monitor the protein sequence and post-translational modifications of AAV capsid proteins. As described herein, N-termini of VP1, VP2 and VP3 of AAVs have been confirmed by mass spectrometry. Acetylations of N-termini of VP1 and VP3 were also identified in the different serotype of AAVs. Direct LC/MS/MS peptide mapping of AAVs has also been developed to provide sequence coverage of VP1, VP2 and VP3 and confirm the N-termini acetylation of VP1 and VP3.

Methods

Materials and Reagents

Dithiothreitol (DTT), 4-vinylpyridine, ultra-pure formic acid, acetic acid, guanidine-HCl, Tris-HCl and Tris base were purchased from Sigma Chemicals (St. Louis, Mo.). Amicon ultra-4 filters were purchased from Millipore (Billerica, Mass.). The porcine sequencing grade trypsin was purchased from Promega (Milwaukee, Wis.). Endoproteinase Lys-C and Asp-N were purchased from Roche (Germany). Slide-A-Lyzer cassettes with 10.000 MWCO were purchased from Pierce (Rockford, Ill.).

Vector Production and Purification

AAV vectors were produced using the transient triple transfection method as previously described (Xiao, 1998 #123). Briefly, HEK293 cells were transfected using polyethyleneimine, PEI, and a 1:1:1 ratio of three plasmids (ITR vector, AAV rep/cap and Ad helper plasmid). The vector plasmid contains the vector genome CBA-EGFP and ITR sequences from AAV2. EGFP expression is driven by the CMV enhancer chicken beta actin hybrid promoter (CBA) as described (Miyazaki, 1989 #124) (Niwa, 1991 #125). The AAV rep/cap helpers contained rep sequences from AAV2 and serotype specific capsid sequences with the nomenclature, rep2/cap2, rep2/cap5, rep2/cap7 etc. The pAd helper used was pHelper (Stratagene/Agilent Technologies, Santa Clara, Calif.). Purification of AAV was performed as described by Qu et al. (2007, *J. Virol. Methods* 140:183-192).

LC/MS Intact Protein Analysis

The AAV virions were concentrated with an Amicon ultra-4 filter (10 kDa MWCO) and denatured with 10% acetic acid followed by direct analysis in an Acquity UPLC-Xevo® QTOF MS instrument (Waters, Milford, Mass.). The separations were performed with a UPLC BEH C4 or C8 column (1.7 μm, 2.1 mm i.d.) at a 0.25 ml/min flow rate. Mobile phase A was 0.1% formic acid in water while mobile phase B was 0.1% formic acid in acetonitrile. The final gradient was as follows: from 10% B to 20% B for 6 minutes, from 20% B to 30% B in 10 min, then from 30% to 38% B for 40 minutes. For MS the capillary voltage and sampling cone voltage were set at 3.5 kV and 45 V respectively. The mass spectra were acquired in the sensitivity mode with m/z range of 500-4000. Assisted calibration with sodium iodide as calibrant was performed for mass calibration. MaxEnt1 in Masslynx software was used for protein deconvolution.

Enzymatic Digestions of AAV2 VPs

The concentrated AAV2 virions were denatured with 6 M Guanidine-HCl, 0.1 M Tris at pH 8.5. The proteins were reduced with 30 mM DTT at 55° C. for 1 hour in darkness and alkylated with 0.07% 4-vinylpyridine at room temperature for 2 hours. The reactions were quenched by the addition of 1M DTT. The samples were dialyzed with Slide-A-Lyzer cassettes (10,000 MWCO) against 25 mM Tris buffer at pH 8.5 for ~18 hours. After dialysis, the samples were split into three aliquots. Each aliquot was digested with trypsin at 1:25 or Lys-C at 1:50 or Asp-N at 1:100 enzyme: protein ratio (wt/wt) for 18 hours at 37° C., respectively.

LC/MS/MS Peptide Mapping

Nano LC/MS/MS was performed in using a NanoAcquity HPLC system (Waters, Milford, Mass.) in conjunction with an Orbitrap Velos mass spectrometer (Thermo-Fisher Scientific, Waltham, Mass.) using home packed nanoLC column (75 μm×10 mm) with Magic C18 with packing material (5 Bruker, Billerica, Mass.) at a 300 nl/min flow rate. The mobile phases A and B were 0.1% formic acid in water and acetonitrile, respectively. The gradient was from 2% B to 60% B in 121 min.

The source parameters for velos were as follows: source voltage: 2.5 kv, capillary temperature 275° C.; S-lens RF level: 55%. Data were acquired using the top-ten data dependent method with accurate ms at 60,000 resolution and 10 MS/MS in ion trap. Mascot was used for database searching against AAV2 viral capsid protein sequences. MS tolerance of 10 ppm and ms/ms tolerance of 0.8 Da were used for the database search.

UPLC/MS/MS Peptide Mapping

The protein digests were also analyzed by UPLC/MS/MS in Acquity UPLC-Xevo qTOF MS. A BEH300 C18 column (2.1×150 mm) was used for separation in the mobile phases with 0.1% formic acid in water/acetonitrile gradient at a flow rate 0.25 ml/min. The mass spectra were acquired in the positive MSe mode in the mass range of 200-2000.

Results

AAV Denaturation Method

AAVs can be denatured through a number of methods using detergent, heat, high salt, or buffer with low or high pHs. Heat denaturation can lead to protein precipitation and as a result reverse phase columns are easily clogged and over pressurized. Denaturation with high salt requires an additional desalting step before LC/MS analysis. Denaturing with 10% acetic acid was used for the LC/MS intact protein analysis, as it allowed for clean mass spectrum. For peptide mapping, either 0.1% RapiGest or 6 M Guanidine HCl can be used as a denaturing reagent.

Intact Protein Analysis Method Development

Initial intact protein analysis of AAV2 was performed using an UPLC BEH C4 column at fast gradient. Under this condition, only one single peak in the total ion chromatogram was observed, with a mass corresponding to VP3 (FIG. **1**A). Without wishing to be bound by theory, it is thought that the absence of VP1 and VP2 is possibly due to low stoichiometry of VP1 and VP2 or suppression of VP1 and VP2 signals by VP3 if all VPs co-elute. Increasing injection or column length, using a shallower gradient, and using alternative columns have been attempted in order to detect VP1 and VP2. Higher loading (1.7 μg) with a shallower gradient at 0.5% B/min resulted in a shoulder peak on the left (FIG. **1**B). The increase in column length from 10 cm to 15 cm did not enhance the separation of the shoulder peak (FIG. **1**C). However, the shoulder peak was further separated from the main peak using a BEH C8 column, with improved signal intensities observed (FIG. **1**D).

As a result, the VP1 and VP2 masses were obtained in this shoulder peak at the signal intensities shown in FIG. **2**A. The masses of VP1 and VP3 correspond to a.a. 2-735 (acetylation) and a.a. 204-735 (acetylation), respectively (FIGS. **2**A&**2**B). No acetylation was observed in VP2 (a.a.139-735). In addition, a minor peak with a smaller mass than VP3 was observed, with a mass corresponding to amino acid sequence 212-735 with one acetylation (FIG. **2**B). These data are consistent with DNA sequences since VP3 contains two ATG initiation codons in AAV2: ***ATGGCTACAGGCAGTG GCGCACCAATGGCAGAC*** (SEQ ID NO:1), resulting in two possible N termini (underlined): MATGSGAPMAD (SEQ ID NO:2). The N-terminal methionine residues were not present in both VP1 and VP3 as measured by intact protein analysis. The acetylation of VP1 and VP3 is not a method-induced artifact (denaturation of AAV by 10% acetic acid) since acetylation of VP1 and VP3 is also observed in an AAV preparation using an alternative denature method

without acetic acid. The intact protein data also confirmed that no glycosylation was present in the viral capsid proteins, even though several N-linked consensus sequences are present [16].

LC/MS/MS Peptide Mapping

To further confirm the N-termini and acetylation observed in the intact protein analysis, peptide mapping was performed using multiple enzymes and analyzed using multiple instruments. Various sample preparation methods, including denaturation methods and desalting steps, have been evaluated. The final digestion method, including denaturation with 6M guanidine HCl, reduction and alkylation with 4-vinylpyridine, and dialysis using slide-A-lyzer followed by enzymatic digestion, created clean peptide mapping with low artificial modifications during the digestion process. As low as 5 μg starting material was tested, yielding complete sequence coverage using nano LC/MS/MS and UPLC/MS/MS.

Mascot search of tryptic digests from nano LC/MS/MS alone yielded 78% sequence coverage with an ion score 13 cut off as shown in FIG. **3**. The two large missing tryptic peptides, T27 and T38 (boxed) from nano LC/MS/MS were found in the LC/MS in Xevo TOF MS with BEH C18 column (FIG. **3**). In addition, most of the T27 and T38 peptide sequences were further confirmed by nano LC/MS/MS of Asp-N digests as shown in Italics in FIG. **3**. The complete N-terminal and C-terminal peptides were covered by Lys-C digests as underlined in FIG. **3**. Therefore, 100% sequence coverage of VP1 was achieved through multiple enzyme digestions and two LC/MS/MS methods.

LC/MS/MS confirmed the N- and C-termini of VP1, VP2 and VP3 and N-terminal acetylation of VP1 and VP3 observed in the intact protein analysis. FIGS. **4**A-**4**C show the MS/MS spectra of the VP1 N-terminal tryptic peptide A(Ac)ADGYLPDWLEDTLSEGIR (SEQ ID NO:4) (FIG. **4**A), VP2 N-terminal Asp-N derived peptide (APGKKRPVEHSPVEP) (SEQ ID NO:15) (FIG. **4**B), and VP3 N-terminal Asp-N peptide A(Ac)TGSGAPM (SEQ ID NO:5) (FIG. **4**C). MS/MS has confirmed the location of acetylation at the N-terminal alanine residues in both VP1 and VP3 peptides. The presence of unmodified y18 and y17 ions, and all detected b ions with 42 Da mass shift in FIG. **4**A indicates the 42 da-modification is located in N-terminal of VP1. Similarly, the presence of unmodified y3 to y8 ions in FIG. **4**C confirmed the location of acetylation at the N-terminal alanine residue.

Comparison of AAV VP N-Termini

In addition to AAV2, AAV1, AAV5, AAV7, AAV9 and AAV Rh10 have also been analyzed by intact protein analysis. The theoretical and predicted masses of VPs in AAVs are shown in Table 2.

TABLE 2

Theoretical Mass vs Experimental Mass for AAV VPs

Serotype	Isoform	Predicted amino acid sequence	Actual amino acid sequence	Theoretical Ms.(Da)	Experimental Ms.(Da)
AAV1	VP1	1-736	2(ac)-736	81286	81291
	VP2	138-736	139-736	66093	66098
	VP3	203-736	204(ac)-736	59517	59520
AAV2	VP1	1-735	2(ac)-735	81856	81856
	VP2	138-735	139-735	66488	66488
	VP3	203-735	204(ac)-735	59974	59974

TABLE 2-continued

Theoretical Mass vs Experimental Mass for AAV VPs

Serotype	Isoform	Predicted amino acid sequence	Actual amino acid sequence	Theoretical Ms.(Da)	Experimental Ms.(Da)
AAV5	VP1	1-724	2(ac)-724	80336	80336
	VP2	137-724	138-724	65283	65284
	VP3	193-724	194(ac)-724	59463	59463
AAV7	VP1	1-737	2(ac)-737	81564	81567
	VP2	138-737	139-737	66372	66374
	VP3	204-737	213(ac)-737	59101	59103
AAV9	VP1	1-736	2(ac)-736	81291	81288
	VP2	138-736	139-736	66210	66209
	VP3	203-736	204(ac)-736	59733	59733
AAVRh10	VP1	1-738	2(ac)-738	81455	81455
	VP2	138-738	139-738	66253	66252
	VP3	204-738	205(ac)-738	59634	59634

N-termini, as well as their posttranslational modifications, are highly conserved among the AAV serotypes analyzed, even though AAV5 is reported as the most diverse AAV serotype sequence, as shown in the sequence alignments in FIG. **5**. In 11 out of 13 AAV serotypes, the N-termini of VP1 share an identical 13 amino acid residue sequence (MAADGYLPDWLED) (SEQ ID NO:6) while all 13 AAV serotypes have identical TAP . . . N-terminal sequences in VP2 (FIG. **5**). LC/MS of AAV2 indicated that T is missing in VP2 at protein level. The N-termini of VP3 are the most diverse among the three viral capsid proteins, with 8 out of 13 AAV serotypes sharing a MA . . . N-terminal sequence. Similar to AAV2, AAV1 and AAV Rh10 also have two ATG initiation codons with the first one as predominant N-terminal based on LC/MS intact protein analysis. Interestingly, though AAV7 has two potential initiation codons (GTGGCTGCAGGCGGTGGCGCACCA ATGGCAGACAATAAC . . .) (SEQ ID NO:7), the second initiation codon (ATG) was favorable based on the intact protein analysis: the VP3' with 213(ac)-737 was a predominant peak while VP3 with 203(ac)-737 was a minor peak.

Conclusions

Applications of LC/MS Intact Protein Analysis and LC/MS/MS Peptide Mapping of AAV VPs in Gene Therapy Research and Development

These results demonstrate that direct LC/MS of different types of AAVs after denaturation was proved to be a simple and effective way to monitor the protein sequence and post-translational modifications with accurate mass measurement in the intact protein level. N-termini of VP1, VP2 and VP3 of AAVs were confirmed by mass spectrometry. Acetylations of N-termini of VP1 and VP3 were also identified in different serotypes of AAVs. Direct LC/MS/MS peptide mapping of AAVs was developed, provided 100% sequence coverage of VP1, VP2 and VP3, and confirmed the N-termini acetylation of VPs. The theoretical masses of predicted sequences of 13 AAV serotypes based on sequence alignment and intact protein analysis of several AAV serotypes are shown in Table 3.

TABLE 3

Predicted Sequences and Masses

	Predicted VP1 sequence	Mass(Da)	Predicted VP2 sequence	Mass(Da)	Predicted VP3 sequence	Mass(Da)
AAV1	2(ac)-736	81286	139-736	66093	204(ac)-736	59517
AAV2	2(ac)-735	81856	139-735	66488	204(ac)-735	59974
AAV3	2(ac)-736	81571	139-736	66319	204(ac)-736	59849
AAV4	2(ac)-734	80550	138-734	65626	198(ac)-734	59529
AAV5	2(ac)-724	80336	138-724	65283	194(ac)-724	59463
AAV6	2(ac)-736	81322	139-736	66096	204(ac)-736	59519
AAV7	2(ac)-737	81564	139-737	66372	213(ac)-737	59101
AAV8	2(ac)-738	81667	139-738	66519	205(ac)-738	59805
AAV9	2(ac)-736	81291	139-736	66210	204(ac)-736	59733
AAV10	2(ac)-738	81477	139-738	66271	205(ac)-738	59638
AAV11	2(ac)-733	80987	139-733	65794	198(ac)-733	59696
AAV12	2(ac)-742	82106	139-742	66905	207(ac)-742	59846
AAVRh10	2(ac)-738	81455	139-738	66253	205(ac)-738	59634

The accurate masses of VP1, VP2 and VP3 of each serotype are unique and therefore intact protein analysis can be used as an identity test to differentiate AAV capsid serotypes. Tables 4-6 show the mass differences of VPs among 13 common AAV serotypes. Shown in regular font are delta masses larger than 10, with delta masses less than 10 bolded.

TABLE 4

Mass Differences of VP1 Among 13 AAV Isotypes

	AAV1											
AAV2	570	AAV2										
AAV3	285	285	AAV3									
AAV4	736	1306	1021	AAV4								
AAV5	950	1520	1235	215	AAV5							
AAV6	36	534	249	772	987	AAV6						
AAV7	277	292	8	1013	1228	241	AAV7					
AAV8	381	189	96	1117	1332	345	104	AAV8				
AAV9	5	585	280	741	955	31	272	376	AAV9			
AAV10	191	379	94	927	1142	155	86	190	186	AAV10		
AAV11	299	869	584	436	651	335	577	681	304	490	AAV11	
AAV12	820	250	535	1555	1770	784	542	439	815	629	1119	AAV12
AAVRh10	169	401	116	905	1119	133	109	212	164	22	468	651

TABLE 5

Mass Differences of VP2 among 13 AAV Isotypes

	AAV1											
AAV2	395	AAV2										
AAV3	226	169	AAV3									
AAV4	467	862	693	AAV4								
AAV5	810	1205	1036	343	AAV5							
AAV6	2	392	224	470	812	AAV6						
AAV7	278	116	52	746	1088	276	AAV7					
AAV8	425	31	199	893	1235	423	147	AAV8				
AAV9	117	278	109	584	927	115	161	308	AAV9			
AAV10	177	217	49	645	987	175	101	248	60	AAV10		
AAV11	299	694	525	168	511	301	578	725	416	476	AAV11	
AAV12	812	417	586	1279	1622	810	533	386	695	635	1111	AAV12
AAVRh10	160	235	66	627	970	157	119	266	43	18	459	652

TABLE 6

Mass Differences of VP3 among 13 AAV Isotypes

	AAV1							
AAV2	457	AAV2						
AAV3	332	125	AAV3					
AAV4	12	445	320	AAV4				
AAV5	54	511	386	66	AAV5			
AAV6	2	455	330	10	56	AAV6		
AAV7	416	673	748	428	362	418	AAV7	
AAV8	288	169	44	276	342	286	704	AAV8

TABLE 6-continued

Mass Differences of VP3 among 13 AAV Isotypes

AAV9	216	241	116	204	270	214	632	72	AAV9			
AAV10	121	336	211	109	175	119	537	167	95	AAV10		
AAV11	179	278	153	167	233	177	595	109	37	58	AAV11	
AAV12	329	128	3	317	383	327	745	41	113	208	150	AAV12
AAVRh10	117	340	215	105	171	115	533	171	99	4	62	212

No masses within 10 Da of all three VPs between two isotypes are observed. Even though both VP2 and VP3 have only a 2 Da difference between AAV1 and AAV6, the mass difference of VP1 between AAV1 and AAV6 is 36, significant enough to be distinguished by an accurate mass measurement. Therefore, intact protein measurement of VP1, VP2 and VP3 is highly specific as an identity test.

These results demonstrate that intact protein analysis and LC/MS/MS can be used to profile VPs to monitor VP expressions, posttranslational modifications, and truncations and to ensure product consistency during VLP production. These two analyses can also be used to confirm site-direct mutagenesis or structural characterization for capsid protein engineering applications.

Example 2: The Role of N Terminal Acetylation of AAV Capsid Proteins

Chemical modifications of cellular proteins are a common means of controlling their functions (Arnesen, T. (2006) *Virology* 353(2): 283-293). N-terminal acetylation (Nt-acetylation), which involves the transfer of an acetyl group from acetyl coenzyme A to the α-amino group of the first amino acid residue of a protein (Brown, J. L., and Roberts, W. K. (1976) J Biol Chem 251: 1009-1014; Arnesen, T. et al. (2009) *Proc Natl Acad Sci USA* 106: 8157-8162), is among the most abundant of protein modifications. Unlike most other protein modifications, Nt-acetylation is irreversible; it occurs mainly during the synthesis of the protein, catalyzed by N-terminal acetyltransferases (NATs) associated with ribosomes (Gautschi, M. et al. (2003) *Mol Cell Biol* 23: 7403-7414; Pestana, A. and Pitot, H. C. (1975) *Biochemistry* 14: 1404-1412; Polevoda, B. et al. (2003) *J Biol Chem* 278: 30686-97). There are several distinct NATs in eukaryotes—NatA-NatF—each composed of one or more subunits and each acetylating a specific subgroup of N-termini depending on the amino acid sequence of the first few amino acids (Jornvall, H. (1975) *J Theor Biol* 55: 1-12; Persson, B. et al. (1985) *Eur J Biochem* 152: 523-527).

Experimental data indicate that proteins with acetylated N-termini are more stable in vivo than non-acetylated proteins; i.e., Nt-acetylation protects proteins from degradation (Hershko, A. et al. (1984) *Proc Natl Acad Sci USA* 81: 7021-7025). One explanation for this might be the discovery in 2004 that another N-terminal modification, ubiquitination, which involves direct attachment of the small protein ubiquitin to the N-terminal amino acid residue, promotes the subsequent degradation of the protein (Ben Saadon, R. et al. (2004) *J Biol Chem* 279: 41414-41421). Conversely, the Nt-acetylation signals can also be part of a quality control mechanism to degrade unfolded or misfolded proteins and to regulate in vivo protein stoichiometries (Hwang, C. S. et al. (2010) *Science* 327: 973-977).

A systematic analysis of the predicted N-terminal processing of cytosolic proteins, versus those destined to be sorted to the secretory pathway, revealed that the cytosolic proteins were profoundly biased in favor of processing, but there is an equal and opposite bias against such modification for secretory proteins (Forte, G. M. A. et al. (2011) *PLoS Biology*, 4 May 2011 Volume 9). Mutations in secretory signal sequences that lead to their acetylation result in mis-sorting to the cytosol in a manner that is dependent upon the N-terminal processing machinery. Hence N-terminal acetylation represents an early determining step in the cellular sorting of nascent polypeptides that represent an extra layer of stringency in order to ensure that proteins destined to remain in the cytosol actually reside in the cytosol. The eukaryotic cell comprises several distinct compartments, called organelles, required to perform specific functions. The proteins in these compartments are synthesized in the cytoplasm and so require complex sorting mechanisms to ensure their delivery to the appropriate organelle. Proteins are modified by acetylation of their amino terminus at a very early stage in their synthesis. There is a profound difference between the likelihood of such a modification on cytoplasmic proteins and on those destined for one of the major organelles, the endoplasmic reticulum (ER): whereas cytoplasmic proteins are typically acetylated, those bound for the ER are largely unmodified. Moreover, when specific ER proteins are engineered to induce their acetylation their targeting to the ER was inhibited (Forte, G. M. A. et al. (2011) *PLoS Biology*, 4 May 2011 Volume 9).

The contractile proteins actin and tropomyosin have been shown to require NatB-mediated Nt-acetylation for proper function, specifically involving actin-tropomyosin binding and actomyosin regulation (Coulton, A. T. et al. (2010) *J Cell Sci* 123: 3235-3243; Polevoda, B. et al. (2003) *J Biol Chem* 278: 30686-97). Thus Nt acetylation of AAV capsid proteins may have importance in the transduction potential of rAAV vectors. If AAV vectors fail to gain entry into the nucleus, they consequently fail to transduce cells. The role of actin filaments and FKBP52 (FK506-binding protein p52) in the translocation of AAV capsids from the endosome to the nucleus is well defined (Zhao, W. et al. (2006) *Virology* 353(2): 283-293). Importantly, Nt-acetylation is essential for the functioning of actin filaments by modulating protein-protein interactions (Coulton, A. T. et al. (2010) *J Cell Sci* 123: 3235-3243; Polevoda, B. et al. (2003) *J Biol Chem* 278: 30686-97).

Though N-terminal acetylation of proteins is a widely known phenomenon, the biological significance of Nt-acetylation on AAV capsid proteins is not well understood. The predicted N-termini of VP1 and VP3 based on DNA sequencing are both methionine followed by alanine. It has been reported that removal of N-terminal methionine by Met-aminopeptidases frequently leads to Nt-acetylation of the resulting N-terminal alanine, valine, serine, threonine, and cysteine residues and that the acetylation of the N-terminus acts as a potential degradation signal [21]. Ubiquitination of viral capsid proteins was suggested as a potential signal for processing of the capsid at the time of virion disassembly [22]. The link between N-acetylation of VP1 and VP3 and viral capsid degradation and uncoating before the nuclear entry is further investigated.

To understand the functional implications of N-terminal acetylation with regard to AAV capsid proteins, site-directed mutagenesis of VP3 N-terminal initiation codons is used to generate AAV mutants.

Methods

AAV capsid proteins are generated with differing amino acids at the 2nd position to the initiating methionine (iMet X) to determine if Nt-acetylation is inhibited or reduced, and the functional consequences are then measured. The ability of the capsid proteins to be trafficked intra-cellularly and/or to acquire post translational modifications such as glycosylation is assessed, and whether this ability affects the infectivity of the assembled AAV particle is subsequently determined. In addition, the impact of acetylation on ubiquitination/degradation and targeting to the lysosome, ER, Golgi, or inner nuclear membrane is determined.

For example, to assay trafficking or targeting, AAV particles with capsid proteins having a mutated 2nd position (e.g., iMet X) are fluorescently labeled and used to infect cells (e.g., HeLa cells). These AAV particles are assayed for one or more of: time of viral particle uptake, colocalization of AAV particles with specific compartmental markers (e.g., Golgi, ER, or lysosomal proteins or other markers), nuclear accumulation (e.g., as assayed by colocalization with a nuclear marker or stain), and/or sensitivity of trafficking to specific inhibitors of early endosomal escape (such as bafilomycin A or ammonium chloride), as compared to fluorescently labeled wild-type AAV particles used to infect the same cell line (see, e.g., Bartlett, J. S. et al. (2000) *J. Virol.* 74:2777-2785 for a description of such assays).

To assay infectivity, AAV particles with capsid proteins having a mutated 2nd position (e.g., iMet X) are used to infect cells (e.g., HeLa cells), and their transduction efficiency is compared to wild-type AAV particles (e.g., having the same AAV serotype and infecting the same type of cells).

To assay glycosylation, AAV particles with capsid proteins having a mutated 2nd position (e.g., iMet X) are used to infect cells (e.g., HeLa cells). AAV particles from infected cells are subjected to one or more assays including without limitation chemical detection of glycosylation (e.g., applying a commercially available digoxigenin (DIG) glycan detection and/or fluorescent glycoprotein detection kit on denatured and electrophoretically separated capsid proteins) and mass spectrometry (e.g., FT-ICR MS), as compared to wild-type AAV particles used to infect the same cell line (see, e.g., Murray, S. et al. (2006) *J. Virol.* 80:6171-6176 for a description of such assays).

To assay ubiquitination, AAV particles with capsid proteins having a mutated 2nd position are used to infect cells (e.g., HeLa cells). AAV particles are immunoprecipitated from infected cells with an anti-capsid antibody, then subjected to Western blotting with an anti-ubiquitin antibody and compared to wild-type AAV particles used to infect cells in the same manner. Mutant AAV particles may also be used in in vitro ubiquitination assays, as compared to wild-type AAV particles (see, e.g., Yan, Z. et al. (2002) *J. Virol.* 76:2043-2053).

Example 3: The Role of Deamidation of AAV2 Capsid Proteins

Sequence analysis of the AAV2 capsid protein revealed potential deamidation sites, as underlined in the following amino acid sequence:

(SEQ ID NO: 3)

MAADGYLPDWLEDTLSEGIRQWWKLKPGPPPPKPAERHKDDSRGLVLPGYK

YLGPFNGLDKGEPVNEADAAALEHDKAYDRQLDSGDNPYLKYNHADA

EFQERLKEDTSFGGNLGRAVFQAKKRVLEPLGLVEEPVKTAPGKKRPVEHS

PVEPDSSSGTGKAGQQPARKRLNFGQTGDADSVPDPQPLGQPPAAPSGLGT

NTMATGSGAPMADNNEGADGVGNSSGNWHCDSTWMGDRVITTSTRTWALPT

YNNHLYKQISSQSGASNDNHYFGYSTPWGYFDFNRFHCHFSPRDWQRLINN

NWGFRPKRLNFKLFNIQVKEVTQNDGTTTIANNLTSTVQVFTDSEYQLPYV

LGSAHQGCLPPFPADVFMVPQYGYLTLNNGSQAVGRSSFYCLEYFPSQMLR

TGNNFTFSYTFEDVPFHSSYAHSQSLDRLMNPLIDQYLYYLSRTNTPSGTT

TQSRLQFSQAGASDIRDQSRNWLPGPCYRQQRVSKTSADNNNSEYSWTGAT

KYHLNGRDSLVNPGPAMASHKDDEEKFFPQSGVLIFGKQGSEKTNVDIEKV

MITDEEEIRTTNPVATEQYGSVSTNLQRGNRQAATADVNTQGVLPGMVWQD

RDVYLQGPIWAKIPHTDGHFHPSPLMGGFGLKHPPPQILIKNTPVPANPST

TFSAAKFASFITQYSTGQVSVEIEWELQKENSKRWNPEIQYTSNYNKSVNV

DFTVDTNGVYSEPRPIGTRYLTRNL.

In particular, a potential deamidation site is found at N57/G58 in the phospholipase A2 domain (Ca++ binding site), as bolded and italicized in the above sequence. The following experiments were aimed at exploring whether deamidation at N57 can lead to reduced potency and/or truncation of AAV2, as well as whether different AAV production methods may have different effects on deamidation. For example, the producer cell line method (see Martin et al., (2013) *Human Gene Therapy Methods* 24:253-269; U.S. PG Pub. No. US2004/0224411; and Liu, X. L. et al. (1999) *Gene Ther.* 6:293-299) may induce a higher level of deamidation at N57, as compared to the triple transfection method. According to the crystal structure of AAV2, N57 is not shown; however, N382 and N511 are partially exposed, and N715 is fully exposed.

Methods

Enzymatic Digestions of AAV1 and AAV2 VPs

10 μg of each AAV1-EGFP or AAV2-EGFP material (generated from triple transfection as well as producer cell line process) were concentrated using Amicon filters (10 kDa MWCO), denatured with 6 M Guanidine-HCl, 50 mM Tris at pH 8.5. The proteins were reduced with 5 mM DTT at 60° C. for 30 minutes in darkness, alkylated with 15 mM iodoacetamide at room temperature for 30 minutes, and then buffer exchanged into 25 mM Tris pH 7.1 for digestion using Bio-Spin® 6 Tris micro-columns. After buffer exchange, the samples were split into two aliquots. Each aliquot was digested with trypsin at 1:25 or Asp-N at 1:50 enzyme: protein ratio (wt/wt) for 2 hours at 37° C., respectively.

UPLC/MS/MS Peptide Mapping

The protein digests were also analyzed by UPLC/MS/MS in Acquity UPLC-Xevo qTOF MS. BEH300 C18 column (2.1×150 mm) was used for separation in the mobile phases with 0.1% formic acid in water/acetonitrile gradient at a flow rate 0.25 ml/min. The mass spectra were acquired in the positive MSe resolution mode in the mass range of 50-2000.

Determination of Deamidation Levels in AAV VPs

The extracted ion chromatograms (XIC) of peptides containing NG sites (T9, T49, and T67 in AA1 and AAV2 VP) and their corresponding deamidated species were used for calculation of deamidation levels.

In order to compare AAV vectors produced by the triple transfection (TTx) and producer cell line (PCL) methods, AAV1 or AAV2 tagged with EGFP was produced using the TTx or PCL method. Truncated VP1 (tVP1) was found to be present in AAV2-EGFP produced by PCL, but not in the AAV2-EGFP produced by TTx. AAV1-EGFP was not found to have tVP1, regardless of the production method. The in vitro potency of AAV2 produced by the PCL method was also found to be reduced, as compared to AAV2 produced by TTx. Mutant N57K and N57Q AAV2 particles were also found to have reduced potency and disrupted Ca++ binding.

The following table provides the tryptic peptides that were analyzed to examine each potential deamidation site, as well as the corresponding residue.

TABLE 7

Tryptic peptides containing NG sites

Peptide (NG sequence underlined)	Residue
YLGPFNGLDK (SEQ ID NO: 9)	N57
EVTQNDGTTTIANNLTSTVQVFTDSEYQLPYVLGSAHQGCLP PFPADVFMVPQYGYLTLNNGSQAVGRSSFYCLEYFPSQMLR (SEQ ID NO: 10)	N382
YNLNGR (AAV1) (SEQ ID NO: 11)	N511
YHLNGR (AAV2) (SEQ ID NO: 12)	N511
SANVDFTVDNNGLYTEPR (AAV1) (SEQ ID NO: 13)	N715
SVNVDFTVDTNGVYSEPR (AAV2) (SEQ ID NO: 14)	N715

As shown in Table 7, the T9 peptide YLGPFNGLDK (SEQ ID NO: 9) was used to monitor N57, the T38 peptide EVTQNDGTTTIANNLTSTVQVFTDSEYQLPYVLG-SAHQGCLPPFPADVFMVPQYGYLTLN NGSQAVGRSSFYCLEYFPSQMLR (SEQ ID NO: 10) was used to monitor N382, the T49 peptides YNLNGR (SEQ ID NO: 11) and YHLNGR (SEQ ID NO: 12) were used to monitor N511 in AAV1 or AAV2 (respectively), and the T67 peptides SANVDFTVDNNGLYTEPR (SEQ ID NO: 13) and SVNVDFTVDTNGVYSEPR (SEQ ID NO: 14) were used to monitor N715 in AAV1 or AAV2 (respectively).

LC/MS/MS analysis was used to compare the percentage of deamidation in AAV1 and AAV2 particles produced by the TTx and PCL methods. The results from the T9 peptide are shown in FIGS. **6**A & **6**B. The results from the T49 peptide are shown in FIGS. **7**A & **7**B. The results from the T67 peptide are shown in FIGS. **8**A & **8**B. These results are summarized in Table 8. The T38 peptide was not detected due to its size.

TABLE 8

Summary of LC/MS/MS results

		% Deamidation		
		N57	N511	N715
AAV1	TTx	7.9	30.9	18.1
	PCL	11.3	27.4	18.7
AAV2	TTx	6.7	39.6	27.4
	PCL	18.4	42.3	28.0

In particular, AAV2 produced by PCL showed nearly a 3-fold increase in deamidation as compared to AAV2 produced by TTx. These results suggest that deamidation decreases AAV potency, as the in vitro potency of AAV2 produced by PCL is reduced.

Conclusions

Taken together, Examples 1-3 demonstrate methods for analyzing intact proteins of viral particles (e.g., AAV capsid proteins) using LC/MS. Molecular weights were measured accurately, and these techniques may be also used to assess N-termini and/or modifications of viral capsid proteins. Moreover, these methods are adaptable as capsid serotype identity assays useful in gene therapy, e.g., as an analytical platform. These results further establish a correlation between capsid protein structure (e.g., truncations, deamidation, etc.) and potency, suggesting that point mutations at key sites may be used to engineer more effective vectors.

Example 4: Elucidating the Role of N Terminal Acetylation of AAV Capsid Proteins

As discussed above, the N-termini of AAV capsid proteins are highly conserved across serotypes (FIG. **5**). The techniques described in Example 1 allow for interrogation of VP expression and posttranslational modifications. The role and biological significance of N-terminal acetylation of AAV capsid proteins was next examined.

Results

To elucidate the potential role of deacetylation of AAV capsid proteins, AAV5 deacetylation variants were tested. An AAV5 particle expressing eGFP under the CBA promoter (AAV5-CBA-Egfp) was compared to AAV5 variants with the amino acid adjacent to the initiating methionine (iMET) mutated for VP1 and VP3 (deAC-AAV5-CBA-eGFPs). Three amino acids predicted to have a low likelihood of acetylation by NatA, NatC, or NatD were chosen for generating variants: Gly, Leu, and Pro, as illustrated in Table 9 below.

TABLE 9

N-terminal acetylation frequency

N-term aa	Transferase	NT-AC FREQUENCY
MET-ALA Normally found in VP1 & VP 3	Nat A	High
MET-SER Normally found in VP1 & VP3 for AAV5	Nat A	High
AAV variants		
MET-GLY	Nat A	Low
MET-LEU	NatC	Low
MET-PRO	NatD/other	Low

The following AAV5 deacetylated (deAC) mutants were generated:

S2GVP1—Ser changed to Gly at position 2 in AAV5VP1
S2LVP1—Ser changed to Leu at position 2 in AAV5VP1
S2PVP1—Ser changed to Pro at position 2 in AAV5VP1
S2GVP3—Ser changed to Gly at position 2 in AAV5VP3
S2LVP3—Ser changed to Leu at position 2 in AAV5VP3
S2PVP3—Ser changed to Pro at position 2 in AAV5VP3
S2PVP1/VP3—Ser changed to Pro at position 2 in both AAV5 VP1 and VP3
S2GVP1/VP3—Ser changed to Gly at position 2 in both AAV5 VP1 and VP3
S2LVP1/VP3—Ser changed to Leu at position 2 in both AAV5 VP1 and VP3

These variants were generated using the TTX method as described above. All AAV5 variants showed good productivity, with yields greater than 10^{13} total VG. All AAV5

variants also showed the expected VP1:VP2:VP3 protein ratio by SYPRO protein gel analysis (FIG. **9**). Next, LC/MS was used to confirm that all AAV5 variants had decreased acetylation, as shown in Table 10.

TABLE 10

LC/MS analysis of AAV5 variant acetylation

	mutants	VP1 Theo.	VP1 Exp.	Δmass (VP1)	VP2 Theo.	VP2 Exp.	Δmass (VP2)	VP3 Theo.	VP3 Exp.	Δmass (VP3)	note
1	deAC-AAV5 (S2GVP1)/ CBA-eGFP	80234	nd		65283	65293	10	59463	59472	9	VP1 not detectable
2	deAC-AAV5 (S2LVP1)/ CBA-eGFP	80346	80501	181	65283	65292	9	59463	59471	8	VP1 incorrect
3	deAC-AAV5 (S2GVP3)/ CBA-eGFP	80234	nd		65253	65261	8	59391	59398	7	confirmed
4	deAC-AAV5 (S2LVP3)/ CBA-eGFP	80336	80363	27	65309	65309	0	59447	59620	173	VP3 incorrect
5	deAC-AAV5 (S2PVP1VP3)/ CBA-eGFP	80314	80324	10	65293	65300	7	59431	59438	7	confirmed
6	deAC-AAV5 (S2GVP1VP3)/ CBA-eGFP	80234	80243	9	65253	65261	8	59391	59398	7	confirmed
7	deAC-AAV5 (S2PVP3)/ CBA-eGFP	80336	80346	10	65293	65292	1	59431	59430	1	confirmed
8	deAC-AAV5 (S2PVP1)/ CBA-eGFP	80314	80313	1	65283	65291	8	59463	59470	7	confirmed
9	deAC-AAV5 (S2L VP1VP3)/ CBA-eGFP	80346	nd		65309	65318	9	59447	59629	182	VP3 incorrect

nd = not determined

These LC/MS analyses confirmed that AAV5 variants were deacetylated. The variants S2LVP1, S2LVP3, and S2LVP1/VP3 all showed increased mass (increased from 173 to 182) in VP1 and VP3 proteins, suggesting that changing the second N-terminal amino acid to a leucine in VP1 or VP3 alters the protein, resulting in an increase in mass.

Next, AAV5 variants were assayed in an in vitro transduction assay using eGFP as a reporter gene (FIG. **10**). The assay was designed to evaluate transduction by AAV5 deacetylated mutant variants at 10^6 multiplicity of infection (MOI), comparing each variant to the parental, unmodified AAV5 particle. Three cell lines were used: 293, HuH7, and HeLa cells. Following infection, cells were assayed to determine vector genome copy number (vg/μg cellular protein) and eGFP expression (by ELISA). Vector genome copy number (vg/μg protein) represents the efficiency at which the AAV5 variant enters the cell, and eGFP represents the efficiency of capsid intracellular trafficking, since transgene expression requires the capsid/vector DNA to efficiently traffic to the nucleus (FIG. **10**). Vector genomes were quantified by TaqMan analysis.

FIG. **11** shows that, based on vector genome analyses, AAV5 deacetylated mutant vectors infected all three test cell lines at similar, but reduced, levels as compared to the parental unmodified AAV5 particles. FIG. **12** shows that AAV5 deacetylated mutant vectors all resulted in reduced eGFP expression in all three cell lines, as compared to transduction with parental unmodified AAV5.

Conclusions

As predicted, no acetylation was observed in N-terminal Ser to Pro/Leu/Gly mutant variants when examined by LC/MS. AAV5 deAC variants showed robust vector production, and AAV5 deAC variants infected cells at levels comparable to parental AAV5. However, functional protein levels in cells infected with deAC variants were greatly reduced when compared to the parental AAV5. These data suggest that tropism is minimally affected by a lack of N-terminal deacetylation in VP1/VP3, but downstream processing (e.g., trafficking and/or degradation) is significantly affected. Since the variants tested demonstrated reduced in vitro activity, one of skill in the art may appreciate that variants characterized by reduced or eliminated acetylation could be employed, inter alia, when decreased levels of transduction are desirable.

Example 5: Assessment of Deamidation of AAV Capsid Proteins

Examples 1 and 3 demonstrate techniques that allow the interrogation of post-translational modifications of AAV capsid proteins and explore the role of deamidation of the AAV2 capsid. The following Example tested whether deamidation reduces potency and/or induces truncation of capsid proteins, and whether different manufacturing processes can induce different levels of deamidation.

Methods

AAV particles were generated and deamidation status assayed as described in Example 3.

Results

As described in Example 3, a potential deamidation site is found at N57/G58 in the phospholipase A2 domain (Ca++ binding site) in VP1 of the AAV2 capsid. The N57/G58 motif is conserved across AAV serotypes (FIG. **13**). Example 3 showed that AAV2 produced by PCL exhibited nearly a 3-fold increase in deamidation as compared to AAV2 produced by TTx (see FIGS. **6**A & **6**B and Table 8).

In examining VP1, VP2, and VP3 production by protein gels, a truncated VP1 protein (tVP1) was detected only in AAV2 capsid proteins produced by the PCL method (FIG. **14**).

A series of AAV2 deamidation mutants was generated next. These mutants targeted the Gly residue in the canonical NG sequence. Mutations targeting the A35 residue (see FIG. **13**), the N-terminal amino acid for tVP1 were also generated, as shown in Table 11. The pAF277 and pAF279 mutants bearing multiple mutations did not package.

TABLE 11

Deamidation mutants

Name	mutation	avg drp/cell
pAF274	G58K	4.54E+03
pAF275	G58D	5.00E+03
pAF276	G58Q	5.41E+03
pAF277	G58, 383, 512, 716K	7.2
pAF278	A35N	6.89E+03
pAF279	A35N, G58, 383, 512, 765K	2.2
293	—	0.9
PIM45	Control	6.28E+03

K = positive charge (basic)
D = negative charge (acidic)
Q = polar

Deamidation of variants were next analyzed by LC/MS as described in Example 3 above. The AAV2A35N and AAV2G58D variants had altered deamidation as compared to the parental AAV2 (FIG. **15**). In particular, the AAV2A35N mutant had increased deamidation (17.8%) as compared to parental AAV2 (5.7%). The AAV2G58D variant had reduced deamidation (1.1%) as compared to parental AAV2. SYPRO protein gel analysis demonstrated that the AAV2 deamidation mutants exhibited the correct VP1:VP2:VP2 ratio (FIG. **16**).

Next, AAV2 deamidation variants were assayed in an in vitro transduction assay using eGFP as a reporter gene (FIG. **17**). The assay was designed to evaluate transduction by AAV2 deamidation mutant variants at 10^5 multiplicity of infection (MOI), comparing each variant to the parental, unmodified AAV2 particle. Three cell lines were used: 293, HuH7, and HeLa cells. Following infection, cells were assayed to determine vector genome copy number (vg/μg cellular protein) and eGFP expression (by ELISA). Vector genome copy number (vg/μg protein) represents the efficiency at which the AAV2 variant enters the cell, and eGFP represents the efficiency of capsid intracellular trafficking, since transgene expression requires the capsid/vector DNA to efficiently traffic to the nucleus (FIG. **17**). Vector genomes were quantified by TaqMan analysis.

Vector genome analysis indicated that AAV2 deamidation mutant variants infected all cell lines tested at levels comparable to that of parental AAV2 vectors (FIG. **18**). Importantly, the AAV2A35N variant was found to be more potent than the parental AAV2 vector for transduction in all three cell lines (FIG. **19**). The AAV2G58D variant was found to be more potent than the parental AAV2 vector in HuH7 cells (FIG. **19**).

Conclusions

In summary, AAV2 deamidation mutant vectors infect cells at levels comparable to the parent AAV2 particles (e.g., comparable vg/μg cellular protein). However, based on analysis of eGFP levels in transduced cells, the AAV2A35N variant had higher potency than the parental AAV2 in all cell lines tested, and the AAV2G58D variant had higher potency than the parental AAV2 in HuH7 cells (a liver-derived cell line). These results suggest that the A35N mutation may be effective in increasing vector potency for transducing many cell types, and that the G58D mutation may also be effective in increasing potency in certain cell types, e.g., liver cells.

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SEQUENCES

All polypeptide sequences are presented N-terminal to C-terminal unless otherwise noted. All nucleic sequences are presented 5' to 3' unless otherwise noted.

Nucleotide sequence of potential AAV2 VP3 initiation codons (ATG codons underlined) (SEQ ID NO: 1)

ATGGCTACAGGCAGTGGCGCACCAATGGCAGAC

Polypeptide sequence corresponding to potential AAV2 VP3 initiation codons (methionines underlined) (SEQ ID NO: 2)

MATGSGAPMAD

AAV2 VP1 polypeptide sequence (SEQ ID NO: 3)

MAADGYLPDWLEDTLSEGIRQWWKLKPGPPPPKPAERHKDDSRGLVLPGYK

YLGPFNGLDKGEPVNEADAAALEHDKAYDRQLDSGDNPYLKYNHADAEFQE

-continued

RLKEDTSFGGNLGRAVFQAKKRVLEPLGLVEEPVKTAPGKKRPVEHSPVEP

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LYKQISSQSGASNDNHYFGYSTPWGYFDFNRFHCHFSPRDWQRLINNNWGF

RPKRLNFKLFNIQVKEVTQNDGTTTIANNLTSTVQVFTDSEYQLPYVLGSA

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EEEIRTTNPVATEQYGSVSTNLQRGNRQAATADVNTQGVLPGMVWQDRDVY

LQGPIWAKIPHTDGHFHPSPLMGGFGLKHPPPQILIKNTPVPANPSTTFSA

AKFASFITQYSTGQVSVEIEWELQKENSKRWNPEIQYTSNYNKSVNVDFTV

DTNGVYSEPRPIGTRYLTRNL

VP1 N-terminal tryptic peptide (N-terminal alanine is acetylated) (SEQ ID NO: 4)

AADGYLPDWLEDTLSEGIR

VP3 N-terminal Asp-N peptide (N-terminal alanine is acetylated) (SEQ ID NO: 5)

ATGSGAPM

Common VP1 N-terminal sequence (SEQ ID NO: 6)

MAADGYLPDWLED

Nucleotide sequence of potential AAV7 VP3 initiation codons (start codons underlined) (SEQ ID NO: 7)

GTGGCTGCAGGCGGTGGCGCACCAATGGCAGACAATAAC

Nucleotide sequence of mutated ITR (SEQ ID NO: 8)

CACTCCCTCTCTGCGCGCTCGCTCGCTCACTGAGGCCGGGCGACCAAAGGT

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Lys Pro Ala Glu Arg His Lys Asp Asp Ser Arg Gly Leu Val Leu Pro
35 40 45

Gly Tyr Lys Tyr Leu Gly Pro Phe Asn Gly Leu Asp Lys Gly Glu Pro
50 55 60

Val Asn Glu Ala Asp Ala Ala Ala Leu Glu His Asp Lys Ala Tyr Asp
65 70 75 80

Arg Gln Leu Asp Ser Gly Asp Asn Pro Tyr Leu Lys Tyr Asn His Ala
85 90 95

Asp Ala Glu Phe Gln Glu Arg Leu Lys Glu Asp Thr Ser Phe Gly Gly
100 105 110

Asn Leu Gly Arg Ala Val Phe Gln Ala Lys Lys Arg Val Leu Glu Pro
115 120 125

Leu Gly Leu Val Glu Glu Pro Val Lys Thr Ala Pro Gly Lys Lys Arg
130 135 140

Pro Val Glu His Ser Pro Val Glu Pro Asp Ser Ser Ser Gly Thr Gly
145 150 155 160

Lys Ala Gly Gln Gln Pro Ala Arg Lys Arg Leu Asn Phe Gly Gln Thr
165 170 175

Gly Asp Ala Asp Ser Val Pro Asp Pro Gln Pro Leu Gly Gln Pro Pro
180 185 190

Ala Ala Pro Ser Gly Leu Gly Thr Asn Thr Met Ala Thr Gly Ser Gly
195 200 205

Ala Pro Met Ala Asp Asn Asn Glu Gly Ala Asp Gly Val Gly Asn Ser
210 215 220

Ser Gly Asn Trp His Cys Asp Ser Thr Trp Met Gly Asp Arg Val Ile
225 230 235 240

Thr Thr Ser Thr Arg Thr Trp Ala Leu Pro Thr Tyr Asn Asn His Leu
245 250 255

Tyr Lys Gln Ile Ser Ser Gln Ser Gly Ala Ser Asn Asp Asn His Tyr
260 265 270

Phe Gly Tyr Ser Thr Pro Trp Gly Tyr Phe Asp Phe Asn Arg Phe His
275 280 285

Cys His Phe Ser Pro Arg Asp Trp Gln Arg Leu Ile Asn Asn Asn Trp
290 295 300

Gly Phe Arg Pro Lys Arg Leu Asn Phe Lys Leu Phe Asn Ile Gln Val
305 310 315 320

Lys Glu Val Thr Gln Asn Asp Gly Thr Thr Thr Ile Ala Asn Asn Leu
325 330 335

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Thr Ser Thr Val Gln Val Phe Thr Asp Ser Glu Tyr Gln Leu Pro Tyr
            340                 345                 350

Val Leu Gly Ser Ala His Gln Gly Cys Leu Pro Pro Phe Pro Ala Asp
        355                 360                 365

Val Phe Met Val Pro Gln Tyr Gly Tyr Leu Thr Leu Asn Asn Gly Ser
    370                 375                 380

Gln Ala Val Gly Arg Ser Ser Phe Tyr Cys Leu Glu Tyr Phe Pro Ser
385                 390                 395                 400

Gln Met Leu Arg Thr Gly Asn Asn Phe Thr Phe Ser Tyr Thr Phe Glu
                405                 410                 415

Asp Val Pro Phe His Ser Ser Tyr Ala His Ser Gln Ser Leu Asp Arg
            420                 425                 430

Leu Met Asn Pro Leu Ile Asp Gln Tyr Leu Tyr Tyr Leu Ser Arg Thr
        435                 440                 445

Asn Thr Pro Ser Gly Thr Thr Thr Gln Ser Arg Leu Gln Phe Ser Gln
    450                 455                 460

Ala Gly Ala Ser Asp Ile Arg Asp Gln Ser Arg Asn Trp Leu Pro Gly
465                 470                 475                 480

Pro Cys Tyr Arg Gln Gln Arg Val Ser Lys Thr Ser Ala Asp Asn Asn
                485                 490                 495

Asn Ser Glu Tyr Ser Trp Thr Gly Ala Thr Lys Tyr His Leu Asn Gly
            500                 505                 510

Arg Asp Ser Leu Val Asn Pro Gly Pro Ala Met Ala Ser His Lys Asp
        515                 520                 525

Asp Glu Glu Lys Phe Phe Pro Gln Ser Gly Val Leu Ile Phe Gly Lys
    530                 535                 540

Gln Gly Ser Glu Lys Thr Asn Val Asp Ile Glu Lys Val Met Ile Thr
545                 550                 555                 560

Asp Glu Glu Glu Ile Arg Thr Thr Asn Pro Val Ala Thr Glu Gln Tyr
                565                 570                 575

Gly Ser Val Ser Thr Asn Leu Gln Arg Gly Asn Arg Gln Ala Ala Thr
            580                 585                 590

Ala Asp Val Asn Thr Gln Gly Val Leu Pro Gly Met Val Trp Gln Asp
        595                 600                 605

Arg Asp Val Tyr Leu Gln Gly Pro Ile Trp Ala Lys Ile Pro His Thr
    610                 615                 620

Asp Gly His Phe His Pro Ser Pro Leu Met Gly Gly Phe Gly Leu Lys
625                 630                 635                 640

His Pro Pro Pro Gln Ile Leu Ile Lys Asn Thr Pro Val Pro Ala Asn
                645                 650                 655

Pro Ser Thr Thr Phe Ser Ala Ala Lys Phe Ala Ser Phe Ile Thr Gln
            660                 665                 670

Tyr Ser Thr Gly Gln Val Ser Val Glu Ile Glu Trp Glu Leu Gln Lys
        675                 680                 685

Glu Asn Ser Lys Arg Trp Asn Pro Glu Ile Gln Tyr Thr Ser Asn Tyr
    690                 695                 700

Asn Lys Ser Val Asn Val Asp Phe Thr Val Asp Thr Asn Gly Val Tyr
705                 710                 715                 720

Ser Glu Pro Arg Pro Ile Gly Thr Arg Tyr Leu Thr Arg Asn Leu
                725                 730                 735
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<210> SEQ ID NO 4
<211> LENGTH: 19
<212> TYPE: PRT

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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Construct
<220> FEATURE:
<221> NAME/KEY: ACETYLATION
<222> LOCATION: 1

<400> SEQUENCE: 4

Ala Ala Asp Gly Tyr Leu Pro Asp Trp Leu Glu Asp Thr Leu Ser Glu
1 5 10 15

Gly Ile Arg

<210> SEQ ID NO 5
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Construct
<220> FEATURE:
<221> NAME/KEY: ACETYLATION
<222> LOCATION: 1

<400> SEQUENCE: 5

Ala Thr Gly Ser Gly Ala Pro Met
1 5

<210> SEQ ID NO 6
<211> LENGTH: 13
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Construct

<400> SEQUENCE: 6

Met Ala Ala Asp Gly Tyr Leu Pro Asp Trp Leu Glu Asp
1 5 10

<210> SEQ ID NO 7
<211> LENGTH: 39
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Construct

<400> SEQUENCE: 7

Gly Thr Gly Gly Cys Thr Gly Cys Ala Gly Gly Cys Gly Gly Thr Gly
1 5 10 15

Gly Cys Gly Cys Ala Cys Cys Ala Ala Thr Gly Gly Cys Ala Gly Ala
20 25 30

Cys Ala Ala Thr Ala Ala Cys
35

<210> SEQ ID NO 8
<211> LENGTH: 78
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Construct

<400> SEQUENCE: 8

Cys Ala Cys Thr Cys Cys Cys Thr Cys Thr Cys Thr Gly Cys Gly Cys
1 5 10 15

Gly Cys Thr Cys Gly Cys Thr Cys Gly Cys Thr Cys Ala Cys Thr Gly
20 25 30

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Ala Gly Gly Cys Cys Gly Gly Gly Cys Gly Ala Cys Cys Ala Ala Ala
35 40 45

Gly Gly Thr Cys Gly Cys Cys Cys Ala Cys Gly Cys Cys Cys Gly Gly
50 55 60

Gly Cys Thr Thr Thr Gly Cys Cys Cys Gly Gly Gly Cys Gly
65 70 75

<210> SEQ ID NO 9
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Construct

<400> SEQUENCE: 9

Tyr Leu Gly Pro Phe Asn Gly Leu Asp Lys
1 5 10

<210> SEQ ID NO 10
<211> LENGTH: 83
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Construct

<400> SEQUENCE: 10

Glu Val Thr Gln Asn Asp Gly Thr Thr Thr Ile Ala Asn Asn Leu Thr
1 5 10 15

Ser Thr Val Gln Val Phe Thr Asp Ser Glu Tyr Gln Leu Pro Tyr Val
20 25 30

Leu Gly Ser Ala His Gln Gly Cys Leu Pro Pro Phe Pro Ala Asp Val
35 40 45

Phe Met Val Pro Gln Tyr Gly Tyr Leu Thr Leu Asn Asn Gly Ser Gln
50 55 60

Ala Val Gly Arg Ser Ser Phe Tyr Cys Leu Glu Tyr Phe Pro Ser Gln
65 70 75 80

Met Leu Arg

<210> SEQ ID NO 11
<211> LENGTH: 6
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Construct

<400> SEQUENCE: 11

Tyr Asn Leu Asn Gly Arg
1 5

<210> SEQ ID NO 12
<211> LENGTH: 6
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Construct

<400> SEQUENCE: 12

Tyr His Leu Asn Gly Arg
1 5

<210> SEQ ID NO 13
<211> LENGTH: 18

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<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Construct

<400> SEQUENCE: 13

Ser Ala Asn Val Asp Phe Thr Val Asp Asn Asn Gly Leu Tyr Thr Glu
1 5 10 15

Pro Arg

<210> SEQ ID NO 14
<211> LENGTH: 18
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Construct

<400> SEQUENCE: 14

Ser Val Asn Val Asp Phe Thr Val Asp Thr Asn Gly Val Tyr Ser Glu
1 5 10 15

Pro Arg

<210> SEQ ID NO 15
<211> LENGTH: 15
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Construct

<400> SEQUENCE: 15

Ala Pro Gly Lys Lys Arg Pro Val Glu His Ser Pro Val Glu Pro
1 5 10 15

<210> SEQ ID NO 16
<211> LENGTH: 735
<212> TYPE: PRT
<213> ORGANISM: Adeno-associated virus 2

<400> SEQUENCE: 16

Met Ala Ala Asp Gly Tyr Leu Pro Asp Trp Leu Glu Asp Thr Leu Ser
1 5 10 15

Glu Gly Ile Arg Gln Trp Trp Lys Leu Lys Pro Gly Pro Pro Pro Pro
20 25 30

Lys Pro Ala Glu Arg His Lys Asp Asp Ser Arg Gly Leu Val Leu Pro
35 40 45

Gly Tyr Lys Tyr Leu Gly Pro Phe Asn Gly Leu Asp Lys Gly Glu Pro
50 55 60

Val Asn Glu Ala Asp Ala Ala Ala Leu Glu His Asp Lys Ala Tyr Asp
65 70 75 80

Arg Gln Leu Asp Ser Gly Asp Asn Pro Tyr Leu Lys Tyr Asn His Ala
85 90 95

Asp Ala Glu Phe Gln Glu Arg Leu Lys Glu Asp Thr Ser Phe Gly Gly
100 105 110

Asn Leu Gly Arg Ala Val Phe Gln Ala Lys Lys Arg Val Leu Glu Pro
115 120 125

Leu Gly Leu Val Glu Glu Pro Val Lys Thr Ala Pro Gly Lys Lys Arg
130 135 140

Pro Val Glu His Ser Pro Val Glu Pro Asp Ser Ser Ser Gly Thr Gly
145 150 155 160

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Lys Ala Gly Gln Gln Pro Ala Arg Lys Arg Leu Asn Phe Gly Gln Thr
                165                 170                 175

Gly Asp Ala Asp Ser Val Pro Asp Pro Gln Pro Leu Gly Gln Pro Pro
            180                 185                 190

Ala Ala Pro Ser Gly Leu Gly Thr Asn Thr Met Ala Thr Gly Ser Gly
        195                 200                 205

Ala Pro Met Ala Asp Asn Asn Glu Gly Ala Asp Gly Val Gly Asn Ser
    210                 215                 220

Ser Gly Asn Trp His Cys Asp Ser Thr Trp Met Gly Asp Arg Val Ile
225                 230                 235                 240

Thr Thr Ser Thr Arg Thr Trp Ala Leu Pro Thr Tyr Asn Asn His Leu
                245                 250                 255

Tyr Lys Gln Ile Ser Ser Gln Ser Gly Ala Ser Asn Asp Asn His Tyr
            260                 265                 270

Phe Gly Tyr Ser Thr Pro Trp Gly Tyr Phe Asp Phe Asn Arg Phe His
        275                 280                 285

Cys His Phe Ser Pro Arg Asp Trp Gln Arg Leu Ile Asn Asn Asn Trp
    290                 295                 300

Gly Phe Arg Pro Lys Arg Leu Asn Phe Lys Leu Phe Asn Ile Gln Val
305                 310                 315                 320

Lys Glu Val Thr Gln Asn Asp Gly Thr Thr Thr Ile Ala Asn Asn Leu
                325                 330                 335

Thr Ser Thr Val Gln Val Phe Thr Asp Ser Glu Tyr Gln Leu Pro Tyr
            340                 345                 350

Val Leu Gly Ser Ala His Gln Gly Cys Leu Pro Pro Phe Pro Ala Asp
        355                 360                 365

Val Phe Met Val Pro Gln Tyr Gly Tyr Leu Thr Leu Asn Asn Gly Ser
    370                 375                 380

Gln Ala Val Gly Arg Ser Ser Phe Tyr Cys Leu Glu Tyr Phe Pro Ser
385                 390                 395                 400

Gln Met Leu Arg Thr Gly Asn Asn Phe Thr Phe Ser Tyr Thr Phe Glu
                405                 410                 415

Asp Val Pro Phe His Ser Ser Tyr Ala His Ser Gln Ser Leu Asp Arg
            420                 425                 430

Leu Met Asn Pro Leu Ile Asp Gln Tyr Leu Tyr Tyr Leu Ser Arg Thr
        435                 440                 445

Asn Thr Pro Ser Gly Thr Thr Thr Gln Ser Arg Leu Gln Phe Ser Gln
    450                 455                 460

Ala Gly Ala Ser Asp Ile Arg Asp Gln Ser Arg Asn Trp Leu Pro Gly
465                 470                 475                 480

Pro Cys Tyr Arg Gln Gln Arg Val Ser Lys Thr Ser Ala Asp Asn Asn
                485                 490                 495

Asn Ser Glu Tyr Ser Trp Thr Gly Ala Thr Lys Tyr His Leu Asn Gly
            500                 505                 510

Arg Asp Ser Leu Val Asn Pro Gly Pro Ala Met Ala Ser His Lys Asp
        515                 520                 525

Asp Glu Glu Lys Phe Phe Pro Gln Ser Gly Val Leu Ile Phe Gly Lys
    530                 535                 540

Gln Gly Ser Glu Lys Thr Asn Val Asp Ile Glu Lys Val Met Ile Thr
545                 550                 555                 560

Asp Glu Glu Glu Ile Arg Thr Thr Asn Pro Val Ala Thr Glu Gln Tyr
                565                 570                 575
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Gly Ser Val Ser Thr Asn Leu Gln Arg Gly Asn Arg Gln Ala Ala Thr
            580                 585                 590

Ala Asp Val Asn Thr Gln Gly Val Leu Pro Gly Met Val Trp Gln Asp
        595                 600                 605

Arg Asp Val Tyr Leu Gln Gly Pro Ile Trp Ala Lys Ile Pro His Thr
    610                 615                 620

Asp Gly His Phe His Pro Ser Pro Leu Met Gly Gly Phe Gly Leu Lys
625                 630                 635                 640

His Pro Pro Pro Gln Ile Leu Ile Lys Asn Thr Pro Val Pro Ala Asn
                645                 650                 655

Pro Ser Thr Thr Phe Ser Ala Ala Lys Phe Ala Ser Phe Ile Thr Gln
            660                 665                 670

Tyr Ser Thr Gly Gln Val Ser Val Glu Ile Glu Trp Glu Leu Gln Lys
        675                 680                 685

Glu Asn Ser Lys Arg Trp Asn Pro Glu Ile Gln Tyr Thr Ser Asn Tyr
    690                 695                 700

Asn Lys Ser Val Asn Val Asp Phe Thr Val Asp Thr Asn Gly Val Tyr
705                 710                 715                 720

Ser Glu Pro Arg Pro Ile Gly Thr Arg Tyr Leu Thr Arg Asn Leu
                725                 730                 735


<210> SEQ ID NO 17
<211> LENGTH: 239
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Construct

<400> SEQUENCE: 17

Met Ala Ala Asp Gly Tyr Leu Pro Asp Trp Leu Glu Asp Asn Leu Ser
1               5                   10                  15

Glu Gly Ile Arg Glu Trp Trp Asp Leu Lys Pro Gly Ala Pro Lys Pro
            20                  25                  30

Lys Ala Asn Gln Gln Lys Gln Asp Asp Gly Arg Gly Leu Val Leu Pro
        35                  40                  45

Gly Tyr Lys Tyr Leu Gly Pro Phe Asn Gly Leu Asp Lys Gly Glu Pro
    50                  55                  60

Val Asn Ala Ala Asp Ala Ala Ala Leu Glu His Asp Lys Ala Tyr Asp
65                  70                  75                  80

Gln Gln Leu Lys Ala Gly Asp Asn Pro Tyr Leu Arg Tyr Asn His Ala
                85                  90                  95

Asp Ala Glu Phe Gln Glu Arg Leu Gln Glu Asp Thr Ser Phe Gly Gly
            100                 105                 110

Asn Leu Gly Arg Ala Val Phe Gln Ala Lys Lys Arg Val Leu Glu Pro
        115                 120                 125

Leu Gly Leu Val Glu Glu Gly Ala Lys Thr Ala Pro Gly Lys Lys Arg
    130                 135                 140

Pro Val Glu Pro Ser Pro Gln Arg Ser Pro Asp Ser Ser Thr Gly Ile
145                 150                 155                 160

Gly Lys Lys Gly Gln Gln Pro Ala Lys Lys Arg Leu Asn Phe Gly Gln
                165                 170                 175

Thr Gly Asp Ser Glu Ser Val Pro Asp Pro Gln Pro Ile Gly Glu Pro
            180                 185                 190

Pro Ala Gly Pro Ser Gly Leu Gly Ser Gly Thr Met Ala Ala Gly Gly
        195                 200                 205
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Gly Ala Pro Met Ala Asp Asn Asn Glu Gly Ala Asp Gly Val Gly Ser
    210                 215                 220

Ser Ser Gly Asn Trp His Cys Asp Ser Thr Trp Leu Gly Asp Arg
225                 230                 235


<210> SEQ ID NO 18
<211> LENGTH: 239
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Construct

<400> SEQUENCE: 18

Met Ala Ala Asp Gly Tyr Leu Pro Asp Trp Leu Glu Asp Asn Leu Ser
1               5                   10                  15

Glu Gly Ile Arg Glu Trp Trp Asp Leu Lys Pro Gly Ala Pro Lys Pro
            20                  25                  30

Lys Ala Asn Gln Gln Lys Gln Asp Asp Gly Arg Gly Leu Val Leu Pro
        35                  40                  45

Gly Tyr Lys Tyr Leu Gly Pro Phe Asn Gly Leu Asp Lys Gly Glu Pro
    50                  55                  60

Val Asn Ala Ala Asp Ala Ala Ala Leu Glu His Asp Lys Ala Tyr Asp
65                  70                  75                  80

Gln Gln Leu Lys Ala Gly Asp Asn Pro Tyr Leu Arg Tyr Asn His Ala
                85                  90                  95

Asp Ala Glu Phe Gln Glu Arg Leu Gln Glu Asp Thr Ser Phe Gly Gly
            100                 105                 110

Asn Leu Gly Arg Ala Val Phe Gln Ala Lys Lys Arg Val Leu Glu Pro
        115                 120                 125

Leu Gly Leu Val Glu Glu Ala Ala Lys Thr Ala Pro Gly Lys Lys Arg
    130                 135                 140

Pro Val Glu Pro Ser Pro Gln Arg Ser Pro Asp Ser Ser Thr Gly Ile
145                 150                 155                 160

Gly Lys Lys Gly Gln Gln Pro Ala Lys Lys Arg Leu Asn Phe Gly Gln
                165                 170                 175

Thr Gly Glu Ser Glu Ser Val Pro Asp Pro Gln Pro Ile Gly Glu Pro
            180                 185                 190

Pro Ala Gly Pro Ser Gly Leu Gly Ser Gly Thr Met Ala Ala Gly Gly
        195                 200                 205

Gly Ala Pro Met Ala Asp Asn Asn Glu Gly Ala Asp Gly Val Gly Ser
    210                 215                 220

Ser Ser Gly Asn Trp His Cys Asp Ser Thr Trp Leu Gly Asp Arg
225                 230                 235


<210> SEQ ID NO 19
<211> LENGTH: 239
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Construct

<400> SEQUENCE: 19

Met Ala Ala Asp Gly Tyr Leu Pro Asp Trp Leu Glu Asp Asn Leu Ser
1               5                   10                  15

Glu Gly Ile Arg Glu Trp Trp Ala Leu Lys Pro Gly Ala Pro Lys Pro
            20                  25                  30

Lys Ala Asn Gln Gln Lys Gln Asp Asp Gly Arg Gly Leu Val Leu Pro
        35                  40                  45
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Gly Tyr Lys Tyr Leu Gly Pro Phe Asn Gly Leu Asp Lys Gly Glu Pro
    50                  55                  60

Val Asn Ala Ala Asp Ala Ala Ala Leu Glu His Asp Lys Ala Tyr Asp
65                  70                  75                  80

Gln Gln Leu Gln Ala Gly Asp Asn Pro Tyr Leu Arg Tyr Asn His Ala
                85                  90                  95

Asp Ala Glu Phe Gln Glu Arg Leu Gln Glu Asp Thr Ser Phe Gly Gly
            100                 105                 110

Asn Leu Gly Arg Ala Val Phe Gln Ala Lys Lys Arg Val Leu Glu Pro
        115                 120                 125

Leu Gly Leu Val Glu Glu Gly Ala Lys Thr Ala Pro Gly Lys Lys Arg
    130                 135                 140

Pro Val Glu Pro Ser Pro Gln Arg Ser Pro Asp Ser Ser Thr Gly Ile
145                 150                 155                 160

Gly Lys Lys Gly Gln Gln Pro Ala Arg Lys Arg Leu Asn Phe Gly Gln
                165                 170                 175

Thr Gly Asp Ser Glu Ser Val Pro Asp Pro Gln Pro Leu Gly Glu Pro
            180                 185                 190

Pro Ala Ala Pro Ser Gly Val Gly Pro Asn Thr Met Ala Ala Gly Gly
        195                 200                 205

Gly Ala Pro Met Ala Asp Asn Asn Glu Gly Ala Asp Gly Val Gly Ser
    210                 215                 220

Ser Ser Gly Asn Trp His Cys Asp Ser Thr Trp Leu Gly Asp Arg
225                 230                 235


<210> SEQ ID NO 20
<211> LENGTH: 239
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Construct

<400> SEQUENCE: 20

Met Ala Ala Asp Gly Tyr Leu Pro Asp Trp Leu Glu Asp Asn Leu Ser
1               5                   10                  15

Glu Gly Ile Arg Glu Trp Trp Asp Leu Lys Pro Gly Ala Pro Lys Pro
            20                  25                  30

Lys Ala Asn Gln Gln Lys Gln Asp Asn Gly Arg Gly Leu Val Leu Pro
        35                  40                  45

Gly Tyr Lys Tyr Leu Gly Pro Phe Asn Gly Leu Asp Lys Gly Glu Pro
    50                  55                  60

Val Asn Ala Ala Asp Ala Ala Ala Leu Glu His Asp Lys Ala Tyr Asp
65                  70                  75                  80

Gln Gln Leu Lys Ala Gly Asp Asn Pro Tyr Leu Arg Tyr Asn His Ala
                85                  90                  95

Asp Ala Glu Phe Gln Glu Arg Leu Gln Glu Asp Thr Ser Phe Gly Gly
            100                 105                 110

Asn Leu Gly Arg Ala Val Phe Gln Ala Lys Lys Arg Val Leu Glu Pro
        115                 120                 125

Leu Gly Leu Val Glu Glu Gly Ala Lys Thr Ala Pro Ala Lys Lys Arg
    130                 135                 140

Pro Val Glu Pro Ser Pro Gln Arg Ser Pro Asp Ser Ser Thr Gly Ile
145                 150                 155                 160

Gly Lys Lys Gly Gln Gln Pro Ala Arg Lys Arg Leu Asn Phe Gly Gln
                165                 170                 175
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Thr Gly Asp Ser Glu Ser Val Pro Asp Pro Gln Pro Leu Gly Glu Pro
            180                 185                 190

Pro Ala Ala Pro Ser Ser Val Gly Ser Gly Thr Val Ala Ala Gly Gly
        195                 200                 205

Gly Ala Pro Met Ala Asp Asn Asn Glu Gly Ala Asp Gly Val Gly Asn
    210                 215                 220

Ala Ser Gly Asn Trp His Cys Asp Ser Thr Trp Leu Gly Asp Arg
225                 230                 235


<210> SEQ ID NO 21
<211> LENGTH: 238
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Construct

<400> SEQUENCE: 21

Met Ala Ala Asp Gly Tyr Leu Pro Asp Trp Leu Glu Asp Asn Leu Ser
1               5                   10                  15

Glu Gly Ile Arg Glu Trp Trp Asp Leu Lys Pro Gly Ala Pro Lys Pro
            20                  25                  30

Lys Ala Asn Gln Gln Lys Gln Asp Asp Gly Arg Gly Leu Val Leu Pro
        35                  40                  45

Gly Tyr Lys Tyr Leu Gly Pro Phe Asn Gly Leu Asp Lys Gly Glu Pro
    50                  55                  60

Val Asn Ala Ala Asp Ala Ala Ala Leu Glu His Asp Lys Ala Tyr Asp
65                  70                  75                  80

Gln Gln Leu Lys Ala Gly Asp Asn Pro Tyr Leu Arg Tyr Asn His Ala
                85                  90                  95

Asp Ala Glu Phe Gln Glu Arg Leu Gln Glu Asp Thr Ser Phe Gly Gly
            100                 105                 110

Asn Leu Gly Arg Ala Val Phe Gln Ala Lys Lys Arg Val Leu Glu Pro
        115                 120                 125

Leu Gly Leu Val Glu Glu Gly Ala Lys Thr Ala Pro Gly Lys Lys Arg
    130                 135                 140

Pro Val Glu Gln Ser Pro Gln Glu Pro Asp Ser Ser Ser Gly Ile Gly
145                 150                 155                 160

Lys Thr Gly Gln Gln Pro Ala Lys Lys Arg Leu Asn Phe Gly Gln Thr
                165                 170                 175

Gly Asp Ser Glu Ser Val Pro Asp Pro Gln Pro Leu Gly Glu Pro Pro
            180                 185                 190

Ala Thr Pro Ala Ala Val Gly Pro Thr Thr Met Ala Ser Gly Gly Gly
        195                 200                 205

Ala Pro Met Ala Asp Asn Asn Glu Gly Ala Asp Gly Val Gly Asn Ala
    210                 215                 220

Ser Gly Asn Trp His Cys Asp Ser Thr Trp Leu Gly Asp Arg
225                 230                 235


<210> SEQ ID NO 22
<211> LENGTH: 238
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Construct
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<400> SEQUENCE: 22

Met Ala Ala Asp Gly Tyr Leu Pro Asp Trp Leu Glu Asp Asn Leu Ser
1 5 10 15

Glu Gly Ile Arg Glu Trp Trp Asp Leu Lys Pro Gly Ala Pro Lys Pro
20 25 30

Lys Ala Asn Gln Gln Lys Gln Asp Asp Gly Arg Gly Leu Val Leu Pro
35 40 45

Gly Tyr Lys Tyr Leu Gly Pro Phe Asn Gly Leu Asp Lys Gly Glu Pro
50 55 60

Val Asn Ala Ala Asp Ala Ala Ala Leu Glu His Asp Lys Ala Tyr Asp
65 70 75 80

Gln Gln Leu Lys Ala Gly Asp Asn Pro Tyr Leu Arg Tyr Asn His Ala
85 90 95

Asp Ala Glu Phe Gln Glu Arg Leu Gln Glu Asp Thr Ser Phe Gly Gly
100 105 110

Asn Leu Gly Arg Ala Val Phe Gln Ala Lys Lys Arg Val Leu Glu Pro
115 120 125

Phe Gly Leu Val Glu Glu Gly Ala Lys Thr Ala Pro Gly Lys Lys Arg
130 135 140

Pro Val Glu Gln Ser Pro Gln Glu Pro Asp Ser Ser Ser Gly Ile Gly
145 150 155 160

Lys Thr Gly Gln Gln Pro Ala Lys Lys Arg Leu Asn Phe Gly Gln Thr
165 170 175

Gly Asp Ser Glu Ser Val Pro Asp Pro Gln Pro Leu Gly Glu Pro Pro
180 185 190

Ala Thr Pro Ala Ala Val Gly Pro Thr Thr Met Ala Ser Gly Gly Gly
195 200 205

Ala Pro Met Ala Asp Asn Asn Glu Gly Ala Asp Gly Val Gly Asn Ala
210 215 220

Ser Gly Asn Trp His Cys Asp Ser Thr Trp Leu Gly Asp Arg
225 230 235

<210> SEQ ID NO 23
<211> LENGTH: 238
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Construct

<400> SEQUENCE: 23

Met Ala Ala Asp Gly Tyr Leu Pro Asp Trp Leu Glu Asp Thr Leu Ser
1 5 10 15

Glu Gly Ile Arg Gln Trp Trp Lys Leu Lys Pro Gly Pro Pro Pro Pro
20 25 30

Lys Pro Ala Glu Arg His Lys Asp Asp Ser Arg Gly Leu Val Leu Pro
35 40 45

Gly Tyr Lys Tyr Leu Gly Pro Phe Asn Gly Leu Asp Lys Gly Glu Pro
50 55 60

Val Asn Glu Ala Asp Ala Ala Ala Leu Glu His Asp Lys Ala Tyr Asp
65 70 75 80

Arg Gln Leu Asp Ser Gly Asp Asn Pro Tyr Leu Lys Tyr Asn His Ala
85 90 95

Asp Ala Glu Phe Gln Glu Arg Leu Lys Glu Asp Thr Ser Phe Gly Gly
100 105 110

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Asn Leu Gly Arg Ala Val Phe Gln Ala Lys Lys Arg Val Leu Glu Pro
115 120 125

Leu Gly Leu Val Glu Glu Pro Val Lys Thr Ala Pro Gly Lys Lys Arg
130 135 140

Pro Val Glu His Ser Pro Val Glu Pro Asp Ser Ser Ser Gly Thr Gly
145 150 155 160

Lys Ala Gly Gln Gln Pro Ala Arg Lys Arg Leu Asn Phe Gly Gln Thr
165 170 175

Gly Asp Ala Asp Ser Val Pro Asp Pro Gln Pro Leu Gly Gln Pro Pro
180 185 190

Ala Ala Pro Ser Gly Leu Gly Thr Asn Thr Met Ala Thr Gly Ser Gly
195 200 205

Ala Pro Met Ala Asp Asn Asn Glu Gly Ala Asp Gly Val Gly Asn Ser
210 215 220

Ser Gly Asn Trp His Cys Asp Ser Thr Trp Met Gly Asp Arg
225 230 235

<210> SEQ ID NO 24
<211> LENGTH: 238
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Construct

<400> SEQUENCE: 24

Met Ala Ala Asp Gly Tyr Leu Pro Asp Trp Leu Glu Asp Asn Leu Ser
1 5 10 15

Glu Gly Ile Arg Glu Trp Trp Ala Leu Lys Pro Gly Val Pro Gln Pro
20 25 30

Lys Ala Asn Gln Gln His Gln Asp Asn Arg Arg Gly Leu Val Leu Pro
35 40 45

Gly Tyr Lys Tyr Leu Gly Pro Gly Asn Gly Leu Asp Lys Gly Glu Pro
50 55 60

Val Asn Glu Ala Asp Ala Ala Ala Leu Glu His Asp Lys Ala Tyr Asp
65 70 75 80

Gln Gln Leu Lys Ala Gly Asp Asn Pro Tyr Leu Lys Tyr Asn His Ala
85 90 95

Asp Ala Glu Phe Gln Glu Arg Leu Gln Glu Asp Thr Ser Phe Gly Gly
100 105 110

Asn Leu Gly Arg Ala Val Phe Gln Ala Lys Lys Arg Ile Leu Glu Pro
115 120 125

Leu Gly Leu Val Glu Glu Ala Ala Lys Thr Ala Pro Gly Lys Lys Gly
130 135 140

Ala Val Asp Gln Ser Pro Gln Glu Pro Asp Ser Ser Ser Gly Val Gly
145 150 155 160

Lys Ser Gly Lys Gln Pro Ala Arg Lys Arg Leu Asn Phe Gly Gln Thr
165 170 175

Gly Asp Ser Glu Ser Val Pro Asp Pro Gln Pro Leu Gly Glu Pro Pro
180 185 190

Ala Ala Pro Thr Ser Leu Gly Ser Asn Thr Met Ala Ser Gly Gly Gly
195 200 205

Ala Pro Met Ala Asp Asn Asn Glu Gly Ala Asp Gly Val Gly Asn Ser
210 215 220

Ser Gly Asn Trp His Cys Asp Ser Gln Trp Leu Gly Asp Arg
225 230 235

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<210> SEQ ID NO 25
<211> LENGTH: 232
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Construct

<400> SEQUENCE: 25

Met Ala Ala Asp Gly Tyr Leu Pro Asp Trp Leu Glu Asp Asn Leu Ser
1 5 10 15

Glu Gly Ile Arg Glu Trp Trp Asp Leu Lys Pro Gly Ala Pro Lys Pro
20 25 30

Lys Ala Asn Gln Gln Lys Gln Asp Asp Gly Arg Gly Leu Val Leu Pro
35 40 45

Gly Tyr Lys Tyr Leu Gly Pro Phe Asn Gly Leu Asp Lys Gly Glu Pro
50 55 60

Val Asn Ala Ala Asp Ala Ala Ala Leu Glu His Asp Lys Ala Tyr Asp
65 70 75 80

Gln Gln Leu Lys Ala Gly Asp Asn Pro Tyr Leu Arg Tyr Asn His Ala
85 90 95

Asp Ala Glu Phe Gln Glu Arg Leu Gln Glu Asp Thr Ser Phe Gly Gly
100 105 110

Asn Leu Gly Arg Ala Val Phe Gln Ala Lys Lys Arg Val Leu Glu Pro
115 120 125

Leu Gly Leu Val Glu Glu Gly Ala Lys Thr Ala Pro Gly Lys Lys Arg
130 135 140

Pro Leu Glu Ser Pro Gln Glu Pro Asp Ser Ser Ser Gly Ile Gly Lys
145 150 155 160

Lys Gly Lys Gln Pro Ala Arg Lys Arg Leu Asn Phe Glu Glu Asp Thr
165 170 175

Gly Ala Gly Asp Gly Pro Pro Glu Gly Ser Asp Thr Ser Ala Met Ser
180 185 190

Ser Asp Ile Glu Met Arg Ala Ala Pro Gly Gly Asn Ala Val Asp Ala
195 200 205

Gly Gln Gly Ser Asp Gly Val Gly Asn Ala Ser Gly Asp Trp His Cys
210 215 220

Asp Ser Thr Trp Ser Glu Gly Lys
225 230

<210> SEQ ID NO 26
<211> LENGTH: 241
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Construct

<400> SEQUENCE: 26

Met Ala Ala Asp Gly Tyr Leu Pro Asp Trp Leu Glu Asp Asn Leu Ser
1 5 10 15

Glu Gly Ile Arg Glu Trp Trp Ala Leu Lys Pro Gly Ala Pro Gln Pro
20 25 30

Lys Ala Asn Gln Gln His Gln Asp Asn Gly Arg Gly Leu Val Leu Pro
35 40 45

Gly Tyr Lys Tyr Leu Gly Pro Phe Asn Gly Leu Asp Lys Gly Glu Pro
50 55 60

Val Asn Glu Ala Asp Ala Ala Ala Leu Glu His Asp Lys Ala Tyr Asp
65 70 75 80

-continued

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Lys Gln Leu Glu Gln Gly Asp Asn Pro Tyr Leu Lys Tyr Asn His Ala
                85                  90                  95

Asp Ala Glu Phe Gln Gln Arg Leu Ala Thr Asp Thr Ser Phe Gly Gly
            100                 105                 110

Asn Leu Gly Arg Ala Val Phe Gln Ala Lys Lys Arg Ile Leu Glu Pro
        115                 120                 125

Leu Gly Leu Val Glu Glu Gly Val Lys Thr Ala Pro Gly Lys Lys Arg
    130                 135                 140

Pro Leu Glu Lys Thr Pro Asn Arg Pro Thr Asn Pro Asp Ser Gly Lys
145                 150                 155                 160

Ala Pro Ala Lys Lys Lys Gln Lys Asp Gly Glu Pro Ala Asp Ser Ala
                165                 170                 175

Arg Arg Thr Leu Asp Phe Glu Asp Ser Gly Ala Gly Asp Gly Pro Pro
            180                 185                 190

Glu Gly Ser Ser Ser Gly Glu Met Ser His Asp Ala Glu Met Arg Ala
        195                 200                 205

Ala Pro Gly Gly Asn Ala Val Glu Ala Gly Gln Gly Ala Asp Gly Val
    210                 215                 220

Gly Asn Ala Ser Gly Asp Trp His Cys Asp Ser Thr Trp Ser Glu Gly
225                 230                 235                 240

Arg
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<210> SEQ ID NO 27
<211> LENGTH: 232
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Construct

<400> SEQUENCE: 27

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Met Thr Asp Gly Tyr Leu Pro Asp Trp Leu Glu Asp Asn Leu Ser Glu
1               5                   10                  15

Gly Val Arg Glu Trp Trp Ala Leu Gln Pro Gly Ala Pro Lys Pro Lys
            20                  25                  30

Ala Asn Gln Gln His Gln Asp Asn Ala Arg Gly Leu Val Leu Pro Gly
        35                  40                  45

Tyr Lys Tyr Leu Gly Pro Gly Asn Gly Leu Asp Lys Gly Glu Pro Val
    50                  55                  60

Asn Ala Ala Asp Ala Ala Ala Leu Glu His Asp Lys Ala Tyr Asp Gln
65                  70                  75                  80

Gln Leu Lys Ala Gly Asp Asn Pro Tyr Leu Lys Tyr Asn His Ala Asp
                85                  90                  95

Ala Glu Phe Gln Gln Arg Leu Gln Gly Asp Thr Ser Phe Gly Gly Asn
            100                 105                 110

Leu Gly Arg Ala Val Phe Gln Ala Lys Lys Arg Val Leu Glu Pro Leu
        115                 120                 125

Gly Leu Val Glu Gln Ala Gly Glu Thr Ala Pro Gly Lys Lys Arg Pro
    130                 135                 140

Leu Ile Glu Ser Pro Gln Gln Pro Asp Ser Ser Thr Gly Ile Gly Lys
145                 150                 155                 160

Lys Gly Lys Gln Pro Ala Lys Lys Lys Leu Val Phe Glu Asp Glu Thr
                165                 170                 175

Gly Ala Gly Asp Gly Pro Pro Glu Gly Ser Thr Ser Gly Ala Met Ser
            180                 185                 190
```

-continued

Asp Asp Ser Glu Met Arg Ala Ala Ala Gly Gly Ala Ala Val Glu Gly
195 200 205

Gly Gln Gly Ala Asp Gly Val Gly Asn Ala Ser Gly Asp Trp His Cys
210 215 220

Asp Ser Thr Trp Ser Glu Gly His
225 230

<210> SEQ ID NO 28
<211> LENGTH: 228
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Construct

<400> SEQUENCE: 28

Met Ser Phe Val Asp His Pro Pro Asp Trp Leu Glu Glu Val Gly Glu
1 5 10 15

Gly Leu Arg Glu Phe Leu Gly Leu Glu Ala Gly Pro Pro Lys Pro Lys
20 25 30

Pro Asn Gln Gln His Gln Asp Gln Ala Arg Gly Leu Val Leu Pro Gly
35 40 45

Tyr Asn Tyr Leu Gly Pro Gly Asn Gly Leu Asp Arg Gly Glu Pro Val
50 55 60

Asn Arg Ala Asp Glu Val Ala Arg Glu His Asp Ile Ser Tyr Asn Glu
65 70 75 80

Gln Leu Glu Ala Gly Asp Asn Pro Tyr Leu Lys Tyr Asn His Ala Asp
85 90 95

Ala Glu Phe Gln Glu Lys Leu Ala Asp Asp Thr Ser Phe Gly Gly Asn
100 105 110

Leu Gly Lys Ala Val Phe Gln Ala Lys Lys Arg Val Leu Glu Pro Phe
115 120 125

Gly Leu Val Glu Glu Gly Ala Lys Thr Ala Pro Thr Gly Lys Arg Ile
130 135 140

Asp Asp His Phe Pro Lys Arg Lys Lys Ala Arg Thr Glu Glu Asp Ser
145 150 155 160

Lys Pro Ser Thr Ser Ser Asp Ala Glu Ala Gly Pro Ser Gly Ser Gln
165 170 175

Gln Leu Gln Ile Pro Ala Gln Pro Ala Ser Ser Leu Gly Ala Asp Thr
180 185 190

Met Ser Ala Gly Gly Gly Gly Pro Leu Gly Asp Asn Asn Gln Gly Ala
195 200 205

Asp Gly Val Gly Asn Ala Ser Gly Asp Trp His Cys Asp Ser Thr Trp
210 215 220

Met Gly Asp Arg
225

<210> SEQ ID NO 29
<211> LENGTH: 238
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Construct

<400> SEQUENCE: 29

Met Ala Ala Asp Gly Tyr Leu Pro Asp Trp Leu Glu Asp Asn Leu Ser
1 5 10 15

Glu Gly Ile Arg Glu Trp Trp Ala Leu Lys Pro Gly Ala Pro Gln Pro
20 25 30

-continued

```
Lys Ala Asn Gln Gln His Gln Asp Asn Ala Arg Gly Leu Val Leu Pro
        35                  40                  45

Gly Tyr Lys Tyr Leu Gly Pro Gly Asn Gly Leu Asp Lys Gly Glu Pro
    50                  55                  60

Val Asn Ala Ala Asp Ala Ala Ala Leu Glu His Asp Lys Ala Tyr Asp
65                  70                  75                  80

Gln Gln Leu Lys Ala Gly Asp Asn Pro Tyr Leu Lys Tyr Asn His Ala
                85                  90                  95

Asp Ala Glu Phe Gln Glu Arg Leu Lys Glu Asp Thr Ser Phe Gly Gly
            100                 105                 110

Asn Leu Gly Arg Ala Val Phe Gln Ala Lys Lys Arg Leu Leu Glu Pro
        115                 120                 125

Leu Gly Leu Val Glu Glu Ala Ala Lys Thr Ala Pro Gly Lys Lys Arg
    130                 135                 140

Pro Val Glu Gln Ser Pro Gln Glu Pro Asp Ser Ser Ala Gly Ile Gly
145                 150                 155                 160

Lys Ser Gly Ala Gln Pro Ala Lys Lys Arg Leu Asn Phe Gly Gln Thr
                165                 170                 175

Gly Asp Thr Glu Ser Val Pro Asp Pro Gln Pro Ile Gly Glu Pro Pro
            180                 185                 190

Ala Ala Pro Ser Gly Val Gly Ser Leu Thr Met Ala Ser Gly Gly Gly
        195                 200                 205

Ala Pro Val Ala Asp Asn Asn Glu Gly Ala Asp Gly Val Gly Ser Ser
    210                 215                 220

Ser Gly Asn Trp His Cys Asp Ser Gln Trp Leu Gly Asp Arg
225                 230                 235


<210> SEQ ID NO 30
<211> LENGTH: 246
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Construct
<220> FEATURE:
<221> NAME/KEY: VARIANT
<222> LOCATION: 24, 148, 151, 152, 154, 155, 168, 169, 170, 171, 172,
      208, 209
<223> OTHER INFORMATION: Xaa = Any Amino Acid

<400> SEQUENCE: 30

Met Ala Ala Asp Gly Tyr Leu Pro Asp Trp Leu Glu Asp Asn Leu Ser
1               5                   10                  15

Glu Gly Ile Arg Glu Trp Trp Xaa Leu Lys Pro Gly Ala Pro Lys Pro
            20                  25                  30

Lys Ala Asn Gln Gln Lys Gln Asp Asp Gly Arg Gly Leu Val Leu Pro
        35                  40                  45

Gly Tyr Lys Tyr Leu Gly Pro Phe Asn Gly Leu Asp Lys Gly Glu Pro
    50                  55                  60

Val Asn Ala Ala Asp Ala Ala Ala Leu Glu His Asp Lys Ala Tyr Asp
65                  70                  75                  80

Gln Gln Leu Lys Ala Gly Asp Asn Pro Tyr Leu Arg Tyr Asn His Ala
                85                  90                  95

Asp Ala Glu Phe Gln Glu Arg Leu Gln Glu Asp Thr Ser Phe Gly Gly
            100                 105                 110

Asn Leu Gly Arg Ala Val Phe Gln Ala Lys Lys Arg Val Leu Glu Pro
        115                 120                 125
```

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Leu Gly Leu Val Glu Glu Gly Ala Lys Thr Ala Pro Gly Lys Lys Arg
130 135 140

Pro Val Glu Xaa Ser Pro Xaa Xaa Gln Xaa Xaa Pro Asp Ser Ser Ser
145 150 155 160

Gly Ile Gly Lys Lys Gly Gln Xaa Xaa Xaa Xaa Xaa Gln Pro Ala Lys
165 170 175

Lys Arg Leu Asn Phe Gly Gln Thr Gly Asp Ser Glu Ser Val Pro Asp
180 185 190

Pro Gln Pro Leu Gly Glu Pro Pro Ala Ala Pro Ser Gly Leu Gly Xaa
195 200 205

Xaa Thr Met Ala Ala Gly Gly Gly Ala Pro Met Ala Asp Asn Asn Glu
210 215 220

Gly Ala Asp Gly Val Gly Asn Ala Ser Gly Asn Trp His Cys Asp Ser
225 230 235 240

Thr Trp Leu Gly Asp Arg
245

<210> SEQ ID NO 31
<211> LENGTH: 61
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Construct

<400> SEQUENCE: 31

Met Ala Ala Asp Gly Tyr Leu Pro Asp Trp Leu Glu Asp Asn Leu Ser
1 5 10 15

Glu Gly Ile Arg Glu Trp Trp Asp Leu Lys Pro Gly Ala Pro Lys Pro
20 25 30

Lys Ala Asn Gln Gln Lys Gln Asp Asp Gly Arg Gly Leu Val Leu Pro
35 40 45

Gly Tyr Lys Tyr Leu Gly Pro Phe Asn Gly Leu Asp Lys
50 55 60

<210> SEQ ID NO 32
<211> LENGTH: 61
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Construct

<400> SEQUENCE: 32

Met Ala Ala Asp Gly Tyr Leu Pro Asp Trp Leu Glu Asp Asn Leu Ser
1 5 10 15

Glu Gly Ile Arg Glu Trp Trp Ala Leu Lys Pro Gly Ala Pro Lys Pro
20 25 30

Lys Ala Asn Gln Gln Lys Gln Asp Asp Gly Arg Gly Leu Val Leu Pro
35 40 45

Gly Tyr Lys Tyr Leu Gly Pro Phe Asn Gly Leu Asp Lys
50 55 60

<210> SEQ ID NO 33
<211> LENGTH: 61
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Construct

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<400> SEQUENCE: 33

Met Ala Ala Asp Gly Tyr Leu Pro Asp Trp Leu Glu Asp Asn Leu Ser
1 5 10 15

Glu Gly Ile Arg Glu Trp Trp Asp Leu Lys Pro Gly Ala Pro Lys Pro
20 25 30

Lys Ala Asn Gln Gln Lys Gln Asp Asn Gly Arg Gly Leu Val Leu Pro
35 40 45

Gly Tyr Lys Tyr Leu Gly Pro Phe Asn Gly Leu Asp Lys
50 55 60

<210> SEQ ID NO 34
<211> LENGTH: 61
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Construct

<400> SEQUENCE: 34

Met Ala Ala Asp Gly Tyr Leu Pro Asp Trp Leu Glu Asp Thr Leu Ser
1 5 10 15

Glu Gly Ile Arg Gln Trp Trp Lys Leu Lys Pro Gly Pro Pro Pro Pro
20 25 30

Lys Pro Ala Glu Arg His Lys Asp Asp Ser Arg Gly Leu Val Leu Pro
35 40 45

Gly Tyr Lys Tyr Leu Gly Pro Phe Asn Gly Leu Asp Lys
50 55 60

<210> SEQ ID NO 35
<211> LENGTH: 61
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Construct

<400> SEQUENCE: 35

Met Ala Ala Asp Gly Tyr Leu Pro Asp Trp Leu Glu Asp Asn Leu Ser
1 5 10 15

Glu Gly Ile Arg Glu Trp Trp Ala Leu Lys Pro Gly Val Pro Gln Pro
20 25 30

Lys Ala Asn Gln Gln His Gln Asp Asn Arg Arg Gly Leu Val Leu Pro
35 40 45

Gly Tyr Lys Tyr Leu Gly Pro Gly Asn Gly Leu Asp Lys
50 55 60

<210> SEQ ID NO 36
<211> LENGTH: 61
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Construct

<400> SEQUENCE: 36

Met Ala Ala Asp Gly Tyr Leu Pro Asp Trp Leu Glu Asp Asn Leu Ser
1 5 10 15

Glu Gly Ile Arg Glu Trp Trp Ala Leu Lys Pro Gly Ala Pro Gln Pro
20 25 30

Lys Lys Ala Asn Gln Gln His Gln Asp Asn Gly Arg Gly Leu Val Leu
35 40 45

-continued

Pro Gly Lys Tyr Leu Gly Pro Phe Asn Gly Leu Asp Lys
50 55 60

<210> SEQ ID NO 37
<211> LENGTH: 60
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Construct

<400> SEQUENCE: 37

Met Thr Asp Gly Tyr Leu Pro Asp Trp Leu Glu Asp Asn Leu Ser Glu
1 5 10 15

Gly Val Arg Glu Trp Trp Ala Leu Gln Pro Gly Ala Pro Lys Pro Lys
20 25 30

Ala Asn Gln Gln His Gln Asp Asn Ala Arg Gly Leu Val Leu Pro Gly
35 40 45

Tyr Lys Tyr Leu Gly Pro Gly Asn Gly Leu Asp Lys
50 55 60

<210> SEQ ID NO 38
<211> LENGTH: 60
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Construct

<400> SEQUENCE: 38

Met Ser Phe Val Asp His Pro Pro Asp Trp Leu Glu Glu Val Gly Glu
1 5 10 15

Gly Leu Arg Glu Phe Leu Gly Leu Glu Ala Gly Pro Pro Lys Pro Lys
20 25 30

Pro Asn Gln Gln His Gln Asp Gln Ala Arg Gly Leu Val Leu Pro Gly
35 40 45

Tyr Asn Tyr Leu Gly Pro Gly Asn Gly Leu Asp Arg
50 55 60

<210> SEQ ID NO 39
<211> LENGTH: 61
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
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<400> SEQUENCE: 39

Met Ala Ala Asp Gly Tyr Leu Pro Asp Trp Leu Glu Asp Asn Leu Ser
1 5 10 15

Glu Gly Ile Arg Glu Trp Trp Ala Leu Lys Pro Gly Ala Pro Gln Pro
20 25 30

Lys Ala Asn Gln Gln His Gln Asp Asn Ala Arg Gly Leu Val Leu Pro
35 40 45

Gly Tyr Lys Tyr Leu Gly Pro Gly Asn Gly Leu Asp Lys
50 55 60

<210> SEQ ID NO 40
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<212> TYPE: PRT
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Construct
<220> FEATURE:
<221> NAME/KEY: VARIANT

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<222> LOCATION: 24
<223> OTHER INFORMATION: Xaa = Any Amino Acid

<400> SEQUENCE: 40

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Met Ala Ala Asp Gly Tyr Leu Pro Asp Trp Leu Glu Asp Asn Leu Ser
1               5                  10                  15

Glu Gly Ile Arg Glu Trp Trp Xaa Leu Lys Pro Gly Ala Pro Lys Pro
            20                  25                  30

Lys Ala Asn Gln Gln Lys Gln Asp Asp Gly Arg Gly Leu Val Leu Pro
        35                  40                  45

Gly Tyr Lys Tyr Leu Gly Pro Phe Asn Gly Leu Asp Lys
    50                  55                  60
```

What is claimed is:

1. A method to determine the serotype of an adeno-associated virus (AAV) particle comprising

a) denaturing the AAV particle,

b) directly subjecting the denatured AAV particle to liquid chromatography/mass spectrometry (LC/MS) intact protein analysis, and

c) determining the masses of VP1, VP2 and VP3 of the AAV particle;

wherein the specific combination of masses of VP1, VP2 and VP3 are indicative of the AAV serotype,

and wherein the method is performed in the absence of a gel separation step.

2. A method to determine the serotype of a viral particle comprising

a) denaturing the viral particle,

b) directly subjecting the denatured viral particle to liquid chromatography/mass spectrometry (LC/MS) intact protein analysis, and

c) determining the masses of one or more capsid proteins of the viral particle;

wherein the specific combination of masses of the one or more capsid proteins are indicative of the virus serotype,

and wherein the method is performed in the absence of a gel separation step.

3. The method of claim **1**, wherein the calculated masses of VP1, VP2 and VP3 are compared to the theoretical masses of VP1, VP2 and VP3 of one or more AAV serotypes.

4. The method of claim **1**, wherein the AAV particle is denatured with acetic acid, guanidine hydrochloride, and/or an organic solvent.

5. The method of claim **1**, wherein the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography.

6. The method of claim **1**, wherein the liquid chromatography is reverse phase chromatography.

7. The method of claim **6**, wherein the reverse phase chromatography is a C4 or C8 reverse chromatography.

8. The method of claim **1**, wherein the liquid chromatography is ultra-performance liquid chromatography (UPLC).

9. The method of claim **1**, wherein the mass spectrometry comprises assisted calibration.

10. The method of claim **9**, wherein sodium iodide is used as a calibrant.

11. The method of claim **1**, wherein the AAV particle is a recombinant AAV (rAAV) particle.

12. The method of claim **1**, wherein the AAV particle comprises an AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an AAV LK03 capsid, an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid, an AAV DJ8 capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV/human bocavirus virus 1), an AAV2HBKO capsid, an AAVPHP.B capsid or an AAVPHP.eB capsid.

13. The method of claim **2**, wherein the calculated masses of the one or more capsid proteins are compared to the theoretical masses of the one or more capsid proteins of one or more virus serotypes.

14. The method of claim **2**, wherein the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography.

15. The method of claim **2**, wherein the liquid chromatography is reverse phase chromatography.

16. The method of claim **15**, wherein the reverse phase chromatography is a C4 or C8 reverse chromatography.

17. The method of claim **2**, wherein the liquid chromatography is ultra-performance liquid chromatography (UPLC).

18. The method of claim **2**, wherein the mass spectrometry comprises assisted calibration.

19. The method of claim **18**, wherein sodium iodide is used as a calibrant.

20. The method of claim **2**, wherein the viral particle comprises a viral vector encoding a heterologous transgene.

* * * * *

Exhibit R

US012123880B2

(12) United States Patent

Jin et al.

(10) Patent No.: US 12,123,880 B2
(45) Date of Patent: *Oct. 22, 2024

(54) **METHODS FOR DETECTING AAV**

(71) Applicant: **Genzyme Corporation**, Cambridge, MA (US)

(72) Inventors: **Xiaoying Jin**, Cambridge, MA (US); **Catherine O'Riordan**, Cambridge, MA (US); **Lin Liu**, Cambridge, MA (US); **Kate Zhang**, Cambridge, MA (US)

(73) Assignee: **Genzyme Corporation**, Cambridge, MA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **18/321,542**

(22) Filed: **May 22, 2023**

(65) **Prior Publication Data**

US 2024/0044910 A1 Feb. 8, 2024

Related U.S. Application Data

(62) Division of application No. 16/325,653, filed as application No. PCT/US2017/046814 on Aug. 14, 2017, now Pat. No. 11,698,377.
(Continued)

(51) **Int. Cl.**
G01N 33/68 (2006.01)
C12N 15/86 (2006.01)
(Continued)

(52) **U.S. Cl.**
CPC ***G01N 33/6848*** (2013.01); ***C12N 15/86*** (2013.01); ***G01N 30/72*** (2013.01);
(Continued)

(58) **Field of Classification Search**
None
See application file for complete search history.

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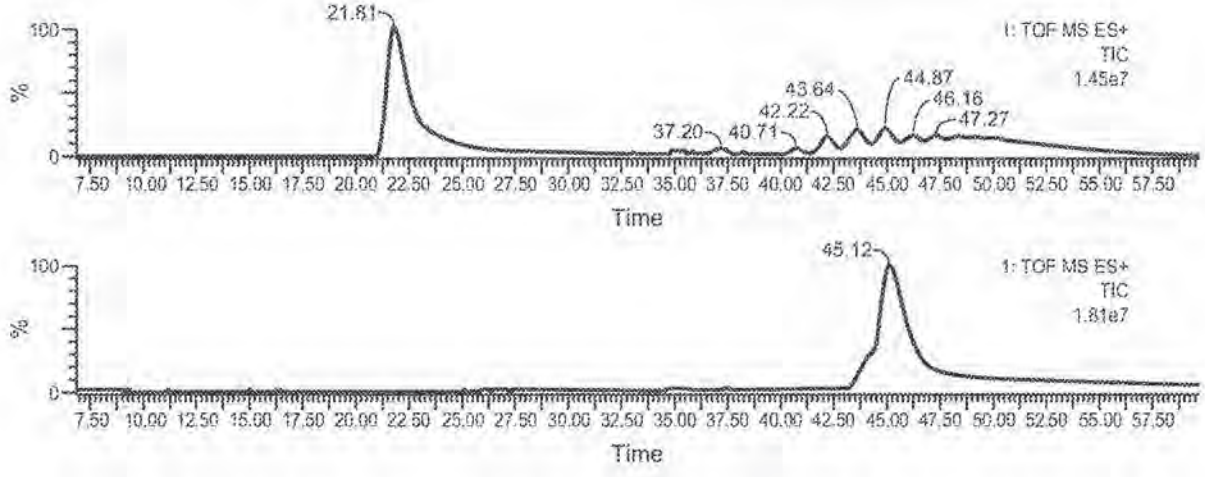
Primary Examiner — Xiaoyun R Xu
(74) *Attorney, Agent, or Firm* — MORRISON & FOERSTER LLP

(57) **ABSTRACT**

Provided herein are methods for determining the serotype of a virus particle and/or or determining the heterogeneity of a virus particle (e.g., an AAV particle). In other embodiments, the invention provides methods to determine the heterogeneity of AAV particles. In some aspects, the invention provides viral particles (e.g., rAAV particles) with improved stability and/or improved transduction efficiency by increasing the acetylation and/or deamidation of capsid proteins.

21 Claims, 27 Drawing Sheets

Specification includes a Sequence Listing.



Related U.S. Application Data

(60) Provisional application No. 62/375,314, filed on Aug. 15, 2016.

(51) **Int. Cl.**
G01N 30/72 (2006.01)
G01N 30/02 (2006.01)

(52) **U.S. Cl.**
CPC *C12N 2750/14123* (2013.01); *C12N 2750/14143* (2013.01); *G01N 2030/027* (2013.01)

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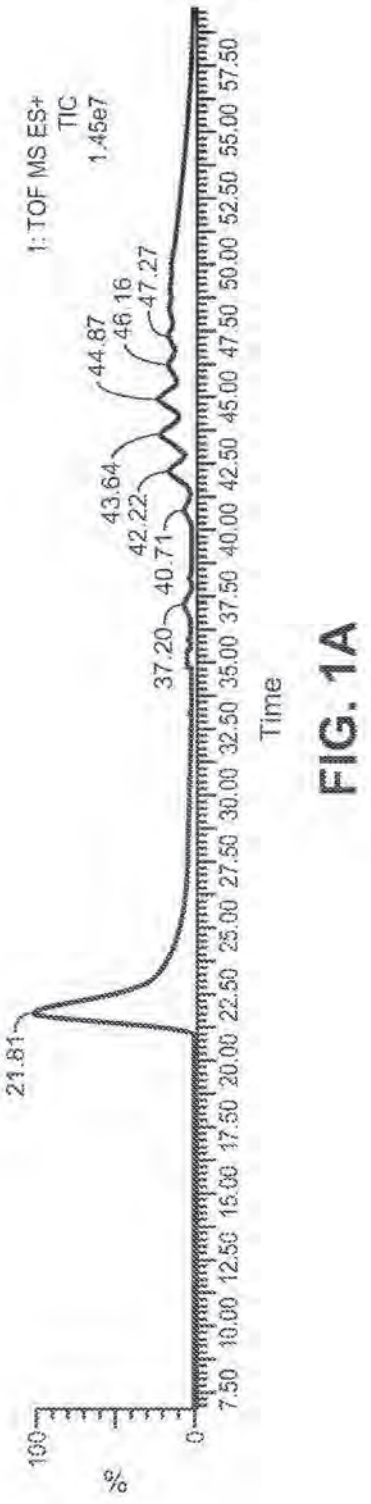

1: TOF MS ES+
TIC
1.45e7
21.81
37.20
40.71
42.22
43.64
44.87
46.16
47.27
Time
%
100
0
7.50 10.00 12.50 15.00 17.50 20.00 22.50 25.00 27.50 30.00 32.50 35.00 37.50 40.00 42.50 45.00 47.50 50.00 52.50 55.00 57.50

FIG. 1A

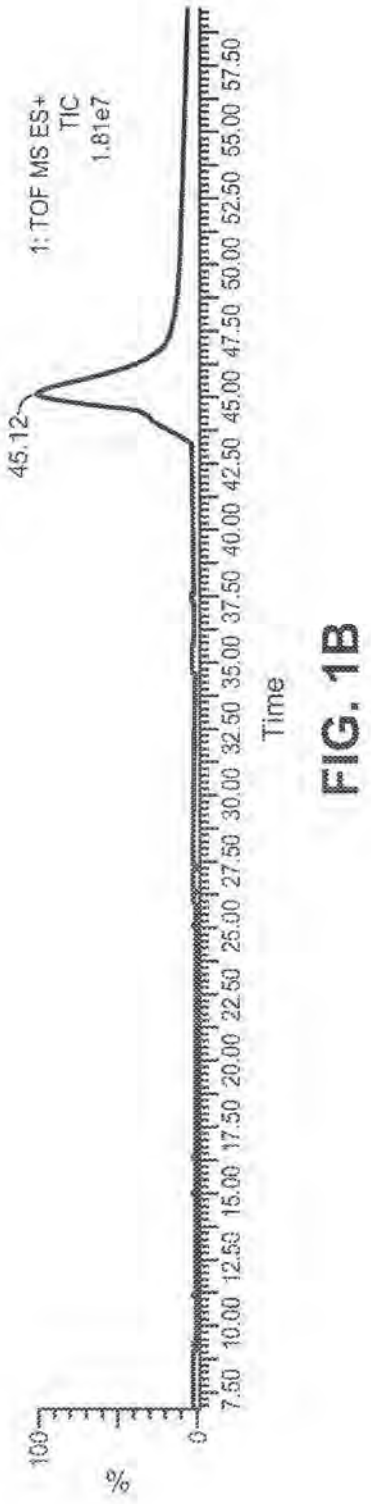
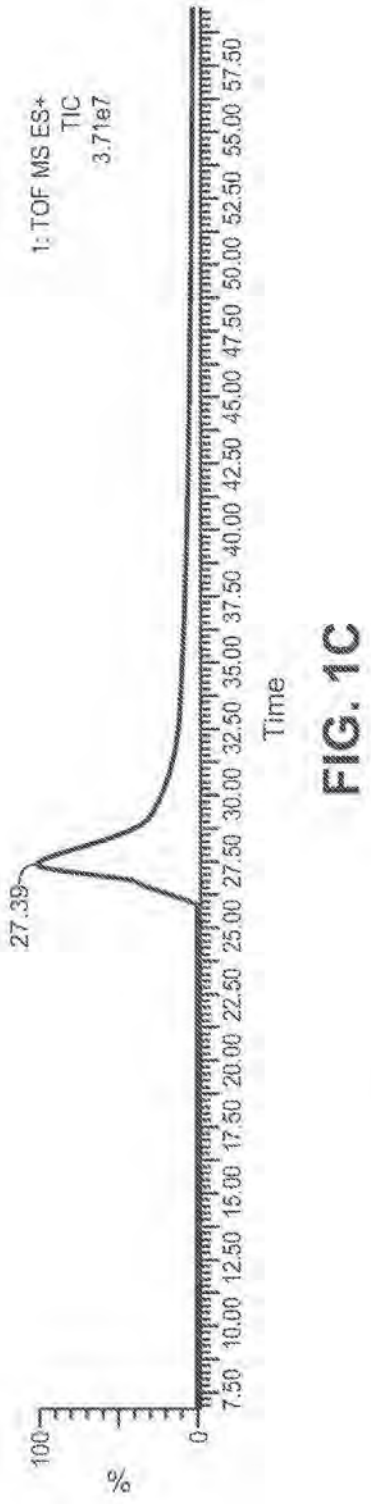

1: TOF MS ES+
TIC
1.81e7
45.12
Time
%
100
0
7.50 10.00 12.50 15.00 17.50 20.00 22.50 25.00 27.50 30.00 32.50 35.00 37.50 40.00 42.50 45.00 47.50 50.00 52.50 55.00 57.50

FIG. 1B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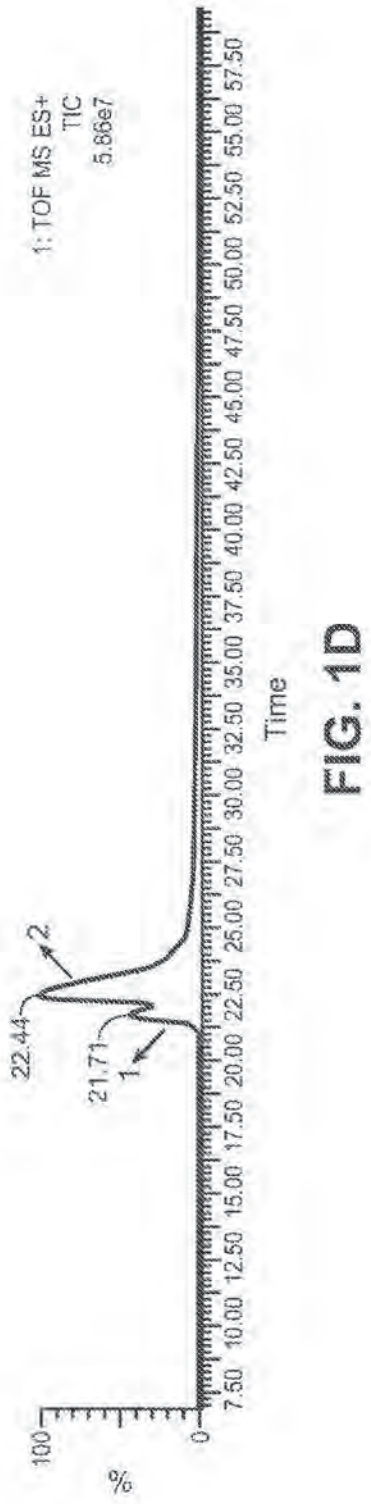
TIC
3.71e7
27.39
100
%
0
7.50 10.00 12.50 15.00 17.50 20.00 22.50 25.00 27.50 30.00 32.50 35.00 37.50 40.00 42.50 45.00 47.50 50.00 52.50 55.00 57.50
Time

FIG. 1C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TIC
5.86e7
22.44
2
21.71
1
100
%
0
7.50 10.00 12.50 15.00 17.50 20.00 22.50 25.00 27.50 30.00 32.50 35.00 37.50 40.00 42.50 45.00 47.50 50.00 52.50 55.00 57.50
Time

FIG. 1D

FIG. 2A

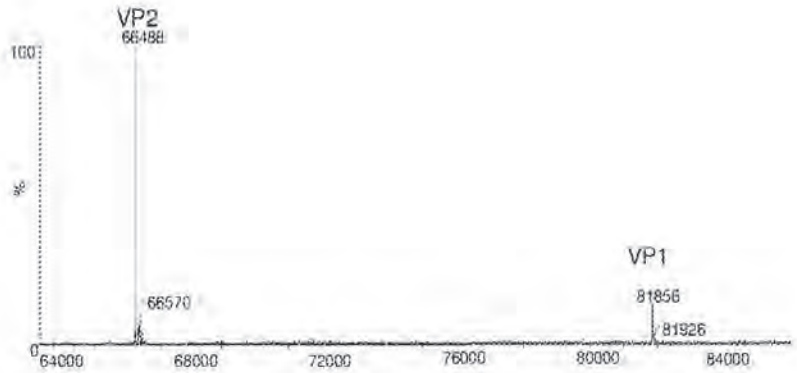
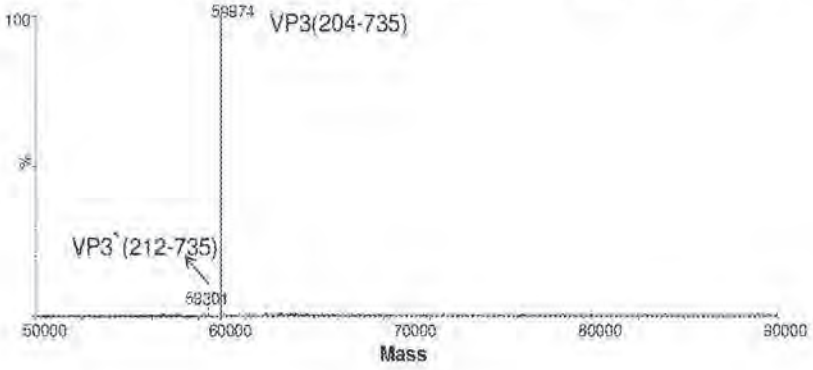

VP2
66488
100
%
VP1
66570
81856
81926
0
64000
68000
72000
76000
80000
84000

FIG. 2B


100
59874
VP3(204-735)
%
VP3`(212-735)
58301
50000
60000
70000
80000
90000
Mass

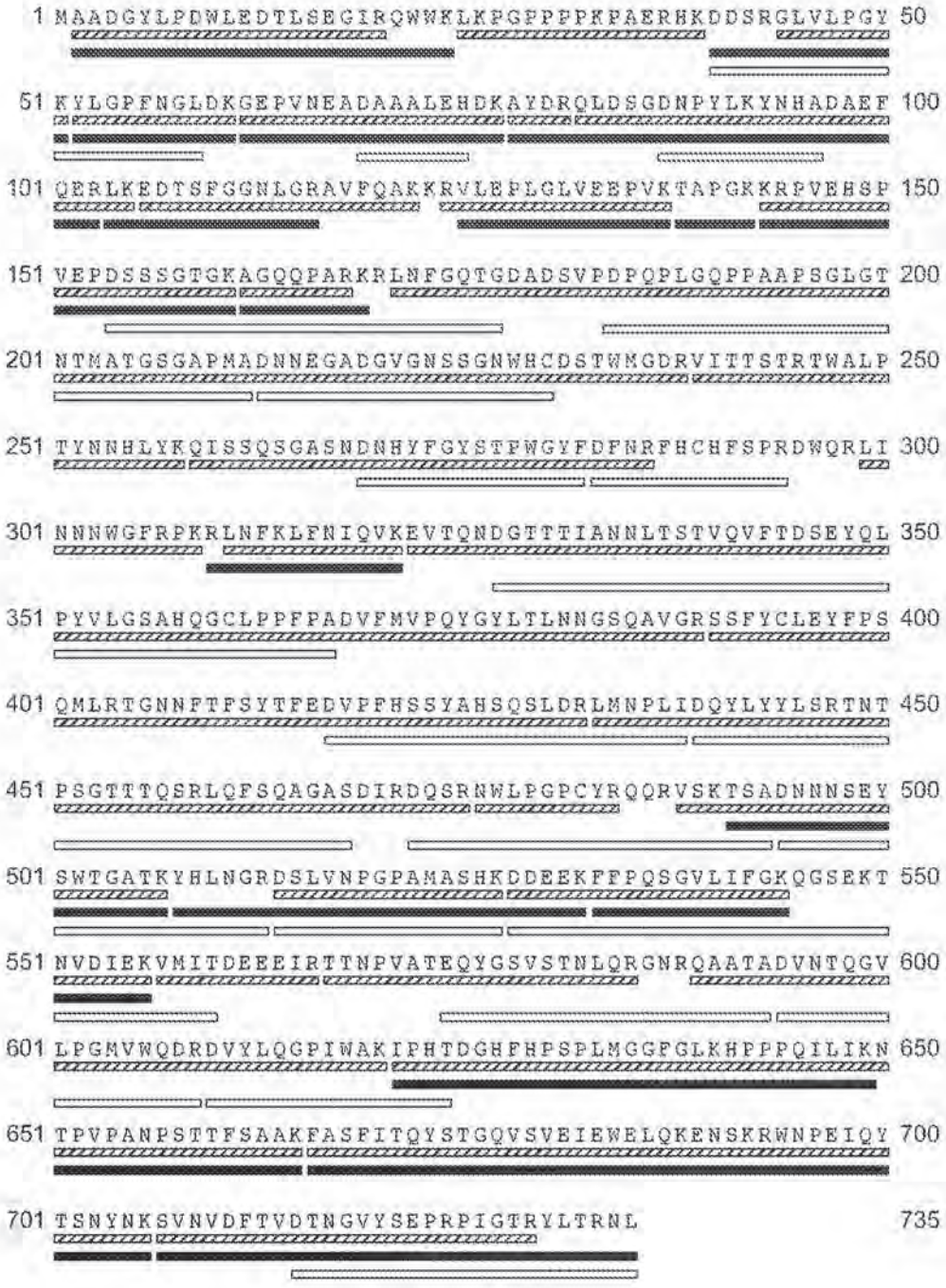

1 MAADGYLPDWLEDTLSEGIRQWWKLKPGPPPPKPAERHKDDSRGLVLPGY 50
51 KYLGPFNGLDKGEPVNEADAAALEHDKAYDRQLDSGDNPYLKYNHADAEF 100
101 QERLKEDTSFGGNLGRAVFQAKKRVLEPLGLVEEPVKTAPGKKRPVEHSP 150
151 VEPDSSSGTGKAGQQPARKRLNFGQTGDADSVPDPQPLGQPPAAPSGLGT 200
201 NTMATGSGAPMADNNEGADGVGNSSGNWHCDSTWMGDRVITTSTRTWALP 250
251 TYNNHLYKQISSQSGASNDNHYFGYSTPWGYFDFNRFHCHFSPRDWQRLI 300
301 NNNWGFRPKRLNFKLFNIQVKEVTQNDGTTTIANNLTSTVQVFTDSEYQL 350
351 PYVLGSAHQGCLPPFPADVFMVPQYGYLTLNNGSQAVGRSSFYCLEYFPS 400
401 QMLRTGNNFTFSYTFEDVPFHSSYAHSQSLDRLMNPLIDQYLYYLSRTNT 450
451 PSGTTTQSRLQFSQAGASDIRDQSRNWLPGPCYRQQRVSKTSADNNNSEY 500
501 SWTGATKYHLNGRDSLVNPGPAMASHKDDEEKFFPQSGVLIFGKQGSEKT 550
551 NVDIEKVMITDEEEIRTTNPVATEQYGSVSTNLQRGNRQAATADVNTQGV 600
601 LPGMVWQDRDVYLQGPIWAKIPHTDGHFHPSPLMGGFGLKHPPPQILIKN 650
651 TPVPANPSTTFSAAKFASFITQYSTGQVSVEIEWELQKENSKRWNPEIQY 700
701 TSNYNKSVNVDFTVDTNGVYSEPRPIGTRYLTRNL 735

FIG. 3

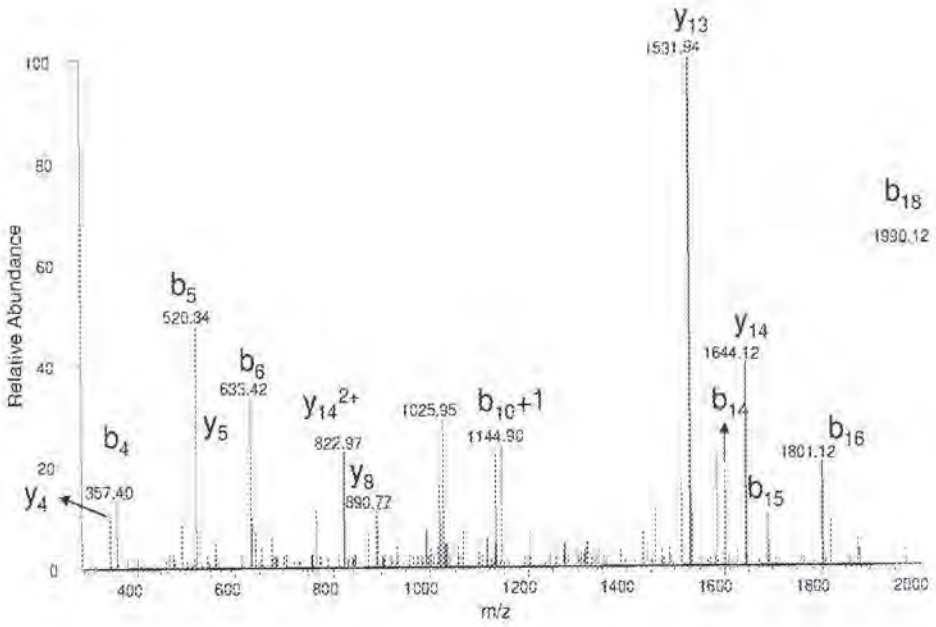

Relative Abundance
m/z
y13
1531.94
b18
1930.12
b5
520.34
b6
633.42
y14
1644.12
y14 2+
822.97
1025.95
b10+1
1144.90
b14
b16
1801.12
b4
y5
y8
890.77
y4
357.40
b15
100
80
60
40
20
0
400
600
800
1000
1200
1400
1600
1800
2000

FIG. 4A

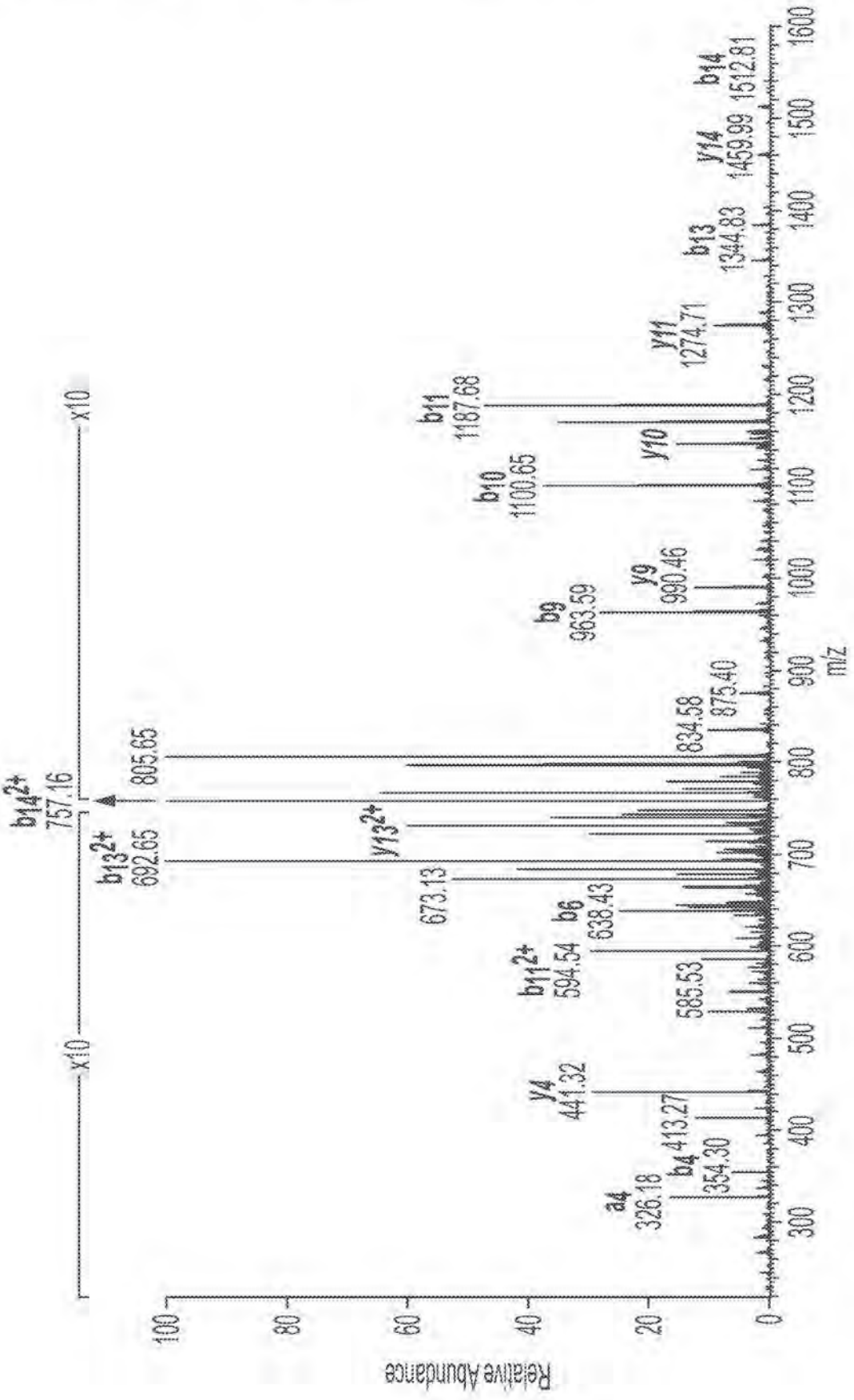

Relative Abundance
m/z
b14 1512.81
y14 1459.99
b13 1344.83
y11 1274.71
b11 1187.68
y10
b10 1100.65
y9 990.46
b9 963.59
834.58
875.40
805.65
b14 2+ 757.16
b13 2+ 692.65
y13 2+
673.13
b6 638.43
b11 2+ 594.54
585.53
y4 441.32
b4 413.27
354.30
a4 326.18
x10
x10

FIG. 4B

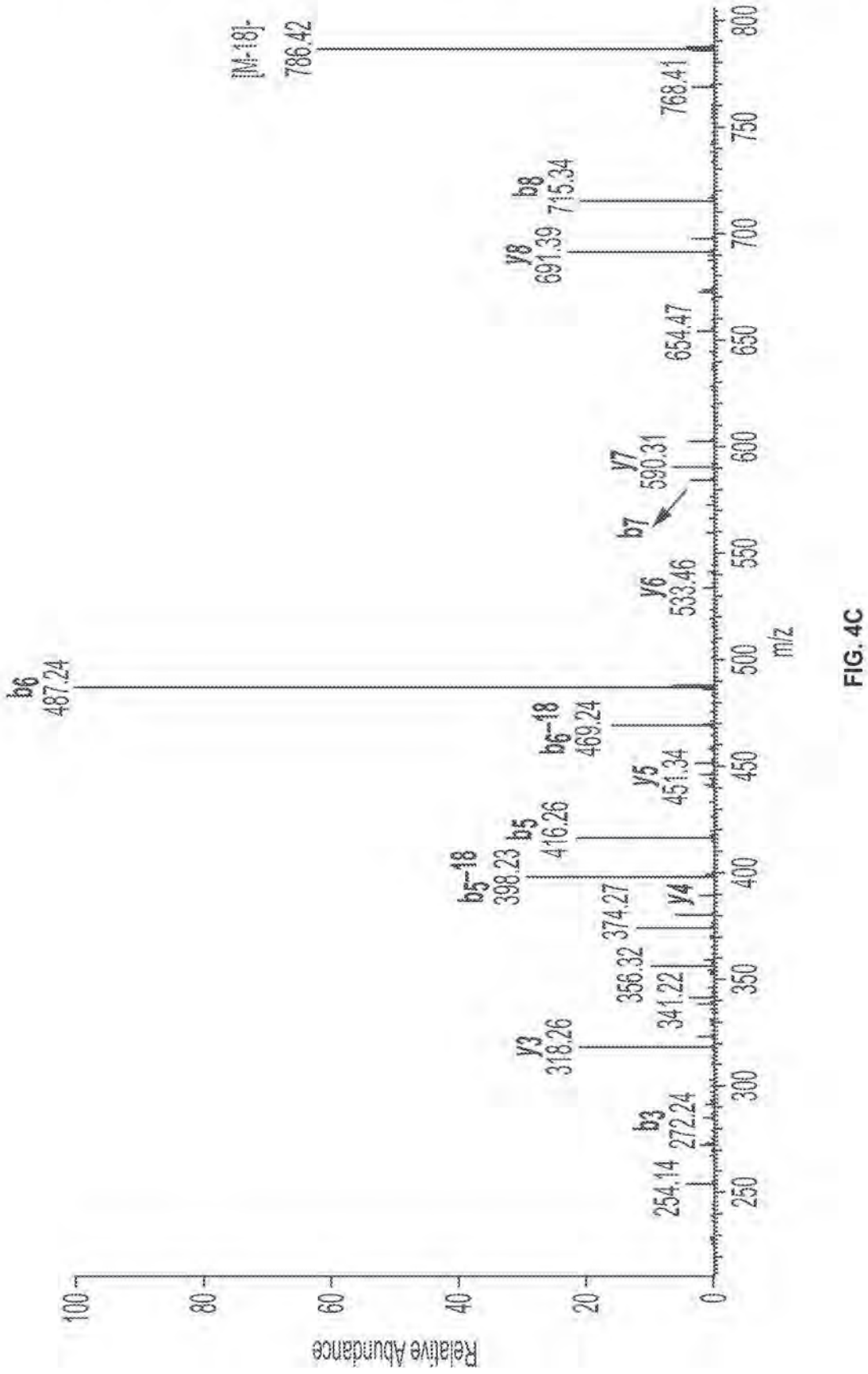

[M-18]-
786.42
768.41
b8
715.34
y8
691.39
654.47
y7
590.31
b7
y6
533.46
b6
487.24
b6−18
469.24
y5
451.34
b5
416.26
b5−18
398.23
y4
374.27
356.32
341.22
y3
318.26
b3
272.24
254.14
Relative Abundance
m/z
100
80
60
40
20
0
800
750
700
650
600
550
500
450
400
350
300
250

FIG. 4C

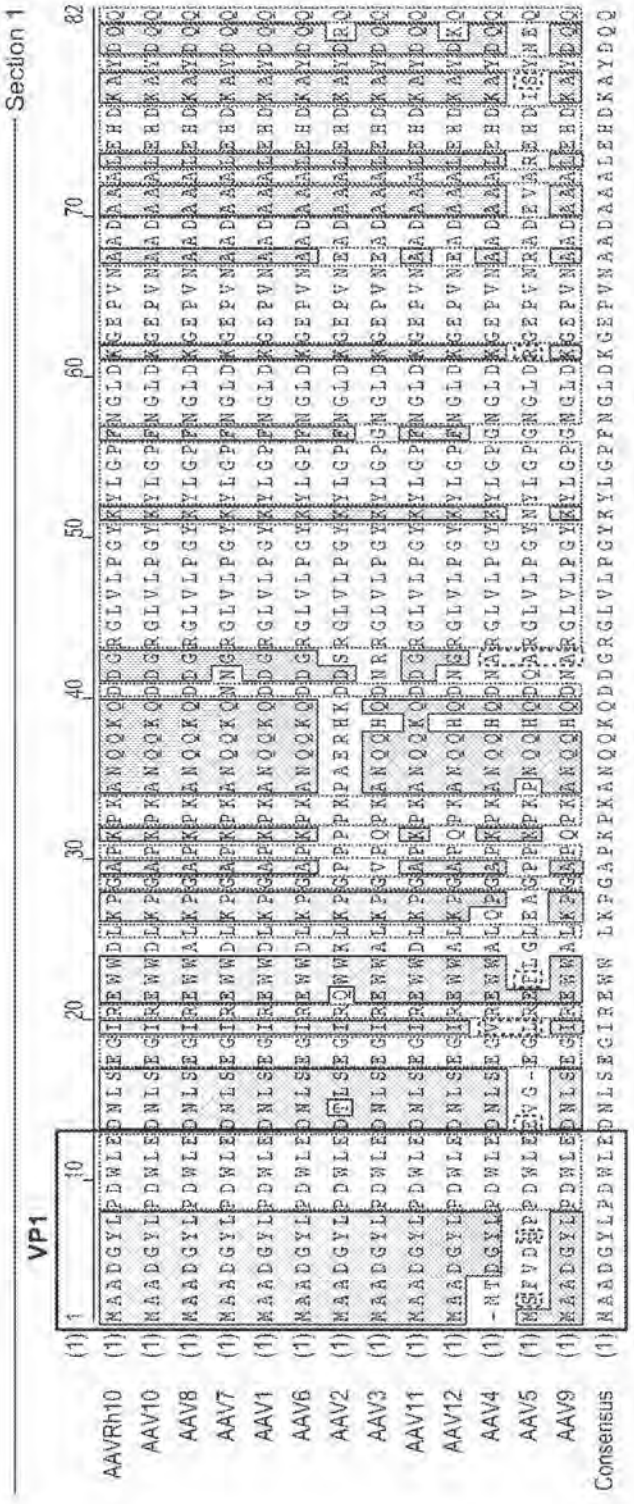

Section 1
VP1
AAVRh10
AAV10
AAV8
AAV7
AAV1
AAV6
AAV2
AAV3
AAV11
AAV12
AAV4
AAV5
AAV9
Consensus

FIG. 5A

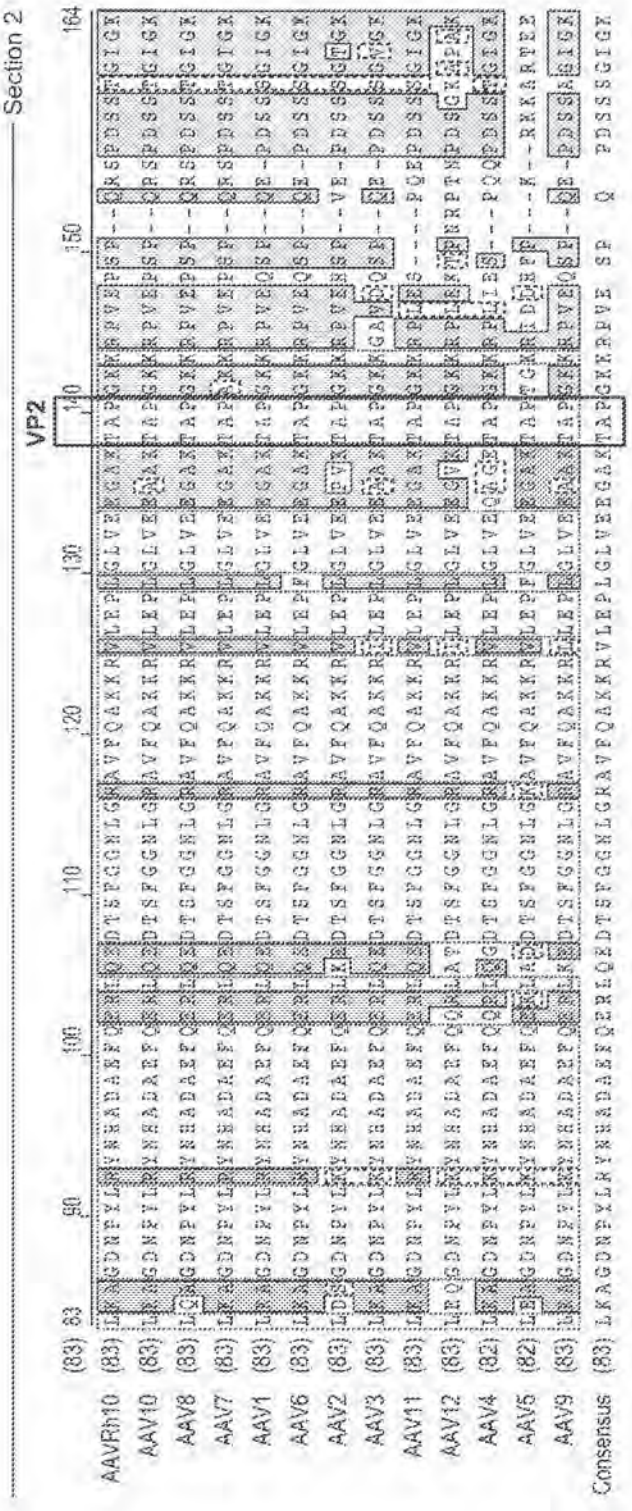

Section 2
VP2
AAVRh10
AAV10
AAV8
AAV7
AAV1
AAV6
AAV2
AAV3
AAV11
AAV12
AAV4
AAV5
AAV9
Consensus

FIG. 5B

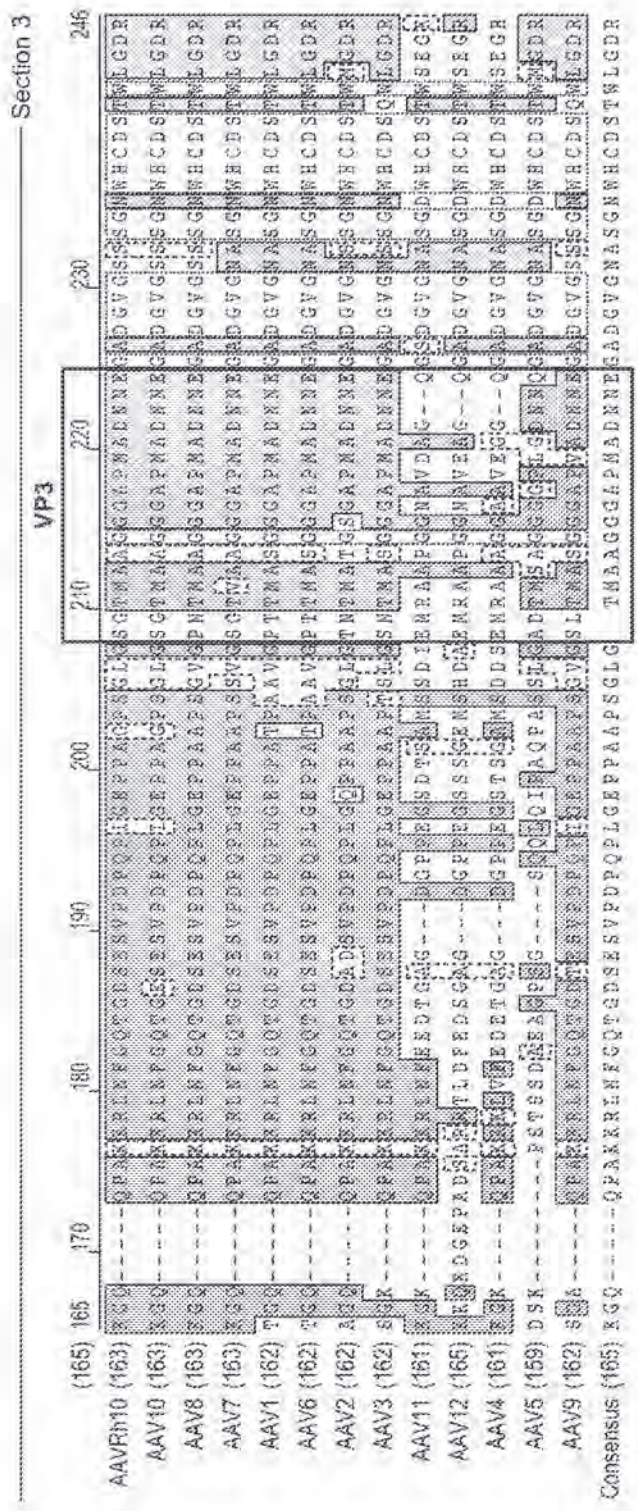

Section 3
VP3
AAVRh10 (163)
AAV10 (163)
AAV8 (163)
AAV7 (163)
AAV1 (162)
AAV6 (162)
AAV2 (162)
AAV3 (162)
AAV11 (161)
AAV12 (165)
AAV4 (161)
AAV5 (159)
AAV9 (162)
Consensus (165)

FIG. 5C

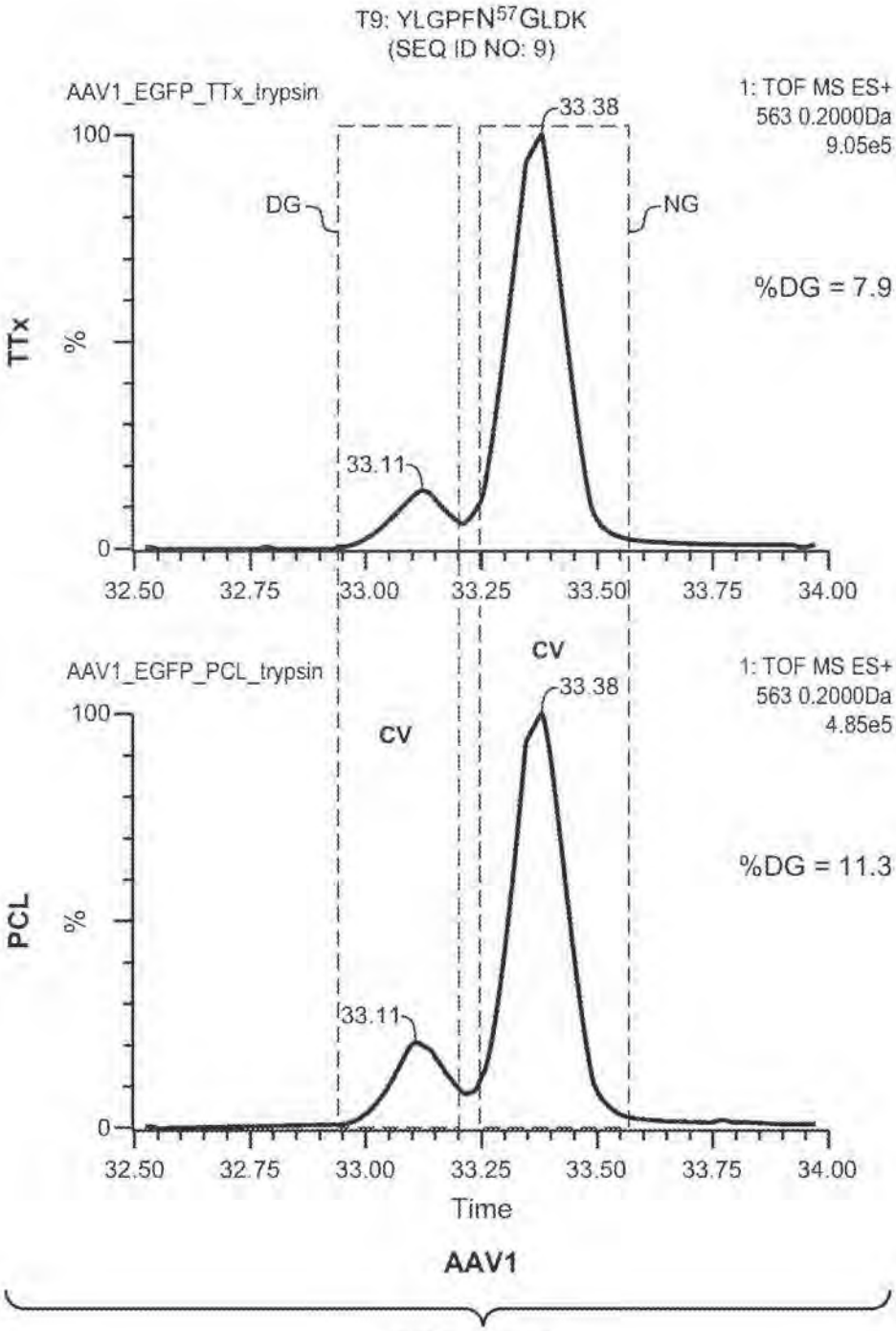

T9: YLGPFN57GLDK
(SEQ ID NO: 9)
AAV1_EGFP_TTx_trypsin
1: TOF MS ES+
563 0.2000Da
9.05e5
33.38
DG
NG
%DG = 7.9
TTx
%
100
0
33.11
32.50
32.75
33.00
33.25
33.50
33.75
34.00
AAV1_EGFP_PCL_trypsin
CV
CV
1: TOF MS ES+
563 0.2000Da
4.85e5
33.38
%DG = 11.3
PCL
%
100
0
33.11
32.50
32.75
33.00
33.25
33.50
33.75
34.00
Time
AAV1

FIG. 6A

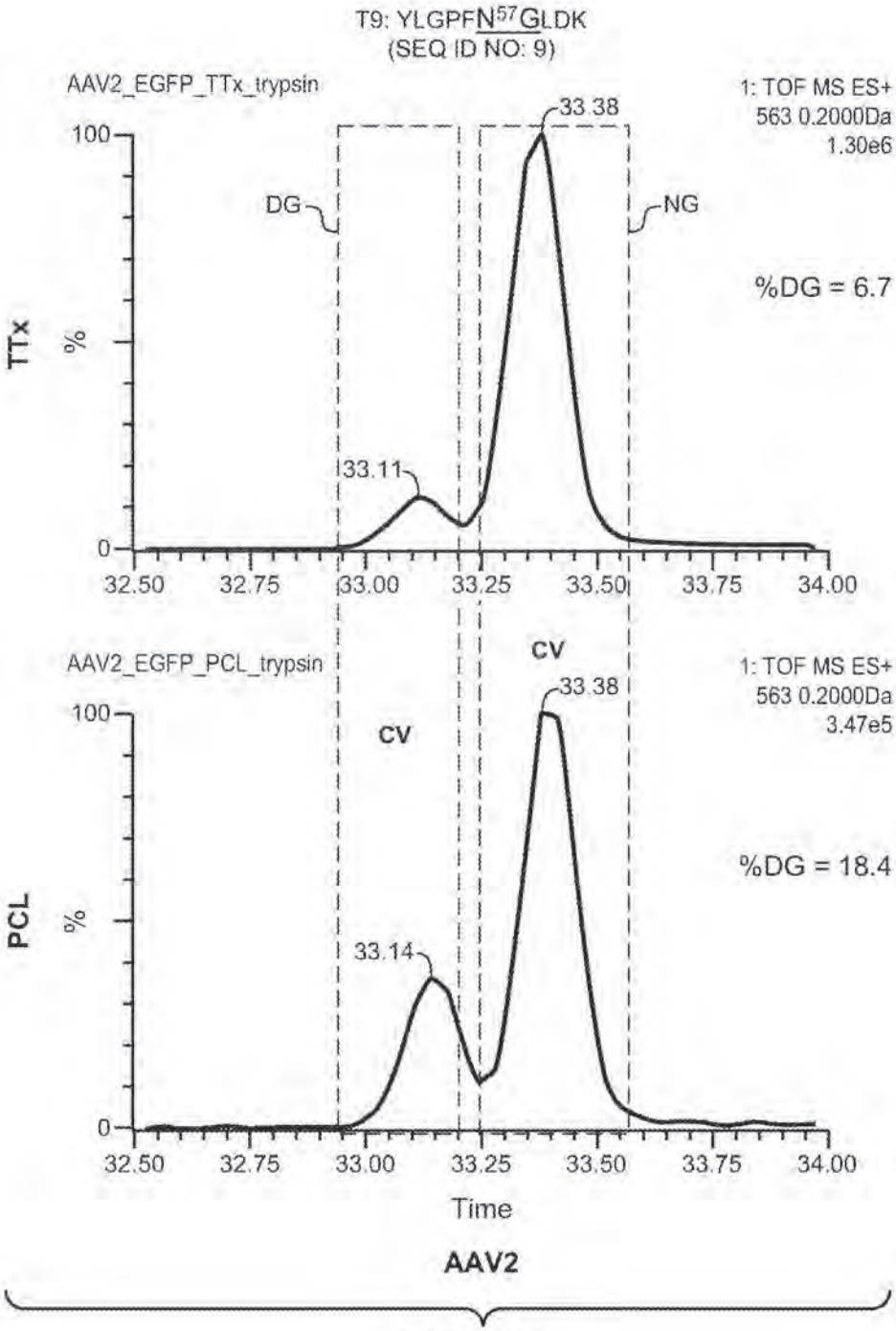

T9: YLGPFN57GLDK
(SEQ ID NO: 9)
AAV2_EGFP_TTx_trypsin
1: TOF MS ES+
563 0.2000Da
1.30e6
33.38
DG
NG
%DG = 6.7
TTx
%
33.11
100
0
32.50
32.75
33.00
33.25
33.50
33.75
34.00
AAV2_EGFP_PCL_trypsin
CV
CV
1: TOF MS ES+
563 0.2000Da
3.47e5
33.38
%DG = 18.4
PCL
%
33.14
100
0
32.50
32.75
33.00
33.25
33.50
33.75
34.00
Time
AAV2

FIG. 6B

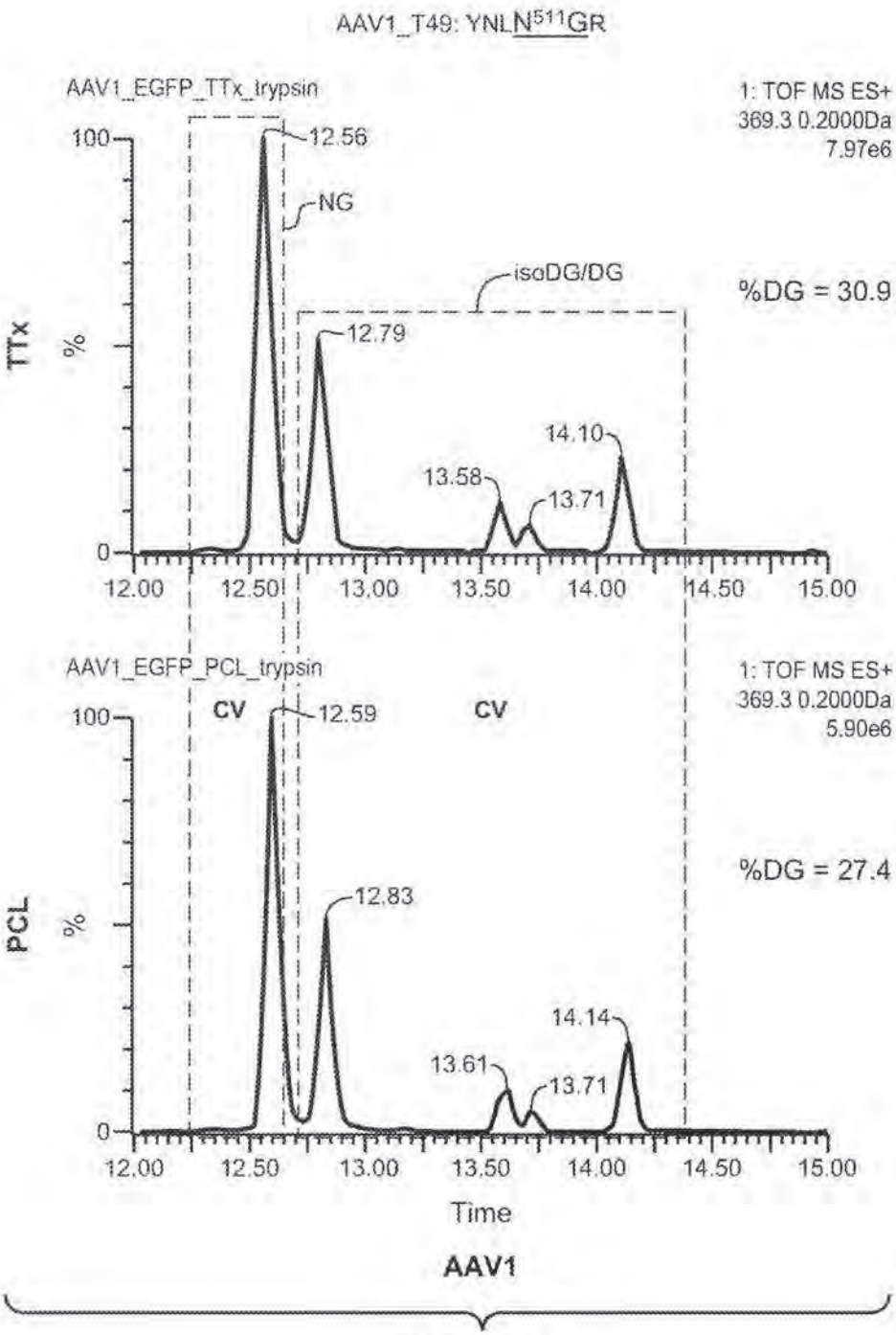

AAV1_T49: YNLN511GR
AAV1_EGFP_TTx_trypsin
1: TOF MS ES+
369.3 0.2000Da
7.97e6
12.56
NG
isoDG/DG
%DG = 30.9
12.79
14.10
13.58
13.71
TTx
%
100
0
12.00
12.50
13.00
13.50
14.00
14.50
15.00
AAV1_EGFP_PCL_trypsin
1: TOF MS ES+
369.3 0.2000Da
5.90e6
CV
12.59
CV
%DG = 27.4
12.83
14.14
13.61
13.71
PCL
Time
AAV1

FIG. 7A

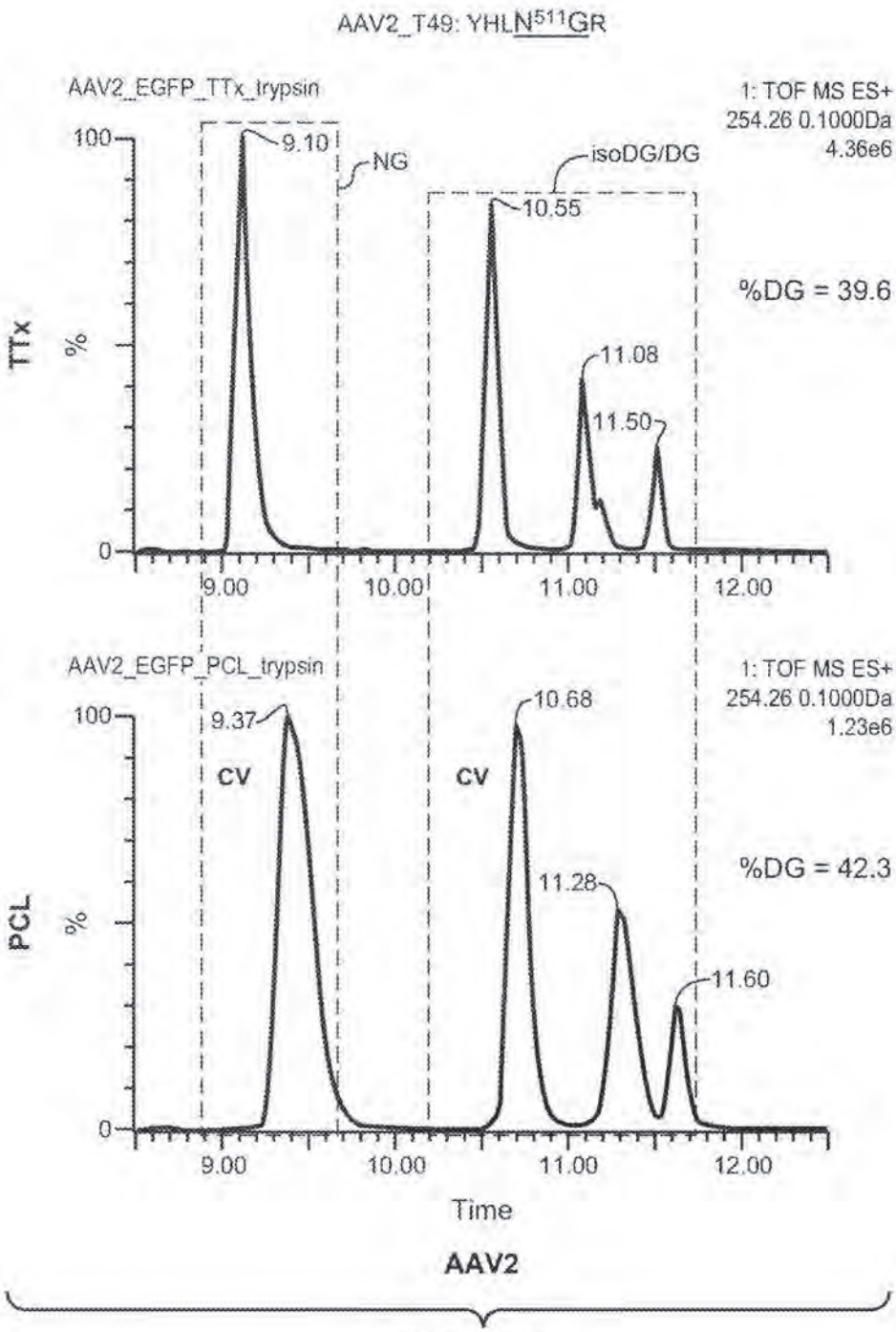

AAV2_T49: YHLN511GR
AAV2_EGFP_TTx_trypsin
1: TOF MS ES+
254.26 0.1000Da
4.36e6
100
9.10
NG
isoDG/DG
10.55
%DG = 39.6
11.08
11.50
TTx
%
0
9.00
10.00
11.00
12.00
AAV2_EGFP_PCL_trypsin
1: TOF MS ES+
254.26 0.1000Da
1.23e6
100
9.37
CV
CV
10.68
%DG = 42.3
11.28
11.60
PCL
%
0
9.00
10.00
11.00
12.00
Time
AAV2

FIG. 7B

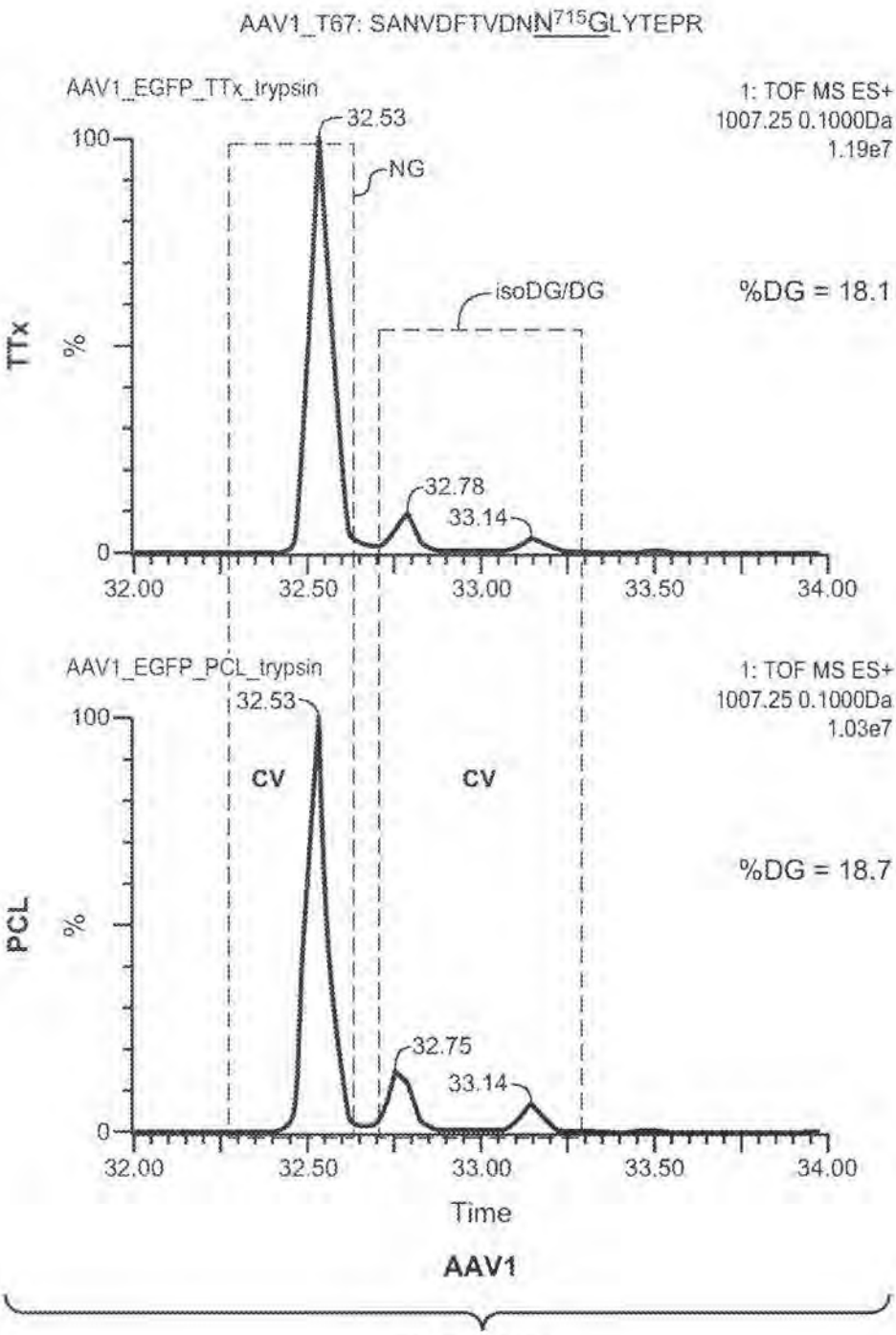

AAV1_T67: SANVDFTVDNN715GLYTEPR
AAV1_EGFP_TTx_trypsin
1: TOF MS ES+
1007.25 0.1000Da
1.19e7
32.53
NG
isoDG/DG
%DG = 18.1
TTx
%
32.78
33.14
100
0
32.00
32.50
33.00
33.50
34.00
AAV1_EGFP_PCL_trypsin
1: TOF MS ES+
1007.25 0.1000Da
1.03e7
32.53
CV
CV
%DG = 18.7
PCL
%
32.75
33.14
Time
AAV1

FIG. 8A

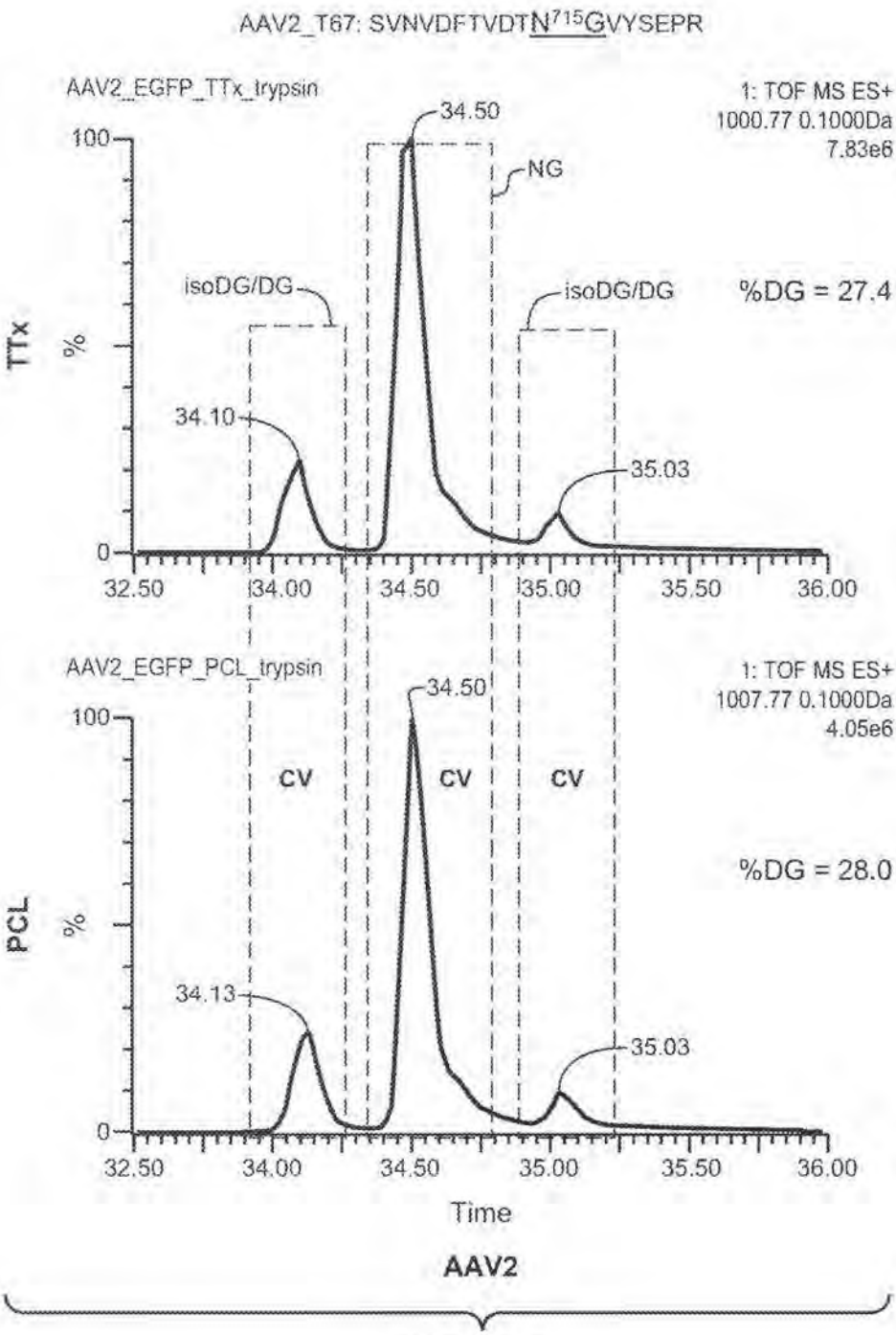
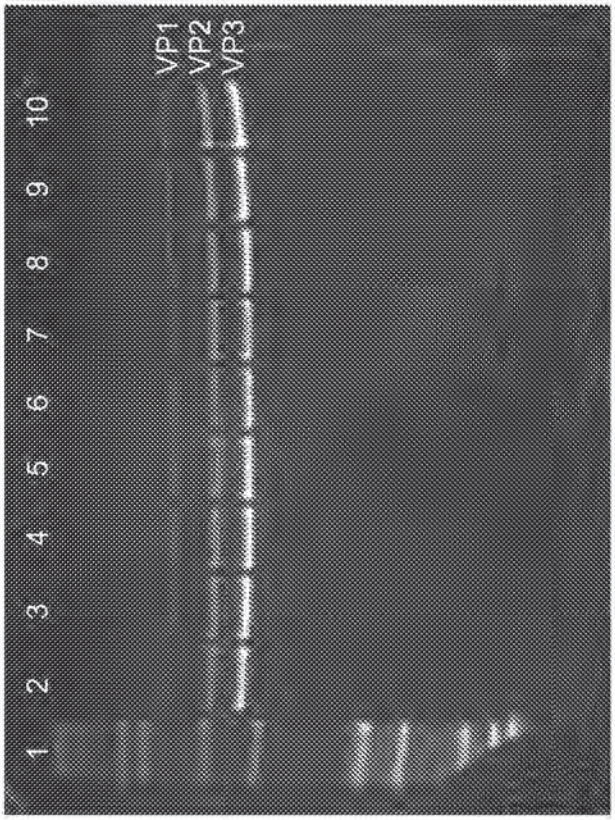

AAV2_T67: SVNVDFTVDTN715GVYSEPR
AAV2_EGFP_TTx_trypsin
1: TOF MS ES+
1000.77 0.1000Da
7.83e6
34.50
NG
isoDG/DG
isoDG/DG
%DG = 27.4
TTx
%
34.10
35.03
100
0
32.50
34.00
34.50
35.00
35.50
36.00
AAV2_EGFP_PCL_trypsin
1: TOF MS ES+
1007.77 0.1000Da
4.05e6
34.50
CV
CV
CV
%DG = 28.0
PCL
%
34.13
35.03
100
0
32.50
34.00
34.50
35.00
35.50
36.00
Time
AAV2

FIG. 8B



Deacetylation AAV5 Mutant Vectors

Lane	Sample
1	Mark12 Marker
2	deAC-AAV5 (S2G)/CBA-eGFP X17052C
3	deAC-AAV5 (S2L)/CBA-eGFP X17052D
4	deAC-AAV5 (S2GVP3)/CBA-eGFP X17068D
5	deAC-AAV5 (S2LVP3)/CBA-eGFP X17065A
6	deAC-AAV5 (S2PVP1VP3)/CBA-eGFP X17065B
7	deAC-AAV5 (S2GVP1VP3)/CBA-eGFP X17065C
8	deAC-AAV5 (S2PVP3)/CBA-eGFP X17065D
9	deAC-AAV5 (S2P)/CBA-eGFP X17065E
10	deAC-AAV5 (S2LVP1VP3)/CBA-eGFP X17072B

(~1E10 VGs/Lane)

FIG. 9

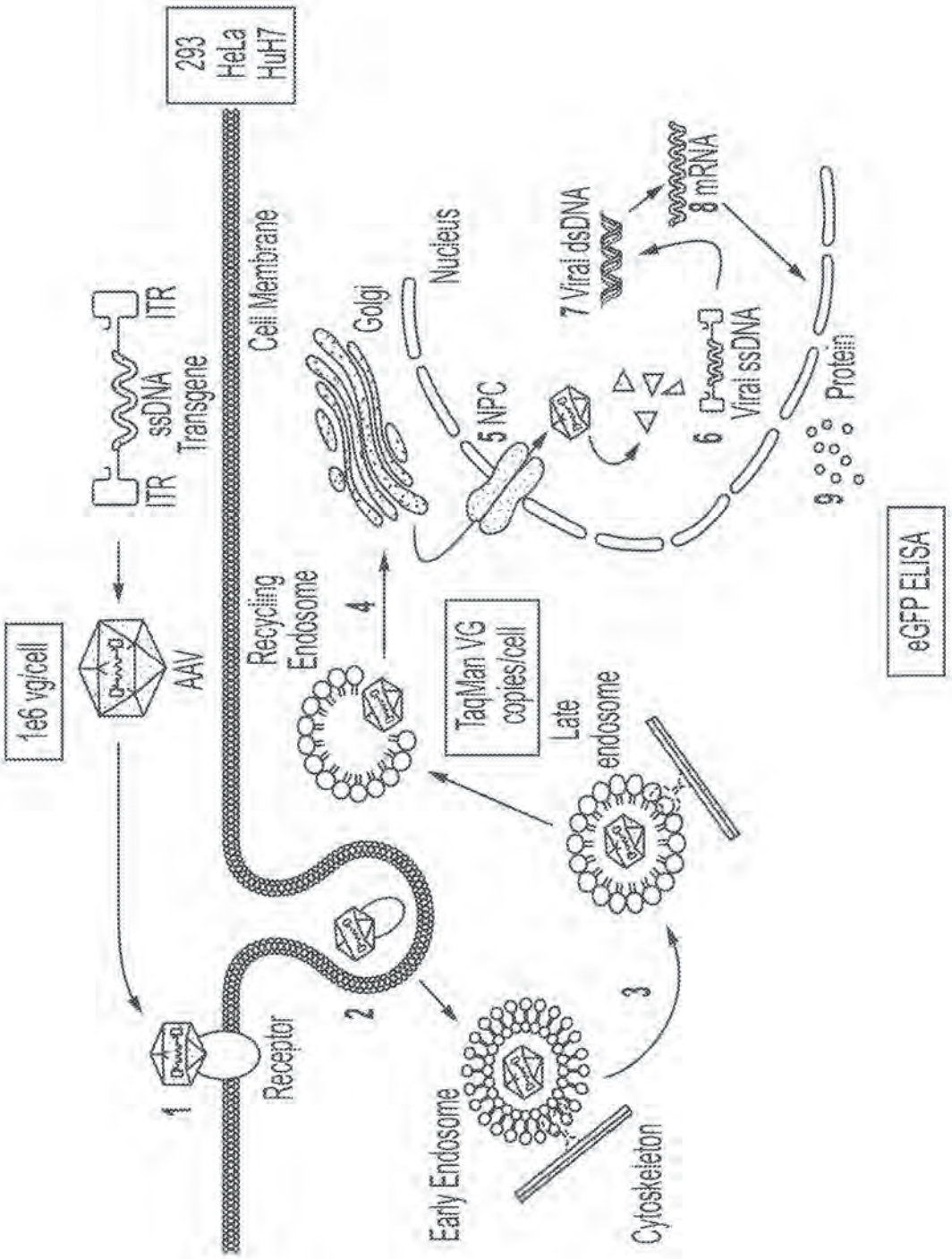

293
HeLa
HuH7
Cell Membrane
ITR
ssDNA
Transgene
ITR
1e6 vg/cell
AAV
Recycling
Endosome
4
Golgi
Nucleus
5 NPC
7 Virai dsDNA
8 mRNA
6
Virai ssDNA
9
Protein
eGFP ELISA
TaqMan VG
copies/cell
Late
endosome
1
Receptor
2
3
Early Endosome
Cytoskeleton

FIG. 10

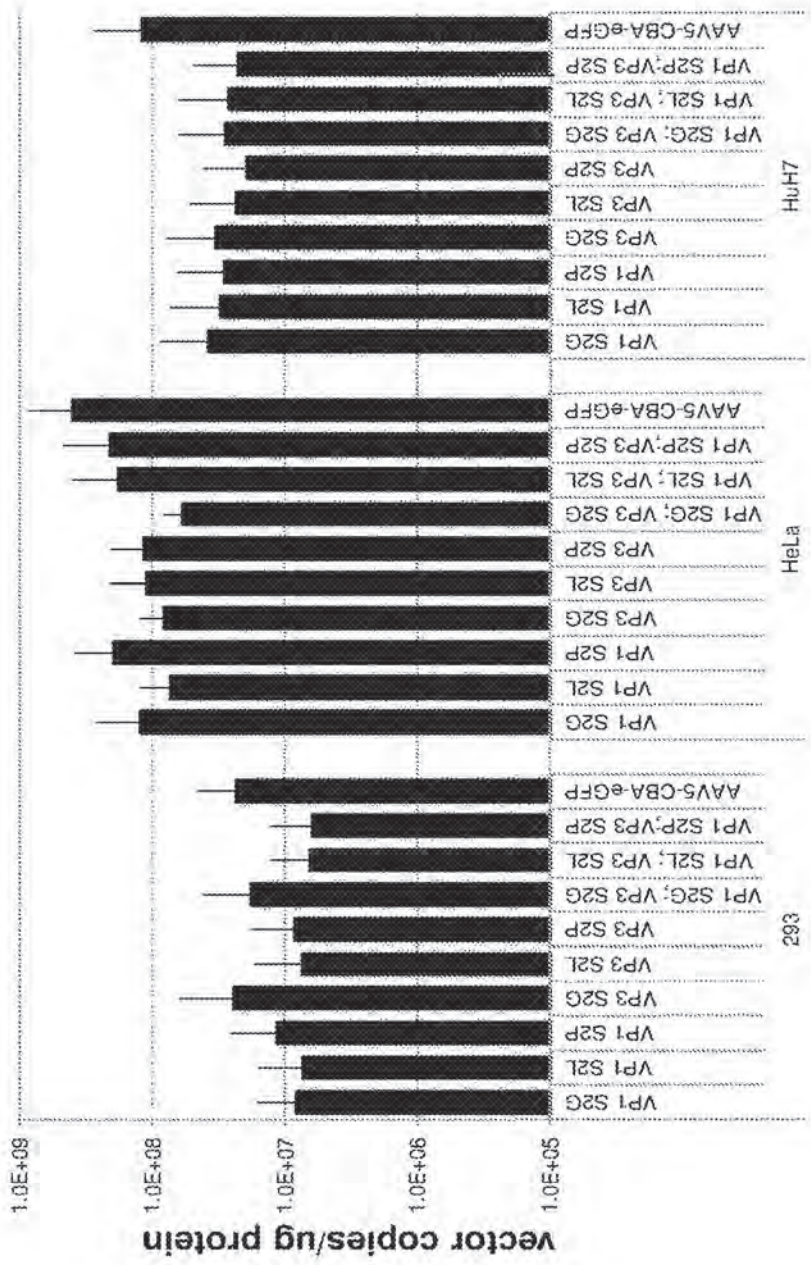

vector copies/ug protein
1.0E+09
1.0E+08
1.0E+07
1.0E+06
1.0E+05
293
HeLa
HuH7
VP1 S2G
VP1 S2L
VP1 S2P
VP3 S2G
VP3 S2L
VP3 S2P
VP1 S2G; VP3 S2G
VP1 S2L; VP3 S2L
VP1 S2P;VP3 S2P
AAV5-CBA-eGFP

FIG. 11

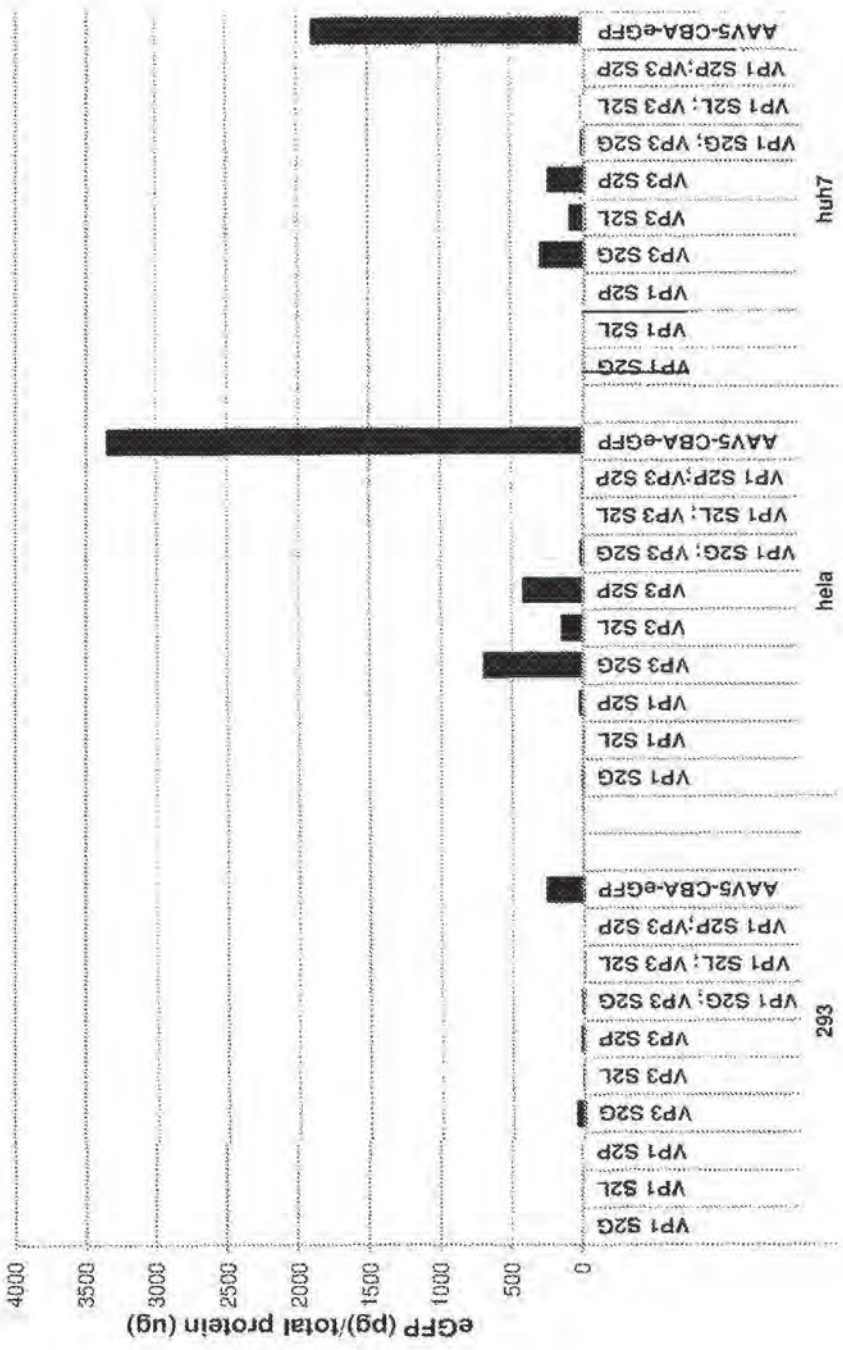

AAV5-CBA-eGFP
VP1 S2P; VP3 S2P
VP1 S2L; VP3 S2L
VP1 S2G; VP3 S2G
VP3 S2P
VP3 S2L
VP3 S2G
VP1 S2P
VP1 S2L
VP1 S2G
huh7
AAV5-CBA-eGFP
VP1 S2P; VP3 S2P
VP1 S2L; VP3 S2L
VP1 S2G; VP3 S2G
VP3 S2P
VP3 S2L
VP3 S2G
VP1 S2P
VP1 S2L
VP1 S2G
hela
AAV5-CBA-eGFP
VP1 S2P; VP3 S2P
VP1 S2L; VP3 S2L
VP1 S2G; VP3 S2G
VP3 S2P
VP3 S2L
VP3 S2G
VP1 S2P
VP1 S2L
VP1 S2G
293
4000
3500
3000
2500
2000
1500
1000
500
0
eGFP (pg)/total protein (ug)

FIG. 12

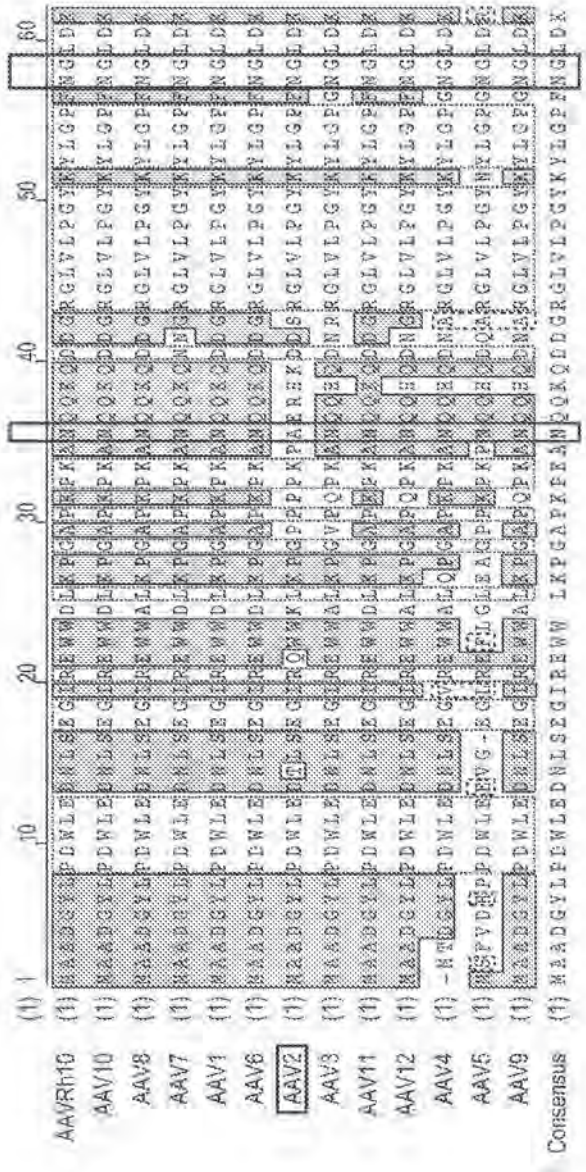

AAVRh10
AAV10
AAV8
AAV7
AAV1
AAV6
AAV2
AAV3
AAV11
AAV12
AAV4
AAV5
AAV9
Consensus

FIG. 13

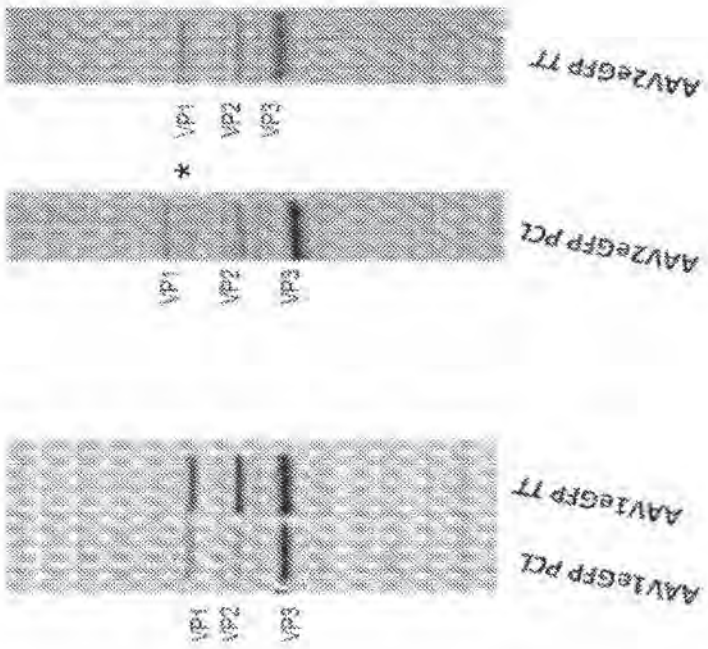

AAV2eGFP TT
VP1
VP2
VP3
*
AAV2eGFP PCL
VP1
VP2
VP3
AAV1eGFP TT
AAV1eGFP PCL
VP1
VP2
VP3

FIG. 14

Deamidation of N57(G)

	% Deamidation on N57	replicate
AAV2(A35N)	17.8	1
AAV2(G58D)	1.1	1
AAV2 control	5.7	2

AAV2(A35N) has been stored at 4C for 1 months

FIG. 15

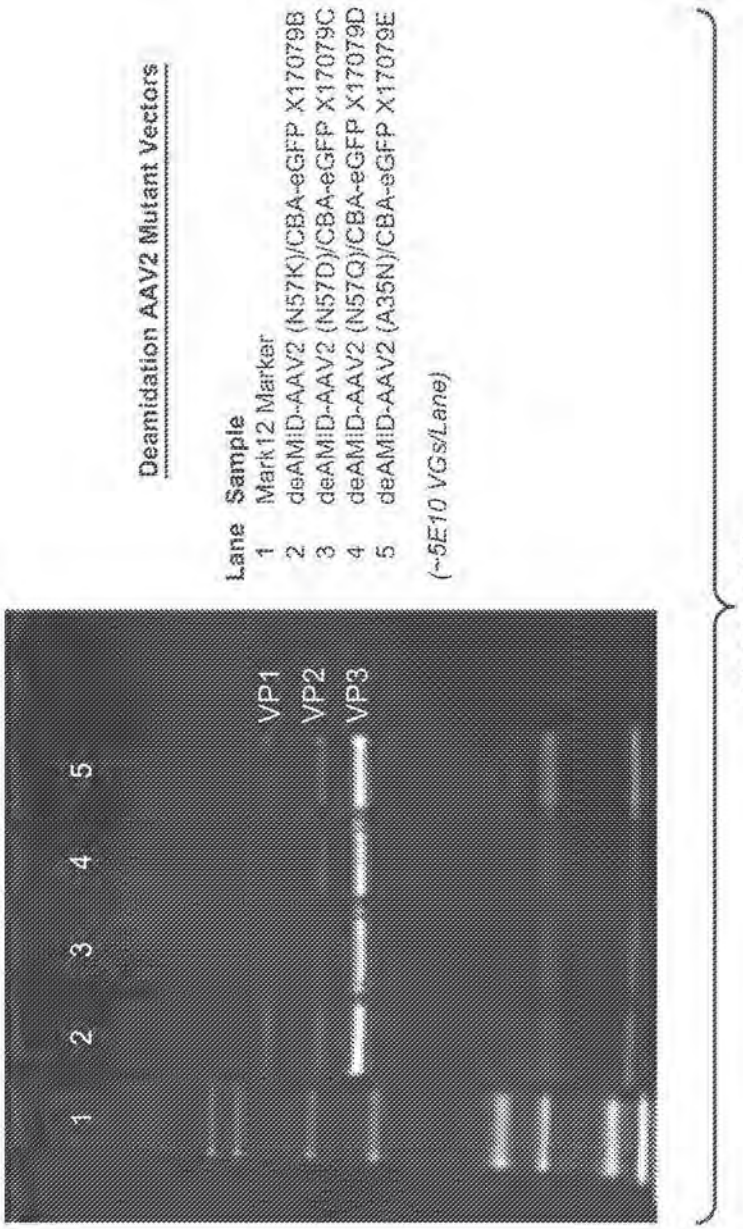

Deamidation AAV2 Mutant Vectors
Lane Sample
1 Mark12 Marker
2 deAMID-AAV2 (N57K)/CBA-eGFP X17079B
3 deAMID-AAV2 (N57D)/CBA-eGFP X17079C
4 deAMID-AAV2 (N57Q)/CBA-eGFP X17079D
5 deAMID-AAV2 (A35N)/CBA-eGFP X17079E
(~5E10 VGs/Lane)
1
2
3
4
5
VP1
VP2
VP3

FIG. 16

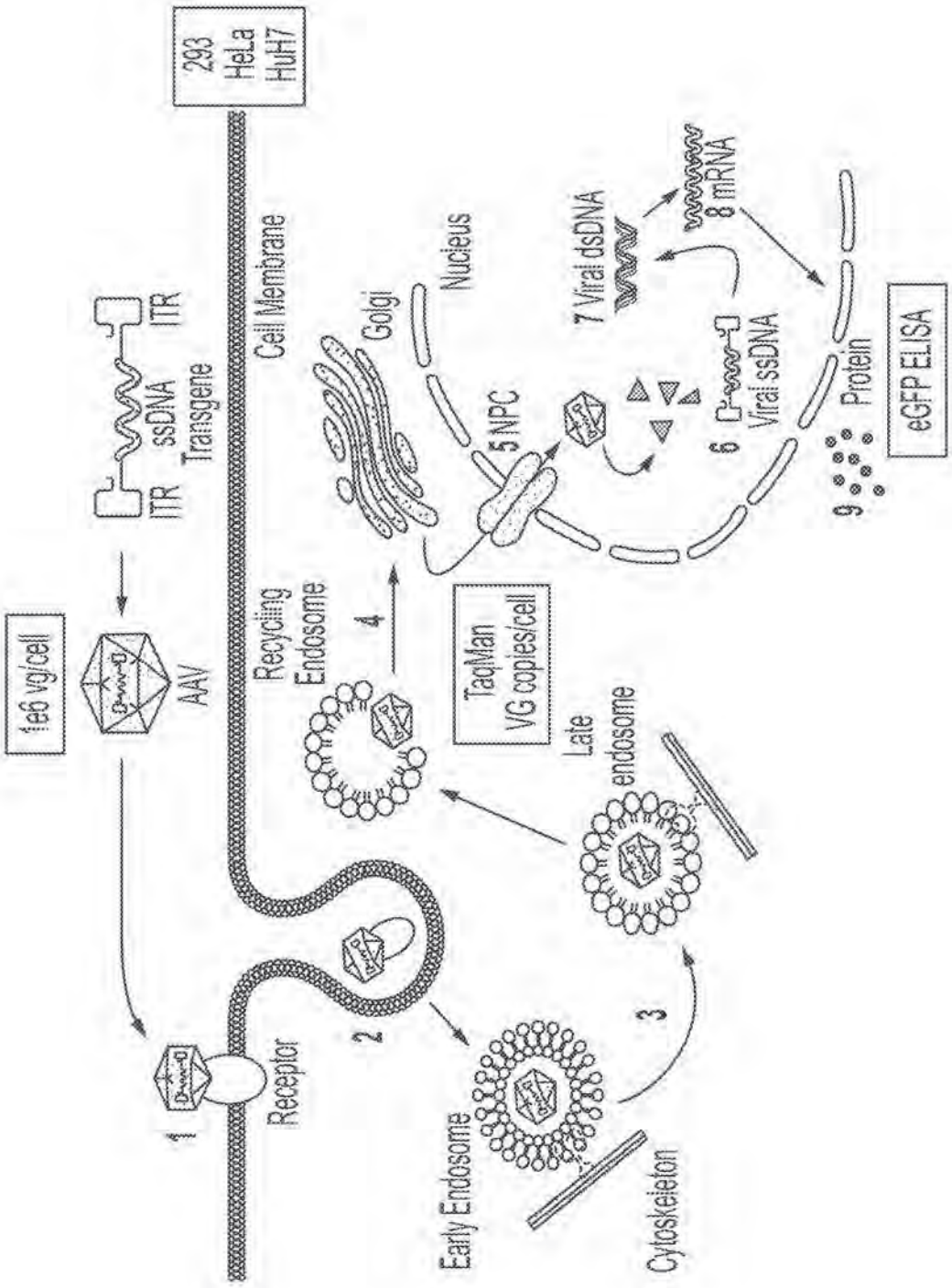

293
HeLa
HuH7
Cell Membrane
ITR
ssDNA
Transgene
ITR
1e6 vg/cell
AAV
Recycling
Endosome
4
TaqMan
VG copies/cell
Golgi
Nucleus
5 NPC
7 Viral dsDNA
8 mRNA
6
Viral ssDNA
9
Protein
eGFP ELISA
Late
endosome
1
Receptor
2
3
Early Endosome
Cytoskeleton

FIG. 17

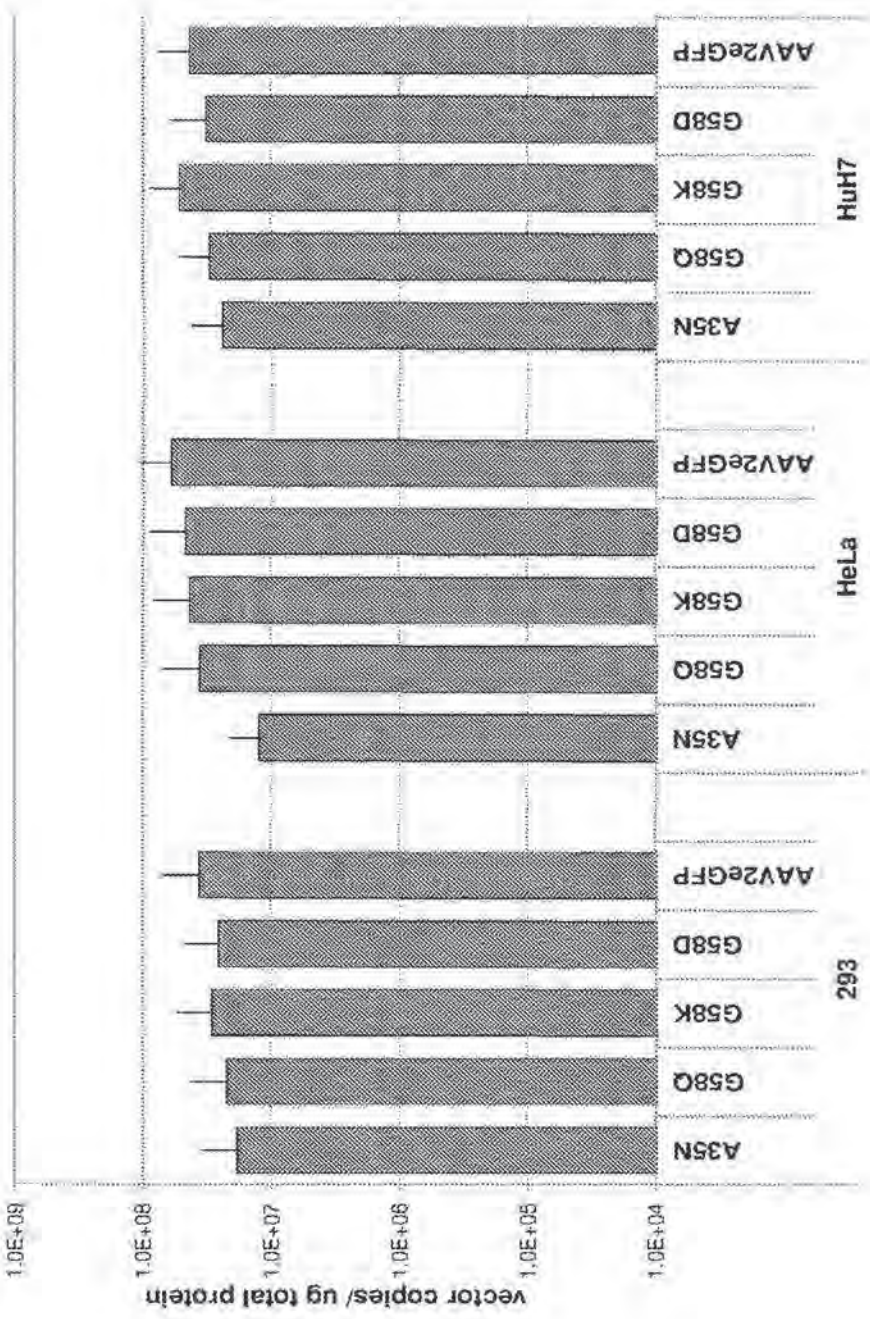

AAV2eGFP
G58D
G58K
G58Q
A35N
HuH7
AAV2eGFP
G58D
G58K
G58Q
A35N
HeLa
AAV2eGFP
G58D
G58K
G58Q
A35N
293
1.0E+09
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
vector copies/ ug total protein

FIG. 18

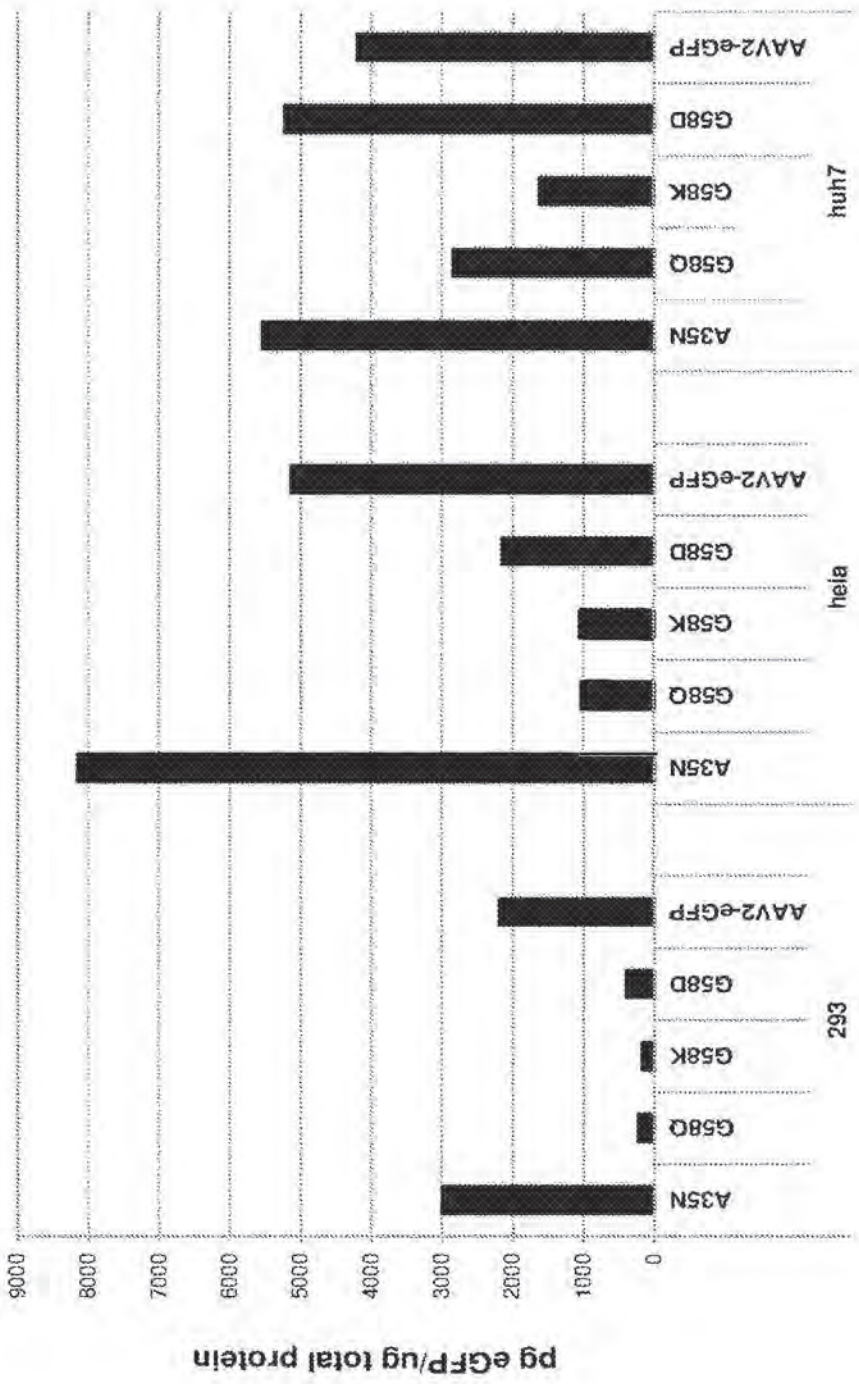

pg eGFP/ug total protein
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
293
A35N
G58Q
G58K
G58D
AAV2-eGFP
hela
A35N
G58Q
G58K
G58D
AAV2-eGFP
huh7
A35N
G58Q
G58K
G58D
AAV2-eGFP

FIG. 19

METHODS FOR DETECTING AAV

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a divisional application of U.S. patent application Ser. No. 16/325,653, which adopts the international filing date of Aug. 14, 2017, which is a National Phase application under 35 U.S.C. § 371 of International Application No. PCT/US2017/046814, filed Aug. 14, 2017, which claims the priority benefit of U.S. Provisional Application No. 62/375,314, filed Aug. 15, 2016, the disclosure of each of which is hereby incorporated by reference in its entirety.

REFERENCE TO AN ELECTRONIC SEQUENCE LISTING

The contents of the electronic Sequence Listing 1597920141 10SEQLIST.xml; Size 56,201 bytes, and date of creation: May 18, 2023) is herein incorporated by reference in its entirety.

FIELD OF THE INVENTION

The present invention relates to methods for serotyping and/or determining the heterogeneity of a viral particle (e.g., an adeno-associated virus (AAV) particle) using mass determination, e.g., by employing liquid chromatography/mass spectrometry (LC/MS) or liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS). In some aspects, the present invention relates to methods to improve the stability of AAV particles.

BACKGROUND OF THE INVENTION

Complete characterization of the viral capsid proteins of viral vectors (e.g., AAV vectors), including their sequence and post-translation modifications, is desired in gene therapy research and development since viral capsid proteins (VPs) are critical for viral infectivity.

Viral vector products such as recombinant Adeno-Associated Virus (rAAV) products are typically identified using molecular tools targeting the nucleic acid transgene. These methods may include polymerase chain reaction (PCR) targeting transgene-specific sequences and Restriction Fragment Length Polymorphism (RFLP) techniques. As rAAV technologies evolve, many facilities are beginning to investigate multiple AAV capsid serotypes encoding their therapeutic transgene in an effort to improve targeted tissue tropism.

Traditional molecular identification methods identify products containing unique transgenes but are unable to discern those that have differing AAV capsid serotypes. Currently, most AAV serotype identity tests are based on SDS-PAGE banding patterns, an antibody-based ELISA, or a Western blot assay. However, the banding patterns and antibodies are not specific enough to differentiate different AAV serotypes. Gel-LC/MS/MS has been reported as a capsid serotype identification method. However, this method involves multiple steps including SDS-PAGE, in-gel digestion, and LC/MS/MS and thus requires multiple days for the analysis while providing limited sequence coverage. Methods for identifying vectors such as rAAV vectors are of interest to gene therapy vectors (see, e.g., U.S. PG Pub. No. US20110275529). Thus, it would be useful to have improved methods of characterizing viral particles.

All references cited herein, including patent applications and publications, are incorporated by reference in their entirety.

BRIEF SUMMARY OF THE INVENTION

Using rAAV as an example, described herein is the use of LC/MS as an analytical tool to specifically identify different viral capsid serotypes (e.g., rAAV capsid serotypes). As part of viral characterization, LC/MS can be used to augment the molecular identification methods. This analytical combination can satisfy regulatory requirements by discerning both the identity of the product's therapeutic transgene and the identity of the capsid serotype. This method can be used e.g., as an AAV serotype identity test or to monitor viral capsid protein heterogeneity in recombinant AAV gene therapy development. It can also be used to confirm VP sequences in capsid engineering research. In addition, this technique can be used to study the impact of post translation modifications, such as N terminal acetylation of viral capsid proteins, on transfection potency and intracellular protein trafficking.

The methods described herein can also be used to design AAV particles for greater stability and/or improved transduction efficiency; for example by altering the amino acid residue at position 2 of VP1 and/or VP3 of the AAV capsid such that the amino acid at position 2 is acetylated to a higher extent compared to a wild type AAV capsid. In some embodiments, the methods can be used to design AAV particles with reduced transduction efficiency; for example by altering the amino acid residue at position 2 of VP1 and/or VP3 of the AAV capsid such that the amino acid at position 2 is deacetylated to a higher or lower extent compared to a wild type AAV capsid.

In some aspects, the invention provides a method to determine the serotype of a viral particle comprising a) denaturing the viral particle, b) subjecting the denatured viral particle to liquid chromatography/mass spectrometry (LC/MS), and c) determining the masses of one or more capsid proteins of the viral particle; wherein the specific combination of masses of the one or more capsid proteins are indicative of the virus serotype. In some embodiments, the calculated masses of the one or more capsid proteins are compared to the theoretical masses of the one or more capsid proteins of one or more virus serotypes.

In some aspects, the invention provides a method of determining the heterogeneity of a viral particle comprising a) denaturing the viral particle, b) subjecting the denatured viral particle to liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS), c) determining the masses of one or more capsid proteins of the viral particle, and d) comparing the masses of step c) with the theoretical masses of the one or more capsid proteins of the virus serotype; wherein a deviation of one or more of the masses of the one or more capsid proteins are indicative of the viral capsid heterogeneity. In some embodiments, the heterogeneity comprises one or more of mixed serotypes, variant capsids, capsid amino acid substitutions, truncated capsids, or modified capsids.

In some embodiments of the above aspects, the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography. In some embodiments, the viral particle comprises a viral vector encoding a heterologous transgene.

In some aspects, the invention provide a method to determine the serotype of a viral particle comprising a) denaturing the viral particle, b) subjecting the denatured

viral particle to reduction and/or alkylation, c) subjecting the denatured viral particle to digestion to generate fragments of one or more capsid proteins of the viral particle, d) subjecting the fragments of the one or more capsid proteins to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), and e) determining the masses of fragments of the one or more capsid proteins of the viral particle; wherein the specific combination of masses of fragments of the one or more capsid proteins are indicative of the viral serotype. In some embodiments, the calculated masses of the fragments of the one or more capsid proteins are compared to the theoretical masses of fragments of the one or more capsid proteins of one or more viral serotypes.

In some aspects, the invention provides a method of determining the heterogeneity of a serotype of a viral particle comprising a) denaturing the viral particle, b) subjecting the denatured viral particle to reduction and/or alkylation, c) subjecting the denatured viral particle to digestion to generate fragments of one or more capsid proteins of the viral particle, d) subjecting the fragments of the one or more capsid proteins to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), e) determining the masses of fragments of the one or more capsid proteins of the viral particle, and f) comparing the masses of step e) with the theoretical masses of fragments of the one or more capsid proteins of the viral serotype; wherein a deviation of one or more of the masses of the one or more capsid proteins are indicative of the viral capsid heterogeneity. In some embodiments, the heterogeneity comprises one or more of mixed serotypes, variant capsids, capsid amino acid substitutions, truncated capsids, or modified capsids. In some embodiments, the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography.

As shown herein the methods can be performed in the absence of a gel separation step (e.g., sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)).

In some embodiments of the above aspects and embodiments, the viral particle comprises a viral vector encoding a heterologous transgene. In some embodiments, the viral particle belongs to a viral family selected from the group consisting of Adenoviridae, Parvoviridae, Retroviridae, Baculoviridae, and Herpesviridae. In some embodiments, the viral particle belongs to a viral genus selected from the group consisting of *Atadenovirus*, *Aviadenovirus*, *Ichtadenovirus*, *Mastadenovirus*, *Siadenovirus*, *Ambidensovirus*, *Brevidensovirus*, *Hepandensovirus*, *Iteradensovirus*, *Penstyldensovirus*, *Amdoparvovirus*, *Aveparvovirus*, *Bocaparvovirus*, *Copiparvovirus*, *Dependoparvovirus*, *Erythroparvovirus*, *Protoparvovirus*, *Tetraparvovirus*, *Alpharetrovirus*, *Betaretrovirus*, *Deltaretrovirus*, *Epsilonretrovirus*, *Gammaretrovirus*, *Lentivirus*, *Spumavirus*, *Alphabaculovirus*, *Betabaculovirus*, *Deltabaculovirus*, *Gammabaculovirus*, *Iltovirus*, *Mardivirus*, *Simplexvirus*, *Varicellovirus*, *Cytomegalovirus*, *Muromegalovirus*, *Proboscivirus*, *Roseolovirus*, *Lymphocryptovirus*, *Macavirus*, *Percavirus*, and *Rhadinovirus*.

In some aspects, the invention provides a method to determine the serotype of an adeno-associated virus (AAV) particle comprising a) denaturing the AAV particle, b) subjecting the denatured AAV particle to liquid chromatography/mass spectrometry (LC/MS), and c) determining the masses of VP1, VP2 and VP3 of the AAV particle; wherein the specific combination of masses of VP1, VP2 and VP3 are indicative of the AAV serotype. In some embodiments, the calculated masses of VP1, VP2 and VP3 are compared to the theoretical masses of VP1, VP2 and VP3 of one or more AAV serotypes.

In some aspects, the invention provides a method of determining the heterogeneity of an AAV particle comprising a) denaturing the AAV particle, b) subjecting the denatured AAV particle to liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS), c) determining the masses of VP1, VP2 and VP3 of the AAV particle, and d) comparing the masses of step c) with the theoretical masses of VP1, VP2 and VP3 of the AAV serotype; wherein a deviation of one or more of the masses of VP1, VP2 or VP3 are indicative of the AAV capsid heterogeneity. In some embodiments, the heterogeneity comprises one or more of mixed serotypes, variant capsids, capsid amino acid substitutions, truncated capsids, or modified capsids.

In some embodiments of the above aspects and embodiments, the AAV particle is denatured with acetic acid, guanidine hydrochloride and/or an organic solvent. In some embodiments, the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography. In some embodiments, the liquid chromatography is reverse phase liquid chromatography. In some embodiments, the reverse phase chromatography is a C4 or C8 reverse chromatography. In some embodiments, the chromatography uses a mobile phase A comprising formic acid in water. In some embodiments, the mobile phase A comprises about 0.1% formic acid. In some embodiments, the chromatography comprises a mobile phase B comprising formic acid in acetonitrile. In some embodiments, the mobile phase B comprises about 0.1% formic acid. In some embodiments, the proportion of mobile phase B in the chromatography increases over time. In some embodiments, the proportion of mobile phase B in the chromatography increases in a stepwise manner. In some embodiments, mobile phase B increases from about 10% to about 20%, from about 20% to about 30%, and from about 30% to about 38%. In some embodiments, mobile phase B increases from about 10% to about 20% in about 6 minutes, from about 20% to about 30% in about 10 minutes, and from about 30% to about 38% in about 40 minutes. In some embodiments, the liquid chromatography is ultra-performance liquid chromatography (UPLC).

In some embodiments of the above aspects and embodiments, the mass spectrometry comprises a capillary voltage of about 3.5 kV. In some embodiments, the mass spectrometry comprises a sampling cone voltage of about 45 V. In some embodiments, the mass spectrometry comprises assisted calibration. In some embodiments, sodium iodide is used as a calibrant.

In some embodiments of the above aspects and embodiments, the N-terminus of VP1 and/or VP3 is acetylated. In some embodiments, the AAV particle is a recombinant AAV (rAAV) particle. In some embodiments, the AAV particle comprises an AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an AAV LK03 capsid, an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid, an AAV DJ8 capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, or a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV/human bocavirus virus 1). In some embodiments, the AAV capsid comprises a tyrosine

mutation or a heparin binding mutation. In some embodiments, the masses of VP1, VP2, and VP3 are compared to the theoretical masses of one or more of AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an AAV LK03 capsid, an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid, an AAV DJ8 capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, or a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV/human bocavirus virus 1), an AAV2HBKO capsid, an AAVPHP.B capsid or an AAVPHP.eB capsid.

In some embodiments of the above aspects and embodiments, the viral particle comprises an AAV1 ITR, an AAV2 ITR, an AAV3 ITR, an AAV4 ITR, an AAV5 ITR, an AAV6 ITR, an AAV7 ITR, an AAV8 ITR, an AAVrh8 ITR, an AAV9 ITR, an AAV10 ITR, an AAVrh10 ITR, an AAV11 ITR, or an AAV12 ITR. In some embodiments, the AAV particle comprises an AAV vector encoding a heterologous transgene.

In some aspects, the invention provides a method to determine the serotype of an adeno-associated virus (AAV) particle comprising a) denaturing the AAV particle, b) subjecting the denatured AAV particle to reduction and/or alkylation, c) subjecting the denatured AAV particle to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle, d) subjecting the fragments of VP1, VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), and e) determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle; wherein the specific combination of masses of fragments of VP1, VP2 and VP3 are indicative of the AAV serotype. In some embodiments, the calculated masses of the fragments of VP1, VP2 and/or VP3 are compared to the theoretical masses of fragments of VP1, VP2 and/or VP3 of one or more AAV serotypes.

In some aspects, the invention provides a method of determining the heterogeneity of a serotype of an AAV particle comprising a) denaturing the AAV particle, b) subjecting the denatured AAV particle to reduction and/or alkylation, c) subjecting the denatured AAV particle to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle, d) subjecting the fragments of VP1, VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), e) determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle, and f) comparing the masses of step e) with the theoretical masses of fragments of VP1, VP2 and VP3 of the AAV serotype; wherein a deviation of one or more of the masses of VP1, VP2 or VP3 are indicative of the AAV capsid heterogeneity. In some embodiments, the heterogeneity comprises one or more of mixed serotypes, variant capsids, capsid amino acid substitutions, truncated capsids, or modified capsids. In some embodiments, the reduction is by subjecting the AAV particle to dithiothreitol, beta-mercaptoethanol, or tris(2-carboxyethyl)phosphine (TCEP). In some embodiments, the alkylation is by subjecting the AAV particle to iodoacetic acid, iodoacetamide, or 4-vinylpyridine. In some embodiments, the digestion is an enzymatic digestion or a chemical digestion. In some embodiments, the enzymatic digestion is an endopeptidase digestion. In some embodiments, the enzymatic digestion is a trypsin digestion, a LysC digestion, an Asp-N digestion or a Glu-C digestion. In some embodiments, the chemical digestion is cyanogen bromide digestion or an acid digestion. In some embodiments, the AAV particle is denatured with acetic acid, guanidine hydrochloride and/or an organic solvent.

In some embodiments of the above aspects and embodiments, the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography. In some embodiments, the liquid chromatography is reverse phase liquid chromatography. In some embodiments, the reverse phase chromatography is a C18 reverse chromatography. In some embodiments, the chromatography uses a mobile phase A comprising formic acid in water. In some embodiments, the mobile phase A comprises about 0.1% formic acid. In some embodiments, the chromatography comprises a mobile phase B comprising formic acid in acetonitrile. In some embodiments, the mobile phase B comprises about 0.1% formic acid. In some embodiments, the proportion of mobile phase B in the chromatography increases over time. In some embodiments, mobile phase B increases from about 2% to about 60%. In some embodiments, mobile phase B increases from about 2% to about 60% in about 121 minutes. In some embodiments, the liquid chromatography is high-performance liquid chromatography (HPLC).

In some embodiments of the above aspects and embodiments, the mass spectrometry comprises a capillary voltage of about 3.5 kV. In some embodiments, the mass spectrometry comprises a sampling cone voltage of about 45 V. In some embodiments, the mass spectrometry comprises assisted calibration. In some embodiments, sodium iodide is used as a calibrant.

In some embodiments of the above aspects and embodiments, the N-terminus of VP1 and/or VP3 is acetylated. In some embodiments, the AAV particle is a recombinant AAV (rAAV) particle. In some embodiments, the AAV particle comprises an AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an AAV LK03 capsid, an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid, an AAV DJ8 capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, or a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV/human bocavirus virus 1). In some embodiments, the AAV capsid comprises a tyrosine mutation or a heparin binding mutation. In some embodiments, the masses of VP1, VP2, and VP3 are compared to the theoretical masses of one or more of AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an AAV LK03 capsid, an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid, an AAV DJ8 capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, or a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV/human bocavirus virus 1).

In some embodiments of the above aspects and embodiments, the viral particle comprises an AAV1 ITR, an AAV2 ITR, an AAV3 ITR, an AAV4 ITR, an AAV5 ITR, an AAV6 ITR, an AAV7 ITR, an AAV8 ITR, an AAVrh8 ITR, an AAV9 ITR, an AAV10 ITR, an AAVrh10 ITR, an AAV11

ITR, or an AAV12 ITR. In some embodiments, the AAV particle comprises an AAV vector encoding a heterologous transgene.

In some embodiments, the invention provides a recombinant AAV (rAAV) particle comprising an amino acid substitution at amino acid residue 2 of VP1 and/or VP3; wherein the amino acid substitution at amino acid residue 2 of VP1 and/or VP3 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of VP1 and/or VP3 of the parent AAV particle. In some embodiments, the substitution results in a higher frequency of N-terminal acetylation or a lower frequency of N-terminal acetylation. In some embodiments, the rAAV particle comprises an amino acid substitution at amino acid residue 2 of VP1; wherein the amino acid substitution at amino acid residue 2 of VP1 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of VP1 of the parent AAV particle. In some embodiments, the rAAV particle comprises an amino acid substitution at amino acid residue 2 of VP3; wherein the amino acid substitution at amino acid residue 2 of VP3 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of VP3 of the parent AAV particle. In some embodiments, amino acid residue 2 is substituted with Cys, Ser, Thr, Val, Gly, Asn, Asp, Glu, Ile, Leu, Phe, Gln, Lys, Met, Pro or Tyr. In some embodiments, amino acid residue 2 is substituted with Ser, Asp or Glu.

In some embodiments of the above aspects and embodiments, the AAV particle comprises an AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV2/2-7m8, AAV DJ, an AAV DJ8 capsid, AAV2 N587A, AAV2 E548A, AAV2 N708A, AAV V708K, goat AAV, AAV1/AAV2 chimeric, bovine AAV, mouse AAV, rAAV2/HBoV1, AAV2HBKO, AAVPHP.B, or AAVPHP.eB serotype capsid. In some embodiments, the AAV capsid further comprises a tyrosine mutation or a heparin binding mutation. In some embodiments, the rAAV particle comprises a rAAV vector. In some embodiments, the rAAV vector comprises one or more AAV ITRs. In some embodiments, the rAAV vector comprises an AAV1 ITR, an AAV2 ITR, an AAV3 ITR, an AAV4 ITR, an AAV5 ITR, an AAV6 ITR, an AAV7 ITR, an AAV8 ITR, an AAVrh8 ITR, an AAV9 ITR, an AAV10 ITR, an AAVrh10 ITR, an AAV11 ITR, or an AAV12 ITR. In some embodiments, the AAV capsid and the AAV ITRs are derived from the same serotype. In some embodiments, the AAV capsid and the AAV ITRs are derived from different serotypes. In some embodiments, the AAV particle comprises an AAV vector encoding a heterologous transgene flanked by one or more AAV ITRs.

In some embodiments of the above aspects and embodiments, the rAAV vector is a self-complementary vector. In some embodiments, the rAAV vector comprises first nucleic acid sequence encoding the transgene and a second nucleic acid sequence encoding a complement of the transgene, wherein the first nucleic acid sequence can form intrastrand base pairs with the second nucleic acid sequence along most or all of its length. In some embodiments, the first nucleic acid sequence and the second nucleic acid sequence are linked by a mutated AAV ITR, wherein the mutated AAV ITR comprises a deletion of the D region and comprises a mutation of the terminal resolution sequence.

In some embodiments of the above aspects and embodiments, the rAAV particle is produced by transfecting a host cell with nucleic acid encoding the rAAV vector and nucleic acid encoding AAV rep and cap functions, and providing nucleic acid encoding AAV helper functions. In some embodiments, the AAV helper functions are provided by transfecting the host cell with nucleic acid encoding the AAV helper functions. In some embodiments, the AAV helper functions are provided by infecting the host cell with an AAV helper virus that provides the AAV helper functions. In some embodiments, the AAV helper virus is an adenovirus, a herpes simplex virus or a baculovirus. In some embodiments, the rAAV particle is produced by an AAV producer cell comprising nucleic acid encoding the rAAV vector and nucleic acid encoding AAV rep and cap functions, and providing nucleic acid encoding AAV helper functions. In some embodiments, the AAV producer cell comprises nucleic acid encoding AAV helper functions. In some embodiments, the AAV helper functions are provided by infecting the AAV producer cells with an AAV helper virus that provides the AAV helper functions. In some embodiments, the AAV helper virus is an adenovirus, a herpes simplex virus, or a baculovirus. In some embodiments, the AAV cap functions provide an amino acid substitution at amino acid residue 2 of VP1 and/or VP3, wherein the amino acid substitution at amino acid residue 2 of VP1 and/or VP3 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of VP1 and/or VP3 of the parent AAV particle.

In some aspects, the invention provides a pharmaceutical composition comprising the rAAV particle as described herein. In some aspects, the invention provides a kit comprising the rAAV particle or the pharmaceutical composition as described herein. In some aspects, the invention provides an article of manufacture comprising the rAAV particle or the pharmaceutical composition as described herein.

In some aspects, the invention provides as AAV capsid protein comprising an amino acid substitution at amino acid residue 2 of a parent AAV capsid protein; wherein the amino acid substitution at amino acid residue 2 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of the parent AAV capsid protein. In some embodiments, the substitution results in a higher frequency of N-terminal acetylation or a lower frequency of N-terminal acetylation. In some embodiments, the AAV capsid protein is VP1 or VP3. In some embodiments, amino acid residue 2 of the AAV capsid protein is substituted with Cys, Ser, Thr, Val, Gly, Asn, Asp, Glu, Ile, Leu, Phe, Gln, Lys, Met, Pro or Tyr. In some embodiments, amino acid residue 2 of the AAV capsid protein is substituted with Ser, Asp or Glu. In some embodiments, the amino acid substitution results in less deamidation of the AAV capsid.

In some embodiments of the above aspects and embodiments, the AAV particle comprises an AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV2/2-7m8, AAV DJ, an AAV DJ8 capsid, AAV2 N587A, AAV2 E548A, AAV2 N708A, AAV V708K, goat AAV, AAV1/AAV2 chimeric, bovine AAV, mouse AAV, rAAV2/HBoV1, AAV2HBKO, AAVPHP.B, or AAVPHP.eB serotype capsid. In some embodiments, the AAV capsid further comprises a tyrosine mutation or a heparin binding mutation.

In some aspects, the invention provides a method of improving stability of a rAAV particle comprising substituting amino acid residue 2 of VP1 and/or VP3 of a parent VP1 and/or VP3; wherein the substituting amino acid residue 2 alters N-terminal acetylation of VP1 and/or VP3, as compared to amino acid residue 2 of the parent VP1 and/or VP3. In some aspects, the invention provides a method of improving assembly of rAAV particles in a cell comprising

substituting amino acid residue 2 of VP1 and/or VP3 or a parental VP1 and/or VP3; wherein substituting amino acid at position 2 alters N-terminal acetylation of VP1 and/or VP3, as compared to amino acid residue 2 of the parent VP1 and/or VP3. In some aspects, the invention provides a method of improving the transduction of rAAV particles in a cell comprising substituting amino acid residue 2 of VP1 and/or VP3 or a parental VP1 and/or VP3; wherein substituting amino acid residue 2 alters N-terminal acetylation of VP1 and/or VP3, as compared to amino acid residue 2 of the parent VP1 and/or VP3. In some embodiments, the substituted amino acid results in a higher frequency of N-terminal acetylation or a lower frequency of N-terminal acetylation. In some embodiments, the amino acid substitution at amino acid residue 2 of VP1 is substituted. In some embodiments, the amino acid substitution at amino acid residue 2 of VP3 is substituted. In some embodiments, amino acid residue 2 is substituted with Cys, Ser, Thr, Val, Gly, Asn, Asp, Glu, Ile, Leu, Phe, Gln, Lys, Met, Pro or Tyr. In some embodiments, amino acid residue 2 is substituted with Ser, Asp or Glu. In some aspects, the invention provides a method of reducing the transduction of rAAV particles in a cell comprising substituting amino acid residue 2 of VP1 and/or VP3; wherein the substituted amino acid at position 2 alters N-terminal acetylation of VP1 and/or VP3, as compared to amino acid residue 2 of the parent VP1 and/or VP3.

In some embodiments of the above aspects and embodiments, the AAV particle comprises an AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV2/2-7m8, AAV DJ, an AAV DJ8 capsid, AAV2 N587A, AAV2 E548A, AAV2 N708A, AAV V708K, goat AAV, AAV1/AAV2 chimeric, bovine AAV, mouse AAV, rAAV2/HBoV1, AAV2HBKO, AAVPHP.B, or AAVPHP.eB serotype capsid. In some embodiments, the AAV capsid further comprises a tyrosine mutation or a heparin binding mutation. In some embodiments, the rAAV particle comprises a rAAV vector. In some embodiments, the rAAV vector comprises one or more AAV ITRs. In some embodiments, the rAAV vector comprises an AAV1 ITR, an AAV2 ITR, an AAV3 ITR, an AAV4 ITR, an AAV5 ITR, an AAV6 ITR, an AAV7 ITR, an AAV8 ITR, an AAVrh8 ITR, an AAV9 ITR, an AAV10 ITR, an AAVrh10 ITR, an AAV11 ITR, or an AAV12 ITR. In some embodiments, the AAV capsid and the AAV ITRs are derived from the same serotype. In some embodiments, the AAV capsid and the AAV ITRs are derived from different serotypes. In some embodiments, the AAV particle comprises an AAV vector encoding a heterologous transgene flanked by one or more AAV ITRs.

In some embodiments of the above aspects and embodiments, the rAAV vector is a self-complementary vector. In some embodiments, the rAAV vector comprises first nucleic acid sequence encoding the transgene and a second nucleic acid sequence encoding a complement of the transgene, wherein the first nucleic acid sequence can form intrastrand base pairs with the second nucleic acid sequence along most or all of its length. In some embodiments, the first nucleic acid sequence and the second nucleic acid sequence are linked by a mutated AAV ITR, wherein the mutated AAV ITR comprises a deletion of the D region and comprises a mutation of the terminal resolution sequence.

In some embodiments of the above aspects and embodiments, the rAAV particle is produced by transfecting a host cell with nucleic acid encoding the rAAV vector and nucleic acid encoding AAV rep and cap functions, and providing nucleic acid encoding AAV helper functions. In some embodiments, the AAV helper functions are provided by transfecting the host cell with nucleic acid encoding the AAV helper functions. In some embodiments, the AAV helper functions are provided by infecting the host cell with an AAV helper virus that provides the AAV helper functions. In some embodiments, the AAV helper virus is an adenovirus, a herpes simplex virus or a baculovirus. In some embodiments, the rAAV particle is produced by an AAV producer cell comprising nucleic acid encoding the rAAV vector and nucleic acid encoding AAV rep and cap functions, and providing nucleic acid encoding AAV helper functions. In some embodiments, the AAV producer cell comprises nucleic acid encoding AAV helper functions. In some embodiments, the AAV helper functions are provided by infecting the AAV producer cells with an AAV helper virus that provides the AAV helper functions. In some embodiments, the AAV helper virus is an adenovirus, a herpes simplex virus, or a baculovirus. In some embodiments, the AAV cap functions provide an amino acid substitution at amino acid residue 2 of VP1 and/or VP3, wherein the amino acid substitution at amino acid residue 2 of VP1 and/or VP3 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of VP1 and/or VP3 of the parent AAV particle.

In some aspects, the invention provides a recombinant AAV (rAAV) particle comprising one or more amino acid substitutions at amino acid residue A35, N57, G58, N382, G383, N511, G512, N715, or G716 of VP1 or VP3 of a parent particle, residue numbering based on VP1 of AAV2; wherein the one or more amino acid substitutions alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some embodiments, the one or more amino acid substitution is at amino acid residue A35, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3 and alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some embodiments, the one or more amino acid substitutions comprises a substitution with Asp at N57 of VP1, N382 of VP3, N511 of VP3, or N715 of VP3; and results in a higher frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some embodiments, the one or more amino acid substitutions comprise a N57K or a N57Q substitution and results in a lower frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some embodiments, the one or more amino acid substitution comprise a substitution with Asp at A35 of VP1 and results in a higher frequency of deamidation as compared to deamidation of VP1 of the parent AAV particle. In some embodiments, the one or more amino acid substitutions is at G58 of VP1, G383 of VP3, G512 of VP3, or G716 of VP3 and results in a lower frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some embodiments, the G58 of VP1 is substituted with Asp. In some embodiments, the rAAV particle is an AAV1 particle or an AAV2 particle.

In some aspects, the invention provides pharmaceutical compositions comprising AAV particles comprising one or more amino acid substitutions at amino acid residue A35, N57, G58, N382, G383, N511, G512, N715, or G716 of VP1 or VP3, residue numbering based on VP1 of AAV2; wherein the amino acid substitution alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some aspects, the invention provides kits comprising AAV particles or compositions comprising AAV particles wherein the AAV particles comprise one or more

amino acid substitutions at amino acid residue A35, N57, G58, N382, G383, N511, G512, N715, or G716 of VP1 or VP3, residue numbering based on VP1 of AAV2; wherein the amino acid substitution alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some aspects, the invention provides articles of manufacture comprising AAV particles or compositions comprising AAV particles wherein the AAV particles comprise one or more amino acid substitutions at amino acid residue A35, N57, G58, N382, G383, N511, G512, N715, or G716 of VP1 or VP3, residue numbering based on VP1 of AAV2; wherein the amino acid substitution alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some aspects, the invention provides an AAV capsid protein comprising an amino acid substitution of a parent AAV capsid protein; wherein the amino acid substitution alters deamidation of the capsid compared to the parent AAV capsid protein.

In some aspects, the invention provides a method of improving the stability of a rAAV particle comprising substituting one or more amino acid residues, wherein the one or more amino acid residues is A35, N57, G58, N382, G383, N511, G512, N715, or G716, residue numbering based on VP1 of AAV2; wherein the amino acid substitution alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some aspects, the invention provides a method of improving the assembly of rAAV particles in a cell comprising substituting one or more amino acid residues, wherein the one or more amino acid residues is A35, N57, G58, N382, G383, N511, G512, N715, or G716, residue numbering based on VP1 of AAV2; wherein the amino acid substitution alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some aspects, the invention provides a method of improving the transduction of rAAV particles in a cell comprising substituting one or more amino acid residues, wherein the one or more amino acid residues is A35, N57, G58, N382, G383, N511, G512, N715, or G716, residue numbering based on VP1 of AAV2; wherein the amino acid substitution alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some embodiments, the one or more amino acid substitutions is at A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3; wherein the amino acid substitution alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some embodiments, the parental Ala residue at position 35 of VP1 is substituted with Asn. In some embodiments, the parental Gly residue at position 58 of VP1 is substituted with Asp. In some embodiments, the rAAV particle is an AAV1 particle or an AAV2 particle.

In some embodiments, the invention provides a method of improving the stability, assembly and/or transduction efficiency of a rAAV particle comprising substituting one or more amino acid residues, wherein the one or more amino acid residues is A35, N57, G58, N382, G383, N511, G512, N715, or G716, residue numbering based on VP1 of AAV2; wherein the amino acid substitution alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle as described above, wherein the AAV particle comprises an AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV2/2-7m8, AAV DJ, an AAV DJ8 capsid, AAV2 N587A, AAV2 E548A, AAV2 N708A, AAV V708K, goat AAV, AAV1/AAV2 chimeric, bovine AAV, mouse AAV, or rAAV2/HBoV1 serotype capsid. In some embodiments, the AAV capsid further comprises a tyrosine mutation or a heparin binding mutation. In some embodiments, the rAAV particle comprises a rAAV vector. In some embodiments, the rAAV vector comprises one or more AAV ITRs. In some embodiments, the rAAV vector comprises an AAV1 ITR, an AAV2 ITR, an AAV3 ITR, an AAV4 ITR, an AAV5 ITR, an AAV6 ITR, an AAV7 ITR, an AAV8 ITR, an AAVrh8 ITR, an AAV9 ITR, an AAV10 ITR, an AAVrh10 ITR, an AAV11 ITR, or an AAV12 ITR. In some embodiments, the AAV capsid and the AAV ITRs are derived from the same serotype. In some embodiments, the AAV capsid and the AAV ITRs are derived from different serotypes. In some embodiments, the AAV particle comprises an AAV vector encoding a heterologous transgene flanked by one or more AAV ITRs.

In some embodiments of the above aspects and embodiments, the rAAV vector is a self-complementary vector. In some embodiments, the rAAV vector comprises first nucleic acid sequence encoding the transgene and a second nucleic acid sequence encoding a complement of the transgene, wherein the first nucleic acid sequence can form intrastrand base pairs with the second nucleic acid sequence along most or all of its length. In some embodiments, the first nucleic acid sequence and the second nucleic acid sequence are linked by a mutated AAV ITR, wherein the mutated AAV ITR comprises a deletion of the D region and comprises a mutation of the terminal resolution sequence.

In some embodiments of the above aspects and embodiments, the rAAV particle is produced by transfecting a host cell with nucleic acid encoding the rAAV vector and nucleic acid encoding AAV rep and cap functions, and providing nucleic acid encoding AAV helper functions. In some embodiments, the AAV helper functions are provided by transfecting the host cell with nucleic acid encoding the AAV helper functions. In some embodiments, the AAV helper functions are provided by infecting the host cell with an AAV helper virus that provides the AAV helper functions. In some embodiments, the AAV helper virus is an adenovirus, a herpes simplex virus or a baculovirus. In some embodiments, the rAAV particle is produced by an AAV producer cell comprising nucleic acid encoding the rAAV vector and nucleic acid encoding AAV rep and cap functions, and providing nucleic acid encoding AAV helper functions. In some embodiments, the AAV producer cell comprises nucleic acid encoding AAV helper functions. In some embodiments, the AAV helper functions are provided by infecting the AAV producer cells with an AAV helper virus that provides the AAV helper functions. In some embodiments, the AAV helper virus is an adenovirus, a herpes simplex virus, or a baculovirus. In some embodiments, the AAV cap functions provide an amino acid substitution of VP1 and/or VP3, wherein the amino acid substitution modulated deamidation of the capsid compared to the parent AAV particle.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. **1**A-D provide total ion Chromatograms of LC/MS of AAV2 VPs. FIG. **1**A: 10 cm long BEH C4 column with 1.7%/min gradient, FIG. **1**B: 10 cm long BEH C4 column with 0.5%/min gradient; FIG. **1**C: 15 cm long BEH C4 column with 0.5%/min gradient, FIG. **1**D: 15 cm long BEH C8 column with 0.5%/min gradient.

FIGS. **2**A&B provide deconvoluted mass spectra from FIG. **1**D peak 1 (FIG. **2**A) and FIG. **1**D peak 2 (FIG. **2**B).

FIG. **3** provides the sequence coverage of AAV2 VP1 (SEQ ID NO:3): green, tryptic peptides, blue, Lys-C peptides, pink, Asp-N peptides.

FIGS. **4**A-**4**C provide MS/MS spectra of AAV2 VP N-terminal peptides. FIG. **4**A: VP1 N-terminal tryptic peptide A(Ac)ADGYLPDWLEDTLSEGIR (SEQ ID NO: 4). FIG. **4**B VP2 N-terminal Asp-N peptide APGKKRPVEHSPVEP (SEQ ID NO: 15). FIG. **4**C: VP-3 N-terminal Asp-N derived peptide A(Ac)TGSGAPM (SEQ ID NO: 5).

FIG. **5**A-**5**C provides the sequence alignment of 13 AAV serotypes black letter/white background: non-similar; blue letter/blue background: conservative; black letter/green background: block of similar; red letter/yellow background: identical; green letter/white background: weakly similar. AAVRh10 (SEQ ID NO: 17); AAV10 (SEQ ID NO: 18); AAV8 (SEQ ID NO: 19); AAV7 (SEQ ID NO: 20); AAV1 (SEQ ID NO: 21); AAV6 (SEQ ID NO: 22); AAV2 (SEQ ID NO: 23); AAV3 (SEQ ID NO: 24); AAV11 (SEQ ID NO: 25); AAV12 (SEQ ID NO: 26); AAV4 (SEQ ID NO: 27); AAV5 (SEQ ID NO: 28); AAV9 (SEQ ID NO: 29); Consensus (SEQ ID NO: 30).

FIGS. **6**A & **6**B show the results of LC/MS/MS analysis comparing the percentage of deamidation in AAV1 and AAV2 particles produced by the TTx and PCL methods. The T9 peptide YLGPF**NG**LDK (SEQ ID NO: 9) was used to monitor potential deamidation site N57 in both AAV1 and AAV2.

FIGS. **7**A & **7**B show the results of LC/MS/MS analysis comparing the percentage of deamidation in AAV1 and AAV2 particles produced by the TTx and PCL methods. The T49 peptides YNL**NG**R (SEQ ID NO: 11) and YHL**NG**GR (SEQ ID NO: 12) were used to monitor potential deamidation site N511 in AAV1 and AAV2, respectively.

FIGS. **8**A & **8**B show the results of LC/MS/MS analysis comparing the percentage of deamidation in AAV1 and AAV2 particles produced by the TTx and PCL methods. The T67 peptides SANVDFTVDN**NG**LYTEPR (SEQ ID NO: 13) and SVNVDFTVDT**NG**VYSEPR (SEQ ID NO: 14) were used to monitor potential deamidation site N715 in AAV1 and AAV2, respectively.

FIG. **9** shows the results of SYPRO protein gel analysis of production and VP1:VP2:VP3 ratio of AAV5 deacetylated mutant variants.

FIG. **10** illustrates an in vitro transduction assay for testing transduction efficiency of AAV5 deacetylated variants.

FIG. **11** shows the efficiency of cell entry by the indicated AAV5 deacetylated variants or parental unmodified AAV5, as measured by vector genome copies/μg protein. Three cell lines were used: 293, HeLa, and HuH7.

FIG. **12** shows eGFP expression (as measured by ELISA) by cells transduced with the indicated AAV5 deacetylated variants as compared to transduction with parental unmodified AAV5. Three cell lines were used: 293, HeLa, and HuH7.

FIG. **13** provides the sequence alignment of 13 AAV serotypes, highlighting the conserved N57G58 deamidation site and the A35 residue in AAV2. AAVRh10 (SEQ ID NO: 31); AAV10 (SEQ ID NO: 31); AAV8 (SEQ ID NO: 32); AAV7 (SEQ ID NO: 33); AAV1 (SEQ ID NO: 31); AAV6 (SEQ ID NO: 31); AAV2 (SEQ ID NO: 34); AAV3 (SEQ ID NO: 35); AAV11 (SEQ ID NO: 31); AAV12 (SEQ ID NO: 36); AAV4 (SEQ ID NO: 37); AAV5 (SEQ ID NO: 38); AAV9 (SEQ ID NO: 39); Consensus (SEQ ID NO: 40).

FIG. **14** shows a protein gel of VP1, VP2, and VP3 capsid proteins from AAV1 or AAV2 particles produced by the PCL or TTx method. *highlights the truncated VP1 (tVP1) protein.

FIG. **15** shows the results of LC/MS analysis of deamidation of the indicated AAV2 mutants, as compared to control AAV2 capsids.

FIG. **16** shows the results of SYPRO protein gel analysis of production and VP1:VP2:VP3 ratio of AAV2 deamidation mutant variants.

FIG. **17** illustrates an in vitro transduction assay for testing transduction efficiency of AAV2 deamidation variants.

FIG. **18** shows the efficiency of cell entry by the indicated AAV2 deamidation variants or parental unmodified AAV2, as measured by vector genome copies/μg protein. Three cell lines were used: 293, HeLa, and HuH7.

FIG. **19** shows eGFP expression (as measured by ELISA) by cells transduced with the indicated AAV2 deamidation variants as compared to transduction with parental unmodified AAV2. Three cell lines were used: 293, HeLa, and HuH7.

DETAILED DESCRIPTION

In some aspects, the invention provides a method to determine the serotype of an adeno-associated virus (AAV) particle(s) comprising: a) denaturing the AAV particle, b) injecting the denatured AAV particle to liquid chromatography/mass spectrometry (LC/MS), and c) determining the masses of VP1, VP2 and VP3 of the AAV particle; wherein the specific combination of masses of VP1, VP2 and VP3 are indicative of the AAV serotype.

In other aspects, the invention provides a method of determining the heterogeneity of an AAV particle comprising: a) denaturing the AAV particle, b) injecting the denatured AAV particle to liquid chromatography/mass spectrometry (LC/MS), and c) determining the masses of VP1, VP2 and VP3 of the AAV particle, and comparing the masses of step c) with the theoretical masses of VP1, VP2 and VP3 of the AAV serotype; wherein a deviation of one or more of the masses of VP1, VP2 or VP3 are indicative of the AAV capsid heterogeneity.

In other aspects, the invention provides a method to determine the serotype of an adeno-associated virus (AAV) particle comprising a) denaturing the AAV particle, b) subjecting the denatured AAV particle to reduction and/or alkylation, c) injecting the denatured AAV particle to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle, d) subjecting the fragments of VP1, VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), and e) determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle; wherein the specific combination of masses of fragments of VP1, VP2 and VP3 are indicative of the AAV serotype.

In other aspects, the invention provides a method of determining the heterogeneity of an AAV particle of a serotype comprising: a) denaturing the AAV particle, b) subjecting the denatured AAV particle to reduction and/or alkylation, c) injecting the denatured AAV particle to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle, d) subjecting the fragments of VP1, VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), e) determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle, and f) comparing the masses of step e) with the theoretical

masses of fragments of VP1, VP2 and VP3 of the AAV serotype; wherein a deviation of one or more of the masses of VP1, VP2 or VP3 are indicative of the AAV capsid heterogeneity.

In some aspects, the invention provides a recombinant AAV (rAAV) particle comprising an amino acid substitution at amino acid residue 2 of VP1 and/or VP3; wherein the amino acid substitution at amino acid residue 2 of VP1 and/or VP3 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of VP1 and/or VP3 of the parent AAV particle.

In some aspects, the invention provides a method of improving the assembly of rAAV particles in a cell comprising substituting amino acid residue 2 of VP1 and/or VP3; wherein the substituted amino acid at position 2 is N-acetylated at a higher frequency than amino acid residue 2 of the parent VP1 and/or VP3. In some aspects, the invention provides a method of improving the transduction of rAAV particles in a cell comprising substituting amino acid residue 2 of VP1 and/or VP3; wherein the substituted amino acid at position 2 is N-acetylated at a higher frequency than amino acid residue 2 of the parent VP1 and/or VP3.

I. General Techniques

The techniques and procedures described or referenced herein are generally well understood and commonly employed using conventional methodology by those skilled in the art, such as, for example, the widely utilized methodologies described in *Molecular Cloning: A Laboratory Manual* (Sambrook et al., 4th ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2012); *Current Protocols in Molecular Biology* (F. M. Ausubel, et al. eds., 2003); the series *Methods in Enzymology* (Academic Press, Inc.); *PCR 2: A Practical Approach* (M. J. MacPherson, B. D. Hames and G. R. Taylor eds., 1995); *Antibodies, A Laboratory Manual* (Harlow and Lane, eds., 1988); *Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications* (R. I. Freshney, 6, ed., J. Wiley and Sons, 2010); *Oligonucleotide Synthesis* (M. J. Gait, ed., 1984); *Methods in Molecular Biology, Humana Press; Cell Biology: A Laboratory Notebook* (J. E. Cellis, ed., Academic Press, 1998); *Introduction to Cell and Tissue Culture* (J. P. Mather and P. E. Roberts, Plenum Press, 1998); *Cell and Tissue Culture: Laboratory Procedures* (A. Doyle, J. B. Griffiths, and D. G. Newell, eds., J. Wiley and Sons, 1993-8); *Handbook of Experimental Immunology* (D. M. Weir and C. C. Blackwell, eds., 1996); *Gene Transfer Vectors for Mammalian Cells* (J. M. Miller and M. P. Calos, eds., 1987); *PCR: The Polymerase Chain Reaction*, (Mullis et al., eds., 1994); *Current Protocols in Immunology* (J. E. Coligan et al., eds., 1991); *Short Protocols in Molecular Biology* (Ausubel et al., eds., J. Wiley and Sons, 2002); *Immunobiology* (C. A. Janeway et al., 2004); *Antibodies* (P. Finch, 1997); *Antibodies: A Practical Approach* (D. Catty., ed., IRL Press, 1988-1989); *Monoclonal Antibodies: A Practical Approach* (P. Shepherd and C. Dean, eds., Oxford University Press, 2000); *Using Antibodies: A Laboratory Manual* (E. Harlow and D. Lane, Cold Spring Harbor Laboratory Press, 1999); *The Antibodies* (M. Zanetti and J. D. Capra, eds., Harwood Academic Publishers, 1995); and *Cancer: Principles and Practice of Oncology* (V. T. DeVita et al., eds., J.B. Lippincott Company, 2011).

II. Definitions

A "vector," as used herein, refers to a recombinant plasmid or virus that comprises a nucleic acid to be delivered into a host cell, either in vitro or in vivo.

The term "polynucleotide" or "nucleic acid" as used herein refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. Thus, this term includes, but is not limited to, single-, double- or multi-stranded DNA or RNA, genomic DNA, cDNA, DNA-RNA hybrids, or a polymer comprising purine and pyrimidine bases, or other natural, chemically or biochemically modified, non-natural, or derivatized nucleotide bases. The backbone of the nucleic acid can comprise sugars and phosphate groups (as may typically be found in RNA or DNA), or modified or substituted sugar or phosphate groups. Alternatively, the backbone of the nucleic acid can comprise a polymer of synthetic subunits such as phosphoramidates and thus can be an oligodeoxynucleoside phosphoramidate ($P—NH_2$) or a mixed phosphoramidate-phosphodiester oligomer. In addition, a double-stranded nucleic acid can be obtained from the single stranded polynucleotide product of chemical synthesis either by synthesizing the complementary strand and annealing the strands under appropriate conditions, or by synthesizing the complementary strand de novo using a DNA polymerase with an appropriate primer.

The terms "polypeptide" and "protein" are used interchangeably to refer to a polymer of amino acid residues, and are not limited to a minimum length. Such polymers of amino acid residues may contain natural or non-natural amino acid residues, and include, but are not limited to, peptides, oligopeptides, dimers, trimers, and multimers of amino acid residues. Both full-length proteins and fragments thereof are encompassed by the definition. The terms also include post-translational modifications of the polypeptide, for example, glycosylation, sialylation, acetylation, phosphorylation, and the like. Furthermore, for purposes of the present invention, a "polypeptide" refers to a protein which includes modifications, such as deletions, additions, and substitutions (generally conservative in nature), to the native sequence, as long as the protein maintains the desired activity. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutations of hosts which produce the proteins or errors due to PCR amplification.

A "recombinant viral vector" refers to a recombinant polynucleotide vector comprising one or more heterologous sequences (i.e., nucleic acid sequence not of viral origin). In the case of recombinant AAV vectors, the recombinant nucleic acid is flanked by at least one, e.g., two, inverted terminal repeat sequences (ITRs).

A "recombinant AAV vector (rAAV vector)" refers to a polynucleotide vector comprising one or more heterologous sequences (i.e., nucleic acid sequence not of AAV origin) that are flanked by at least one, e.g., two, AAV inverted terminal repeat sequences (ITRs). Such rAAV vectors can be replicated and packaged into infectious viral particles when present in a host cell that has been infected with a suitable helper virus (or that is expressing suitable helper functions) and that is expressing AAV rep and cap gene products (i.e. AAV Rep and Cap proteins). When a rAAV vector is incorporated into a larger polynucleotide (e.g., in a chromosome or in another vector such as a plasmid used for cloning or transfection), then the rAAV vector may be referred to as a "pro-vector" which can be "rescued" by replication and encapsidation in the presence of AAV packaging functions and suitable helper functions. A rAAV vector can be in any of a number of forms, including, but not limited to, plasmids, linear artificial chromosomes, complexed with lipids, encapsulated within liposomes, and, in embodiments, encapsidated in a viral particle, particularly an AAV particle.

A rAAV vector can be packaged into an AAV virus capsid to generate a "recombinant adeno-associated viral particle (rAAV particle)".

An "rAAV virus" or "rAAV viral particle" refers to a viral particle composed of at least one AAV capsid protein and an encapsidated rAAV vector genome.

A "parent AAV particle" and "parent AAV capsid protein" as used herein in the context of comparing N-acetylation and/or deamidation refers to an AAV particle or capsid protein into which amino acid modifications are introduced to modulate N-acetylation and/or deamidation (e.g., an AAV particle/capsid protein that is the same as or similar to the AAV particle/capsid of the subject invention but does not comprise the mutations that modulate/alter N-acetylation and/or deamidation as described herein). In some embodiments, the parent AAV particle is a recombinant AAV particle comprising a recombinant AAV genome. In some embodiments, the parent AAV capsid particle or parent AAV capsid protein comprises amino acid substitutions that affect other aspects of the AAV particle. For example, the parent AAV particle may comprise amino acid substitutions that affect the binding of AAV to its receptor, such as affecting binding of AAV2 to heparin sulfate proteoglycan (e.g. an AAV2 HBKO particle). An AAV2 HBKO particle can be mutated to introduce amino acid substitutions that modulate N-acetylation and/or deamidation. Such a mutated AAV particle may then be compared to the parent AAV2 HBKO particle in aspects of the invention as described herein. A parent AAV capsid protein may include a parent VP1 capsid protein, a parent VP2 capsid protein, or a VP3 capsid protein.

As used herein, the term "modulate" or "alter" in reference to a parent molecule means to change a feature of the parent molecule. For example, an AAV particle with altered N-acetylation may show increased or decreased N-acetylation compared to the parent AAV particle and an AAV particle with altered deamidation may show increased or decreased deamidation compared to the parent AAV particle.

"Heterologous" means derived from a genotypically distinct entity from that of the rest of the entity to which it is compared or into which it is introduced or incorporated. For example, a nucleic acid introduced by genetic engineering techniques into a different cell type is a heterologous nucleic acid (and, when expressed, can encode a heterologous polypeptide). Similarly, a cellular sequence (e.g., a gene or portion thereof) that is incorporated into a viral vector is a heterologous nucleotide sequence with respect to the vector.

The term "transgene" refers to a nucleic acid that is introduced into a cell and is capable of being transcribed into RNA and optionally, translated and/or expressed under appropriate conditions. In aspects, it confers a desired property to a cell into which it was introduced, or otherwise leads to a desired therapeutic or diagnostic outcome. In another aspect, it may be transcribed into a molecule that mediates RNA interference, such as siRNA.

The terms "genome particles (gp)," "genome equivalents," or "genome copies" as used in reference to a viral titer, refer to the number of virions containing the recombinant AAV DNA genome, regardless of infectivity or functionality. The number of genome particles in a particular vector preparation can be measured by procedures such as described in the Examples herein, or for example, in Clark et al. (1999) *Hum. Gene Ther.*, 10:1031-1039; Veldwijk et al. (2002) *Mol. Ther.*, 6:272-278.

The terms "infection unit (iu)," "infectious particle," or "replication unit," as used in reference to a viral titer, refer to the number of infectious and replication-competent recombinant AAV vector particles as measured by the infectious center assay, also known as replication center assay, as described, for example, in McLaughlin et al. (1988) *J. Virol.*, 62:1963-1973.

The term "transducing unit (tu)" as used in reference to a viral titer, refers to the number of infectious recombinant AAV vector particles that result in the production of a functional transgene product as measured in functional assays such as described in Examples herein, or for example, in Xiao et al. (1997) *Exp. Neurobiol.*, 144:113-124; or in Fisher et al. (1996) *J. Virol.*, 70:520-532 (LFU assay).

An "inverted terminal repeat" or "ITR" sequence is a term well understood in the art and refers to relatively short sequences found at the termini of viral genomes which are in opposite orientation.

An "AAV inverted terminal repeat (ITR)" sequence, a term well-understood in the art, is an approximately 145-nucleotide sequence that is present at both termini of the native single-stranded AAV genome. The outermost 125 nucleotides of the ITR can be present in either of two alternative orientations, leading to heterogeneity between different AAV genomes and between the two ends of a single AAV genome. The outermost 125 nucleotides also contains several shorter regions of self-complementarity (designated A, A', B, B', C, C' and D regions), allowing intrastrand base-pairing to occur within this portion of the ITR.

A "terminal resolution sequence" or "trs" is a sequence in the D region of the AAV ITR that is cleaved by AAV rep proteins during viral DNA replication. A mutant terminal resolution sequence is refractory to cleavage by AAV rep proteins. "AAV helper functions" refer to functions that allow AAV to be replicated and packaged by a host cell. AAV helper functions can be provided in any of a number of forms, including, but not limited to, helper virus or helper virus genes which aid in AAV replication and packaging. Other AAV helper functions are known in the art such as genotoxic agents.

"AAV helper functions" refer to functions that allow AAV to be replicated and packaged by a host cell. AAV helper functions can be provided in any of a number of forms, including, but not limited to, helper virus or helper virus genes which aid in AAV replication and packaging. Other AAV helper functions are known in the art such as genotoxic agents.

A "helper virus" for AAV refers to a virus that allows AAV (which is a defective parvovirus) to be replicated and packaged by a host cell. A number of such helper viruses have been identified, including adenoviruses, herpesviruses, poxviruses such as vaccinia, and baculovirus. The adenoviruses encompass a number of different subgroups, although Adenovirus type 5 of subgroup C (Ad5) is most commonly used. Numerous adenoviruses of human, non-human mammalian and avian origin are known and are available from depositories such as the ATCC. Viruses of the herpes family, which are also available from depositories such as ATCC, include, for example, herpes simplex viruses (HSV), Epstein-Barr viruses (EBV), cytomegaloviruses (CMV) and pseudorabies viruses (PRV). Baculoviruses available from depositories include *Autographa californica* nuclear polyhedrosis virus.

"Percent (%) sequence identity" with respect to a reference polypeptide or nucleic acid sequence is defined as the percentage of amino acid residues or nucleotides in a candidate sequence that are identical with the amino acid residues or nucleotides in the reference polypeptide or nucleic acid sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum

percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid or nucleic acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software programs, for example, those described in Current Protocols in Molecular Biology (Ausubel et al., eds., 1987), Supp. 30, section 7.7.18, Table 7.7.1, and including BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. A potential alignment program is ALIGN Plus (Scientific and Educational Software, Pennsylvania). Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For purposes herein, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows: 100 times the fraction X/Y, where X is the number of amino acid residues scored as identical matches by the sequence alignment program in that program's alignment of A and B, and where Y is the total number of amino acid residues in B. It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A. For purposes herein, the % nucleic acid sequence identity of a given nucleic acid sequence C to, with, or against a given nucleic acid sequence D (which can alternatively be phrased as a given nucleic acid sequence C that has or comprises a certain % nucleic acid sequence identity to, with, or against a given nucleic acid sequence D) is calculated as follows: 100 times the fraction W/Z, where W is the number of nucleotides scored as identical matches by the sequence alignment program in that program's alignment of C and D, and where Z is the total number of nucleotides in D. It will be appreciated that where the length of nucleic acid sequence C is not equal to the length of nucleic acid sequence D, the % nucleic acid sequence identity of C to D will not equal the % nucleic acid sequence identity of D to C.

An "isolated" molecule (e.g., nucleic acid or protein) or cell means it has been identified and separated and/or recovered from a component of its natural environment.

"Mass spectrometry" refers to the analytical chemistry technique of identifying an amount and/or type of a compound (e.g., a polypeptide) by measuring the mass-to-charge ratio and abundance of gas-phase ions. The term "mass spectrometry" may be used interchangeably herein.

"Heterogeneity" when used in reference to an AAV capsid refers to an AAV capsid characterized by one or more capsid polypeptides observed to deviate from a reference mass of a VP1, VP2, and/or VP3 polypeptide, or fragment thereof. A reference mass may include, without limitation, a theoretical, predicted, or expected mass of a VP1, VP2, and/or VP3 polypeptide, e.g., of a known AAV serotype. For example, an AAV capsid may be said to display heterogeneity if it demonstrates one or more of the following properties (without limitation): a mixed serotype, a variant capsid, a capsid amino acid substitution, a truncated capsid, or a modified capsid.

Reference to "about" a value or parameter herein includes (and describes) embodiments that are directed to that value or parameter per se. For example, description referring to "about X" includes description of "X."

As used herein, the singular form of the articles "a," "an," and "the" includes plural references unless indicated otherwise.

It is understood that aspects and embodiments of the invention described herein include "comprising," "consisting," and/or "consisting essentially of" aspects and embodiments.

III. Methods

Certain aspects of the present disclosure relate to methods of determining the serotype of a viral particle. Other aspects of the present disclosure relate to methods of determining the heterogeneity of a viral particle. As described below, the accurate masses of VP1, VP2 and VP3 of each AAV serotype are unique and can be used to identify or differentiate AAV capsid serotypes. These methods are based in part on the discovery described herein that direct LC/MS of different types of AAVs after denaturation may be used to monitor the protein sequence and post-translational modifications with accurate mass measurement in the intact protein level. Further, acetylations of N-termini of VP1 and VP3 may also be identified and/or monitored in different AAV serotypes. Based on these AAV results and the guidance provided herein, it is contemplated that such methods may readily be applied to profile a variety of viruses, e.g., the viral families, subfamilies, and genera of the present disclosure. The methods of the present disclosure may find use, e.g., in profile VPs to monitor VP expressions, posttranslational modifications, and truncations and to ensure product consistency during VLP production, to confirm site-direct mutagenesis or structural characterization for capsid protein engineering applications, and/or to monitor or detect heterogeneity of a viral particle or preparation.

In some embodiments, the methods include denaturing a viral particle. In some embodiments, a viral particle such as an AAV particle may be denatured using detergent, heat, high salt, or buffering with a low or high pH. In certain embodiments, an AAV particle may be denatured using acetic acid or guanidine hydrochloride. The skilled artisan will recognize that a variety of methods useful for promoting and/or monitoring protein denaturation are available in the art and may suitably select a denaturation method compatible with liquid chromatography/mass spectrometry. For example, if heat denaturation is used, care may be applied to avoid protein precipitation and reverse phase column clogging. Similarly, high salt denaturation may be coupled with a desalting step prior to LC/MS or LC/MS/MS. In other embodiments, high pH denaturation, low pH denaturation, or denaturation using organic solvents is used.

In some embodiments, the methods include subjecting a denatured viral particle of the present disclosure to liquid chromatography/mass spectrometry (LC/MS). As is known in the art, LC/MS utilizes liquid chromatography for physical separation of ions and mass spectrometry for generation of mass spectral data from the ions. Such mass spectral data may be used to determine, e.g., molecular weight or structure, identification of particles by mass, quantity, purity, and so forth. These data may represent properties of the detected ions such as signal strength (e.g., abundance) over time (e.g., retention time), or relative abundance over mass-to-charge ratio.

In some embodiments, liquid chromatography (e.g., used in LC/MS as described herein) is ultra-performance liquid chromatography (UPLC; the term "ultra high performance

liquid chromatography" or UHPLC may be used interchangeably herein). UPLC is known in the art as an LC technique that relies upon a column with reduced particle size (e.g., less than 2 μm) and increased flow velocity to improve chromatographic resolution, efficiency, peak capacity, and sensitivity (see, e.g., Plumb, R. et al. (2004) *Rapid Commun. Mass Spectrom.* 18:2331-2337). In some embodiments, UPLC refers to the use of a column with a particle size less than 2 μm in liquid chromatography. In some embodiments, UPLC refers to the use of a high linear solvent velocity (e.g., as observed when operating at 6000 psi or higher) in liquid chromatography. Exemplary UPLC machines are commercially available (e.g., the ACQUITY UPLC® from Waters; Milford, MA).

In some embodiments, mass spectrometry (e.g., used in LC/MS as described herein) may refer to electrospray ionization mass spectrometry (ESI-MS). ESI-MS is known in the art as a technique that uses electrical energy to analyze ions derived from a solution using mass spectrometry (see, e.g., Yamashita, M. and Fenn, J. B. (1984) *J. Phys. Chem.* 88:4451-4459). Ionic species (or neutral species that are ionized in solution or in gaseous phase) are transferred from a solution to a gaseous phase by dispersal in an aerosol of charged droplets, followed by solvent evaporation that reduces the size of the charged droplets and sample ion ejection from the charge droplets as the solution is passed through a small capillary with a voltage relative to ground (e.g., the wall of the surrounding chamber). In some embodiments, the capillary voltage is from about 2 kV to about 10 kV, or about 2.5 kV to about 6.0 kV. In certain embodiments, liquid chromatography (e.g., used in LC/MS as described herein) uses a capillary voltage of about 3.5 kV. In some embodiments the capillary voltage ranges from about 1 kV to about 10 kV. In other embodiments, mass spectrometry (e.g., used in LC/MS as described herein) may refer to matrix-assisted laser desorption/ionization (MALDI).

In some embodiments, mass spectrometry (e.g., used in LC/MS as described herein) uses a sampling cone and/or skimmer, through which ions may pass before entering the analyzer. In some embodiments, e.g., when applying voltage to the capillary as described above, the sample cone is held at a lower voltage than the capillary voltage. In certain embodiments, liquid chromatography (e.g., used in LC/MS as described herein) uses a sampling cone voltage of about 45 V. In some embodiments the sampling cone voltage ranges from about 0 V to about 200 V.

In some embodiments, mass spectrometry (e.g., used in LC/MS as described herein) uses assisted calibration. Calibration, when used in reference to mass spectrometry, may include the introduction of one or more compounds having a known mass (e.g., a standard) for the purpose of calibrating the instrument with respect to mass detection (e.g., m/z measurements). In some embodiments, assisted calibration may refer to using software to correlate a peak and/or position of a known standard (e.g., a calibrant) to a specific mass-to-charge (m/z) ratio. Once calibrated, the user may then perform mass spectrometry on a sample having one or more unknown compounds, or compounds present at an unknown concentration, within a certain degree of accuracy or error, and/or a desired level of reproducibility, e.g., as compared to a previous or known experimental condition. Various calibrants are known in the art, including without limitation sodium iodide, sodium cesium iodide, polyethylene glycol, and perfluorotributylamine. In certain embodiments, sodium iodide is used as a calibrant. In some embodiments the calibrants are Glu-1-fibrinopeptide B and leucine encephalin peptide to lock mass during LC/MS operation.

In some embodiments, the methods include subjecting a denatured viral particle of the present disclosure, or subjecting digested fragments of a denatured viral particle of the present disclosure, to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS). As is known in the art, LC/MS/MS (the term "liquid chromatography-tandem mass spectrometry" may be used interchangeably herein) utilizes liquid chromatography for physical separation of ions and mass spectrometry for generation of mass spectral data from the ions, where the mass spectrometry uses multiple stages of mass (e.g., m/z) separation, typically separated by a fragmentation step. For example, ions of interest within a range of m/z may be separated out in a first round of MS, fragmented, and then further separated based on individual m/z in a second round of MS. Ion fragmentation may include without limitation a technique such as collision-induced dissociation (CID), higher energy collision dissociation (HCD), electron-capture dissociation (ECD), or electron-transfer dissociation (ETD).

In some embodiments, the methods include subjecting a denatured viral particle of the present disclosure to reduction and/or alkylation. Means to reduce the viral particle include but are not limited to treatment with dithiothreitol, β-mercaptoethanol, or tris(2-carboxyethyl)phosphine (TCEP). Means to alkylate the viral particle include but are not limited to treating the AAV particle with iodoacetic acid, iodoacetamide, or 4-vinylpyridine.

In some embodiments, the methods include subjecting a denatured viral particle of the present disclosure to digestion, e.g., to generate fragments of VP1, VP2 and/or VP3 of an AAV particle. For example, a denatured AAV particle may be subjected to digestion to generate peptide fragments that may be analyzed, e.g., using LC for separation and MS/MS for analysis (see below for greater description). In some embodiments, the digestion is an enzymatic digestion. In some embodiments, the digestion uses chemical digestion such as CNBr treatment of instrument fragmentation (e.g., top down). In some embodiments, the digestion uses chemical digestion such as acid digestion.

In some embodiments, the enzymatic digestion is an endopeptidase digestion. An endopeptidase may include any peptidase that catalyzes the proteolysis of peptide bonds of non-terminal amino acids of a polypeptide. Known endopeptidases may include, without limitation, trypsin, chymotrypsin, AspN, Glu-C, LysC, pepsin, thermolysin, glutamyl endopeptidase, elastase, and neprilysin. In certain embodiments, the enzymatic digestion is a trypsin digestion or a LysC digestion.

In some embodiments, the liquid chromatography (e.g., used in LC/MS or LC/MS/MS as described herein) is reverse phase liquid chromatography (the terms "reversed phase liquid chromatography" or RPLC may be used interchangeably herein with reverse phase liquid chromatography). As is known in the art, reverse phase liquid chromatography may refer to a chromatographic separation using a hydrophobic stationary phase (e.g., a support or substrate such as a column) to adsorb hydrophobic molecules in a polar mobile phase. By decreasing the polarity of the mobile phase (e.g., by adding an organic solvent), one may achieve gradient separation of molecules by hydrophobicity, since more hydrophobic molecules will stay on the column in higher concentrations of organic solvent due to stronger hydrophobic interactions with the column. In some embodiment, separation is by capillary electrophoresis (CE), size exclusion chromatography (SEC), ion exchange chromatography (IEC) such as cation exchange chromatography, hydrophobic interaction chromatography (HIC), hydrophilic

interaction liquid chromatography (HILIC), but not limited to on-line LC/MS such as offline separation before MS; e.g., tips, columns; plates or cartridges.

Generally, a stationary phase suitable for reverse phase liquid chromatography (e.g., a hydrophobic moiety) may be coupled to a support including without limitation a column or resin packed with particles or beads (e.g., porous silica particles or polystyrene). A variety of hydrophobic stationary phases are known in the art, including without limitation hydrophobic alkyl chains, octyl or octadecyl silyl moieties, cyano moieties, and amino moieties. In some embodiments, the stationary phase may include a hydrophobic alkyl chain of a particular length, such as C4, C8, or C18. In certain embodiments, the reverse phase chromatography is a C4 or C8 reverse chromatography (e.g., reverse phase chromatography utilizing a C4 or C8 stationary phase). One of skill in the art may suitably select a stationary phase based on the molecule of interest (e.g., a denatured AAV particle or fragment thereof).

A variety of mobile phases suitable for reverse phase liquid chromatography are known in the art. As described above, a reverse phase liquid chromatography mobile phase may include a mixture of organic (e.g., hydrophobic) and aqueous (e.g., polar) solvents. Increasing the proportion of organic solvent increases its power to elute hydrophobic compounds from the stationary phase. Compound retention and/or selectivity may be altered, e.g., by changing the type or exposure of the stationary phase, adding polar reagents such as end capping reagents, altering the temperature, and/or altering mobile phase characteristics such as the proportion of organic solvent, pH, buffers, and the type of organic solvent used. In some embodiments, the polar component of the mobile phase may include without limitation water or an aqueous buffer. In some embodiments, the polar component of the mobile phase may include without limitation acetonitrile, methanol, ethanol, isopropyl alcohol, tetrahydrofuran (THF), and formic acid.

In some embodiments, two or more mobile phases may be used (e.g., mobile phase A, mobile phase B, etc.) in a gradient or proportion of interest. In certain embodiments, the chromatography uses a mobile phase A comprising formic acid in water. In certain embodiments, the mobile phase A comprises about 0.1% formic acid. In certain embodiments, the mobile phase A comprises about 0.1% to about 5% formic acid. In certain embodiments, the chromatography uses a mobile phase B comprising formic acid in acetonitrile. In certain embodiments, the mobile phase B comprises about 0.1% formic acid.

In some embodiments, the proportion of mobile phase B in the chromatography increases over time. For example, the proportion of mobile phase B in the chromatography may be increased in a stepwise manner. In certain embodiments, mobile phase B increases from about 10% to about 20%, from about 20% to about 30%, and from about 30% to about 38%. In other embodiments, mobile phase B increases from about 2% to about 60%. In other embodiments, mobile phase B increases from about 2% to about 100% from about 1 min to about 200 min In some embodiments, the remainder of the mobile phase is a second mobile phase of the present disclosure, e.g., mobile phase A. In certain embodiments, mobile phase B increases from about 10% to about 20% in about 6 minutes, from about 20% to about 30% in about 10 minutes, and from about 30% to about 38% in about 40 minutes. In other embodiments, mobile phase B increases from about 2% to about 60% in about 121 minutes. One of skill in the art may suitably adjust the mobile phase of interest and the gradient timing used based on the desired chromatographic separation and/or analyte of interest.

In some embodiments, the liquid chromatography is high-performance liquid chromatography (HPLC). HPLC is known in the art as a form of liquid chromatography in which a liquid solvent containing a sample is pressurized as it passes through a column containing solid phase. While traditional or low pressure LC may use gravity to pass a mobile phase through the solid phase, HPLC uses pumps to apply a pressure to the mobile phase and typically uses a solid phase with smaller particles to increase resolution. In some embodiments, the HPLC uses a pressure of between about 50 bar and about 350 bar. In some embodiments, reversed phase HPLC may be used to concentrate and/or desalt proteins (e.g., AAV capsid proteins) for MS analysis.

In some embodiments, one or more parameters including without limitation source voltage, capillary temperature, ESI voltage (if using ESI-MS), CID energy, and the number of MS/MS events may be adjusted, e.g., in LC/MS/MS as used herein, based on the findings described herein. In some embodiments, mass spectrometry (e.g., used in LC/MS/MS as described herein) uses a source voltage (e.g., capillary voltage) of about 2.5 kV. In some embodiments, mass spectrometry (e.g., used in LC/MS/MS as described herein) uses a capillary temperature of about 275° C. In some embodiments, the capillary temperature ranges from about 20° C. to about 400° C.

A variety of mass analyzers suitable for LC/MS and/or LC/MS/MS are known in the art, including without limitation time-of-flight (TOF) analyzers, quadrupole mass filters, quadrupole TOF (QTOF), and ion traps (e.g., a Fourier transform-based mass spectrometer or an Orbitrap). In Orbitrap, a barrel-like outer electrode at ground potential and a spindle-like central electrode are used to trap ions in trajectories rotating elliptically around the central electrode with oscillations along the central axis, confined by the balance of centrifugal and electrostatic forces. The use of such instruments employs a Fourier transform operation to convert a time domain signal (e.g., frequency) from detection of image current into a high resolution mass measurement (e.g., nano LC/MS/MS). Further descriptions and details may be found, e.g., in Scheltema, R. A. et al. (2014) *Mol. Cell Proteomics* 13:3698-3708; Perry, R. H. et al. (2008) *Mass. Spectrom. Rev.* 27:661-699; and Scigelova, M. et al. (2011) *Mol. Cell Proteomics* 10:M111.009431.

As described above, in some embodiments, the MS includes nano LC/MS/MS, e.g., using an Orbitrap mass analyzer. In some embodiments, the ion source may include a stacked-ring ion guide or S-lens. As is known in the art, an S-lens may be employed to focus the ion beam using radio frequency (RF), thereby increasing transmission of ions into the instrument. This may improve sensitivity (e.g., for low-intensity ions) and/or improve the scan rate. In certain embodiments, the S-lens RF level of the mass spectrometry is about 55%. In certain embodiments, the S-lens RF level of the mass spectrometry is about 20% to about 100%.

In some embodiments, masses of viral capsid proteins may be determined, e.g., based on LC/MS and/or LC/MS/MS data. In some embodiments, masses of VP1, VP2 and VP3 of an AAV particle, or of fragments of VP1, VP2 and VP3 of the AAV particle, may be determined, e.g., based on LC/MS and/or LC/MS/MS data. Various methods to determine protein mass and/or identity from MS data are known in the art. For example, peptide mass fingerprinting may be used to determine protein sequence based on MS data, or proteins may be identified based on MS/MS data related to one or more constituent peptides. When using tandem MS,

product ion scanning may be used to analyze m/z data related to one or more peptides of a protein of interest. Software known in the art may then be used, e.g., to match identified peaks to reference or known peaks, to group peaks into isotopomer envelopes, and so forth. Peptide mass values may be compared to a database of known peptide sequences. For example, Mascot may be used to match observed peptides with theoretical database peptides, e.g., resulting from application of a particular digest pattern to an in silico protein database. Other suitable software may include without limitation Proteome Discoverer, ProteinProspector, X!Tandem, Pepfinder, Bonics, or MassLynx™ (Waters). Other software suitable for various steps of MS data analysis may be found, e.g., at www.ms-utils.org/wiki/pmwiki.php/Main/SoftwareList.

In some embodiments, a determined or calculated mass of the present disclosure (e.g., the determined or calculated mass of VP1, VP2 and/or VP3 of the AAV particle) may be compared with a reference, e.g., a theoretical mass of a VP1, VP2, and/or VP3 of one or more AAV serotypes. A reference of the present disclosure may include a theoretical mass of a VP1, VP2, and/or VP3 of one or more of any of the AAV serotypes described herein. For example, in some embodiments, the masses of VP1, VP2, and/or VP3 are compared to the theoretical masses of one or more of an AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an AAV LK03 capsid (see U.S. Pat. No. 9,169,299), an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid (see U.S. Pat. No. 7,588,772), an AAV DJ8 capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, or a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV/human bocavirus virus 1), an AAV2HBKO capsid, an AAVPHP.B capsid or an AAVPHP.eB capsid. In some embodiments, a determined or calculated mass of the present disclosure (e.g., the determined or calculated mass of VP1, VP2 and/or VP3 of the AAV particle) may be compared with a theoretical mass of a VP1, VP2, and/or VP3 of the corresponding AAV serotype.

In some embodiments, the methods of the present disclosure may include determining the heterogeneity of an AAV particle. In some embodiments, a deviation of one or more of the masses of VP1, VP2 and/or VP3 (e.g., from a reference mass, such as a theoretical, predicted, or expected mass) is indicative of the AAV capsid heterogeneity. In some embodiments, heterogeneity may include one or more of the following, without limitation: mixed serotypes, variant capsids, capsid amino acid substitutions, truncated capsids, or modified capsids.

In some embodiments, the use of LC/MS and LC/MS/MS as described herein may be combined. In some embodiments, a method of determining the serotype of an AAV particle may include subjecting a denatured AAV particle to LC/MS (e.g., as described herein) and determining the masses of VP1, VP2 and VP3 of the AAV particle; as well as subjecting fragments of VP1, VP2 and/or VP3 to LC/MS/MS and determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle (the specific combination of masses of fragments of VP1, VP2 and VP3 are indicative of the AAV serotype). In some embodiments, a method of determining the heterogeneity of an AAV particle may include subjecting a denatured AAV particle to LC/MS (e.g., as described herein), determining the masses of VP1, VP2 and VP3 of the AAV particle, and comparing these masses with the theoretical masses of VP1, VP2 and VP3 of the AAV serotype; as well as subjecting fragments of VP1, VP2 and/or VP3 to LC/MS/MS, determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle, and comparing these masses with the theoretical masses of VP1, VP2 and VP3 of the AAV serotype (a deviation of one or more of the masses of VP1, VP2 or VP3 are indicative of the AAV capsid heterogeneity).

In some embodiments, an AAV particle of the present disclosure may be acetylated. For example, in some embodiments, the N-terminus of VP1 and/or VP3 is acetylated. As described in greater detail below, the amino acid at the 2nd position to the initiating methionine (iMet X) of an AAV capsid protein may be mutated in order to determine its effect on N-terminal (Nt-) acetylation, as well as the functional consequences of affecting Nt-acetylation on AAV particle trafficking, transduction, and/or post-translational modification (e.g., glycosylation, ubiquitination, and so forth). In some embodiments, the N-terminus of an AAV capsid protein (e.g., VP1 or VP3) may refer to the first amino acid after the initiating methionine, which in some cases may be removed by, e.g., a Met-aminopeptidase.

In some embodiments, an AAV particle of the present disclosure (e.g., a recombinant AAV or rAAV particle) comprises an amino acid substitution at amino acid residue 2 of VP1 and/or VP3. In some embodiments, the amino acid substitution at amino acid residue 2 of VP1 and/or VP3 leads to a VP1 and/or VP3 with a different frequency or proportion of N-terminal acetylation as compared to a reference (e.g., the parent AAV particle before the amino acid substitution, or an AAV particle with a different amino acid residue 2 of VP1 and/or VP3). In some embodiments, the amino acid substitution at amino acid residue 2 of VP1 and/or VP3 alters N-terminal acetylation as compared to N-terminal acetylation at amino acid residue 2 of VP1 and/or VP3 of the parent AAV particle. For example, in certain embodiments, the amino acid substitution at amino acid residue 2 of VP1 alters N-terminal acetylation as compared to N-terminal acetylation at amino acid residue 2 of VP1 of the parent AAV particle. In certain embodiments, the amino acid substitution at amino acid residue 2 of VP3 alters N-terminal acetylation as compared to N-terminal acetylation at amino acid residue 2 of VP3 of the parent AAV particle. In some embodiments, an amino acid substitution (e.g., an amino acid substitution at amino acid residue 2 of VP1 or VP3) that "alters" N-terminal acetylation results in a higher frequency of N-terminal acetylation or a lower frequency of N-terminal acetylation, e.g., as compared to a VP1 or VP3 without the substitution, such as the parental VP1 or VP3. The VP1 and/or VP3 may belong to any of the exemplary AAV serotypes described herein, including variants or hybrids thereof (e.g., bearing tyrosine mutation or heparin binding mutations). Exemplary assays for N-terminal acetylation include without limitation mass spectrometry, isotope labeling (e.g., with an isotope-labeled acetyl group or precursor thereof), Western blotting with an acetylation-specific antibody, and so forth. In some embodiments, amino acid residue 2 of the AAV capsid protein (e.g., VP1 or VP3) is substituted with Cys, Ser, Thr, Val, Gly, Asn, Asp, Glu, Ile, Leu, Phe, Gln, Lys, Met, Pro or Tyr. In some embodiments, the amino acid substitution results in less deamidation of the AAV capsid.

In some embodiments, an AAV particle of the present disclosure may be deamidated. For example, in some embodiments, N57 of VP1 and/or N382, N511, and/or N715 VP3 is deamidated. As described in greater detail below, an

amino acid selected from A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3 of an AAV capsid protein (e.g., VP1 or VP3) may be mutated in order to determine its effect on deamidation, as well as the functional consequences of affecting deamidation on AAV particle trafficking, transduction, and/or post-translational modification (e.g., glycosylation, ubiquitination, and so forth).

In some embodiments, an AAV particle of the present disclosure (e.g., a recombinant AAV or rAAV particle) comprises an amino acid substitution at one or more amino acid residues selected from A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, and G716 of VP3. In some embodiments, the amino acid substitution at A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, and/or G716 of VP3 leads to a VP1 and/or VP3 with a different frequency or proportion of deamidation as compared to a reference (e.g., the parent AAV particle before the amino acid substitution, or an AAV particle with a different corresponding amino acid residue 2). In some embodiments, an amino acid substitution (e.g., an amino acid substitution at A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3) that "alters" deamidation results in a higher frequency of deamidation or a lower frequency of deamidation, e.g., as compared to a VP1 or VP3 without the substitution, such as the parental VP1 or VP3. The VP1 and/or VP3 may belong to any of the exemplary AAV serotypes described herein, including variants or hybrids thereof (e.g., bearing tyrosine mutation or heparin binding mutations). Exemplary assays for deamidation include without limitation mass spectrometry, HPLC (see, e.g., the ISOQUANT® isoaspartate detection kit from Promega), and so forth. In some embodiments, N57 of VP1, N382 of VP3, N511 of VP3, and/or N715 of VP3 is substituted with Asp, and the amino acid substitution results in a higher frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In other embodiments, the amino acid substitution is N57K or N57Q, and the amino acid substitution results in a lower frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In yet other embodiments, G58 of VP1, G383 of VP3, G512 of VP3, and/or G716 of VP3 is substituted with an amino acid that is not Gly (e.g., Ala, Arg, Asn, Asp, Cys, Glu, Gln, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val), and the amino acid substitution results in a lower frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In yet other embodiments, A35 of VP1 is substituted with Asn and results in a higher frequency of deamidation as compared to deamidation of VP1 of a parent particle.

As used herein "N-acetylation" refers to a process whereby an acetyl group is covalently added to the amino group of the N-terminal amino acid of a protein. Typically, N-terminal acetyltransferases (NATs) transfer an acetyl group from acetyl-coenzyme A (Ac-CoA) to the α-amino group of the first amino acid residue of the protein.

As used here in, "deamidation" refers to a chemical reaction in which an amide functional group in the side chain of asparagine or glutamine is removed or converted to another functional group. For example, asparagine may be converted to aspartic acid or isoaspartic acid. In other examples, glutamine is converted to glutamic acid or pyroglutamic acid (5-oxoproline).

In some embodiments, the AAV particle is N-acetylated to a higher extent compared to a parental AAV capsid protein. In some embodiments, the AAV particle comprises more than about any of 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% more N-acetyl groups compared to a parent AAV particle. In some embodiments, the AAV particle comprises between about any of 5%-10%, 10%-15%, 15%-20%, 20%-25%, 25%-30%, 30%-35%, 35%-40%, 40%-55%, 45%-50%, 50%-55%, 55%-60%, 60%-65%, 65%-70%, 70%-75%, 75%-80%, 80%-85%, 85%-90%, 90%-95%, 95%-100%, 5-25%, 25-50%, 50-75%, 75%-100%, 5-50% or 50%-100% more N-acetyl groups compared to a parent AAV particle. In some embodiments, the AAV particle comprises more than about any of 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 25-fold, 50-fold, 100-fold, 500-fold, or 1000-fold more N-acetyl groups compared to a parent AAV particle. In some embodiments, the AAV particle comprises between about any of 2-fold to 3-fold, 3-fold to 4-fold, 4-fold to 5-fold, 5-fold to 10-fold, 10-fold to 25-fold, 25-fold to 50-fold, 50-fold to 100-fold, 100-fold to 500-fold, 500-fold to 1000-fold, 2-fold to 10-fold, 10-fold to 100-fold, or 100-fold to 1000-fold more N-acetyl groups compared to a parent AAV particle.

In some embodiments, the AAV particle N-acetylated to a lower extent compared to a parental AAV capsid protein. In some embodiments, the AAV particle comprises more than about any of 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% less N-acetyl groups compared to a parent AAV particle. In some embodiments, the AAV particle comprises between about any of 5%-10%, 10%-15%, 15%-20%, 20%-25%, 25%-30%, 30%-35%, 35%-40%, 40%-55%, 45%-50%, 50%-55%, 55%-60%, 60%-65%, 65%-70%, 70%-75%, 75%-80%, 80%-85%, 85%-90%, 90%-95%, 95%-100%, 5-25%, 25-50%, 50-75%, 75%-100%, 5-50% or 50%-100% less N-acetyl groups compared to a parent AAV particle. In some embodiments, the AAV particle comprises more than about any of 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 25-fold, 50-fold, 100-fold, 500-fold, or 1000-fold less N-acetyl groups compared to a parent AAV particle. In some embodiments, the AAV particle comprises between about any of 2-fold to 3-fold, 3-fold to 4-fold, 4-fold to 5-fold, 5-fold to 10-fold, 10-fold to 25-fold, 25-fold to 50-fold, 50-fold to 100-fold, 100-fold to 500-fold, 500-fold to 1000-fold, 2-fold to 10-fold, 10-fold to 100-fold, or 100-fold to 1000-fold less N-acetyl groups compared to a parent AAV particle.

In some embodiments, the AAV particle is deamidated to a higher extent compared to a parental AAV particle. In some embodiments, the AAV particle is more than about any of 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% more deamidated compared to a parent AAV particle. In some embodiments, the AAV particle is deamidated between about any of 5%-10%, 10%-15%, 15%-20%, 20%-25%, 25%-30%, 30%-35%, 35%-40%, 40%-55%, 45%-50%, 50%-55%, 55%-60%, 60%-65%, 65%-70%, 70%-75%, 75%-80%, 80%-85%, 85%-90%, 90%-95%, 95%-100%, 5-25%, 25-50%, 50-75%, 75%-100%, 5-50% or 50%-100% more than a parent AAV particle. In some embodiments, the AAV particle is deamidated more than about any of 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 25-fold, 50-fold, 100-fold, 500-fold, or 1000-fold compared to a parent AAV particle. In some embodiments, the AAV particle is deamidated between about any of 2-fold to 3-fold, 3-fold to 4-fold, 4-fold to 5-fold, 5-fold to 10-fold, 10-fold to 25-fold, 25-fold

to 50-fold, 50-fold to 100-fold, 100-fold to 500-fold, 500-fold to 1000-fold, 2-fold to 10-fold, 10-fold to 100-fold, or 100-fold to 1000-fold more than a parent AAV particle.

In some embodiments, a capsid protein of AAV is deamidated to a lower extent compared to a parental AAV capsid protein. In some embodiments, the AAV particle is more than about any of 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% less deamidated compared to a parent AAV particle. In some embodiments, the AAV particle is deamidated between about any of 5%-10%, 10%-15%, 15%-20%, 20%-25%, 25%-30%, 30%-35%, 35%-40%, 40%-55%, 45%-50%, 50%-55%, 55%-60%, 60%-65%, 65%-70%, 70%-75%, 75%-80%, 80%-85%, 85%-90%, 90%-95%, 95%-100%, 5-25%, 25-50%, 50-75%, 75%-100%, 5-50% or 50%-100% less than a parent AAV particle. In some embodiments, the AAV particle is deamidated more than about any of 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 25-fold, 50-fold, 100-fold, 500-fold, or 1000-fold less than a parent AAV particle. In some embodiments, the AAV particle is deamidated between about any of 2-fold to 3-fold, 3-fold to 4-fold, 4-fold to 5-fold, 5-fold to 10-fold, 10-fold to 25-fold, 25-fold to 50-fold, 50-fold to 100-fold, 100-fold to 500-fold, 500-fold to 1000-fold, 2-fold to 10-fold, 10-fold to 100-fold, or 100-fold to 1000-fold less than a parent AAV particle.

The invention provides any combination of N-acetylation and deamidation. For example, the AAV capsid protein may be N-acetylated to a higher extent than a parent AAV capsid protein and deamidated to a higher extent than a parent AAV capsid protein, the AAV capsid protein may be N-acetylated to a higher extent than a parent AAV capsid protein and deamidated to the same extent than a parent AAV capsid protein, the AAV capsid protein may be N-acetylated to a higher extent than a parent AAV capsid protein and deamidated to a lower extent than a parent AAV capsid protein, the AAV capsid protein may be N-acetylated to the same extent than a parent AAV capsid protein and deamidated to a higher extent than a parent AAV capsid protein, the AAV capsid protein may be N-acetylated to the same extent than a parent AAV capsid protein and deamidated to the same extent than a parent AAV capsid protein, the AAV capsid protein may be N-acetylated to the same extent than a parent AAV capsid protein and deamidated to a lower extent than a parent AAV capsid protein, the AAV capsid protein may be N-acetylated to a lower extent than a parent AAV capsid protein and deamidated to a higher extent than a parent AAV capsid protein, the AAV capsid protein may be N-acetylated to a lower extent than a parent AAV capsid protein and deamidated to the same extent than a parent AAV capsid protein, or the AAV capsid protein may be N-acetylated to a lower extent than a parent AAV capsid protein and deamidated to a lower extent than a parent AAV capsid protein.

IV. Vectors

In certain aspects, the invention relates to viral particles, suitable for use in any of the methods described herein, which may comprise AAV vectors (e.g., rAAV vectors) or vectors derived from another virus. In some embodiments, the viral particle comprises a vector encoding a heterologous nucleic acid, e.g., a heterologous transgene. In some embodiments, the AAV particle comprises an AAV vector genome encoding a heterologous nucleic acid, e.g., a heterologous transgene.

The present invention contemplates the use of a recombinant viral genome for introduction of one or more nucleic acid sequences encoding a therapeutic polypeptide and/or nucleic acid for packaging into a rAAV viral particle. The recombinant viral genome may include any element to establish the expression of the therapeutic polypeptide and/or nucleic acid, for example, a promoter, an ITR of the present disclosure, a ribosome binding element, terminator, enhancer, selection marker, intron, polyA signal, and/or origin of replication.

In some embodiments, the heterologous nucleic acid encodes a therapeutic polypeptide. A therapeutic polypeptide may, e.g., supply a polypeptide and/or enzymatic activity that is absent or present at a reduced level in a cell or organism. Alternatively, a therapeutic polypeptide may supply a polypeptide and/or enzymatic activity that indirectly counteracts an imbalance in a cell or organism. For example, a therapeutic polypeptide for a disorder related to buildup of a metabolite caused by a deficiency in a metabolic enzyme or activity may supply a missing metabolic enzyme or activity, or it may supply an alternate metabolic enzyme or activity that leads to reduction of the metabolite. A therapeutic polypeptide may also be used to reduce the activity of a polypeptide (e.g., one that is overexpressed, activated by a gain-of-function mutation, or whose activity is otherwise misregulated) by acting, e.g., as a dominant-negative polypeptide.

The nucleic acids of the invention may encode polypeptides that are intracellular proteins, anchored in the cell membrane, remain within the cell, or are secreted by the cell transduced with the vectors of the invention. For polypeptides secreted by the cell that receives the vector; the polypeptide can be soluble (i.e., not attached to the cell). For example, soluble polypeptides are devoid of a transmembrane region and are secreted from the cell. Techniques to identify and remove nucleic acid sequences which encode transmembrane domains are known in the art.

The nucleic acids if the invention (e.g. the AAV vector genome) may comprise as a transgene, a nucleic acid encoding a protein or functional RNA that modulates or treats a CNS-associated disorder. The following is a non-limiting list of genes associated with CNS-associated disorders: neuronal apoptosis inhibitory protein (NAIP), nerve growth factor (NGF), glial-derived growth factor (GDNF), brain-derived growth factor (BDNF), ciliary neurotrophic factor (CNTF), tyrosine hydroxylase (TM, GTP-cyclohydrolase (GTPCH), aspartoacylase (ASPA), superoxide dismutase (SOD1), an anti-oxidant, an anti-angiogenic polypeptide, an anti-inflammatory polypeptide, and amino acid decorboxylase (AADC). For example, a useful transgene in the treatment of Parkinson's disease encodes TH, which is a rate limiting enzyme in the synthesis of dopamine. A transgene encoding GTPCII, which generates the TII cofactor tetrahydrobiopterin, may also be used in the treatment of Parkinson's disease. A transgene encoding GDNF or BDNF, or AADC, which facilitates conversion of L-Dopa to DA, may also be used for the treatment of Parkinson's disease. For the treatment of ALS, a useful transgene may encode: GDNF, BDNF or CNTF. Also for the treatment of ALS, a useful transgene may encode a functional RNA, e.g., shRNA, miRNA, that inhibits the expression of SOD1. For the treatment of ischemia a useful transgene may encode NAIP or NGF. A transgene encoding Beta-glucuronidase (GUS) may be useful for the treatment of certain lysosomal storage diseases (e.g., Mucopolysacharidosis type VII (MPS VII)). A transgene encoding a prodrug activation gene, e.g., HSV-Thymidine kinase which converts ganciclovir to a toxic nucleotide which disrupts DNA synthesis and leads to cell death, may be useful for treating certain cancers, e.g.,

when administered in combination with the prodrug. A transgene encoding an endogenous opioid, such a β-endorphin may be useful for treating pain. Examples of antioxidants include without limitation SOD1; SOD2; Catalase; Sirtuins 1, 3, 4, or 5; NRF2; PGC1a; GCL (catalytic subunit); GCL (modifier subunit); adiponectin; glutathione peroxidase 1; and neuroglobin. Examples of anti-angiogenic polypeptides include without limitation angiostatin, endostatin, PEDF, a soluble VEGF receptor, and a soluble PDGF receptor. Examples of anti-inflammatory polypeptides include without limitation IL-10, soluble IL17R, soluble TNF-R, TNF-R-Ig, an IL-1 inhibitor, and an IL18 inhibitor. Other examples of transgenes that may be used in the rAAV vectors of the invention will be apparent to the skilled artisan (See, e.g., Costantini L C, et al., *Gene Therapy* (2000) 7, 93-109).

In some embodiments, the heterologous nucleic acid encodes a therapeutic nucleic acid. In some embodiments, a therapeutic nucleic acid may include without limitation an siRNA, an shRNA, an RNAi, a miRNA, an antisense RNA, a ribozyme or a DNAzyme. As such, a therapeutic nucleic acid may encode an RNA that when transcribed from the nucleic acids of the vector can treat a disorder by interfering with translation or transcription of an abnormal or excess protein associated with a disorder of the invention. For example, the nucleic acids of the invention may encode for an RNA which treats a disorder by highly specific elimination or reduction of mRNA encoding the abnormal and/or excess proteins. Therapeutic RNA sequences include RNAi, small inhibitory RNA (siRNA), micro RNA (miRNA), and/or ribozymes (such as hammerhead and hairpin ribozymes) that can treat disorders by highly specific elimination or reduction of mRNA encoding the abnormal and/or excess proteins.

In some embodiments, the therapeutic polypeptide or therapeutic nucleic acid is used to treat a disorder of the CNS. Without wishing to be bound to theory, it is thought that a therapeutic polypeptide or therapeutic nucleic acid may be used to reduce or eliminate the expression and/or activity of a polypeptide whose gain-of-function has been associated with a disorder, or to enhance the expression and/or activity of a polypeptide to complement a deficiency that has been associated with a disorder (e.g., a mutation in a gene whose expression shows similar or related activity). Non-limiting examples of disorders of the invention that may be treated by a therapeutic polypeptide or therapeutic nucleic acid of the invention (exemplary genes that may be targeted or supplied are provided in parenthesis for each disorder) include stroke (e.g., caspase-3, Beclin1, Ask1, PAR1, HIF1α, PUMA, and/or any of the genes described in Fukuda, A. M. and Badaut, J. (2013) *Genes* (*Basel*) 4:435-456), Huntington's disease (mutant HTT), epilepsy (e.g., SCN1A, NMDAR, ADK, and/or any of the genes described in Boison, D. (2010) *Epilepsia* 51:1659-1668), Parkinson's disease (alpha-synuclein), Lou Gehrig's disease (also known as amyotrophic lateral sclerosis; SOD1), Alzheimer's disease (tau, amyloid precursor protein), corticobasal degeneration or CBD (tau), corticogasal ganglionic degeneration or CBGD (tau), frontotemporal dementia or FTD (tau), progressive supranuclear palsy or PSP (tau), multiple system atrophy or MSA (alpha-synuclein), cancer of the brain (e.g., a mutant or overexpressed oncogene implicated in brain cancer), and lysosomal storage diseases (LSD). Disorders of the invention may include those that involve large areas of the cortex, e.g., more than one functional area of the cortex, more than one lobe of the cortex, and/or the entire cortex. Other non-limiting examples of disorders of the invention that may be treated by a therapeutic polypeptide or therapeutic nucleic acid of the invention include traumatic brain injury, enzymatic dysfunction disorders, psychiatric disorders (including post-traumatic stress syndrome), neurodegenerative diseases, and cognitive disorders (including dementias, autism, and depression). Enzymatic dysfunction disorders include without limitation leukodystrophies (including Canavan's disease) and any of the lysosomal storage diseases described below.

In some embodiments, the therapeutic polypeptide or therapeutic nucleic acid is used to treat a lysosomal storage disease. As is commonly known in the art, lysosomal storage disease are rare, inherited metabolic disorders characterized by defects in lysosomal function. Such disorders are often caused by a deficiency in an enzyme required for proper mucopolysaccharide, glycoprotein, and/or lipid metabolism, leading to a pathological accumulation of lysosomally stored cellular materials. Non-limiting examples of lysosomal storage diseases of the invention that may be treated by a therapeutic polypeptide or therapeutic nucleic acid of the invention (exemplary genes that may be targeted or supplied are provided in parenthesis for each disorder) include Gaucher disease type 2 or type 3 (acid beta-glucosidase, GBA), GM1 gangliosidosis (beta-galactosidase-1, GLB1), Hunter disease (iduronate 2-sulfatase, IDS), Krabbe disease (galactosylceramidase, GALC), a mannosidosis disease (a mannosidase, such as alpha-D-mannosidase, MAN2B1), β mannosidosis disease (beta-mannosidase, MANBA), metachromatic leukodystrophy disease (pseudoarylsulfatase A, ARSA), mucolipidosisII/III disease (N-acetylglucosamine-1-phosphotransferase, GNPTAB), Niemann-Pick A disease (acid sphingomyelinase, ASM), Niemann-Pick C disease (Niemann-Pick C protein, NPC1), Pompe disease (acid alpha-1,4-glucosidase, GAA), Sandhoff disease (hexosaminidase beta subunit, HEXB), Sanfilippo A disease (N-sulfoglucosamine sulfohydrolase, MPS3A), Sanfilippo B disease (N-alpha-acetylglucosaminidase, NAGLU), Sanfilippo C disease (heparin acetyl-CoA:alpha-glucosaminidase N-acetyltransferase, MPS3C), Sanfilippo D disease (N-acetylglucosamine-6-sulfatase, GNS), Schindler disease (alpha-N-acetylgalactosaminidase, NAGA), Sly disease (beta-glucuronidase, GUSB), Tay-Sachs disease (hexosaminidase alpha subunit, HEXA), and Wolman disease (lysosomal acid lipase, LIPA).

Additional lysosomal storage diseases, as well as the defective enzyme associated with each disease, are listed in Table 1 below. In some embodiments, a disease listed in the table below is treated by a therapeutic polypeptide or therapeutic nucleic acid of the invention that complements or otherwise compensates for the corresponding enzymatic defect.

TABLE 1

Lysosomal storage disorders and associated defective enzymes.

Lysosomal storage disease	Defective enzyme
Aspartylglusoaminuria	Aspartylglucosaminidase
Fabry	Alpha-galactosidase A
Infantile Batten Disease (CNL1)	Palmitoyl protein thioesterase
Classic Late Infantile Batten Disease (CNL2)	Tripeptidyl peptidase
Juvenile Batten Disease (CNL3)	Lysosomal transmembrane protein
Batten, other forms (CNL4-CNL8)	multiple gene products
Cystinosis	Cysteine transporter
Farber	Acid ceramidase
Fucosidosis	Acid alpha-L-fucosidase
Galactosidosialidosis	Protective protein/cathepsin A

TABLE 1-continued

Lysosomal storage disorders and associated defective enzymes.

Lysosomal storage disease	Defective enzyme
Gaucher types 1, 2, and 3	Acid beta-glucosidase
GM1 gangliosidosis	Acid beta-galactosidase
Hunter	Iduronate-2-sulfatase
Hurler-Scheie	Alpha-L-iduronidase
Krabbe	Galactocerebrosidase
alpha-mannosidosis	Acid alpha-mannosidase
beta-mannosidosis	Acid beta-mannosidase
Maroteaux-Lamy	Arylsulfatase B
Metachromatic leukodystrophy	Arylsulfatase A
Morquio A	N-acetylgalactosamine-6-sulfate
Morquio B	Acid beta-galactosidase
Mucolipidosis II/III	N-acetylglucosamine-1-phosphotransferase
Niemann-Pick A, B	Acid sphingomyelinase
Niemann-Pick C	NPC-1
Pompe acid	alpha-glucosidase
Sandhoff	beta-hexosaminidase B
Sanfilippo A	Heparan N-sulfatase
Sanfilippo B	alpha-N-acetylglucosaminidase
Sanfilippo C	Acetyl-CoA:alpha-glucosaminide N-acetyltransferase
Sanfilippo D	N-acetylglucosamine-6-sulfate
Schindler disease	alpha-N-acetylgalactosaminidase
Schindler-Kanzaki	alpha-N-acetylgalactosaminidase
Sialidosis	alpha-neuramidase
Sly	beta-glucuronidase
Tay-Sachs	beta-hexosaminidase A
Wolman	Acid lipase

In some embodiments, the heterologous nucleic acid is operably linked to a promoter. Exemplary promoters include, but are not limited to, the cytomegalovirus (CMV) immediate early promoter, the RSV LTR, the MoMLV LTR, the phosphoglycerate kinase-1 (PGK) promoter, a simian virus 40 (SV40) promoter and a CK6 promoter, a transthyretin promoter (TTR), a TK promoter, a tetracycline responsive promoter (TRE), an HBV promoter, an hAAT promoter, a LSP promoter, chimeric liver-specific promoters (LSPs), the E2F promoter, the telomerase (hTERT) promoter; the cytomegalovirus enhancer/chicken beta-actin/Rabbit β-globin promoter (CAG promoter; Niwa et al., *Gene*, 1991, 108(2):193-9) and the elongation factor 1-alpha promoter (EF1-alpha) promoter (Kim et al., *Gene*, 1990, 91(2):217-23 and Guo et al., *Gene Ther.*, 1996, 3(9):802-10). In some embodiments, the promoter comprises a human β-glucuronidase promoter or a cytomegalovirus enhancer linked to a chicken β-actin (CBA) promoter. The promoter can be a constitutive, inducible or repressible promoter. In some embodiments, the invention provides a recombinant vector comprising nucleic acid encoding a heterologous transgene of the present disclosure operably linked to a CBA promoter. Exemplary promoters and descriptions may be found, e.g., in U.S. PG Pub. 20140335054.

Examples of constitutive promoters include, without limitation, the retroviral Rous sarcoma virus (RSV) LTR promoter (optionally with the RSV enhancer), the cytomegalovirus (CMV) promoter (optionally with the CMV enhancer) [see, e.g., Boshart et al., Cell, 41:521-530 (1985)], the SV40 promoter, the dihydrofolate reductase promoter, the 13-actin promoter, the phosphoglycerol kinase (PGK) promoter, and the EF1a promoter [Invitrogen].

Inducible promoters allow regulation of gene expression and can be regulated by exogenously supplied compounds, environmental factors such as temperature, or the presence of a specific physiological state, e.g., acute phase, a particular differentiation state of the cell, or in replicating cells only. Inducible promoters and inducible systems are available from a variety of commercial sources, including, without limitation, Invitrogen, Clontech and Ariad. Many other systems have been described and can be readily selected by one of skill in the art. Examples of inducible promoters regulated by exogenously supplied promoters include the zinc-inducible sheep metallothionine (MT) promoter, the dexamethasone (Dex)-inducible mouse mammary tumor virus (MMTV) promoter, the T7 polymerase promoter system (WO 98/10088); the ecdysone insect promoter (No et al., *Proc. Natl. Acad. Sci. USA*, 93:3346-3351 (1996)), the tetracycline-repressible system (Gossen et al., *Proc. Natl. Acad. Sci. USA*, 89:5547-5551 (1992)), the tetracycline-inducible system (Gossen et al., *Science*, 268:1766-1769 (1995), see also Harvey et al., *Curr. Opin. Chem. Biol.*, 2:512-518 (1998)), the RU486-inducible system (Wang et al., *Nat. Biotech.*, 15:239-243 (1997) and Wang et al., *Gene Ther.*, 4:432-441 (1997)) and the rapamycin-inducible system (Magari et al., *J. Clin. Invest.*, 100:2865-2872 (1997)). Still other types of inducible promoters which may be useful in this context are those which are regulated by a specific physiological state, e.g., temperature, acute phase, a particular differentiation state of the cell, or in replicating cells only.

In another embodiment, the native promoter, or fragment thereof, for the transgene will be used. The native promoter can be used when it is desired that expression of the transgene should mimic the native expression. The native promoter may be used when expression of the transgene must be regulated temporally or developmentally, or in a tissue-specific manner, or in response to specific transcriptional stimuli. In a further embodiment, other native expression control elements, such as enhancer elements, polyadenylation sites or Kozak consensus sequences may also be used to mimic the native expression

In some embodiments, the regulatory sequences impart tissue-specific gene expression capabilities. In some cases, the tissue-specific regulatory sequences bind tissue-specific transcription factors that induce transcription in a tissue specific manner. Such tissue-specific regulatory sequences (e.g., promoters, enhancers, etc.) are well known in the art.

In some embodiments, the vector comprises an intron. For example, in some embodiments, the intron is a chimeric intron derived from chicken beta-actin and rabbit beta-globin. In some embodiments, the intron is a minute virus of mice (MVM) intron.

In some embodiments, the vector comprises a polyadenylation (polyA) sequence. Numerous examples of polyadenylation sequences are known in the art, such as a bovine growth hormone (BGH) Poly(A) sequence (see, e.g., accession number EF592533), an SV40 polyadenylation sequence, and an HSV TK pA polyadenylation sequence.

V. Viral Particles and Methods of Producing Viral Particles

Certain aspects of the present disclosure relate to recombinant viral particles (e.g., rAAV particles).

Based on the guidance provided herein, the techniques of the present disclosure may suitably be adapted by one of skill in the art for use with a variety of different viruses.

In some embodiments, the virus is of the family Adenoviridae, which includes non-enveloped viruses typically known as Adenoviruses. In some embodiments, the virus is of the genus *Atadenovirus*, *Aviadenovirus*, *Ichtadenovirus*, *Mastadenovirus*, or *Siadenovirus*.

In some embodiments, the virus is of the family Parvoviridae, which includes non-enveloped viruses such as AAV and Bocaparvovirus. In some embodiments, the virus is of

the subfamily Densovirinae. In some embodiments, the virus is of the genus *Ambidensovirus, Brevidensovirus, Hepandensovirus, Iteradensovirus*, or *Penstyldensovirus*. In some embodiments, the virus is of the subfamily Parvovirinae. In some embodiments, the virus is of the genus *Amdoparvovirus, Aveparvovirus, Bocaparvovirus, Copiparvovirus, Dependoparvovirus, Erythroparvovirus, Protoparvovirus*, or *Tetraparvovirus*.

In some embodiments, the virus is of the family Retroviridae, which includes enveloped viruses including lentivirus. In some embodiments, the virus is of the subfamily Orthoretrovirinae. In some embodiments, the virus is of the genus *Alpharetrovirus, Betaretrovirus, Deltaretrovirus, Epsilonretrovirus, Gammaretrovirus*, or *Lentivirus*. In some embodiments, the virus is of the subfamily Spumaretrovirinae. In some embodiments, the virus is of the genus *Spumavirus*.

In some embodiments, the virus is of the family Baculoviridae, which includes enveloped viruses including alphabaculovirus. In some embodiments, the virus is of the genus *Alphabaculovirus, Betabaculovirus, Deltabaculovirus*, or *Gammabaculovirus*.

In some embodiments, the virus is of the family Herpesviridae, which includes enveloped viruses such as the simplex viruses HSV-1 and HSV-2. In some embodiments, the virus is of the subfamily Alphaherpesvirinae. In some embodiments, the virus is of the genus *Iltovirus, Mardivirus, Simplexvirus*, or *Varicellovirus*. In some embodiments, the virus is of the subfamily Betaherpesvirinae. In some embodiments, the virus is of the genus *Cytomegalovirus, Muromegalovirus, Proboscivirus*, or *Roseolovirus*. In some embodiments, the virus is of the subfamily Gammaherpesvirinae. In some embodiments, the virus is of the genus *Lymphocryptovirus, Macavirus, Percavirus*, or *Rhadinovirus*.

In some embodiments, the virus is an AAV virus. In an AAV particle, a nucleic acid is encapsidated in the AAV particle. The AAV particle also comprises capsid proteins. In some embodiments, the nucleic acid comprises a heterologous nucleic acid and/or one or more of the following components, operatively linked in the direction of transcription, control sequences including transcription initiation and termination sequences, thereby forming an expression cassette.

In some embodiments, the viral particle comprises an AAV ITR sequence. For example, an expression cassette may be flanked on the 5' and 3' end by at least one functional AAV ITR sequence. By "functional AAV ITR sequences" it is meant that the ITR sequences function as intended for the rescue, replication and packaging of the AAV virion. See Davidson et al., *PNAS*, 2000, 97(7) 3428-32; Passini et al., *J. Virol.*, 2003, 77(12):7034-40; and Pechan et al., *Gene Ther.*, 2009, 16:10-16, all of which are incorporated herein in their entirety by reference. For practicing some aspects of the invention, the recombinant vectors comprise at least all of the sequences of AAV essential for encapsidation and the physical structures for infection by the rAAV. AAV ITRs for use in the vectors of the invention need not have a wild-type nucleotide sequence (e.g., as described in Kotin, *Hum. Gene Ther.*, 1994, 5:793-801), and may be altered by the insertion, deletion or substitution of nucleotides or the AAV ITRs may be derived from any of several AAV serotypes. More than 40 serotypes of AAV are currently known, and new serotypes and variants of existing serotypes continue to be identified. See Gao et al., *PNAS*, 2002, 99(18): 11854-6; Gao et al., *PNAS*, 2003, 100(10):6081-6; and Bossis et al., *J. Virol.*, 2003, 77(12):6799-810. Use of any AAV serotype is considered within the scope of the present invention. In some embodiments, a rAAV vector is a vector derived from an AAV serotype, including without limitation, AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV DJ, AAV DJ8, a goat AAV, bovine AAV, or mouse AAV ITRs or the like. In some embodiments, the nucleic acid in the AAV (e.g., an rAAV vector) comprises an ITR of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV DJ, AAV DJ8, a goat AAV, bovine AAV, or mouse AAV ITRs or the like. In some embodiments, the AAV particle comprises an AAV vector encoding a heterologous transgene flanked by one or more AAV ITRs.

In some embodiments, a rAAV particle comprises an encapsulation protein selected from AAV1, AAV2, AAV3, AAV4, AAV5, AAV6 (e.g., a wild-type AAV6 capsid, or a variant AAV6 capsid such as ShH10, as described in U.S. PG Pub. 2012/0164106), AAV7, AAV8, AAVrh8, AAVrh8R, AAV9 (e.g., a wild-type AAV9 capsid, or a modified AAV9 capsid as described in U.S. PG Pub. 2013/0323226), AAV10, AAVrh10, AAV11, AAV12, a tyrosine capsid mutant, a heparin binding capsid mutant, an AAV2R471A capsid, an AAVAAV2/2-7m8 capsid, an AAV LK03 capsid, an AAV DJ capsid (e.g., an AAV-DJ/8 capsid, an AAV-DJ/9 capsid, or any other of the capsids described in U.S. PG Pub. 2012/0066783), AAV2 N587A capsid, AAV2 E548A capsid, AAV2 N708A capsid, AAV V708K capsid, goat AAV capsid, AAV1/AAV2 chimeric capsid, bovine AAV capsid, mouse AAV capsid, rAAV2/HBoV1 capsid, an AAV2HBKO capsid, an AAVPHP.B capsid or an AAVPHP.eB capsid, or an AAV capsid described in U.S. Pat. No. 8,283,151 or International Publication No. WO/2003/042397. In further embodiments, a rAAV particle comprises capsid proteins of an AAV serotype from Clades A-F.

Certain aspects of the present disclosure relate to an AAV (e.g., a rAAV) capsid protein comprising an amino acid substitution at amino acid residue 2. In some embodiments, the amino acid substitution at amino acid residue 2 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of the parent AAV capsid protein. As described herein, the amino acid at the 2nd position to the initiating methionine (iMet X) of an AAV capsid protein may be examined for effects on N-terminal acetylation, trafficking, transduction, and/or other post-translational modification(s) (e.g., glycosylation, ubiquitination, and so forth). Any assay described herein for examining acetylation, or a functional consequence thereof related to AAV particles, may be used to assess N-terminal acetylation. In some embodiments, amino acid residue 2 of the AAV capsid protein (e.g., VP1 or VP3) is substituted with Cys, Ser, Thr, Val, Gly, Asn, Asp, Glu, Ile, Leu, Phe, Gln, Lys, Met, Pro or Tyr. In some embodiments, the amino acid substitution results in less deamidation of the AAV capsid.

Other aspects of the present disclosure relate to an AAV (e.g., a rAAV) capsid protein comprising an amino acid substitution that alters deamidation. In some embodiments, an amino acid substitution (e.g., an amino acid substitution at A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3) that "alters" deamidation results in a higher frequency of deamidation or a lower frequency of deamidation, e.g., as compared to a VP1 or VP3 without the substitution, such as the parental VP1 or VP3. As described herein, a potential deamidation site of an AAV capsid protein (e.g., VP1 or VP3) may be examined for effects on deami-

dation, trafficking, transduction, and/or other post-translational modification(s) (e.g., glycosylation, ubiquitination, and so forth). Any assay described herein for examining deamidation, or a functional consequence thereof related to AAV particles, may be used to assess deamidation.

Several potential deamidation sites are described herein. In some embodiments, an amino acid substitution that alters deamidation is selected from A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3. For example, in some embodiments, N57 of VP1, N382 of VP3, N511 of VP3, and/or N715 of VP3 is substituted with Asp, and the amino acid substitution results in a higher frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In other embodiments, the amino acid substitution is N57K or N57Q, and the amino acid substitution results in a lower frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In yet other embodiments, G58 of VP1, G383 of VP3, G512 of VP3, and/or G716 of VP3 is substituted with an amino acid that is not Gly (e.g., Ala, Arg, Asn, Asp, Cys, Glu, Gln, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val), and the amino acid substitution results in a lower frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle.

In some embodiments, the AAV capsid protein is VP1, VP2, or VP3. The AAV particle may comprise any of the exemplary AAV capsid serotypes described herein, such as AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV2/2-7m8, AAV DJ, AAV DJ8, AAV2 N587A, AAV2 E548A, AAV2 N708A, AAV V708K, goat AAV, AAV1/AAV2 chimeric, bovine AAV, mouse AAV, or rAAV2/HBoV1. The AAV capsid protein may further comprise any of the capsid protein mutations described herein, such as tyrosine and/or heparin binding mutations.

Other aspects of the present disclosure relate to methods of improving the stability of a rAAV particle. In some embodiments, the methods include substituting amino acid residue 2 of VP1 and/or VP3, e.g., as described herein. For example, in some embodiments, amino acid residue 2 of VP1 is substituted. In other embodiments, amino acid residue 2 of VP3 is substituted. In some embodiments, the substituted amino acid at position 2 is N-acetylated at a higher frequency than amino acid residue 2 of the parent VP1 and/or VP3, e.g., as described herein. In some embodiments, substituting amino acid residue 2 of VP1 and/or VP3 improves the stability of a rAAV particle by at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 100%. In some embodiments, the stability of a rAAV particle with a substituted amino acid at position 2 may be compared to a wild-type or parental AAV capsid, e.g., of the same serotype. For example, in some embodiments, substituting amino acid residue 2 of VP1 and/or VP3 improves the stability of a rAAV particle by any one of about 10% to about 100%, about 20% to about 100%, about 30% to about 100%, about 40% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 10% to about 90%, about 20% to about 90%, about 30% to about 90%, about 40% to about 90%, about 50% to about 90%, about 60% to about 90%, about 70% to about 90%, about 80% to about 90%, about 10% to about 80%, about 20% to about 80%, about 30% to about 80%, about 40% to about 80%, about 50% to about 80%, about 60% to about 80%, about 70% to about 80%, about 10% to about 70%, about 20% to about 70%, about 30% to about 70%, about 40% to about 70%, about 50% to about 70%, about 60% to about 70%, about 10% to about 60%, about 20% to about 60%, about 30% to about 60%, about 40% to about 60%, about 50% to about 60%, about 10% to about 50%, about 20% to about 50%, about 30% to about 50%, about 40% to about 50%, about 10% to about 40%, about 20% to about 40%, about 30% to about 40%, about 10% to about 30%, about 20% to about 30%, or about 10% to about 20%, e.g., as compared to stability of a rAAV particle comprising a wild-type capsid. AAV particle stability may be measured using various assays known in the art, including without limitation differential scanning fluorescence (DSF), differential scanning calorimetry (DSC), other thermal denaturation assays, susceptibility to proteolysis, imaging or structural analysis to observe denaturation (e.g., using electron microscopy), transduction efficiency or another functional assay on AAV particle compositions kept for a designated time interval at a particular temperature (e.g., room temperature, or 4° C., for thermal stability) or treated at a particular pH (e.g., pH stability), and the like.

Other aspects of the present disclosure relate to methods of improving the assembly of a rAAV particle. In some embodiments, the methods include substituting amino acid residue 2 of VP1 and/or VP3, e.g., as described herein. For example, in some embodiments, amino acid residue 2 of VP1 is substituted. In other embodiments, amino acid residue 2 of VP3 is substituted. In some embodiments, the substituted amino acid at position 2 is N-acetylated at a higher frequency than amino acid residue 2 of the parent VP1 and/or VP3, e.g., as described herein. In some embodiments, substituting amino acid residue 2 of VP1 and/or VP3 improves the assembly of a rAAV particle by at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 100%. In some embodiments, the assembly of a rAAV particle with a substituted amino acid at position 2 may be compared to a wild-type or parental AAV capsid, e.g., of the same serotype. For example, in some embodiments, substituting amino acid residue 2 of VP1 and/or VP3 improves the assembly of a rAAV particle by any one of about 10% to about 100%, about 20% to about 100%, about 30% to about 100%, about 40% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 10% to about 90%, about 20% to about 90%, about 30% to about 90%, about 40% to about 90%, about 50% to about 90%, about 60% to about 90%, about 70% to about 90%, about 80% to about 90%, about 10% to about 80%, about 20% to about 80%, about 30% to about 80%, about 40% to about 80%, about 50% to about 80%, about 60% to about 80%, about 70% to about 80%, about 10% to about 70%, about 20% to about 70%, about 30% to about 70%, about 40% to about 70%, about 50% to about 70%, about 60% to about 70%, about 10% to about 60%, about 20% to about 60%, about 30% to about 60%, about 40% to about 60%, about 50% to about 60%, about 10% to about 50%, about 20% to about 50%, about 30% to about

50%, about 40% to about 50%, about 10% to about 40%, about 20% to about 40%, about 30% to about 40%, about 10% to about 30%, about 20% to about 30%, or about 10% to about 20%, e.g., as compared to assembly of a rAAV particle comprising a wild-type capsid. AAV particle assembly may be measured using various assays known in the art, including without limitation, measuring particle production amount and/or rate, quantifying capsid production (e.g., after purification using any of the methods described herein), assaying production of complete vectors vs. empty capsids, measuring transduction efficiency, imaging or structural analysis to observe particle formation (e.g., using electron microscopy), production of AAV capsid proteins (e.g., as assayed by Western blotting), and the like.

Other aspects of the present disclosure relate to methods of improving the transduction of a rAAV particle. In some embodiments, the methods include substituting amino acid residue 2 of VP1 and/or VP3, e.g., as described herein. For example, in some embodiments, amino acid residue 2 of VP1 is substituted. In other embodiments, amino acid residue 2 of VP3 is substituted. In some embodiments, the substituted amino acid at position 2 is N-acetylated at a higher frequency than amino acid residue 2 of the parent VP1 and/or VP3, e.g., as described herein. In some embodiments, substituting amino acid residue 2 of VP1 and/or VP3 improves the transduction of a rAAV particle by at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 100%. In some embodiments, the transduction of a rAAV particle with a substituted amino acid at position 2 may be compared to a wild-type or parental AAV capsid, e.g., of the same serotype. For example, in some embodiments, substituting amino acid residue 2 of VP1 and/or VP3 improves the transduction of a rAAV particle by any one of about 10% to about 100%, about 20% to about 100%, about 30% to about 100%, about 40% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 10% to about 90%, about 20% to about 90%, about 30% to about 90%, about 40% to about 90%, about 50% to about 90%, about 60% to about 90%, about 70% to about 90%, about 80% to about 90%, about 10% to about 80%, about 20% to about 80%, about 30% to about 80%, about 40% to about 80%, about 50% to about 80%, about 60% to about 80%, about 70% to about 80%, about 10% to about 70%, about 20% to about 70%, about 30% to about 70%, about 40% to about 70%, about 50% to about 70%, about 60% to about 70%, about 10% to about 60%, about 20% to about 60%, about 30% to about 60%, about 40% to about 60%, about 50% to about 60%, about 10% to about 50%, about 20% to about 50%, about 30% to about 50%, about 40% to about 50%, about 10% to about 40%, about 20% to about 40%, about 30% to about 40%, about 10% to about 30%, about 20% to about 30%, or about 10% to about 20%, e.g., as compared to transduction of a rAAV particle comprising a wild-type capsid. AAV particle transduction may be measured using various assays known in the art, including without limitation, the transduction efficiency assays described herein. In some embodiments, the invention provide methods of reducing the transduction of a rAAV particle; for example, by substituting amino acid residue 2 of VP1 and/or VP3.

Other aspects of the present disclosure relate to methods of improving the stability of a rAAV particle. In some embodiments, the methods include substituting an amino acid of VP1 and/or VP3 that alters deamidation, e.g., as described herein. For example, in some embodiments, A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3 is substituted. In some embodiments, the substituted amino acid is deamidated at a higher frequency than the amino acid residue of the parent VP1 and/or VP3, e.g., as described herein. In some embodiments, substituting A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, and/or G716 of VP3 improves the stability of a rAAV particle by at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 100%. In some embodiments, the stability of a rAAV particle with a substituted A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3 may be compared to a wild-type or parental AAV capsid, e.g., of the same serotype. For example, in some embodiments, substituting A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, and/or G716 of VP3 improves the stability of a rAAV particle by any one of about 10% to about 100%, about 20% to about 100%, about 30% to about 100%, about 40% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 10% to about 90%, about 20% to about 90%, about 30% to about 90%, about 40% to about 90%, about 50% to about 90%, about 60% to about 90%, about 70% to about 90%, about 80% to about 90%, about 10% to about 80%, about 20% to about 80%, about 30% to about 80%, about 40% to about 80%, about 50% to about 80%, about 60% to about 80%, about 70% to about 80%, about 10% to about 70%, about 20% to about 70%, about 30% to about 70%, about 40% to about 70%, about 50% to about 70%, about 60% to about 70%, about 10% to about 60%, about 20% to about 60%, about 30% to about 60%, about 40% to about 60%, about 50% to about 60%, about 10% to about 50%, about 20% to about 50%, about 30% to about 50%, about 40% to about 50%, about 10% to about 40%, about 20% to about 40%, about 30% to about 40%, about 10% to about 30%, about 20% to about 30%, or about 10% to about 20%, e.g., as compared to stability of a rAAV particle comprising a wild-type capsid. AAV particle stability may be measured using various assays known in the art, including without limitation differential scanning fluorescence (DSF), differential scanning calorimetry (DSC), other thermal denaturation assays, susceptibility to proteolysis, imaging or structural analysis to observe denaturation (e.g., using electron microscopy), transduction efficiency or another functional assay on AAV particle compositions kept for a designated time interval at a particular temperature (e.g., room temperature, or 4° C., for thermal stability) or treated at a particular pH (e.g., pH stability), and the like.

Other aspects of the present disclosure relate to methods of improving the assembly of a rAAV particle. In some embodiments, the methods include substituting an amino acid of VP1 and/or VP3 that alters deamidation, e.g., as described herein. For example, in some embodiments, A35

of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3 is substituted. In some embodiments, the substituted amino acid is deamidated at a higher frequency than the amino acid residue of the parent VP1 and/or VP3, e.g., as described herein. In some embodiments, substituting A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, and/or G716 of VP3 improves the assembly of a rAAV particle by at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 100%. In some embodiments, the stability of a rAAV particle with a substituted A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3 may be compared to a wild-type or parental AAV capsid, e.g., of the same serotype. For example, in some embodiments, substituting A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, and/or G716 of VP3 improves the assembly of a rAAV particle by any one of about 10% to about 100%, about 20% to about 100%, about 30% to about 100%, about 40% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 10% to about 90%, about 20% to about 90%, about 30% to about 90%, about 40% to about 90%, about 50% to about 90%, about 60% to about 90%, about 70% to about 90%, about 80% to about 90%, about 10% to about 80%, about 20% to about 80%, about 30% to about 80%, about 40% to about 80%, about 50% to about 80%, about 60% to about 80%, about 70% to about 80%, about 10% to about 70%, about 20% to about 70%, about 30% to about 70%, about 40% to about 70%, about 50% to about 70%, about 60% to about 70%, about 10% to about 60%, about 20% to about 60%, about 30% to about 60%, about 40% to about 60%, about 50% to about 60%, about 10% to about 50%, about 20% to about 50%, about 30% to about 50%, about 40% to about 50%, about 10% to about 40%, about 20% to about 40%, about 30% to about 40%, about 10% to about 30%, about 20% to about 30%, or about 10% to about 20%, e.g., as compared to assembly of a rAAV particle comprising a wild-type capsid. AAV particle assembly may be measured using various assays known in the art, including without limitation, measuring particle production amount and/or rate, quantifying capsid production (e.g., after purification using any of the methods described herein), assaying production of complete vectors vs. empty capsids, measuring transduction efficiency, imaging or structural analysis to observe particle formation (e.g., using electron microscopy), production of AAV capsid proteins (e.g., as assayed by Western blotting), and the like.

Other aspects of the present disclosure relate to methods of improving the transduction of a rAAV particle. In some embodiments, the methods include substituting an amino acid of VP1 and/or VP3 that alters deamidation, e.g., as described herein. For example, in some embodiments, A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3 is substituted. In some embodiments, the substituted amino acid is deamidated at a higher frequency than the amino acid residue of the parent VP1 and/or VP3, e.g., as described herein. In some embodiments, substituting A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, and/or G716 of VP3 improves the transduction of a rAAV particle by at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 100%. In some embodiments, the stability of a rAAV particle with a substituted A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3 may be compared to a wild-type or parental AAV capsid, e.g., of the same serotype. For example, in some embodiments, substituting A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, and/or G716 of VP3 improves the transduction of a rAAV particle by any one of about 10% to about 100%, about 20% to about 100%, about 30% to about 100%, about 40% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 10% to about 90%, about 20% to about 90%, about 30% to about 90%, about 40% to about 90%, about 50% to about 90%, about 60% to about 90%, about 70% to about 90%, about 80% to about 90%, about 10% to about 80%, about 20% to about 80%, about 30% to about 80%, about 40% to about 80%, about 50% to about 80%, about 60% to about 80%, about 70% to about 80%, about 10% to about 70%, about 20% to about 70%, about 30% to about 70%, about 40% to about 70%, about 50% to about 70%, about 60% to about 70%, about 10% to about 60%, about 20% to about 60%, about 30% to about 60%, about 40% to about 60%, about 50% to about 60%, about 10% to about 50%, about 20% to about 50%, about 30% to about 50%, about 40% to about 50%, about 10% to about 40%, about 20% to about 40%, about 30% to about 40%, about 10% to about 30%, about 20% to about 30%, or about 10% to about 20%, e.g., as compared to transduction of a rAAV particle comprising a wild-type capsid. AAV particle transduction may be measured using various assays known in the art, including without limitation, the transduction efficiency assays described herein.

In some aspects, the invention provides viral particles comprising a recombinant self-complementing genome (e.g., a self-complementary or self-complimenting rAAV vector). AAV viral particles with self-complementing vector genomes and methods of use of self-complementing AAV genomes are described in U.S. Pat. Nos. 6,596,535; 7,125,717; 7,465,583; 7,785,888; 7,790,154; 7,846,729; 8,093,054; and 8,361,457; and Wang Z., et al., (2003) *Gene Ther* 10:2105-2111, each of which are incorporated herein by reference in its entirety. A rAAV comprising a self-complementing genome will quickly form a double stranded DNA molecule by virtue of its partially complementing sequences (e.g., complementing coding and non-coding strands of a heterologous nucleic acid). In some embodiments, the vector comprises a first nucleic acid sequence encoding a heterologous nucleic acid and a second nucleic acid sequence encoding a complement of the nucleic acid, where the first nucleic acid sequence can form intrastrand base pairs with the second nucleic acid sequence along most or all of its length.

In some embodiments, the first heterologous nucleic acid sequence and a second heterologous nucleic acid sequence are linked by a mutated ITR (e.g., the right ITR). In some

embodiments, the ITR comprises the polynucleotide sequence 5'-CACTCCCTCTCTGCGCGCTCGCTCGCT-CACTGAGGCC GGGCGACCAAAGGTCGCC-CACGCCCGGGCTTTGCCCGGGCG-3' (SEQ ID NO:8). The mutated ITR comprises a deletion of the D region comprising the terminal resolution sequence. As a result, on replicating an AAV viral genome, the rep proteins will not cleave the viral genome at the mutated ITR and as such, a recombinant viral genome comprising the following in 5' to 3' order will be packaged in a viral capsid: an AAV ITR, the first heterologous polynucleotide sequence including regulatory sequences, the mutated AAV ITR, the second heterologous polynucleotide in reverse orientation to the first heterologous polynucleotide and a third AAV ITR.

Different AAV serotypes are used to optimize transduction of particular target cells or to target specific cell types within a particular target tissue (e.g., a diseased tissue). A rAAV particle can comprise viral proteins and viral nucleic acids of the same serotype or a mixed serotype. For example, a rAAV particle may contain one or more ITRs and capsid derived from the same AAV serotype, or a rAAV particle may contain one or more ITRs derived from a different AAV serotype than capsid of the rAAV particle.

In some embodiments, the AAV capsid comprises a mutation, e.g., the capsid comprises a mutant capsid protein. In some embodiments, the mutation is a tyrosine mutation or a heparin binding mutation. In some embodiments, a mutant capsid protein maintains the ability to form an AAV capsid. In some embodiments, the rAAV particle comprises an AAV2 or AAV5 tyrosine mutant capsid (see, e.g., Zhong L. et al., (2008) *Proc Natl Acad Sci USA* 105(22):7827-7832), such as a mutation in Y444 or Y730 (numbering according to AAV2). In further embodiments, the rAAV particle comprises capsid proteins of an AAV serotype from Clades A-F (Gao, et al., *J. Virol.* 2004, 78(12):6381).

In some embodiments, a capsid protein comprises one or more amino acid substitutions at one or more positions that interact with a heparin sulfate proteoglycan or at one or more positions corresponding to amino acids 484, 487, 527, 532, 585 or 588, numbering based on VP1 numbering of AAV2. Heparan sulfate proteoglycan (HSPG) is known in the art to act as the cellular receptor for AAV2 particles (Summerford, C. and Samulski, R. J. (1998) *J. Virol.* 72(2):1438-45). Binding between an AAV2 particle and HSPG at the cell membrane serves to attach the particle to the cell. Other cell surface proteins such as fibroblast growth factor receptor and αvβ5 integrin may also facilitate cellular infection. After binding, an AAV2 particle may enter the cell through mechanisms including receptor mediated endocytosis via clathrin-coated pits. An AAV2 particle may be released from an endocytic vesicle upon endosomal acidification. This allows the AAV2 particle to travel to the perinuclear region and then the cell nucleus. AAV3 particles are also known to bind heparin (Rabinowitz, J. E., et al. (2002) *J. Virol.* 76(2):791-801).

The binding between AAV2 capsid proteins and HSPG is known to occur via electrostatic interactions between basic AAV2 capsid protein residues and negatively charged glycosaminoglycan residues (Opie, S R et al., (2003) *J. Virol.* 77:6995-7006; Kern, A et al., (2003) *J. Virol.* 77:11072-11081). Specific capsid residues implicated in these interactions include R484, R487, K527, K532, R585, and R588. Mutations in these residues have been shown to reduce AAV2 binding to Hela cells and heparin itself (Opie, S R et al., (2003) *J. Virol.* 77:6995-7006; Kern, A et al., (2003) *J. Virol.* 77:11072-11081; WO 2004/027019 A2, U.S. Pat. No. 7,629,322). Further, without wishing to be bound to theory, it is thought that amino acid substitution(s) at one or more of the residues corresponding to amino acids 484, 487, 527, 532, 585 or 588, numbering based on VP1 numbering of AAV2 may modulate the transduction properties of AAV capsid types that do not bind to HSPG, or may modulate the transduction properties of AAV capsid types independent from their ability to bind HSPG. In some embodiments, the one or more amino acid substitutions comprises a substitution at position R484, R487, K527, K532, R585 and/or R588 of VP1, VP2 and/or VP3, numbering based on VP1 of AAV2.

In some embodiments, the one or more amino acid substitutions reduce binding of the rAAV particle to the heparin sulfate proteoglycan by about at least 10%, about at least 25%, about at least 50%, about at least 75%, or about at least 100%. In some embodiments, the one or more amino acid substitutions reduce binding of the rAAV particle to the heparin sulfate proteoglycan by about at least 10%, about at least 15%, about at least 20%, about at least 25%, about at least 30%, about at least 35%, about at least 40%, about at least 45%, about at least 50%, about at least 55%, about at least 60%, about at least 65%, about at least 70%, about at least 75%, about at least 80%, about at least 85%, about at least 90%, about at least 95%, or about at least 100% (as compared to binding of a rAAV particle comprising a wild-type capsid). In some embodiments, the one or more amino acid substitutions reduce binding of the rAAV particle to the heparin sulfate proteoglycan by any one of about 10% to about 100%, about 20% to about 100%, about 30% to about 100%, about 40% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 10% to about 90%, about 20% to about 90%, about 30% to about 90%, about 40% to about 90%, about 50% to about 90%, about 60% to about 90%, about 70% to about 90%, about 80% to about 90%, about 10% to about 80%, about 20% to about 80%, about 30% to about 80%, about 40% to about 80%, about 50% to about 80%, about 60% to about 80%, about 70% to about 80%, about 10% to about 70%, about 20% to about 70%, about 30% to about 70%, about 40% to about 70%, about 50% to about 70%, about 60% to about 70%, about 10% to about 60%, about 20% to about 60%, about 30% to about 60%, about 40% to about 60%, about 50% to about 60%, about 10% to about 50%, about 20% to about 50%, about 30% to about 50%, about 40% to about 50%, about 10% to about 40%, about 20% to about 40%, about 30% to about 40%, about 10% to about 30%, about 20% to about 30%, or about 10% to about 20%, (as compared to binding of a rAAV particle comprising a wild-type capsid). In some embodiments, the one or more amino acid substitutions results in no detectable binding of the rAAV particle to the heparin sulfate proteoglycan compared to binding of a wild-type rAAV particle. Means to measure binding of AAV particles to HSPG are known in the art; e.g., binding to a heparin sulfate chromatography media or binding to a cell known to express HSPG on its surface. For example, see Opie, S R et al., (2003) *J. Virol.* 77:6995-7006 and Kern, A et al., (2003) *J. Virol.* 77:11072-11081. In some embodiments, the one or more amino acid substitutions improve the transduction efficiency of the rAAV particle to a cell (e.g., a cell in the eye or CNS) by about at least 10%, about at least 15%, about at least 20%, about at least 25%, about at least 30%, about at least 35%, about at least 40%, about at least 45%, about at least 50%, about at least 55%, about at least 60%, about at least 65%, about at least 70%, about at least 75%, about at least 80%, about at least 85%, about at least 90%, about at least 95%, or about at least

100% (as compared to transduction efficiency of a rAAV particle comprising a wild-type capsid). In some embodiments, the one or more amino acid substitutions improve the transduction efficiency of the rAAV particle to a cell (e.g., a cell in the eye or CNS) by any one of about 10% to about 100%, about 20% to about 100%, about 30% to about 100%, about 40% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 10% to about 90%, about 20% to about 90%, about 30% to about 90%, about 40% to about 90%, about 50% to about 90%, about 60% to about 90%, about 70% to about 90%, about 80% to about 90%, about 10% to about 80%, about 20% to about 80%, about 30% to about 80%, about 40% to about 80%, about 50% to about 80%, about 60% to about 80%, about 70% to about 80%, about 10% to about 70%, about 20% to about 70%, about 30% to about 70%, about 40% to about 70%, about 50% to about 70%, about 60% to about 70%, about 10% to about 60%, about 20% to about 60%, about 30% to about 60%, about 40% to about 60%, about 50% to about 60%, about 10% to about 50%, about 20% to about 50%, about 30% to about 50%, about 40% to about 50%, about 10% to about 40%, about 20% to about 40%, about 30% to about 40%, about 10% to about 30%, about 20% to about 30%, or about 10% to about 20%, (as compared to transduction efficiency of a rAAV particle comprising a wild-type capsid). Means to measure transduction efficiency of AAV particles to a cell (e.g., a cell in culture or part of a tissue) are known in the art. For example, a population of cells (e.g., in culture or part of a tissue) may be infected with a concentration of rAAV particles containing a vector that, when expressed in the cells, produces an assayable reporter (e.g., GFP fluorescence, sFLT production, etc.).

AAV Capsid Proteins

In some aspects, the invention provides an AAV capsid protein comprising an amino acid substitution at amino acid residue 2; wherein the amino acid substitution at amino acid residue 2 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of the parent AAV capsid protein. In some embodiments, the AAV capsid protein is VP1 or VP3. In some embodiments, amino acid residue 2 of the AAV capsid protein (e.g., VP1 or VP3) is substituted with Cys, Ser, Thr, Val, Gly, Asn, Asp, Glu, Ile, Leu, Phe, Gln, Lys, Met, Pro or Tyr. In some embodiments, the amino acid substitution results in less deamidation of the AAV capsid protein. Non-limiting examples of AAV capsid proteins of the invention include VP1 and/or VP3 of any of the following AAV serotypes: AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV2/2-7m8, AAV DJ, AAV DJ8, AAV2 N587A, AAV2 E548A, AAV2 N708A, AAV V708K, goat AAV, AAV1/AAV2 chimeric, bovine AAV, mouse AAV, or rAAV2/HBoV1 serotype capsid. In some embodiments, the AAV capsid further comprises a tyrosine mutation or a heparin binding mutation.

Production of AAV Particles

Numerous methods are known in the art for production of rAAV vectors, including transfection, stable cell line production, and infectious hybrid virus production systems which include adenovirus-AAV hybrids, herpesvirus-AAV hybrids (Conway, J E et al., (1997) *J. Virology* 71(11):8780-8789) and baculovirus-AAV hybrids (Urabe, M. et al., (2002) *Human Gene Therapy* 13(16):1935-1943; *Kotin, R.* (2011) *Hum Mol Genet.* 20(R1): R2-R6). rAAV production cultures for the production of rAAV viral particles all require: 1) suitable host cells, 2) suitable helper virus function, 3) AAV rep and cap genes and gene products; 4) a nucleic acid (such as a therapeutic nucleic acid) flanked by at least one AAV ITR sequences (e.g., an AAV genome encoding GNPTAB); and 5) suitable media and media components to support rAAV production. In some embodiments, the suitable host cell is a primate host cell. In some embodiments, the suitable host cell is a human-derived cell lines such as HeLa, A549, 293, or Perc.6 cells. In some embodiments, the suitable helper virus function is provided by wild-type or mutant adenovirus (such as temperature sensitive adenovirus), herpes virus (HSV), baculovirus, or a plasmid construct providing helper functions. In some embodiments, the AAV rep and cap gene products may be from any AAV serotype. In general, but not obligatory, the AAV rep gene product is of the same serotype as the ITRs of the rAAV vector genome as long as the rep gene products may function to replicated and package the rAAV genome. Suitable media known in the art may be used for the production of rAAV vectors. These media include, without limitation, media produced by Hyclone Laboratories and JRH including Modified Eagle Medium (MEM), Dulbecco's Modified Eagle Medium (DMEM), custom formulations such as those described in U.S. Pat. No. 6,566,118, and Sf-900 II SFM media as described in U.S. Pat. No. 6,723,551, each of which is incorporated herein by reference in its entirety, particularly with respect to custom media formulations for use in production of recombinant AAV vectors. In some embodiments, the AAV helper functions are provided by adenovirus or HSV. In some embodiments, the AAV helper functions are provided by baculovirus and the host cell is an insect cell (e.g., *Spodoptera frugiperda* (Sf9) cells). In some embodiments, the AAV cap functions provide an amino acid substitution at amino acid residue 2 of VP1 and/or VP3, wherein the amino acid substitution at amino acid residue 2 of VP1 and/or VP3 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of VP1 and/or VP3 of the parent AAV particle. In some embodiments, amino acid residue 2 of the AAV capsid protein (e.g., VP1 or VP3) is substituted with Cys, Ser, Thr, Val, Gly, Asn, Asp, Glu, Ile, Leu, Phe, Gln, Lys, Met, Pro or Tyr. In some embodiments, the amino acid substitution results in less deamidation of the AAV capsid.

One method for producing rAAV particles is the triple transfection method. Briefly, a plasmid containing a rep gene and a capsid gene, along with a helper adenoviral plasmid, may be transfected (e.g., using the calcium phosphate method) into a cell line (e.g., HEK-293 cells), and virus may be collected and optionally purified. As such, in some embodiments, the rAAV particle was produced by triple transfection of a nucleic acid encoding the rAAV vector, a nucleic acid encoding AAV rep and cap, and a nucleic acid encoding AAV helper virus functions into a host cell, wherein the transfection of the nucleic acids to the host cells generates a host cell capable of producing rAAV particles.

In some embodiments, rAAV particles may be produced by a producer cell line method (see Martin et al., (2013) *Human Gene Therapy Methods* 24:253-269; U.S. PG Pub. No. US2004/0224411; and Liu, X. L. et al. (1999) *Gene Ther.* 6:293-299). Briefly, a cell line (e.g., a HeLa, 293, A549, or Perc.6 cell line) may be stably transfected with a plasmid containing a rep gene, a capsid gene, and a vector genome comprising a promoter-heterologous nucleic acid sequence (e.g., GNPTAB). Cell lines may be screened to select a lead clone for rAAV production, which may then be expanded to a production bioreactor and infected with a helper virus (e.g., an adenovirus or HSV) to initiate rAAV

production. Virus may subsequently be harvested, adenovirus may be inactivated (e.g., by heat) and/or removed, and the rAAV particles may be purified. As such, in some embodiments, the rAAV particle was produced by a producer cell line comprising one or more of nucleic acid encoding the rAAV vector, a nucleic acid encoding AAV rep and cap, and a nucleic acid encoding AAV helper virus functions. As described herein, the producer cell line method may be advantageous for the production of rAAV particles with an oversized genome, as compared to the triple transfection method.

In some embodiments, the nucleic acid encoding AAV rep and cap genes and/or the rAAV genome are stably maintained in the producer cell line. In some embodiments, nucleic acid encoding AAV rep and cap genes and/or the rAAV genome is introduced on one or more plasmids into a cell line to generate a producer cell line. In some embodiments, the AAV rep, AAV cap, and rAAV genome are introduced into a cell on the same plasmid. In other embodiments, the AAV rep, AAV cap, and rAAV genome are introduced into a cell on different plasmids. In some embodiments, a cell line stably transfected with a plasmid maintains the plasmid for multiple passages of the cell line (e.g., 5, 10, 20, 30, 40, 50 or more than 50 passages of the cell). For example, the plasmid(s) may replicate as the cell replicates, or the plasmid(s) may integrate into the cell genome. A variety of sequences that enable a plasmid to replicate autonomously in a cell (e.g., a human cell) have been identified (see, e.g., Krysan, P. J. et al. (1989) *Mol. Cell Biol.* 9:1026-1033). In some embodiments, the plasmid(s) may contain a selectable marker (e.g., an antibiotic resistance marker) that allows for selection of cells maintaining the plasmid. Selectable markers commonly used in mammalian cells include without limitation blasticidin, G418, hygromycin B, zeocin, puromycin, and derivatives thereof. Methods for introducing nucleic acids into a cell are known in the art and include without limitation viral transduction, cationic transfection (e.g., using a cationic polymer such as DEAE-dextran or a cationic lipid such as lipofectamine), calcium phosphate transfection, microinjection, particle bombardment, electroporation, and nanoparticle transfection (for more details, see e.g., Kim, T. K. and Eberwine, J. H. (2010) *Anal. Bioanal. Chem.* 397:3173-3178).

In some embodiments, the nucleic acid encoding AAV rep and cap genes and/or the rAAV genome are stably integrated into the genome of the producer cell line. In some embodiments, nucleic acid encoding AAV rep and cap genes and/or the rAAV genome is introduced on one or more plasmids into a cell line to generate a producer cell line. In some embodiments, the AAV rep, AAV cap, and rAAV genome are introduced into a cell on the same plasmid. In other embodiments, the AAV rep, AAV cap, and rAAV genome are introduced into a cell on different plasmids. In some embodiments, the plasmid(s) may contain a selectable marker (e.g., an antibiotic resistance marker) that allows for selection of cells maintaining the plasmid. Methods for stable integration of nucleic acids into a variety of host cell lines are known in the art. For example, repeated selection (e.g., through use of a selectable marker) may be used to select for cells that have integrated a nucleic acid containing a selectable marker (and AAV cap and rep genes and/or a rAAV genome). In other embodiments, nucleic acids may be integrated in a site-specific manner into a cell line to generate a producer cell line. Several site-specific recombination systems are known in the art, such as FLP/FRT (see, e.g., O'Gorman, S. et al. (1991) *Science* 251:1351-1355), Cre/loxP (see, e.g., Sauer, B. and Henderson, N. (1988) *Proc. Natl. Acad. Sci.* 85:5166-5170), and phi C31-att (see, e.g., Groth, A. C. et al. (2000) *Proc. Natl. Acad. Sci.* 97:5995-6000).

In some embodiments, the producer cell line is derived from a primate cell line (e.g., a non-human primate cell line, such as a Vero or FRhL-2 cell line). In some embodiments, the cell line is derived from a human cell line. In some embodiments, the producer cell line is derived from HeLa, 293, A549, or PERC.6® (Crucell) cells. For example, prior to introduction and/or stable maintenance/integration of nucleic acid encoding AAV rep and cap genes and/or the oversized rAAV genome into a cell line to generate a producer cell line, the cell line is a HeLa, 293, A549, or PERC.6® (Crucell) cell line, or a derivative thereof.

In some embodiments, the producer cell line is adapted for growth in suspension. As is known in the art, anchorage-dependent cells are typically not able to grow in suspension without a substrate, such as microcarrier beads. Adapting a cell line to grow in suspension may include, for example, growing the cell line in a spinner culture with a stirring paddle, using a culture medium that lacks calcium and magnesium ions to prevent clumping (and optionally an antifoaming agent), using a culture vessel coated with a siliconizing compound, and selecting cells in the culture (rather than in large clumps or on the sides of the vessel) at each passage. For further description, see, e.g., ATCC frequently asked questions document (available on the world wide web at atcc.org/Global/FAQs/9/1/Adapting%20a%20monolayer%20cell%20line%20to%20suspension-40.aspx) and references cited therein.

Suitable AAV production culture media of the present invention may be supplemented with serum or serum-derived recombinant proteins at a level of 0.5%-20% (v/v or w/v). Alternatively, as is known in the art, AAV vectors may be produced in serum-free conditions which may also be referred to as media with no animal-derived products. One of ordinary skill in the art may appreciate that commercial or custom media designed to support production of AAV vectors may also be supplemented with one or more cell culture components know in the art, including without limitation glucose, vitamins, amino acids, and or growth factors, in order to increase the titer of AAV in production cultures.

AAV production cultures can be grown under a variety of conditions (over a wide temperature range, for varying lengths of time, and the like) suitable to the particular host cell being utilized. As is known in the art, AAV production cultures include attachment-dependent cultures which can be cultured in suitable attachment-dependent vessels such as, for example, roller bottles, hollow fiber filters, microcarriers, and packed-bed or fluidized-bed bioreactors. AAV vector production cultures may also include suspension-adapted host cells such as HeLa, 293, and SF-9 cells which can be cultured in a variety of ways including, for example, spinner flasks, stirred tank bioreactors, and disposable systems such as the Wave bag system.

AAV vector particles of the invention may be harvested from AAV production cultures by lysis of the host cells of the production culture or by harvest of the spent media from the production culture, provided the cells are cultured under conditions known in the art to cause release of AAV particles into the media from intact cells, as described more fully in U.S. Pat. No. 6,566,118). Suitable methods of lysing cells are also known in the art and include for example multiple freeze/thaw cycles, sonication, microfluidization, and treatment with chemicals, such as detergents and/or proteases.

In a further embodiment, the AAV particles are purified. The term "purified" as used herein includes a preparation of

AAV particles devoid of at least some of the other components that may also be present where the AAV particles naturally occur or are initially prepared from. Thus, for example, isolated AAV particles may be prepared using a purification technique to enrich it from a source mixture, such as a culture lysate or production culture supernatant. Enrichment can be measured in a variety of ways, such as, for example, by the proportion of DNase-resistant particles (DRPs) or genome copies (gc) present in a solution, or by infectivity, or it can be measured in relation to a second, potentially interfering substance present in the source mixture, such as contaminants, including production culture contaminants or in-process contaminants, including helper virus, media components, and the like.

In some embodiments, the AAV production culture harvest is clarified to remove host cell debris. In some embodiments, the production culture harvest is clarified by filtration through a series of depth filters including, for example, a grade DOHC Millipore Millistak+HC Pod Filter, a grade A1HC Millipore Millistak+HC Pod Filter, and a 0.2 m Filter Opticap XL1O Millipore Express SHC Hydrophilic Membrane filter. Clarification can also be achieved by a variety of other standard techniques known in the art, such as, centrifugation or filtration through any cellulose acetate filter of 0.2 m or greater pore size known in the art.

In some embodiments, the AAV production culture harvest is further treated with Benzonase® to digest any high molecular weight DNA present in the production culture. In some embodiments, the Benzonase® digestion is performed under standard conditions known in the art including, for example, a final concentration of 1-2.5 units/ml of Benzonase® at a temperature ranging from ambient to 37° C. for a period of 30 minutes to several hours.

AAV particles may be isolated or purified using one or more of the following purification steps: equilibrium centrifugation; flow-through anionic exchange filtration; tangential flow filtration (TFF) for concentrating the AAV particles; AAV capture by apatite chromatography; heat inactivation of helper virus; AAV capture by hydrophobic interaction chromatography; buffer exchange by size exclusion chromatography (SEC); nanofiltration; and AAV capture by anionic exchange chromatography, cationic exchange chromatography, or affinity chromatography. These steps may be used alone, in various combinations, or in different orders. In some embodiments, the method comprises all the steps in the order as described below. Methods to purify AAV particles are found, for example, in Xiao et al., (1998) *Journal of Virology* 72:2224-2232; U.S. Pat. Nos. 6,989,264 and 8,137,948; and WO 2010/148143.

Pharmaceutical Compositions

In some embodiments, an AAV particle of the present disclosure (e.g., a rAAV particle) is in a pharmaceutical composition. The pharmaceutical compositions may be suitable for any mode of administration described herein or known in the art. In some embodiments, the pharmaceutical composition comprises rAAV particles modified to improve the stability and/or improve the transduction efficiency of rAAV particles; for example, for use in substituting the amino acid residue at position 2 of VP1 and/or VP3 to improve acetylation of rAAV capsid proteins. In some embodiments, the pharmaceutical composition comprises rAAV particles modified to modulate the stability and/or the transduction efficiency of rAAV particles (e.g., increase stability and/or transduction efficiency or decrease stability and/or transduction efficiency); for example, for use in substituting the amino acid residues that modulate deamidation (e.g., increase deamidation or decrease deamidation).

In some embodiments, the rAAV particle is in a pharmaceutical composition comprising a pharmaceutically acceptable excipient. As is well known in the art, pharmaceutically acceptable excipients are relatively inert substances that facilitate administration of a pharmacologically effective substance and can be supplied as liquid solutions or suspensions, as emulsions, or as solid forms suitable for dissolution or suspension in liquid prior to use. For example, an excipient can give form or consistency, or act as a diluent. Suitable excipients include but are not limited to stabilizing agents, wetting and emulsifying agents, salts for varying osmolarity, encapsulating agents, pH buffering substances, and buffers. Such excipients include any pharmaceutical agent suitable for direct delivery to the eye which may be administered without undue toxicity. Pharmaceutically acceptable excipients include, but are not limited to, sorbitol, any of the various TWEEN compounds, and liquids such as water, saline, glycerol and ethanol. Pharmaceutically acceptable salts can be included therein, for example, mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. A thorough discussion of pharmaceutically acceptable excipients is available in REMINGTON'S PHARMACEUTICAL SCIENCES (Mack Pub. Co., N.J. 1991). In some embodiments, the pharmaceutical composition comprising a rAAV particle described herein and a pharmaceutically acceptable carrier is suitable for administration to human. Such carriers are well known in the art (see, e.g., Remington's Pharmaceutical Sciences, 15th Edition, pp. 1035-1038 and 1570-1580).

Such pharmaceutically acceptable carriers can be sterile liquids, such as water and oil, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, and the like. Saline solutions and aqueous dextrose, polyethylene glycol (PEG) and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. The pharmaceutical composition may further comprise additional ingredients, for example preservatives, buffers, tonicity agents, antioxidants and stabilizers, nonionic wetting or clarifying agents, viscosity-increasing agents, and the like. The pharmaceutical compositions described herein can be packaged in single unit dosages or in multidosage forms. The compositions are generally formulated as sterile and substantially isotonic solution.

Kits and Articles of Manufacture

The present invention also provides kits or articles of manufacture comprising any of the rAAV particles and/or pharmaceutical compositions of the present disclosure. The kits or articles of manufacture may comprise any of the rAAV particles or rAAV particle compositions of the invention. In some embodiments the kits are used to improve the stability and/or improve the transduction efficiency of rAAV particles; for example, for use in substituting the amino acid residue at position 2 of VP1 and/or VP3 to improve acetylation of rAAV capsid proteins. In some embodiments the kits are used to modulate the stability and/or the transduction efficiency of rAAV particles (e.g., increase stability and/or transduction efficiency or decrease stability and/or transduction efficiency); for example, for use in substituting the amino acid residues that modulate deamidation (e.g., increase deamidation or decrease deamidation).

In some embodiments, the kits or articles of manufacture further include instructions for administration of a composition of rAAV particles. The kits or articles of manufacture described herein may further include other materials desir-

able from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for performing any methods described herein. Suitable packaging materials may also be included and may be any packaging materials known in the art, including, for example, vials (such as sealed vials), vessels, ampules, bottles, jars, flexible packaging (e.g., sealed Mylar or plastic bags), and the like. These articles of manufacture may further be sterilized and/or sealed.

In some embodiments, the kits or articles of manufacture further contain one or more of the buffers and/or pharmaceutically acceptable excipients described herein (e.g., as described in REMINGTON'S PHARMACEUTICAL SCIENCES (Mack Pub. Co., N.J. 1991). In some embodiments, the kits or articles of manufacture include one or more pharmaceutically acceptable excipients, carriers, solutions, and/or additional ingredients described herein. The kits or articles of manufacture described herein can be packaged in single unit dosages or in multidosage forms. The contents of the kits or articles of manufacture are generally formulated as sterile and can be lyophilized or provided as a substantially isotonic solution.

EXAMPLES

The invention will be more fully understood by reference to the following examples. They should not, however, be construed as limiting the scope of the invention. It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

Example 1: Direct LC/MS and LC/MS/MS for Complete Characterization of Recombinant AAV Viral Capsid Protein

Recombinant adeno-associated viruses (rAAVs) have become popular gene therapy vectors due to their nonpathogenic nature, ability to infect both dividing and non-dividing cells and long term gene expression. Currently, AAV-based gene therapies are used in clinical trials for numerous disease targets, such as muscular dystrophy, hemophilia, Parkinson's disease, Leber's congenital amaurosis and macular degeneration.

AAV is a small and nonenveloped parvovirus with a single stranded DNA genome encapsulated in an icosahedra shell. Each capsid includes sixty copies of three viral capsid proteins VP1 (87 kDa), VP2 (73 kDa) and VP3 (62 kDa) in an approximately 1:1:10 ratio. The three viral capsid proteins are expressed from the same open reading frame by using alternative splicing and an atypical start codon and thus have overlapping sequences. VP1 has ~137 additional N-terminal amino acid residues compared to VP3 while VP2 has ~65 additional N-terminal amino acid residues compared to VP3. At least 13 AAV serotypes and ~150 gene sequences have been isolated from human and non-human primate tissues; AAV serotypes differ in the amino acid sequence of viral capsid proteins and their corresponding cellular receptors and co-receptors for targeting.

The AAV capsid, in addition to protecting the genome inside, plays an important role in mediating receptor binding, escape of virus from endosome, and transport of viral DNA into nucleus in the viral infection cycle, thus directly impacting viral infectivity. It has been shown that the VP1 N-terminus contains a phospholipase PLA2 domain (a.a. 52-97) which is critical in endosomal escaping of virus [1-3]. N-termini of VP1 and VP2 also contain three basic amino acid clusters as nuclear localization signals. These sequences are highly conserved among different AAV serotypes. Mutations of these amino acids have been shown to reduce or abolish infectivity completely [4]. In addition, each AAV serotype has corresponding sequence-specific receptors and co-receptors. For example, heparin sulfate proteoglycan was identified as a major receptor of AAV2 and several other co-receptors, including $\alpha V\beta 5$ integrin, fibroblast growth factor receptor 1, and hepatocyte growth receptor have been identified [5-8]. Mutation analysis of AAV2 capsid proteins has identified a group of basic amino acids (Arginine484, 487, 585, and Lysine532) as a heparin-binding motif which contributes to the heparin and HeLa cell binding [9]. NGR domain in AAV2 was identified as an integrin $\alpha 5\beta 1$ binding domain which is essential for viral cell entry [10]. In summary, viral capsid protein sequences are important in cellular targeting and trafficking in the viral infection cycle. Since different production conditions may cause different expression levels of viral capsid proteins, post-translational modifications, and truncations, the viral capsid proteins need to be characterized and monitored to ensure the product consistency in gene therapy development programs.

Traditionally, SDS-PAGE has been used to characterize the AAV viral capsid proteins, providing rough molecular weight information such as 87 kDa, 73 kDa and 62 kDa. No sequence information was obtained from Edman sequencing, possibly due to the blocked N-termini of viral capsid proteins, except VP2. Although X-ray structures of multiple AAVs have been solved, only the VP3 region sequence was observed in the crystal structures. Fifteen N-terminal amino acid residues of VP3 were still missing in the X-ray structure, possibly due to its intrinsic disorder [11-13]. It is possible that the lack of information of VP1 and VP2 N-terminal regions in the atomic structure might be due to low stoichiometry of VP1 and VP2 in the capsid. In addition, N-termini of VP1 and VP2 are buried inside the capsid and are not accessible to antibodies in the native state as reported in the some literature [3, 14, 15]. Conventionally, a Gel-LC/MS method (SDS-PAGE, in-gel tryptic digestion and LC/MS/MS) was used in characterization of VPs [16-18]. However, N-termini of VP1, VP2 and VP3 have not been confirmed using this approach, since this method failed to obtain 100% sequence coverage of VPs due to the limited recovery of peptide from gel.

Direct analysis using MALDI-TOF MS was reported for several virus capsid proteins including tobacco mosaic virus U2 after dissociation with organic acid [19]. Direct peptide mapping after amide hydrogen exchange and mass spectrometry have been used to study the pH-induced structural changes in the capsid of brome mosaic virus (BMV) [20]. Since AAVs are nonenveloped viruses containing only capsid proteins and genome, AAVs capsids could be directly analyzed by RP-LC/MS of proteins and LC/MS/MS of peptide mapping to achieve 100% sequence coverage after capsid dissociation without SDS-PAGE separation. The DNA fragments could elute in the void volume and thus have no interference on protein/peptide detection by LC/MS. In order to investigate these methods, direct LC/MS of different types of AAVs after denaturation was used to monitor the protein sequence and post-translational modifications of AAV capsid proteins. As described herein, N-termini of VP1, VP2 and VP3 of AAVs have been confirmed by mass spectrometry. Acetylations of N-termini of VP1 and

VP3 were also identified in the different serotype of AAVs. Direct LC/MS/MS peptide mapping of AAVs has also been developed to provide sequence coverage of VP1, VP2 and VP3 and confirm the N-termini acetylation of VP1 and VP3.

Methods

Materials and Reagents

Dithiothreitol (DTT), 4-vinylpyridine, ultra-pure formic acid, acetic acid, guanidine-HCl, Tris-HCl and Tris base were purchased from Sigma Chemicals (St. Louis, MO). Amicon ultra-4 filters were purchased from Millipore (Billerica, MA). The porcine sequencing grade trypsin was purchased from Promega (Milwaukee, WI). Endoproteinase Lys-C and Asp-N were purchased from Roche (Germany). Slide-A-Lyzer cassettes with 10,000 MWCO were purchased from Pierce (Rockford, IL).

Vector Production and Purification

AAV vectors were produced using the transient triple transfection method as previously described (Xiao, 1998 #123). Briefly, HEK293 cells were transfected using polyethyleneimine, PEI, and a 1:1:1 ratio of three plasmids (ITR vector, AAV rep/cap and Ad helper plasmid). The vector plasmid contains the vector genome CBA-EGFP and ITR sequences from AAV2. EGFP expression is driven by the CMV enhancer chicken beta actin hybrid promoter (CBA) as described (Miyazaki, 1989 #124) (Niwa, 1991 #125). The AAV rep/cap helpers contained rep sequences from AAV2 and serotype specific capsid sequences with the nomenclature, rep2/cap2, rep2/cap5, rep2/cap7 etc. The pAd helper used was pHelper (Stratagene/Agilent Technologies, Santa Clara, CA). Purification of AAV was performed as described by Qu et al. (2007, *J. Virol. Methods* 140:183-192).

LC/MS Intact Protein Analysis

The AAV virions were concentrated with an Amicon ultra-4 filter (10 kDa MWCO) and denatured with 10% acetic acid followed by direct analysis in an Acquity UPLC-Xevo® QTOF MS instrument (Waters, Milford, MA). The separations were performed with a UPLC BEH C4 or C8 column (1.7 μm, 2.1 mm i.d.) at a 0.25 ml/min flow rate. Mobile phase A was 0.1% formic acid in water while mobile phase B was 0.1% formic acid in acetonitrile. The final gradient was as follows: from 10% B to 20% B for 6 minutes, from 20% B to 30% B in 10 min, then from 30% to 38% B for 40 minutes. For MS the capillary voltage and sampling cone voltage were set at 3.5 kV and 45 V respectively. The mass spectra were acquired in the sensitivity mode with m/z range of 500-4000. Assisted calibration with sodium iodide as calibrant was performed for mass calibration. MaxEnt1 in Masslynx software was used for protein deconvolution.

Enzymatic Digestions of AAV2 VPs

The concentrated AAV2 virions were denatured with 6 M Guanidine-HCl, 0.1 M Tris at pH 8.5. The proteins were reduced with 30 mM DTT at 55° C. for 1 hour in darkness and alkylated with 0.07% 4-vinylpyridine at room temperature for 2 hours. The reactions were quenched by the addition of 1M DTT. The samples were dialyzed with Slide-A-Lyzer cassettes (10,000 MWCO) against 25 mM Tris buffer at pH 8.5 for ~18 hours. After dialysis, the samples were split into three aliquots. Each aliquot was digested with trypsin at 1:25 or Lys-C at 1:50 or Asp-N at 1:100 enzyme:protein ratio (wt/wt) for 18 hours at 37° C., respectively.

LC/MS/MS Peptide Mapping

Nano LC/MS/MS was performed in using a NanoAcquity HPLC system (Waters, Milford, MA) in conjunction with an Orbitrap Velos mass spectrometer (Thermo-Fisher Scientific, Waltham, MA) using home packed nanoLC column (75 μm×10 mm) with Magic C18 with packing material (5 μm, Bruker, Billerica, MA) at a 300 nl/min flow rate. The mobile phases A and B were 0.1% formic acid in water and acetonitrile, respectively. The gradient was from 2% B to 60% B in 121 min.

The source parameters for velos were as follows: source voltage: 2.5 kv, capillary temperature 275° C.; S-lens RF level: 55%. Data were acquired using the top-ten data dependent method with accurate ms at 60,000 resolution and 10 MS/MS in ion trap. Mascot was used for database searching against AAV2 viral capsid protein sequences. MS tolerance of 10 ppm and ms/ms tolerance of 0.8 Da were used for the database search.

UPLC/MS/MS Peptide Mapping

The protein digests were also analyzed by UPLC/MS/MS in Acquity UPLC-Xevo qTOF MS. A BEH300 C18 column (2.1×150 mm) was used for separation in the mobile phases with 0.1% formic acid in water/acetontitrile gradient at a flow rate 0.25 ml/min. The mass spectra were acquired in the positive MSe mode in the mass range of 200-2000.

Results

AAV Denaturation Method

AAVs can be denatured through a number of methods using detergent, heat, high salt, or buffer with low or high pHs. Heat denaturation can lead to protein precipitation and as a result reverse phase columns are easily clogged and over pressurized. Denaturation with high salt requires an additional desalting step before LC/MS analysis. Denaturing with 10% acetic acid was used for the LC/MS intact protein analysis, as it allowed for clean mass spectrum. For peptide mapping, either 0.1% RapiGest or 6 M Guanidine HCl can be used as a denaturing reagent.

Intact Protein Analysis Method Development

Initial intact protein analysis of AAV2 was performed using an UPLC BEH C4 column at fast gradient. Under this condition, only one single peak in the total ion chromatogram was observed, with a mass corresponding to VP3 (FIG. **1**A). Without wishing to be bound by theory, it is thought that the absence of VP1 and VP2 is possibly due to low stoichiometry of VP1 and VP2 or suppression of VP1 and VP2 signals by VP3 if all VPs co-elute. Increasing injection or column length, using a shallower gradient, and using alternative columns have been attempted in order to detect VP1 and VP2. Higher loading (1.7 μg) with a shallower gradient at 0.5% B/min resulted in a shoulder peak on the left (FIG. **1**B). The increase in column length from 10 cm to 15 cm did not enhance the separation of the shoulder peak (FIG. **1**C). However, the shoulder peak was further separated from the main peak using a BEH C8 column, with improved signal intensities observed (FIG. **1**D).

As a result, the VP1 and VP2 masses were obtained in this shoulder peak at the signal intensities shown in FIG. **2**A. The masses of VP1 and VP3 correspond to a.a. 2-735(acetylation) and a.a. 204-735(acetylation), respectively (FIGS. **2**A&**2**B). No acetylation was observed in VP2 (a.a.139-735). In addition, a minor peak with a smaller mass than VP3 was observed, with a mass corresponding to amino acid sequence 212-735 with one acetylation (FIG. **2**B). These data are consistent with DNA sequences since VP3 contains two ATG initiation codons in AAV2: ***ATGGCTACAGG CAGTGGCGCA CCAATGGCAGAC*** (SEQ ID NO:1), resulting in two possible N termini (underlined): MATGSGAPMAD (SEQ ID NO:2). The N-terminal methionine residues were not present in both VP1 and VP3 as measured by intact protein analysis. The acetylation of VP1 and VP3 is not a method-induced artifact (denaturation of AAV by 10% acetic acid) since acetylation of VP1 and VP3 is also observed

in an AAV preparation using an alternative denature method without acetic acid. The intact protein data also confirmed that no glycosylation was present in the viral capsid proteins, even though several N-linked consensus sequences are present [16].

LC/MS/MS Peptide Mapping

To further confirm the N-termini and acetylation observed in the intact protein analysis, peptide mapping was performed using multiple enzymes and analyzed using multiple instruments. Various sample preparation methods, including denaturation methods and desalting steps, have been evaluated. The final digestion method, including denaturation with 6M guanidine HCl, reduction and alkylation with 4-vinylpyridine, and dialysis using slide-A-lyzer followed by enzymatic digestion, created clean peptide mapping with low artificial modifications during the digestion process. As low as 5 μg starting material was tested, yielding complete sequence coverage using nano LC/MS/MS and UPLC/MS/MS.

Mascot search of tryptic digests from nano LC/MS/MS alone yielded 78% sequence coverage with an ion score 13 cut off as shown in FIG. **3**. The two large missing tryptic peptides, T27 and T38 (boxed) from nano LC/MS/MS were found in the LC/MS in Xevo TOF MS with BEH C18 column (FIG. **3**). In addition, most of the T27 and T38 peptide sequences were further confirmed by nano LC/MS/MS of Asp-N digests as shown in Italics in FIG. **3**. The complete N-terminal and C-terminal peptides were covered by Lys-C digests as underlined in FIG. **3**. Therefore, 100% sequence coverage of VP1 was achieved through multiple enzyme digestions and two LC/MS/MS methods.

LC/MS/MS confirmed the N- and C-termini of VP1, VP2 and VP3 and N-terminal acetylation of VP1 and VP3 observed in the intact protein analysis. FIGS. **4**A-**4**C show the MS/MS spectra of the VP1 N-terminal tryptic peptide A(Ac)ADGYLPDWLEDTLSEGIR (SEQ ID NO:4) (FIG. **4**A), VP2 N-terminal Asp-N derived peptide (APGKKRPVEHSPVEP) (SEQ ID NO:15) (FIG. **4**B), and VP3 N-terminal Asp-N peptide A(Ac)TGSGAPM (SEQ ID NO:5) (FIG. **4**C). MS/MS has confirmed the location of acetylation at the N-terminal alanine residues in both VP1 and VP3 peptides. The presence of unmodified y18 and y17 ions, and all detected b ions with 42 Da mass shift in FIG. **4**A indicates the 42 da-modification is located in N-terminal of VP1. Similarly, the presence of unmodified y3 to y8 ions in FIG. **4**C confirmed the location of acetylation at the N-terminal alanine residue.

Comparison of AAV VP N-Termini

In addition to AAV2, AAV1, AAV5, AAV7, AAV9 and AAV Rh10 have also been analyzed by intact protein analysis. The theoretical and predicted masses of VPs in AAVs are shown in Table 2.

TABLE 2

Theoretical Mass vs Experimental Mass for AAV VPs

Serotype	Isoform	Predicted amino acid sequence	Actual amino acid sequence	Theoretical Ms.(Da)	Experimental Ms.(Da)
AAV1	VP1	1-736	2(ac)-736	81286	81291
	VP2	138-736	139-736	66093	66098
	VP3	203-736	204(ac)-736	59517	59520
AAV2	VP1	1-735	2(ac)-735	81856	81856
	VP2	138-735	139-735	66488	66488
	VP3	203-735	204(ac)-735	59974	59974
AAV5	VP1	1-724	2(ac)-724	80336	80336
	VP2	137-724	138-724	65283	65284
	VP3	193-724	194(ac)-724	59463	59463
AAV7	VP1	1-737	2(ac)-737	81564	81567
	VP2	138-737	139-737	66372	66374
	VP3	204-737	213(ac)-737	59101	59103
AAV9	VP1	1-736	2(ac)-736	81291	81288
	VP2	138-736	139-736	66210	66209
	VP3	203-736	204(ac)-736	59733	59733
AAVRh10	VP1	1-738	2(ac)-738	81455	81455
	VP2	138-738	139-738	66253	66252
	VP3	204-738	205(ac)-738	59634	59634

N-termini, as well as their posttranslational modifications, are highly conserved among the AAV serotypes analyzed, even though AAV5 is reported as the most diverse AAV serotype sequence, as shown in the sequence alignments in FIG. **5**. In 11 out of 13 AAV serotypes, the N-termini of VP1 share an identical 13 amino acid residue sequence (MAADGYLPDWLED) (SEQ ID NO:6) while all 13 AAV serotypes have identical TAP . . . N-terminal sequences in VP2 (FIG. **5**). LC/MS of AAV2 indicated that T is missing in VP2 at protein level. The N-termini of VP3 are the most diverse among the three viral capsid proteins, with 8 out of 13 AAV serotypes sharing a MA . . . N-terminal sequence. Similar to AAV2, AAV1 and AAV Rh10 also have two ATG initiation codons with the first one as predominant N-terminal based on LC/MS intact protein analysis. Interestingly, though AAV7 has two potential initiation codons (**GTG** GCTGCAGGCGGTGGCGCACCA**ATG** GCAGACAAT AAC . . .) (SEQ ID NO:7), the second initiation codon (ATG) was favorable based on the intact protein analysis: the VP3' with 213(ac)-737 was a predominant peak while VP3 with 203(ac)-737 was a minor peak.

Conclusions

Applications of LC/MS Intact Protein Analysis and LC/MS/MS Peptide Mapping of AAV VPs in Gene Therapy Research and Development

These results demonstrate that direct LC/MS of different types of AAVs after denaturation was proved to be a simple and effective way to monitor the protein sequence and post-translational modifications with accurate mass measurement in the intact protein level. N-termini of VP1, VP2 and VP3 of AAVs were confirmed by mass spectrometry. Acetylations of N-termini of VP1 and VP3 were also identified in different serotypes of AAVs. Direct LC/MS/MS peptide mapping of AAVs was developed, provided 100% sequence coverage of VP1, VP2 and VP3, and confirmed the N-termini acetylation of VPs. The theoretical masses of predicted sequences of 13 AAV serotypes based on sequence alignment and intact protein analysis of several AAV serotypes are shown in Table 3.

TABLE 3

Predicted Sequences and Masses

	Predicted VP1 sequence	Mass(Da)	Predicted VP2 sequence	Mass(Da)	Predicted VP3 sequence	Mass(Da)
AAV1	2(ac)-736	81286	139-736	66093	204(ac)-736	59517
AAV2	2(ac)-735	81856	139-735	66488	204(ac)-735	59974
AAV3	2(ac)-736	81571	139-736	66319	204(ac)-736	59849
AAV4	2(ac)-734	80550	138-734	65626	198(ac)-734	59529
AAV5	2(ac)-724	80336	138-724	65283	194(ac)-724	59463
AAV6	2(ac)-736	81322	139-736	66096	204(ac)-736	59519
AAV7	2(ac)-737	81564	139-737	66372	213(ac)-737	59101
AAV8	2(ac)-738	81667	139-738	66519	205(ac)-738	59805
AAV9	2(ac)-736	81291	139-736	66210	204(ac)-736	59733
AAV10	2(ac)-738	81477	139-738	66271	205(ac)-738	59638
AAV11	2(ac)-733	80987	139-733	65794	198(ac)-733	59696
AAV12	2(ac)-742	82106	139-742	66905	207(ac)-742	59846
AAVRh10	2(ac)-738	81455	139-738	66253	205(ac)-738	59634

The accurate masses of VP1, VP2 and VP3 of each serotype are unique and therefore intact protein analysis can be used as an identity test to differentiate AAV capsid serotypes. Tables 4-6 show the mass differences of VPs among 13 common AAV serotypes. Shown in regular font are delta masses larger than 10, with delta masses less than 10 bolded.

TABLE 4

Mass Differences of VP1 Among 13 AAV Isotypes

	AAV1	AAV2	AAV3	AAV4	AAV5	AAV6	AAV7	AAV8	AAV9	AAV10	AAV11	AAV12
AAV2	570											
AAV3	285	285										
AAV4	736	1306	1021									
AAV5	950	1520	1235	215								
AAV6	36	534	249	772	987							
AAV7	277	292	**8**	1013	1228	241						
AAV8	381	189	96	1117	1332	345	104					
AAV9	**5**	585	280	741	955	31	272	376				
AAV10	191	379	94	927	1142	155	86	190	186			
AAV11	299	869	584	436	651	335	577	681	304	490		
AAV12	820	250	535	1555	1770	784	542	439	815	629	1119	
AAVRh10	169	401	116	905	1119	133	109	212	164	22	468	651

TABLE 5

Mass Differences of VP2 among 13 AAV Isotypes

	AAV1	AAV2	AAV3	AAV4	AAV5	AAV6	AAV7	AAV8	AAV9	AAV10	AAV11	AAV12
AAV2	395											
AAV3	226	169										
AAV4	467	862	693									
AAV5	810	1205	1036	343								
AAV6	**2**	392	224	470	812							
AAV7	278	116	52	746	1088	276						
AAV8	425	31	199	893	1235	423	147					
AAV9	117	278	109	584	927	115	161	308				
AAV10	177	217	49	645	987	175	101	248	60			
AAV11	299	694	525	168	511	301	578	725	416	476		
AAV12	812	417	586	1279	1622	810	533	386	695	635	1111	
AAVRh10	160	235	66	627	970	157	119	266	43	18	459	652

TABLE 6

Mass Differences of VP3 among 13 AAV Isotypes

	AAV1	AAV2	AAV3	AAV4	AAV5	AAV6	AAV7	AAV8	AAV9	AAV10	AAV11	AAV12
AAV2	457											
AAV3	332	125										
AAV4	12	445	320									
AAV5	54	511	386	66								
AAV6	**2**	455	330	**10**	56							
AAV7	416	673	748	428	362	418						
AAV8	288	169	44	276	342	286	704					
AAV9	216	241	116	204	270	214	632	72				
AAV10	121	336	211	109	175	119	537	167	95			
AAV11	179	278	153	167	233	177	595	109	37	58		
AAV12	329	128	**3**	317	383	327	745	41	113	208	150	
AAVRh10	117	340	215	105	171	115	533	171	99	**4**	62	212

No masses within 10 Da of all three VPs between two isotypes are observed. Even though both VP2 and VP3 have only a 2 Da difference between AAV1 and AAV6, the mass difference of VP1 between AAV1 and AAV6 is 36, significant enough to be distinguished by an accurate mass measurement. Therefore, intact protein measurement of VP1, VP2 and VP3 is highly specific as an identity test.

These results demonstrate that intact protein analysis and LC/MS/MS can be used to profile VPs to monitor VP expressions, posttranslational modifications, and truncations and to ensure product consistency during VLP production. These two analyses can also be used to confirm site-direct mutagenesis or structural characterization for capsid protein engineering applications.

Example 2: The Role of N Terminal Acetylation of AAV Capsid Proteins

Chemical modifications of cellular proteins are a common means of controlling their functions (Arnesen, T. (2006) *Virology* 353(2): 283-293). N-terminal acetylation (Nt-acetylation), which involves the transfer of an acetyl group from acetyl coenzyme A to the α-amino group of the first amino acid residue of a protein (Brown, J. L. and Roberts, W. K. (1976) *J Biol Chem* 251: 1009-1014; Arnesen, T. et al. (2009) *Proc Natl Acad Sci USA* 106: 8157-8162), is among the most abundant of protein modifications. Unlike most other protein modifications, Nt-acetylation is irreversible; it occurs mainly during the synthesis of the protein, catalyzed by N-terminal acetyltransferases (NATs) associated with ribosomes (Gautschi, M. et al. (2003) *Mol Cell Biol* 23: 7403-7414; Pestana, A. and Pitot, H. C. (1975) *Biochemistry* 14: 1404-1412; Polevoda, B. et al. (2003) *J Biol Chem* 278: 30686-97). There are several distinct NATs in eukaryotes—NatA-NatF—each composed of one or more subunits and each acetylating a specific subgroup of N-termini depending on the amino acid sequence of the first few amino acids (Jornvall, H. (1975) *J Theor Biol* 55: 1-12; Persson, B. et al. (1985) *Eur J Biochem* 152: 523-527).

Experimental data indicate that proteins with acetylated N-termini are more stable in vivo than non-acetylated proteins; i.e., Nt-acetylation protects proteins from degradation (Hershko, A. et al. (1984) *Proc Natl Acad Sci USA* 81: 7021-7025). One explanation for this might be the discovery in 2004 that another N-terminal modification, ubiquitination, which involves direct attachment of the small protein ubiquitin to the N-terminal amino acid residue, promotes the subsequent degradation of the protein (Ben Saadon, R. et al. (2004) *J Biol Chem* 279: 41414-41421). Conversely, the Nt-acetylation signals can also be part of a quality control mechanism to degrade unfolded or misfolded proteins and to regulate in vivo protein stoichiometries (Hwang, C. S. et al. (2010) *Science* 327: 973-977).

A systematic analysis of the predicted N-terminal processing of cytosolic proteins, versus those destined to be sorted to the secretory pathway, revealed that the cytosolic proteins were profoundly biased in favor of processing, but there is an equal and opposite bias against such modification for secretory proteins (Forte, G. M. A. et al. (2011) *PLoS Biology*, 4 May 2011 Volume 9). Mutations in secretory signal sequences that lead to their acetylation result in mis-sorting to the cytosol in a manner that is dependent upon the N-terminal processing machinery. Hence N-terminal acetylation represents an early determining step in the cellular sorting of nascent polypeptides that represent an extra layer of stringency in order to ensure that proteins destined to remain in the cytosol actually reside in the cytosol. The eukaryotic cell comprises several distinct compartments, called organelles, required to perform specific functions. The proteins in these compartments are synthesized in the cytoplasm and so require complex sorting mechanisms to ensure their delivery to the appropriate organelle. Proteins are modified by acetylation of their amino terminus at a very early stage in their synthesis. There is a profound difference between the likelihood of such a modification on cytoplasmic proteins and on those destined for one of the major organelles, the endoplasmic reticulum (ER): whereas cytoplasmic proteins are typically acetylated, those bound for the ER are largely unmodified. Moreover, when specific ER proteins are engineered to induce their acetylation their targeting to the ER was inhibited (Forte, G. M. A. et al. (2011) *PLoS Biology*, 4 May 2011 Volume 9).

The contractile proteins actin and tropomyosin have been shown to require NatB-mediated Nt-acetylation for proper function, specifically involving actin-tropomyosin binding and actomyosin regulation (Coulton, A. T. et al. (2010) *J Cell Sci* 123: 3235-3243; Polevoda, B. et al. (2003) *J Biol Chem* 278: 30686-97). Thus Nt acetylation of AAV capsid proteins may have importance in the transduction potential of rAAV vectors. If AAV vectors fail to gain entry into the nucleus, they consequently fail to transduce cells. The role of actin filaments and FKBP52 (FK506-binding protein p52) in the translocation of AAV capsids from the endosome (o the nucleus is well defined (Zhao, W. et al. (2006) *Virology* 353(2): 283-293). Importantly, Nt-acetylation is essential for the functioning of actin filaments by modulating protein-protein interactions (Coulton, A. T. et al. (2010) *J Cell Sci* 123: 3235-3243; Polevoda, B. et al. (2003) *J Biol Chem* 278: 30686-97).

Though N-terminal acetylation of proteins is a widely known phenomenon, the biological significance of Nt-acetylation on AAV capsid proteins is not well understood. The predicted N-termini of VP1 and VP3 based on DNA sequencing are both methionine followed by alanine. It has been reported that removal of N-terminal methionine by Met-aminopeptidases frequently leads to Nt-acetylation of the resulting N-terminal alanine, valine, serine, threonine, and cysteine residues and that the acetylation of the N-terminus acts as a potential degradation signal [21]. Ubiquitination of viral capsid proteins was suggested as a potential signal for processing of the capsid at the time of virion disassembly [22]. The link between N-acetylation of VP1 and VP3 and viral capsid degradation and uncoating before the nuclear entry is further investigated.

To understand the functional implications of N-terminal acetylation with regard to AAV capsid proteins, site-directed mutagenesis of VP3 N-terminal initiation codons is used to generate AAV mutants.

Methods

AAV capsid proteins are generated with differing amino acids at the 2nd position to the initiating methionine (iMet X) to determine if Nt-acetylation is inhibited or reduced, and the functional consequences are then measured. The ability of the capsid proteins to be trafficked intra-cellularly and/or to acquire post translational modifications such as glycosylation is assessed, and whether this ability affects the infectivity of the assembled AAV particle is subsequently determined. In addition, the impact of acetylation on ubiquitination/degradation and targeting to the lysosome, ER, Golgi, or inner nuclear membrane is determined.

For example, to assay trafficking or targeting, AAV particles with capsid proteins having a mutated 2nd position (e.g., iMet X) are fluorescently labeled and used to infect cells (e.g., HeLa cells). These AAV particles are assayed for one or more of: time of viral particle uptake, colocalization of AAV particles with specific compartmental markers (e.g., Golgi, ER, or lysosomal proteins or other markers), nuclear accumulation (e.g., as assayed by colocalization with a nuclear marker or stain), and/or sensitivity of trafficking to specific inhibitors of early endosomal escape (such as bafilomycin A or ammonium chloride), as compared to fluorescently labeled wild-type AAV particles used to infect the same cell line (see, e.g., Bartlett, J. S. et al. (2000) *J. Virol.* 74:2777-2785 for a description of such assays).

To assay infectivity, AAV particles with capsid proteins having a mutated 2nd position (e.g., iMet X) are used to infect cells (e.g., HeLa cells), and their transduction efficiency is compared to wild-type AAV particles (e.g., having the same AAV serotype and infecting the same type of cells).

To assay glycosylation, AAV particles with capsid proteins having a mutated 2nd position (e.g., iMet X) are used to infect cells (e.g., HeLa cells). AAV particles from infected cells are subjected to one or more assays including without limitation chemical detection of glycosylation (e.g., applying a commercially available digoxigenin (DIG) glycan detection and/or fluorescent glycoprotein detection kit on denatured and electrophoretically separated capsid proteins) and mass spectrometry (e.g., FT-ICR MS), as compared to wild-type AAV particles used to infect the same cell line (see, e.g., Murray, S. et al. (2006) *J. Virol.* 80:6171-6176 for a description of such assays).

To assay ubiquitination, AAV particles with capsid proteins having a mutated 2nd position are used to infect cells (e.g., HeLa cells). AAV particles are immunoprecipitated from infected cells with an anti-capsid antibody, then subjected to Western blotting with an anti-ubiquitin antibody and compared to wild-type AAV particles used to infect cells in the same manner. Mutant AAV particles may also be used in in vitro ubiquitination assays, as compared to wild-type AAV particles (see, e.g., Yan, Z. et al. (2002) *J. Virol.* 76:2043-2053).

Example 3: The Role of Deamidation of AAV2 Capsid Proteins

Sequence analysis of the AAV2 capsid protein revealed potential deamidation sites, as underlined in the following amino acid sequence:

(SEQ ID NO: 3)

MAADGYLPDWLEDTLSEGIRQWWKLKPGPPPPKPAERHKDDSRGLVLPG

YKLGPFNGLDKGEPVNEADAAALEHDKAYIRQLDSGDN

PYLKYNHADA EPQERLKEDTSFGGNLGRAVFQAKKRVLEPLGLVEEPV

KTAPGKKRPVEHSPVEPDSSSGTGKAGQQPARKRLNFGQTGDADSVPDP

QPLGQPPAAPSGLGTNTMATGSGAPMADNNEGADGVGNSSGNWHCDSTW

MGDRVITTSTRTWALPTYNNHLYKQISSQSGASNDNHYFGYSTPWGYFD

FNRFHCHFSPRDWQRLINNNWGFRPKRLNFKLFNIQVKEVTQNDGTTTI

ANNLTSTVQVFTDSEYQLPYVLGSAHQGCLPPFPADVFMVPQYGYLTLN

NGSQAVGRSSFYCLEYFPSQMLRTGNNFTFSYTFEDVPFHSSYAHSQSL

DRLMNPLIDQYLYYLSRTNTPSGTTTQSRLQFSQAGASDIRDQSRNWLP

GPCYRQQRVSKTSADNNNSEYSWTGATKYHLNGRDSLVNPGPAMASHKD

DEEKFFPQSGVLIFGKQGSEKTNVDIEKVMITDEEEIRTTNPVATEQYG

SVSTNLQRGNRQAATADVNTQGVLPGMVWQDRDVYLQGPIWAKIPHTDG

HFHPSPLMGGFGLKHPPPQILIKNTPVPANPSTTFSAAKFASFITQYST

GQVSVEIEWELQKENSKRWNPEIQYTSNYNKSVNVDFTVDTNGVYSEPR

PIGTRYLTRNL

In particular, a potential deamidation site is found at N57/G58 in the phospholipase A2 domain (Ca++ binding site), as bolded and italicized in the above sequence. The following experiments were aimed at exploring whether deamidation at N57 can lead to reduced potency and/or truncation of AAV2, as well as whether different AAV production methods may have different effects on deamidation. For example, the producer cell line method (see Martin et al., (2013) *Human Gene Therapy Methods* 24:253-269; U.S. PG Pub. No. US2004/0224411; and Liu, X. L. et al. (1999) *Gene Ther.* 6:293-299) may induce a higher level of deamidation at N57, as compared to the triple transfection method. According to the crystal structure of AAV2, N57 is not shown; however, N382 and N511 are partially exposed, and N715 is fully exposed.

Methods

Enzymatic Digestions of AAV1 and AAV2 VPs

10 μg of each AAV1-EGFP or AAV2-EGFP material (generated from triple transfection as well as producer cell line process) were concentrated using Amicon filters (10 kDa MWCO), denatured with 6 M Guanidine-HCl, 50 mM Tris at pH 8.5. The proteins were reduced with 5 mM DTT at 60° C. for 30 minutes in darkness, alkylated with 15 mM iodoacetamide at room temperature for 30 minutes, and then buffer exchanged into 25 mM Tris pH 7.1 for digestion using Bio-Spin® 6 Tris micro-columns. After buffer exchange, the

samples were split into two aliquots. Each aliquot was digested with trypsin at 1:25 or Asp-N at 1:50 enzyme: protein ratio (wt/wt) for 2 hours at 37° C., respectively.

UPLC/MS/MS Peptide Mapping

The protein digests were also analyzed by UPLC/MS/MS in Acquity UPLC-Xevo qTOF MS. BEH300 C18 column (2.1×150 mm) was used for separation in the mobile phases with 0.1% formic acid in water/acetontitrile gradient at a flow rate 0.25 ml/min. The mass spectra were acquired in the positive MSe resolution mode in the mass range of 50-2000.

Determination of Deamidation Levels in AAV VPs

The extracted ion chromatograms (XIC) of peptides containing NG sites (T9, T49, and T67 in AA1 and AAV2 VP) and their corresponding deamidated species were used for calculation of deamidation levels.

In order to compare AAV vectors produced by the triple transfection (TTx) and producer cell line (PCL) methods, AAV1 or AAV2 tagged with EGFP was produced using the TTx or PCL method. Truncated VP1 (tVP1) was found to be present in AAV2-EGFP produced by PCL, but not in the AAV2-EGFP produced by TTx. AAV1-EGFP was not found to have tVP1, regardless of the production method. The in vitro potency of AAV2 produced by the PCL method was also found to be reduced, as compared to AAV2 produced by TTx. Mutant N57K and N57Q AAV2 particles were also found to have reduced potency and disrupted Ca++ binding.

The following table provides the tryptic peptides that were analyzed to examine each potential deamidation site, as well as the corresponding residue.

TABLE 7

Tryptic peptides containing NG sites

Peptide (NG sequence underlined)	Residue
YLGPF**NG**LDK (SEQ ID NO: 9)	N57
EVTQNDGTTTIANNLTSTVQVFTDSEYQLPYVLGSAHQGCLPPFPADVFMV PQYGYLTLN**NG**SQAVGRSSFYCLEYFPSQMLR (SEQ ID NO: 10)	N382
YNL**NG**R (AAV1) (SEQ ID NO: 11)	N511
YHL**NG**R (AAV2) (SEQ ID NO: 12)	N511
SANVDFTVDN**NG**LYTEPR (AAV1) (SEQ ID NO: 13)	N715
SVNVDFTVDT**NG**VYSEPR (AAV2) (SEQ ID NO: 14)	N715

As shown in Table 7, the T9 peptide YLGPF **NG** LDK (SEQ ID NO: 9) was used to monitor N57, the T38 peptide EVTQNDGTTTIANNLTSTVQVFTDSEYQLPYVLGSA-HQGCLPPFPADVFMVPQYGYLTL N **NG** SQAVGRSS-FYCLEYFPSQMLR (SEQ ID NO: 10) was used to monitor N382, the T49 peptides YNL **NG** R (SEQ ID NO: 11) and YHL **NG** R (SEQ ID NO: 12) were used to monitor N511 in AAV1 or AAV2 (respectively), and the T67 peptides SANVDFTVDN **NG** LYTEPR (SEQ ID NO: 13) and SVNVDFTVDT **NG** VYSEPR (SEQ ID NO: 14) were used to monitor N715 in AAV1 or AAV2 (respectively).

LC/MS/MS analysis was used to compare the percentage of deamidation in AAV1 and AAV2 particles produced by the TTx and PCL methods. The results from the T9 peptide are shown in FIGS. **6**A & **6**B. The results from the T49 peptide are shown in FIGS. **7**A & **7**B. The results from the T67 peptide are shown in FIGS. **8**A & **8**B. These results are summarized in Table 8. The T38 peptide was not detected due to its size.

TABLE 8

Summary of LC/MS/MS results

		% Deamidation		
		N57	N511	N715
AAV1	TTx	7.9	30.9	18.1
	PCL	11.3	27.4	18.7
AAV2	TTx	6.7	39.6	27.4
	PCL	18.4	42.3	28.0

In particular, AAV2 produced by PCL showed nearly a 3-fold increase in deamidation as compared to AAV2 produced by TTx. These results suggest that deamidation decreases AAV potency, as the in vitro potency of AAV2 produced by PCL is reduced.

Conclusions

Taken together, Examples 1-3 demonstrate methods for analyzing intact proteins of viral particles (e.g., AAV capsid proteins) using LC/MS. Molecular weights were measured accurately, and these techniques may be also used to assess N-termini and/or modifications of viral capsid proteins. Moreover, these methods are adaptable as capsid serotype identity assays useful in gene therapy, e.g., as an analytical platform. These results further establish a correlation between capsid protein structure (e.g., truncations, deamidation, etc.) and potency, suggesting that point mutations at key sites may be used to engineer more effective vectors.

Example 4: Elucidating the Role of N Terminal Acetylation of AAV Capsid Proteins

As discussed above, the N-termini of AAV capsid proteins are highly conserved across serotypes (FIG. **5**). The techniques described in Example 1 allow for interrogation of VP expression and posttranslational modifications. The role and biological significance of N-terminal acetylation of AAV capsid proteins was next examined.

Results

To elucidate the potential role of deacetylation of AAV capsid proteins, AAV5 deacetylation variants were tested. An AAV5 particle expressing eGFP under the CBA promoter (AAV5-CBA-Egfp) was compared to AAV5 variants with the amino acid adjacent to the initiating methionine (iMET) mutated for VP1 and VP3 (deAC-AAV5-CBA-eGFPs). Three amino acids predicted to have a low likelihood of acetylation by NatA, NatC, or NatD were chosen for generating variants: Gly, Leu, and Pro, as illustrated in Table 9 below.

TABLE 9

N-terminal acetylation frequency

N-term aa	Transferase	NT-AC FREQUENCY
MET-ALA Normally found in VP1 & VP3	NatA	High
MET-SER Normally found in	NatA	High

TABLE 9-continued

N-terminal acetylation frequency

N-term aa	Transferase	NT-AC FREQUENCY
VP1 & VP3 for AAV5 AAV variants		
MET-GLY	NatA	Low
MET-LEU	NatC	Low
MET-PRO	NatD/other	Low

The following AAV5 deacetylated (deAC) mutants were generated:

S2GVP1—Ser changed to Gly at position 2 in AAV5VP1

S2LVP1—Ser changed to Leu at position 2 in AAV5VP1

S2PVP1—Ser changed to Pro at position 2 in AAV5VP1

S2GVP3—Ser changed to Gly at position 2 in AAV5VP3

S2LVP3—Ser changed to Leu at position 2 in AAV5VP3

S2PVP3—Ser changed to Pro at position 2 in AAV5VP3

S2PVP1/VP3—Ser changed to Pro at position 2 in both AAV5 VP1 and VP3

S2GVP1/VP3—Ser changed to Gly at position 2 in both AAV5 VP1 and VP3

S2LVP1/VP3—Ser changed to Leu at position 2 in both AAV5 VP1 and VP3

These variants were generated using the TTX method as described above. All AAV5 variants showed good productivity, with yields greater than 10^{13} total VG. All AAV5 variants also showed the expected VP1:VP2:VP3 protein ratio by SYPRO protein gel analysis (FIG. **9**). Next, LC/MS was used to confirm that all AAV5 variants had decreased acetylation, as shown in Table 10.

TABLE 10

LC/MS analysis of AAV5 variant acetylation

	mutants	VP1 Theo.	VP1 Exp.	Δmass (VP1)	VP2 Theo.	VP2 Exp.	Δmass (VP2)	VP3 Theo.	VP3 Exp.	Δmass (VP3)	note
1	deAC-AAV5 (S2GVP1)/ CBA-eGFP	80234	nd		65283	65293	10	59463	59472	9	VP1 not detectable
2	deAC-AAV5 (S2LVP1)/ CBA-eGFP	80346	80501	181	65283	65292	9	59463	59471	8	VP1 incorrect
3	deAC-AAV5 (S2GVP3)/ CBA-eGFP	80234	nd		65253	65261	8	59391	59398	7	confirmed
4	deAC-AAV5 (S2LVP3)/ CBA-eGFP	80336	80363	27	65309	65309	0	59447	59620	173	VP3 incorrect
5	deAC-AAV5 (S2PVP1VP3)/ CBA-eGFP	80314	80324	10	65293	65300	7	59431	59438	7	confirmed
6	deAC-AAV5 (S2GVP1VP3)/ CBA-eGFP	80234	80243	9	65253	65261	8	59391	59398	7	confirmed
7	deAC-AAV5 (S2PVP3)/ CBA-eGFP	80336	80346	10	65293	65292	1	59431	59430	1	confirmed
8	deAC-AAV5 (S2PVP1)/ CBA-eGFP	80314	80313	1	65283	65291	8	59463	59470	7	confirmed
9	deAC-AAV5 (S2L VP1VP3)/ CBA-eGFP	80346	nd		65309	65318	9	59447	59629	182	VP3 incorrect

nd = not determined

These LC/MS analyses confirmed that AAV5 variants were deacetylated. The variants S2LVP1, S2LVP3, and S2LVP1/VP3 all showed increased mass (increased from 173 to 182) in VP1 and VP3 proteins, suggesting that changing the second N-terminal amino acid to a leucine in VP1 or VP3 alters the protein, resulting in an increase in mass.

Next, AAV5 variants were assayed in an in vitro transduction assay using eGFP as a reporter gene (FIG. **10**). The assay was designed to evaluate transduction by AAV5 deacetylated mutant variants at 10^6 multiplicity of infection (MOI), comparing each variant to the parental, unmodified AAV5 particle. Three cell lines were used: 293, HuH7, and HeLa cells. Following infection, cells were assayed to determine vector genome copy number (vg/μg cellular protein) and eGFP expression (by ELISA). Vector genome copy number (vg/μg protein) represents the efficiency at which the AAV5 variant enters the cell, and eGFP represents the efficiency of capsid intracellular trafficking, since transgene expression requires the capsid/vector DNA to efficiently traffic to the nucleus (FIG. **10**). Vector genomes were quantified by TaqMan analysis.

FIG. **11** shows that, based on vector genome analyses, AAV5 deacetylated mutant vectors infected all three test cell

lines at similar, but reduced, levels as compared to the parental unmodified AAV5 particles. FIG. **12** shows that AAV5 deacetylated mutant vectors all resulted in reduced eGFP expression in all three cell lines, as compared to transduction with parental unmodified AAV5.

Conclusions

As predicted, no acetylation was observed in N-terminal Ser to Pro/Leu/Gly mutant variants when examined by LC/MS. AAV5 deAC variants showed robust vector production, and AAV5 deAC variants infected cells at levels comparable to parental AAV5. However, functional protein levels in cells infected with deAC variants were greatly reduced when compared to the parental AAV5. These data suggest that tropism is minimally affected by a lack of N-terminal deacetylation in VP1/VP3, but downstream processing (e.g., trafficking and/or degradation) is significantly affected. Since the variants tested demonstrated reduced in vitro activity, one of skill in the art may appreciate that variants characterized by reduced or eliminated acetylation could be employed, inter alia, when decreased levels of transduction are desirable.

Example 5: Assessment of Deamidation of AAV Capsid Proteins

Examples 1 and 3 demonstrate techniques that allow the interrogation of post-translational modifications of AAV capsid proteins and explore the role of deamidation of the AAV2 capsid. The following Example tested whether deamidation reduces potency and/or induces truncation of capsid proteins, and whether different manufacturing processes can induce different levels of deamidation.

Methods

AAV particles were generated and deamidation status assayed as described in Example 3.

Results

As described in Example 3, a potential deamidation site is found at N57/G58 in the phospholipase A2 domain (Ca++ binding site) in VP1 of the AAV2 capsid. The N57/G58 motif is conserved across AAV serotypes (FIG. **13**). Example 3 showed that AAV2 produced by PCL exhibited nearly a 3-fold increase in deamidation as compared to AAV2 produced by TTx (see FIGS. **6**A & **6**B and Table 8).

In examining VP1, VP2, and VP3 production by protein gels, a truncated VP1 protein (tVP1) was detected only in AAV2 capsid proteins produced by the PCL method (FIG. **14**).

A series of AAV2 deamidation mutants was generated next. These mutants targeted the Gly residue in the canonical NG sequence. Mutations targeting the A35 residue (see FIG. **13**), the N-terminal amino acid for tVP1 were also generated, as shown in Table 11. The pAF277 and pAF279 mutants bearing multiple mutations did not package.

TABLE 11

Deamidation mutants

Name	mutation	avg drp/cell
pAF274	G58K	4.54E+03
pAF275	G58D	5.00E+03
pAF276	G58Q	5.41E+03
pAF277	G58, 383, 512, 716K	1.2
pAF278	A35N	6.89E+03
pAF279	A35N, G58, 383, 512, 765K	2.2
293	—	0.9
PIM45	Control	6.28E+03

K = positive charge (basic)
D = negative charge (acidic)
Q = polar

Deamidation of variants were next analyzed by LC/MS as described in Example 3 above. The AAV2A35N and AAV2G58D variants had altered deamidation as compared to the parental AAV2 (FIG. **15**). In particular, the AAV2A35N mutant had increased deamidation (17.8%) as compared to parental AAV2 (5.7%). The AAV2G58D variant had reduced deamidation (1.1%) as compared to parental AAV2. SYPRO protein gel analysis demonstrated that the AAV2 deamidation mutants exhibited the correct VP1:VP2:VP2 ratio (FIG. **16**).

Next, AAV2 deamidation variants were assayed in an in vitro transduction assay using eGFP as a reporter gene (FIG. **17**). The assay was designed to evaluate transduction by AAV2 deamidation mutant variants at 10^6 multiplicity of infection (MOI), comparing each variant to the parental, unmodified AAV2 particle. Three cell lines were used: 293, HuH7, and HeLa cells. Following infection, cells were assayed to determine vector genome copy number (vg/μg cellular protein) and eGFP expression (by ELISA). Vector genome copy number (vg/μg protein) represents the efficiency at which the AAV2 variant enters the cell, and eGFP represents the efficiency of capsid intracellular trafficking, since transgene expression requires the capsid/vector DNA to efficiently traffic to the nucleus (FIG. **17**). Vector genomes were quantified by TaqMan analysis.

Vector genome analysis indicated that AAV2 deamidation mutant variants infected all cell lines tested at levels comparable to that of parental AAV2 vectors (FIG. **18**). Importantly, the AAV2A35N variant was found to be more potent than the parental AAV2 vector for transduction in all three cell lines (FIG. **19**). The AAV2G58D variant was found to be more potent than the parental AAV2 vector in HuH7 cells (FIG. **19**).

Conclusions

In summary, AAV2 deamidation mutant vectors infect cells at levels comparable to the parent AAV2 particles (e.g., comparable vg/μg cellular protein). However, based on analysis of eGFP levels in transduced cells, the AAV2A35N variant had higher potency than the parental AAV2 in all cell lines tested, and the AAV2G58D variant had higher potency than the parental AAV2 in HuH7 cells (a liver-derived cell line). These results suggest that the A35N mutation may be effective in increasing vector potency for transducing many cell types, and that the G58D mutation may also be effective in increasing potency in certain cell types, e.g., liver cells.

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SEQUENCES

All polypeptide sequences are presented N-terminal to C-terminal unless otherwise noted.

All nucleic sequences are presented 5' to 3' unless otherwise noted.

Nucleotide sequence of potential AAV2 VP3 initiation codons (ATG codons underlined)
(SEQ ID NO: 1)
ATGGCTACAGGCAGTGGCGCACCAATGGCAGAC

Polypeptide sequence corresponding to potential AAV2 VP3 initiation codons (methionines underlined)
(SEQ ID NO: 2)
MATGSGAPMAD

AAV2 VP1 polypeptide sequence
(SEQ ID NO: 3)
MAADGYLPDWLEDTLSEGIRQWWKLKPGPPPPKPAERHKDDSRGLVLPG
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GLKHPPPQILIKNTPVPANPSTTFSAAKFASFITQYSTGQVSVEIEWEL
QKENSKRWNPEIQYTSNYNKSVNVDFTVDINGVYSEPRPIGTRYLTRNL
VP1 N-terminal tryptic peptide (N-terminal alanine is acetylated)
(SEQ ID NO: 4)
AADGYLPDWLEDTLSEGIR

VP3 N-terminal Asp-N peptide (N-terminal alanine is acetylated)
(SEQ ID NO: 5)
ATGSGAPM

Common VP1 N-terminal sequence
(SEQ ID NO: 6)
MAADGYLPDWLED

Nucleotide sequence of potential AAV7 VP3 initiation codons (start codons underlined)
(SEQ ID NO: 7)
GTGGCTGCAGGCGGTGGCGCACCAATGGCAGACAATAAC

Nucleotide sequence of mutated ITR
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SEQUENCE LISTING

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KGEPVNAADA AALEHDKAYD QQLQAGDNPY LRYNHADAEF QERLQEDTSF GGNLGRAVFQ  120
AKKRVLEPLG LVEEGAKTAP GKKRPVEPSP QRSPDSSTGI GKKGQQPARK RLNFGQTGDS  180
ESVPDPQPLG EPPAAPSGVG PNTMAAGGGA PMADNNEGAD GVGSSSGNWH CDSTWLGDR   239

SEQ ID NO: 20          moltype = AA  length = 239
FEATURE                Location/Qualifiers
REGION                 1..239
                       note = Synthetic Construct
source                 1..239
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                       organism = synthetic construct
SEQUENCE: 20
MAADGYLPDW LEDNLSEGIR EWWDLKPGAP KPKANQQKQD NGRGLVLPGY KYLGPFNGLD  60
KGEPVNAADA AALEHDKAYD QQLKAGDNPY LRYNHADAEF QERLQEDTSF GGNLGRAVFQ  120
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AKKRVLEPLG LVEEGAKTAP AKKRPVEPSP QRSPDSSTGI GKKGQQPARK RLNFGQTGDS  180
ESVPDPQPLG EPPAAPSSVG SGTVAAGGGA PMADNNEGAD GVGNASGNWH CDSTWLGDR   239

SEQ ID NO: 21          moltype = AA  length = 238
FEATURE                Location/Qualifiers
REGION                 1..238
                       note = Synthetic Construct
source                 1..238
                       mol_type = protein
                       organism = synthetic construct
SEQUENCE: 21
MAADGYLPDW LEDNLSEGIR EWWDLKPGAP KPKANQQKQD DGRGLVLPGY KYLGPFNGLD  60
KGEPVNAADA AALEHDKAYD QQLKAGDNPY LRYNHADAEF QERLQEDTSF GGNLGRAVFQ  120
AKKRVLEPLG LVEEGAKTAP GKKRPVEQSP QEPDSSSGIG KTGQQPAKKR LNFGQTGDSE  180
SVPDPQPLGE PPATPAAVGP TTMASGGGAP MADNNEGADG VGNASGNWHC DSTWLGDR    238

SEQ ID NO: 22          moltype = AA  length = 238
FEATURE                Location/Qualifiers
REGION                 1..238
                       note = Synthetic Construct
source                 1..238
                       mol_type = protein
                       organism = synthetic construct
SEQUENCE: 22
MAADGYLPDW LEDNLSEGIR EWWDLKPGAP KPKANQQKQD DGRGLVLPGY KYLGPFNGLD  60
KGEPVNAADA AALEHDKAYD QQLKAGDNPY LRYNHADAEF QERLQEDTSF GGNLGRAVFQ  120
AKKRVLEPFG LVEEGAKTAP GKKRPVEQSP QEPDSSSGIG KTGQQPAKKR LNFGQTGDSE  180
SVPDPQPLGE PPATPAAVGP TTMASGGGAP MADNNEGADG VGNASGNWHC DSTWLGDR    238

SEQ ID NO: 23          moltype = AA  length = 238
FEATURE                Location/Qualifiers
REGION                 1..238
                       note = Synthetic Construct
source                 1..238
                       mol_type = protein
                       organism = synthetic construct
SEQUENCE: 23
MAADGYLPDW LEDTLSEGIR QWWKLKPGPP PPKPAERHKD DSRGLVLPGY KYLGPFNGLD  60
KGEPVNEADA AALEHDKAYD RQLDSGDNPY LKYNHADAEF QERLKEDTSF GGNLGRAVFQ  120
AKKRVLEPLG LVEEPVKTAP GKKRPVEHSP VEPDSSSGTG KAGQQPARKR LNFGQTGDAD  180
SVPDPQPLGQ PPAAPSGLGT NTMATGSGAP MADNNEGADG VGNSSGNWHC DSTWMGDR    238

SEQ ID NO: 24          moltype = AA  length = 238
FEATURE                Location/Qualifiers
REGION                 1..238
                       note = Synthetic Construct
source                 1..238
                       mol_type = protein
                       organism = synthetic construct
SEQUENCE: 24
MAADGYLPDW LEDNLSEGIR EWWALKPGVP QPKANQQHQD NRRGLVLPGY KYLGPGNGLD  60
KGEPVNEADA AALEHDKAYD QQLKAGDNPY LKYNHADAEF QERLQEDTSF GGNLGRAVFQ  120
AKKRILEPLG LVEEAAKTAP GKKGAVDQSP QEPDSSSGVG KSGKQPARKR LNFGQTGDSE  180
SVPDPQPLGE PPAAPTSLGS NTMASGGGAP MADNNEGADG VGNSSGNWHC DSQWLGDR    238

SEQ ID NO: 25          moltype = AA  length = 232
FEATURE                Location/Qualifiers
REGION                 1..232
                       note = Synthetic Construct
source                 1..232
                       mol_type = protein
                       organism = synthetic construct
SEQUENCE: 25
MAADGYLPDW LEDNLSEGIR EWWDLKPGAP KPKANQQKQD DGRGLVLPGY KYLGPFNGLD  60
KGEPVNAADA AALEHDKAYD QQLKAGDNPY LRYNHADAEF QERLQEDTSF GGNLGRAVFQ  120
AKKRVLEPLG LVEEGAKTAP GKKRPLESPQ EPDSSSGIGK KGKQPARKRL NFEEDTGAGD  180
GPPEGSDTSA MSSDIEMRAA PGGNAVDAGQ GSDGVGNASG DWHCDSTWSE GK          232

SEQ ID NO: 26          moltype = AA  length = 241
FEATURE                Location/Qualifiers
REGION                 1..241
                       note = Synthetic Construct
source                 1..241
                       mol_type = protein
                       organism = synthetic construct
SEQUENCE: 26
MAADGYLPDW LEDNLSEGIR EWWALKPGAP QPKANQQHQD NGRGLVLPGY KYLGPFNGLD  60
KGEPVNEADA AALEHDKAYD KQLEQGDNPY LKYNHADAEF QQRLATDTSF GGNLGRAVFQ  120
AKKRILEPLG LVEEGVKTAP GKKRPLEKTP NRPTNPDSGK APAKKKQKDG EPADSARRTL  180
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DPEDSGAGDG PPEGSSSGEM SHDAEMRAAP GGNAVEAGQG ADGVGNASGD WHCDSTWSEG  240
R                                                                  241

SEQ ID NO: 27          moltype = AA  length = 232
FEATURE                Location/Qualifiers
REGION                 1..232
                       note = Synthetic Construct
source                 1..232
                       mol_type = protein
                       organism = synthetic construct
SEQUENCE: 27
MTDGYLPDWL EDNLSEGVRE WWALQPGAPK PKANQQHQDN ARGLVLPGYK YLGPGNGLDK  60
GEPVNAADAA ALEHDKAYDQ QLKAGDNPYL KYNHADAEFQ QRLQGDTSFG GNLGRAVFQA  120
KKRVLEPLGL VEQAGETAPG KKRPLIESPQ QPDSSTGIGK KGKQPAKKKL VFEDETGAGD  180
GPPEGSTSGA MSDDSEMRAA AGGAAVEGGQ GADGVGNASG DWHCDSTWSE GH          232

SEQ ID NO: 28          moltype = AA  length = 228
FEATURE                Location/Qualifiers
REGION                 1..228
                       note = Synthetic Construct
source                 1..228
                       mol_type = protein
                       organism = synthetic construct
SEQUENCE: 28
MSFVDHPPDW LEEVGEGLRE FLGLEAGPPK PKPNQQHQDQ ARGLVLPGYN YLGPGNGLDR  60
GEPVNRADEV AREHDISYNE QLEAGDNPYL KYNHADAEFQ EKLADDTSFG GNLGKAVFQA  120
KKRVLEPFGL VEEGAKTAPT GKRIDDHFPK RKKARTEEDS KPSTSSDAEA GPSGSQQLQI  180
PAQPASSLGA DTMSAGGGGP LGDNNQGADG VGNASGDWHC DSTWMGDR               228

SEQ ID NO: 29          moltype = AA  length = 238
FEATURE                Location/Qualifiers
REGION                 1..238
                       note = Synthetic Construct
source                 1..238
                       mol_type = protein
                       organism = synthetic construct
SEQUENCE: 29
MAADGYLPDW LEDNLSEGIR EWWALKPGAP QPKANQQHQD NARGLVLPGY KYLGPGNGLD  60
KGEPVNAADA AALEHDKAYD QQLKAGDNPY LKYNHADAEF QERLKEDTSF GGNLGRAVPQ  120
AKKRLLEPLG LVEEAAKTAP GKKRPVEQSP QEPDSSAGIG KSGAQPAKKR LNPGQTGDTE  180
SVPDPQPIGE PPAAPSGVGS LTMASGGGAP VADNNEGADG VGSSSGNWHC DSQWLGDR    238

SEQ ID NO: 30          moltype = AA  length = 246
FEATURE                Location/Qualifiers
REGION                 1..246
                       note = Synthetic Construct
source                 1..246
                       mol_type = protein
                       organism = synthetic construct
SEQUENCE: 30
MAADGYLPDW LEDNLSEGIR EWWXLKPGAP KPKANQQKQD DGRGLVLPGY KYLGPFNGLD  60
KGEPVNAADA AALEHDKAYD QQLKAGDNPY LRYNHADAEF QERLQEDTSF GGNLGRAVPQ  120
AKKRVLEPLG LVEEGAKTAP GKKRPVEXSP XXQXXPDSSS GIGKKGQXXX XXQPAKKRLN  180
FGQTGDSESV PDPQPLGEPP AAPSGLGXXT MAAGGGAPMA DNNEGADGVG NASGNWHCDS  240
TWLGDR                                                             246

SEQ ID NO: 31          moltype = AA  length = 61
FEATURE                Location/Qualifiers
REGION                 1..61
                       note = Synthetic Construct
source                 1..61
                       mol_type = protein
                       organism = synthetic construct
SEQUENCE: 31
MAADGYLPDW LEDNLSEGIR EWWDLKPGAP KPKANQQKQD DGRGLVLPGY KYLGPFNGLD  60
K                                                                  61

SEQ ID NO: 32          moltype = AA  length = 61
FEATURE                Location/Qualifiers
REGION                 1..61
                       note = Synthetic Construct
source                 1..61
                       mol_type = protein
                       organism = synthetic construct
SEQUENCE: 32
MAADGYLPDW LEDNLSEGIR EWWALKPGAP KPKANQQKQD DGRGLVLPGY KYLGPFNGLD  60
K                                                                  61

SEQ ID NO: 33          moltype = AA  length = 61
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FEATURE               Location/Qualifiers
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source                1..61
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SEQUENCE: 33
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K                                                                  61

SEQ ID NO: 34         moltype = AA  length = 61
FEATURE               Location/Qualifiers
REGION                1..61
                      note = Synthetic Construct
source                1..61
                      mol_type = protein
                      organism = synthetic construct
SEQUENCE: 34
MAADGYLPDW LEDTLSEGIR QWWKLKPGPP PPKPAERHKD DSRGLVLPGY KYLGPFNGLD  60
K                                                                  61

SEQ ID NO: 35         moltype = AA  length = 61
FEATURE               Location/Qualifiers
REGION                1..61
                      note = Synthetic Construct
source                1..61
                      mol_type = protein
                      organism = synthetic construct
SEQUENCE: 35
MAADGYLPDW LEDNLSEGIR EWWALKPGVP QPKANQQHQD NRRGLVLPGY KYLGPGNGLD  60
K                                                                  61

SEQ ID NO: 36         moltype = AA  length = 61
FEATURE               Location/Qualifiers
REGION                1..61
                      note = Synthetic Construct
source                1..61
                      mol_type = protein
                      organism = synthetic construct
SEQUENCE: 36
MAADGYLPDW LEDNLSEGIR EWWALKPGAP QPKKANQQHQ DNGRGLVLPG KYLGPFNGLD  60
K                                                                  61

SEQ ID NO: 37         moltype = AA  length = 60
FEATURE               Location/Qualifiers
REGION                1..60
                      note = Synthetic Construct
source                1..60
                      mol_type = protein
                      organism = synthetic construct
SEQUENCE: 37
MTDGYLPDWL EDNLSEGVRE WWALQPGAPK PKANQQHQDN ARGLVLPGYK YLGPGNGLDK  60

SEQ ID NO: 38         moltype = AA  length = 60
FEATURE               Location/Qualifiers
REGION                1..60
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source                1..60
                      mol_type = protein
                      organism = synthetic construct
SEQUENCE: 38
MSFVDHPPDW LEEVGEGLRE FLGLEAGPPK PKPNQQHQDQ ARGLVLPGYN YLGPGNGLDR  60

SEQ ID NO: 39         moltype = AA  length = 61
FEATURE               Location/Qualifiers
REGION                1..61
                      note = Synthetic Construct
source                1..61
                      mol_type = protein
                      organism = synthetic construct
SEQUENCE: 39
MAADGYLPDW LEDNLSEGIR EWWALKPGAP QPKANQQHQD NARGLVLPGY KYLGPGNGLD  60
K                                                                  61

SEQ ID NO: 40         moltype = AA  length = 61
FEATURE               Location/Qualifiers
REGION                1..61
                      note = Synthetic Construct
source                1..61
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mol_type = protein
organism = synthetic construct

SEQUENCE: 40
MAADGYLPDW LEDNLSEGIR EWWXLKPGAP KPKANQQKQD DGRGLVLPGY KYLGPFNGLD 60
K 61

What is claimed is:

1. A method of analyzing a preparation of AAV particles, the method comprising

a) denaturing the AAV particles,

b) subjecting the denatured AAV particles to liquid chromatography/mass spectroscopy (LC/MS) intact protein analysis, and

c) determining the masses of one or more viral proteins (VPs) of the particles in the preparation,

wherein the method is performed in the absence of a gel separation step.

2. The method of claim **1**, wherein the VPs comprise VP1, VP2, and VP3 capsid proteins and one more variants of VP1, VP2, or VP3 capsid proteins.

3. The method of claim **1**, wherein the AAV particles are denatured with acetic acid, guanidine hydrochloride and/or an organic solvent.

4. The method of claim **1**, wherein the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography.

5. The method of claim **1**, wherein the liquid chromatography is reverse phase liquid chromatography.

6. The method of claim **5**, wherein the reverse phase chromatography is performed with a C4 column.

7. The method of claim **1**, wherein the liquid chromatography is ultra-performance liquid chromatography (UPLC).

8. The method of claim **1**, wherein the mass spectrometry comprises assisted calibration.

9. The method of claim **8**, wherein sodium iodide is used as a calibrant.

10. A method of determining post-translational modifications of viral proteins (VPs) in a preparation of viral particles, the method comprising

a) denaturing the viral particles,

b) subjecting the denatured viral particles to liquid chromatography/mass spectroscopy (LC/MS) intact protein analysis, and

c) determining the masses of one or more VPs of the viral particles

wherein a deviation of one or more of the masses of the one or more VPs from the theoretical masses of VPs that have not undergone post-translational modifications is indicative of post-translational modifications of the VPs,

and wherein the method is performed in the absence of a gel separation step.

11. The method of claim **10**, wherein the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography.

12. The method of claim **10**, wherein the viral particles comprise a viral vector encoding a heterologous transgene.

13. The method of claim **10**, wherein the liquid chromatography is reverse phase chromatography.

14. The method of claim **13**, wherein the reverse phase chromatography is a C4 or C8 reverse chromatography.

15. The method of claim **10**, wherein the liquid chromatography is ultra-performance liquid chromatography (UPLC).

16. The method of claim **10**, wherein the mass spectrometry comprises assisted calibration.

17. The method of claim **16**, wherein sodium iodide is used as a calibrant.

18. A method of determining the extent of deamidation of viral proteins (VPs) in a preparation of viral particles, the method comprising

a) denaturing the viral particles,

b) subjecting the denatured viral particles to liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) intact protein analysis, and

c) determining the percentage of deamidated VPs in the preparation,

wherein the method is performed in the absence of a gel separation step.

19. The method of claim **18**, wherein the liquid chromatography is reverse phase chromatography, wherein the reverse phase chromatography is performed with a C4 column.

20. The method of claim **18**, wherein the liquid chromatography is reverse phase chromatography, wherein the reverse phase chromatography is performed with a C8 column.

21. The method of claim **2**, wherein the one more variants of VP1, VP2, or VP3 capsid proteins comprise one or more post-translational modifications, wherein the method comprises determining the masses of the VP1, VP2, and VP3 capsid proteins and the one more variants of VP1, VP2, or VP3 capsid proteins, and wherein a deviation of one or more of the masses of the variants of VP1, VP2, or VP3 capsid proteins from the theoretical masses of VP1, VP2, and VP3 capsid proteins that have not undergone post-translational modifications is indicative of post-translational modifications of the variants of VP1, VP2, or VP3 capsid proteins.

* * * * *

Exhibit S

US012298313B1

(12) United States Patent

Jin et al.

(10) Patent No.: US 12,298,313 B1
(45) Date of Patent: *May 13, 2025

(54) **METHODS FOR DETECTING AAV**

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(73) Assignee: **Genzyme Corporation**, Cambridge, MA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **19/013,863**

(22) Filed: **Jan. 8, 2025**

Related U.S. Application Data

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(Continued)

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C12N 15/86	(2006.01)
G01N 30/72	(2006.01)
G01N 30/02	(2006.01)

(52) **U.S. Cl.**

CPC ***G01N 33/6848*** (2013.01); ***C12N 15/86*** (2013.01); ***G01N 30/72*** (2013.01); *C12N 2750/14123* (2013.01); *C12N 2750/14143* (2013.01); *G01N 2030/027* (2013.01)

(58) **Field of Classification Search**

None

See application file for complete search history.

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Primary Examiner — Xiaoyun R Xu

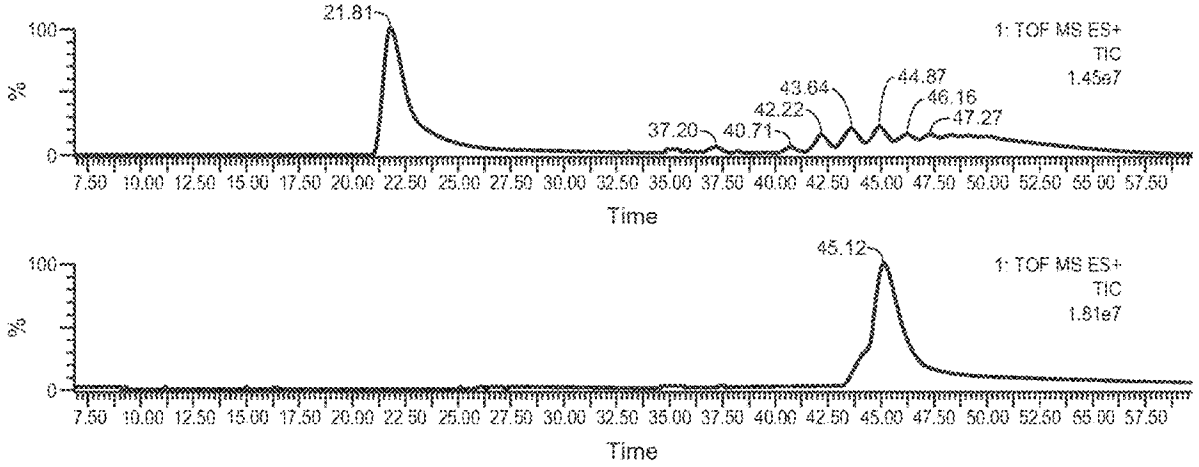
(74) *Attorney, Agent, or Firm* — MORRISON & FOERSTER LLP

(57) **ABSTRACT**

Provided herein are methods for determining the serotype of a virus particle and/or or determining the heterogeneity of a virus particle (e.g., an AAV particle). In other embodiments, the invention provides methods to determine the heterogeneity of AAV particles. In some aspects, the invention provides viral particles (e.g., rAAV particles) with improved stability and/or improved transduction efficiency by increasing the acetylation and/or deamidation of capsid proteins.

27 Claims, 27 Drawing Sheets

Specification includes a Sequence Listing.



Related U.S. Application Data

(60) Provisional application No. 62/375,314, filed on Aug. 15, 2016.

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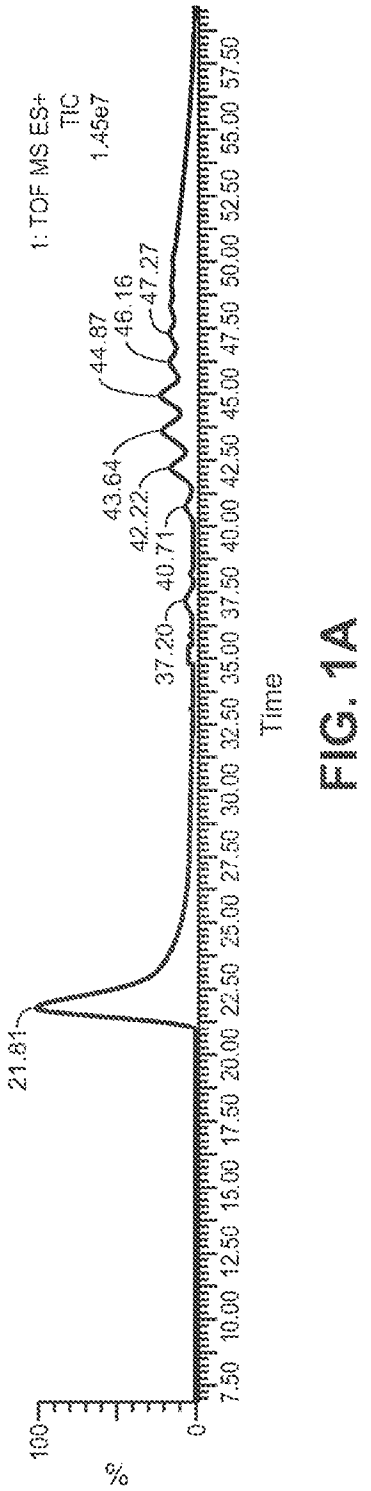

1: TOF MS ES+
TIC
1.45e7
21.81
37.20
40.71
42.22
43.64
44.87
46.16
47.27
100
%
0
7.50 10.00 12.50 15.00 17.50 20.00 22.50 25.00 27.50 30.00 32.50 35.00 37.50 40.00 42.50 45.00 47.50 50.00 52.50 55.00 57.50
Time

FIG. 1A

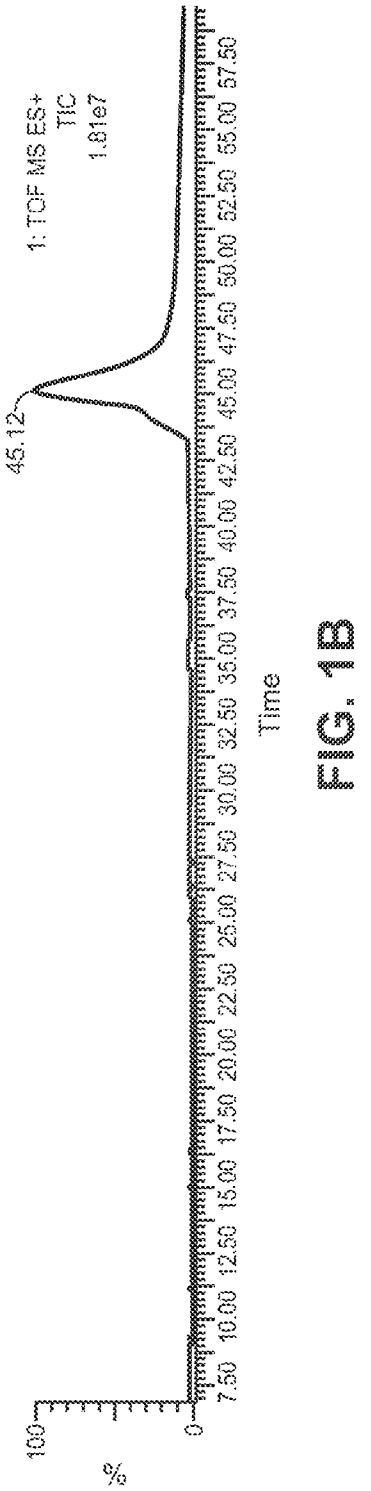

1: TOF MS ES+
TIC
1.81e7
45.12
100
%
0
7.50 10.00 12.50 15.00 17.50 20.00 22.50 25.00 27.50 30.00 32.50 35.00 37.50 40.00 42.50 45.00 47.50 50.00 52.50 55.00 57.50
Time

FIG. 1B

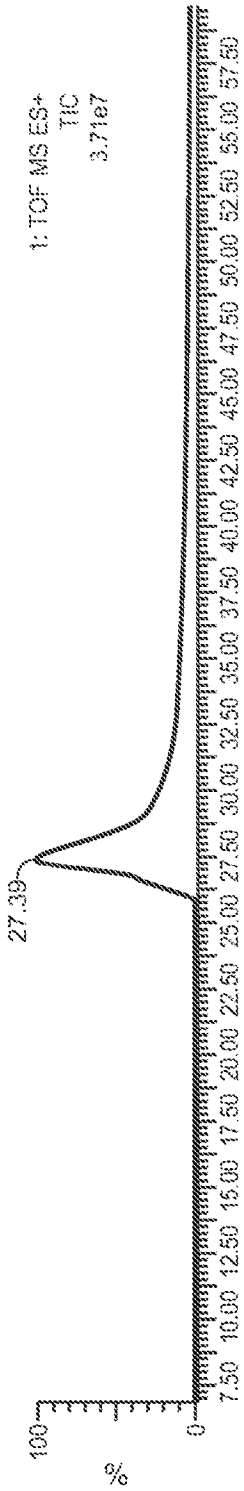

1: TOF MS ES+
TIC
3.71e7
27.39
100
%
0
7.50 10.00 12.50 15.00 17.50 20.00 22.50 25.00 27.50 30.00 32.50 35.00 37.50 40.00 42.50 45.00 47.50 50.00 52.50 55.00 57.50
Time

FIG. 1C

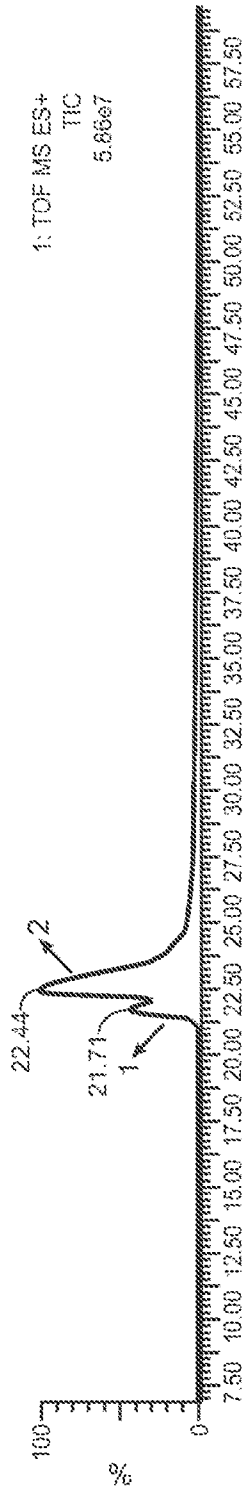

1: TOF MS ES+
TIC
5.86e7
22.44
2
21.71
1
100
%
0
7.50 10.00 12.50 15.00 17.50 20.00 22.50 25.00 27.50 30.00 32.50 35.00 37.50 40.00 42.50 45.00 47.50 50.00 52.50 55.00 57.50
Time

FIG. 1D

FIG. 2A

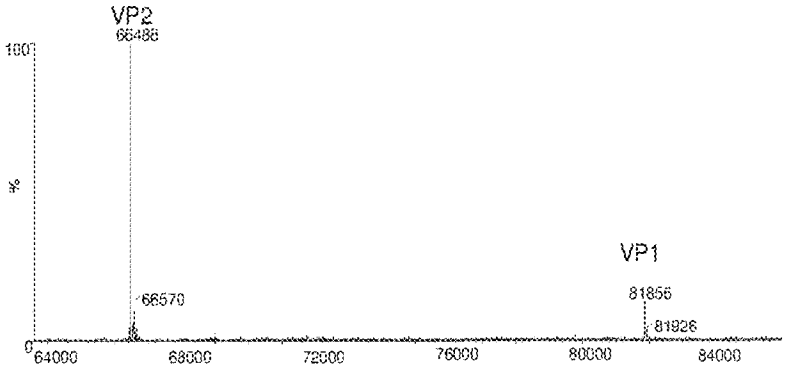
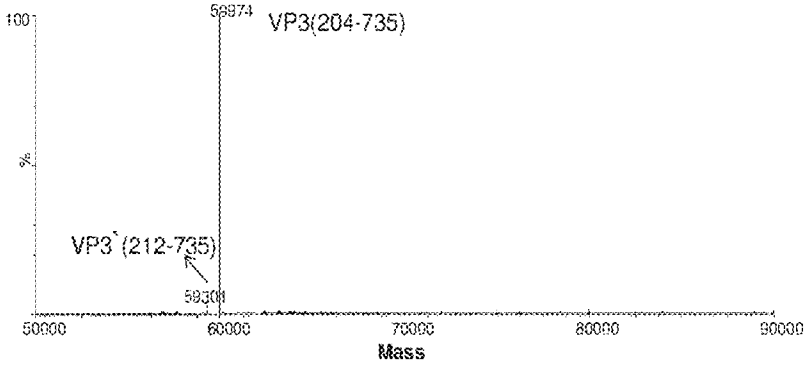

VP2
66488
100
%
VP1
66570
81856
81926
0
64000
68000
72000
76000
80000
84000

FIG. 2B


59874
VP3(204-735)
100
%
VP3`(212-735)
59301
50000
60000
70000
80000
90000
Mass

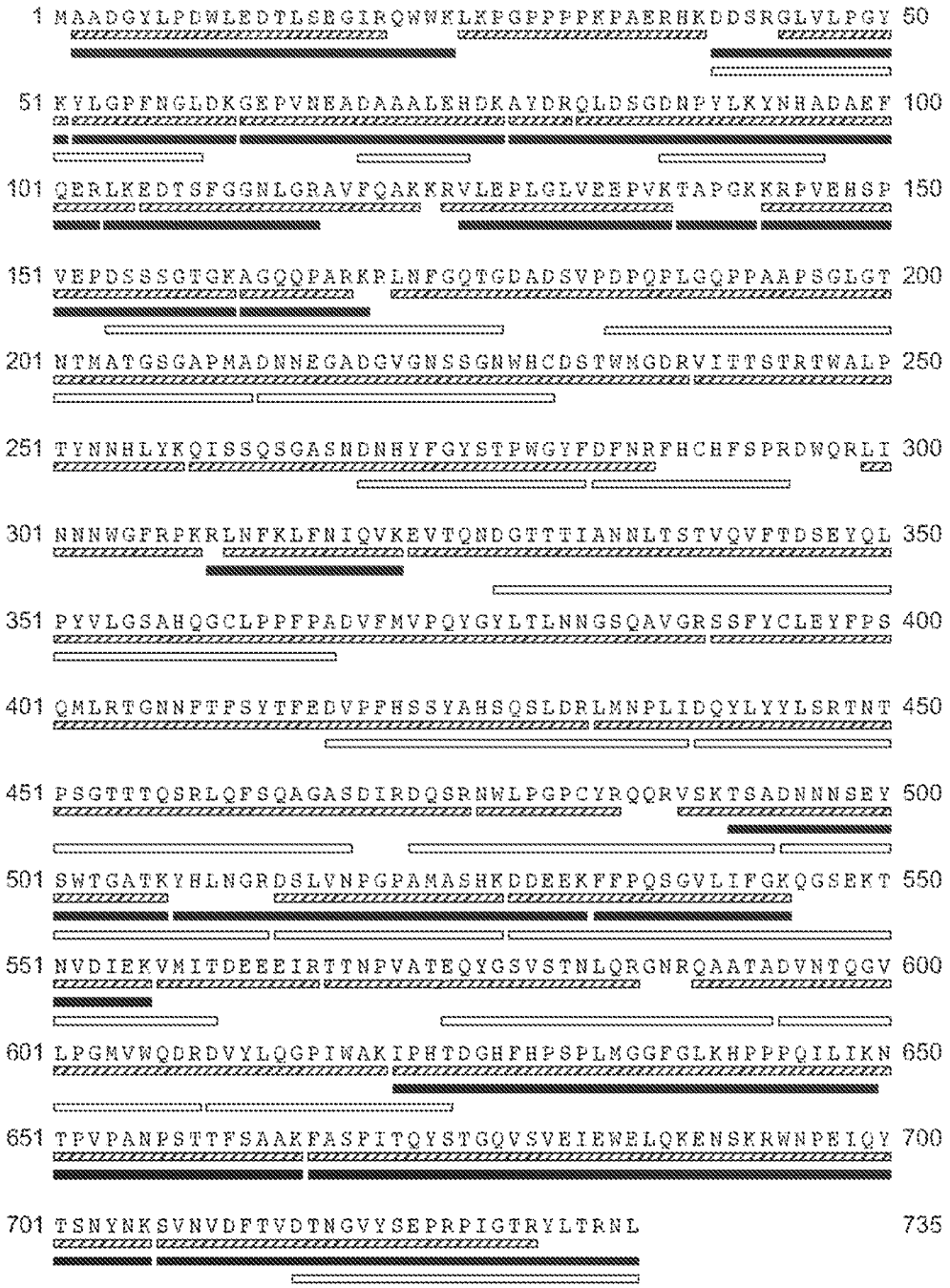

1 MAADGYLPDWLEDTLSEGIRQWWKLKPGPPPPKPAERHKDDSRGLVLPGY 50
51 KYLGPFNGLDKGEPVNEADAAALEHDKAYDRQLDSGDNPYLKYNHADAEF 100
101 QERLKEDTSFGGNLGRAVFQAKKRVLEPLGLVEEPVKTAPGKKRPVEHSP 150
151 VEPDSSSGTGKAGQQPARKRLNFGQTGDADSVPDPQPLGQPPAAPSGLGT 200
201 NTMATGSGAPMADNNEGADGVGNSSGNWHCDSTWMGDRVITTSTRTWALP 250
251 TYNNHLYKQISSQSGASNDNHYFGYSTPWGYFDFNRFHCHFSPRDWQRLI 300
301 NNNWGFRPKRLNFKLFNIQVKEVTQNDGTTTIANNLTSTVQVFTDSEYQL 350
351 PYVLGSAHQGCLPPFPADVFMVPQYGYLTLNNGSQAVGRSSFYCLEYFPS 400
401 QMLRTGNNFTFSYTFEDVPFHSSYAHSQSLDRLMNPLIDQYLYYLSRTNT 450
451 PSGTTTQSRLQFSQAGASDIRDQSRNWLPGPCYRQQRVSKTSADNNNSEY 500
501 SWTGATKYHLNGRDSLVNPGPAMASHKDDEEKFFPQSGVLIFGKQGSEKT 550
551 NVDIEKVMITDEEEIRTTNPVATEQYGSVSTNLQRGNRQAATADVNTQGV 600
601 LPGMVWQDRDVYLQGPIWAKIPHTDGHFHPSPLMGGFGLKHPPPQILIKN 650
651 TPVPANPSTTFSAAKFASFITQYSTGQVSVEIEWELQKENSKRWNPEIQY 700
701 TSNYNKSVNVDFTVDTNGVYSEPRPIGTRYLTRNL 735

FIG. 3

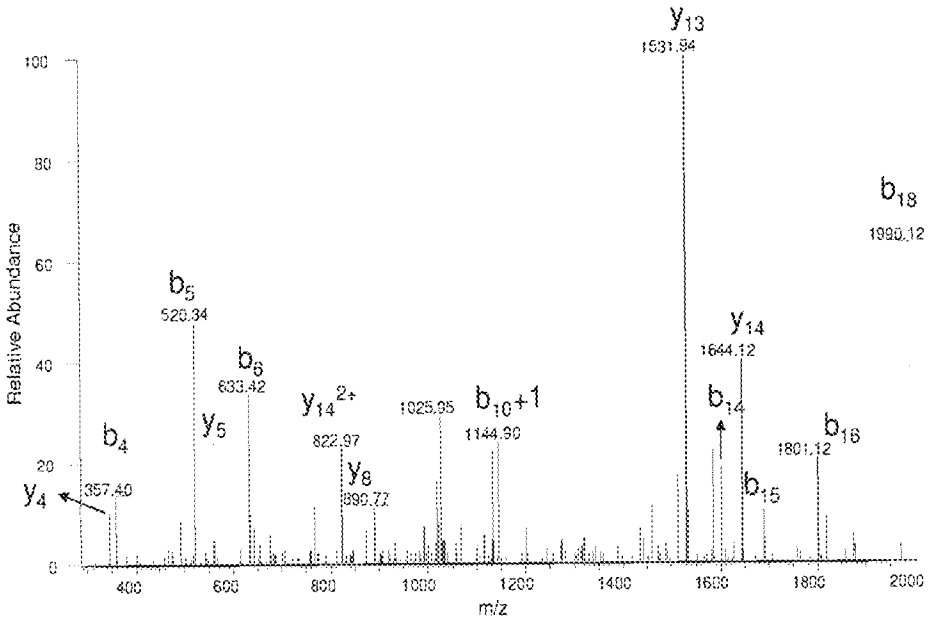

Relative Abundance
m/z
100
80
60
40
20
0
400
600
800
1000
1200
1400
1600
1800
2000
y13
1531.84
b18
1990.12
b5
520.34
b6
633.42
y14
1644.12
y14 2+
822.97
1025.95
b10+1
1144.90
b14
b16
1801.12
b4
y5
y8
899.77
y4
357.40
b15

FIG. 4A

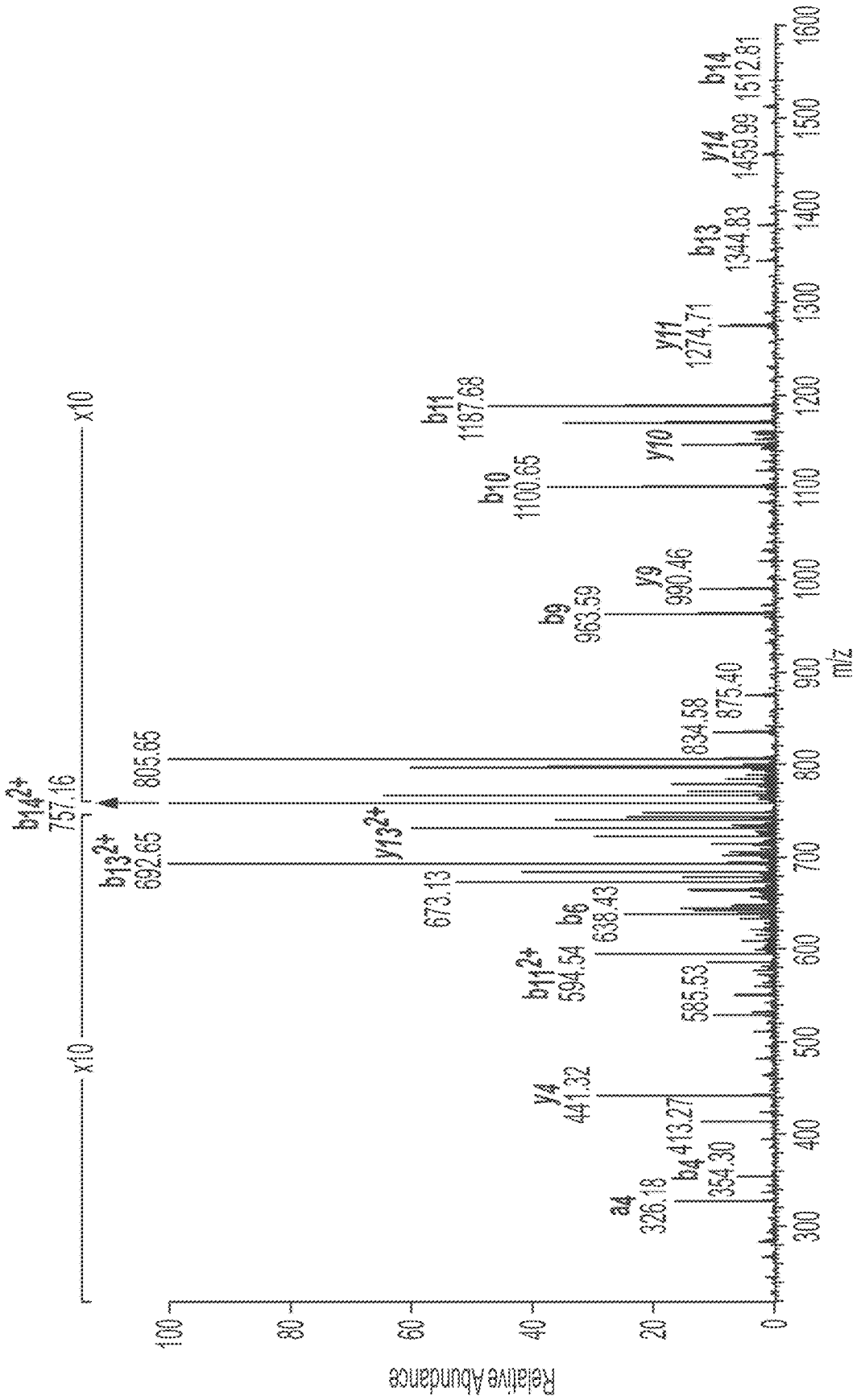

Relative Abundance
m/z
100
80
60
40
20
0
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
1500
1600
x10
x10
b14 2+ 757.16
805.65
b13 2+ 692.65
y13 2+
673.13
a4 326.18
b4 413.27 354.30
y4 441.32
b11 2+ 594.54
b6 638.43
585.53
b9 963.59
y9 990.46
b10 1100.65
b11 1187.68
y10
y11 1274.71
b13 1344.83
y14 1459.99
b14 1512.81
834.58
875.40

FIG. 4B

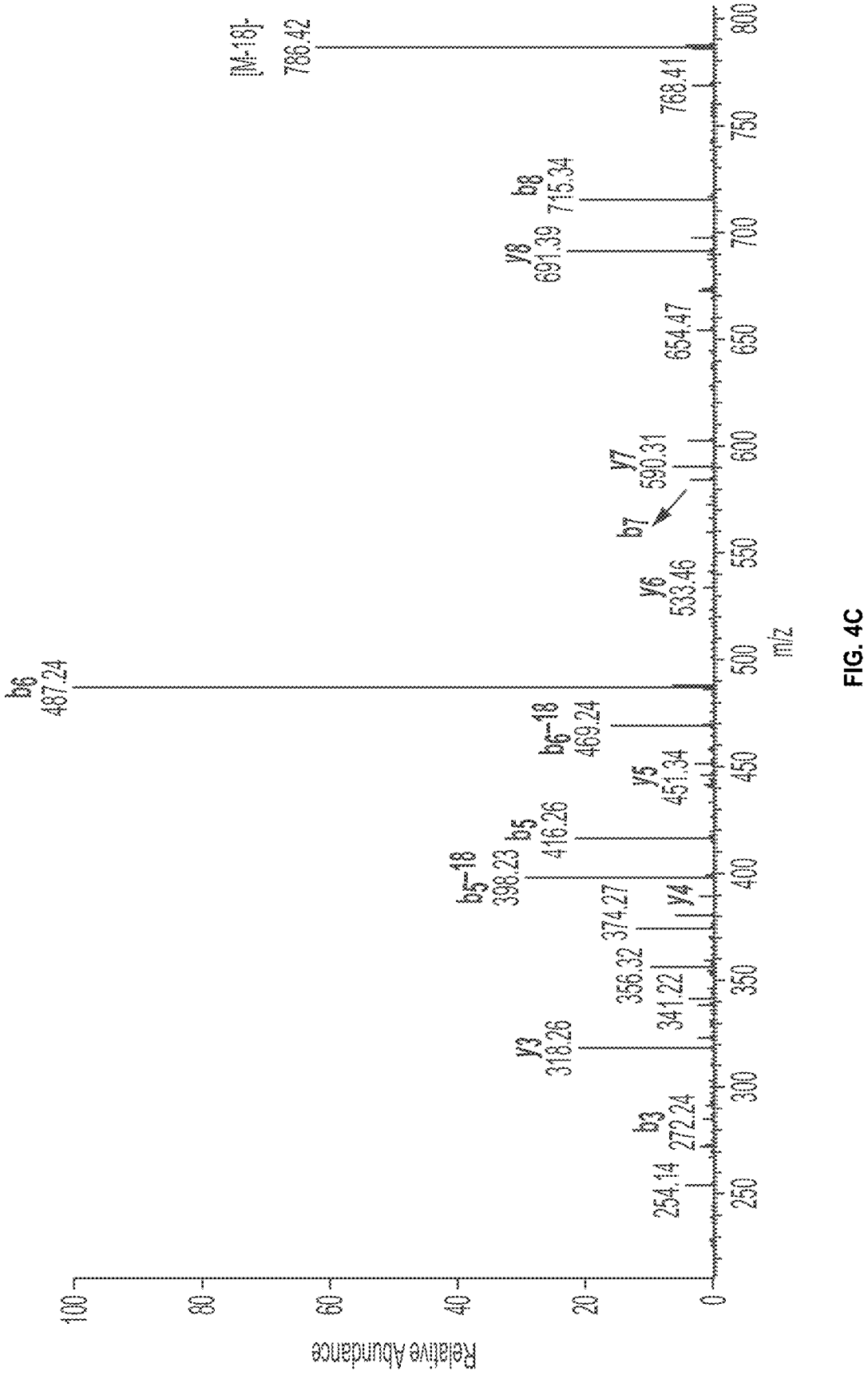

[M-18]-
786.42
768.41
b8
715.34
y8
691.39
654.47
y7
590.31
b7
y6
533.46
b6
487.24
b6−18
469.24
y5
451.34
b5
416.26
b5−18
398.23
374.27
y4
356.32
341.22
y3
318.26
b3
272.24
254.14
Relative Abundance
m/z
0
20
40
60
80
100
250
300
350
400
450
500
550
600
650
700
750
800

FIG. 4C

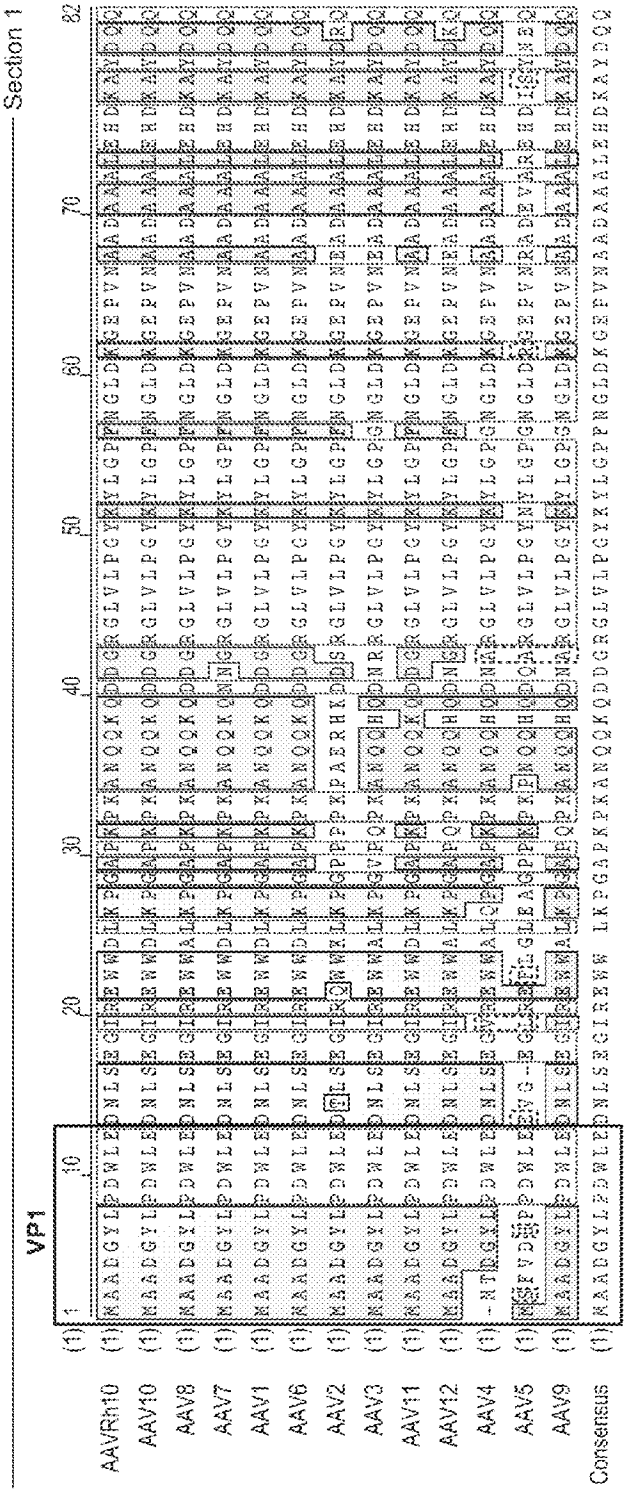

Section 1
VP1
AAVRh10
AAV10
AAV8
AAV7
AAV1
AAV6
AAV2
AAV3
AAV11
AAV12
AAV4
AAV5
AAV9
Consensus

FIG. 5A

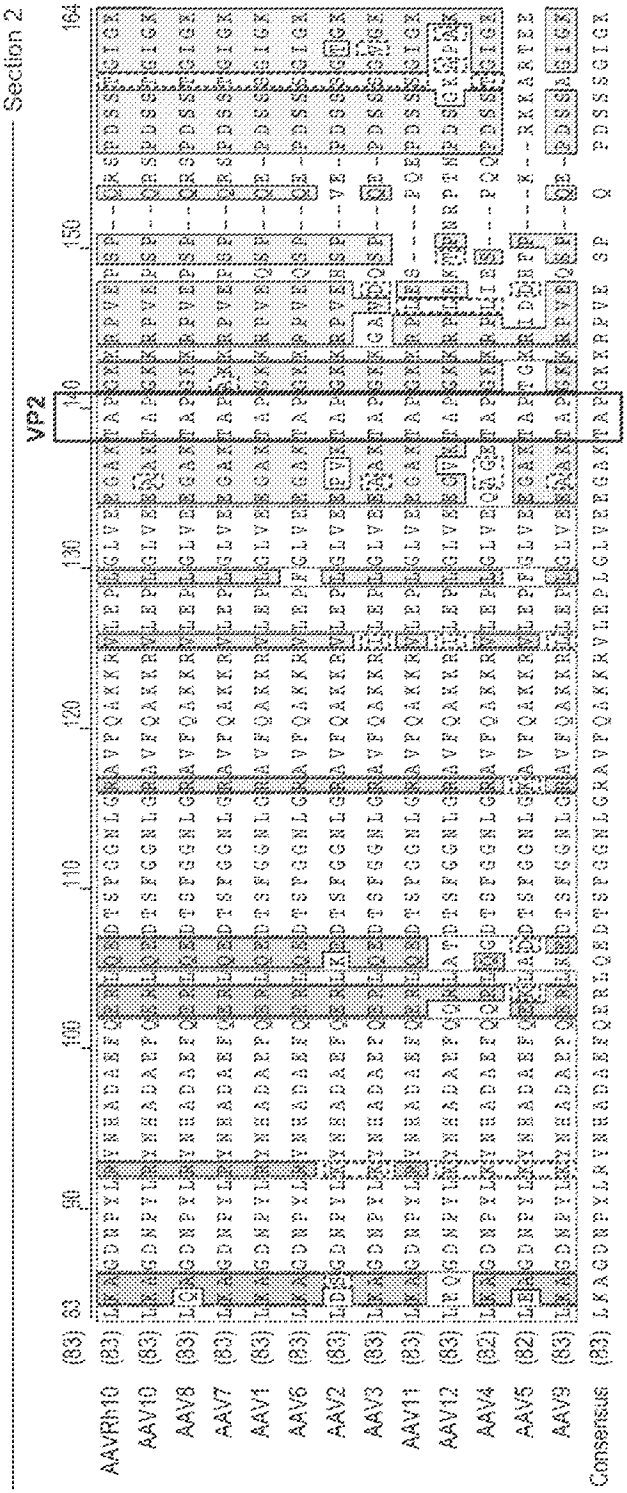

Section 2
VP2
AAVRh10
AAV10
AAV8
AAV7
AAV1
AAV6
AAV2
AAV3
AAV11
AAV12
AAV4
AAV5
AAV9
Consensus

FIG. 5B

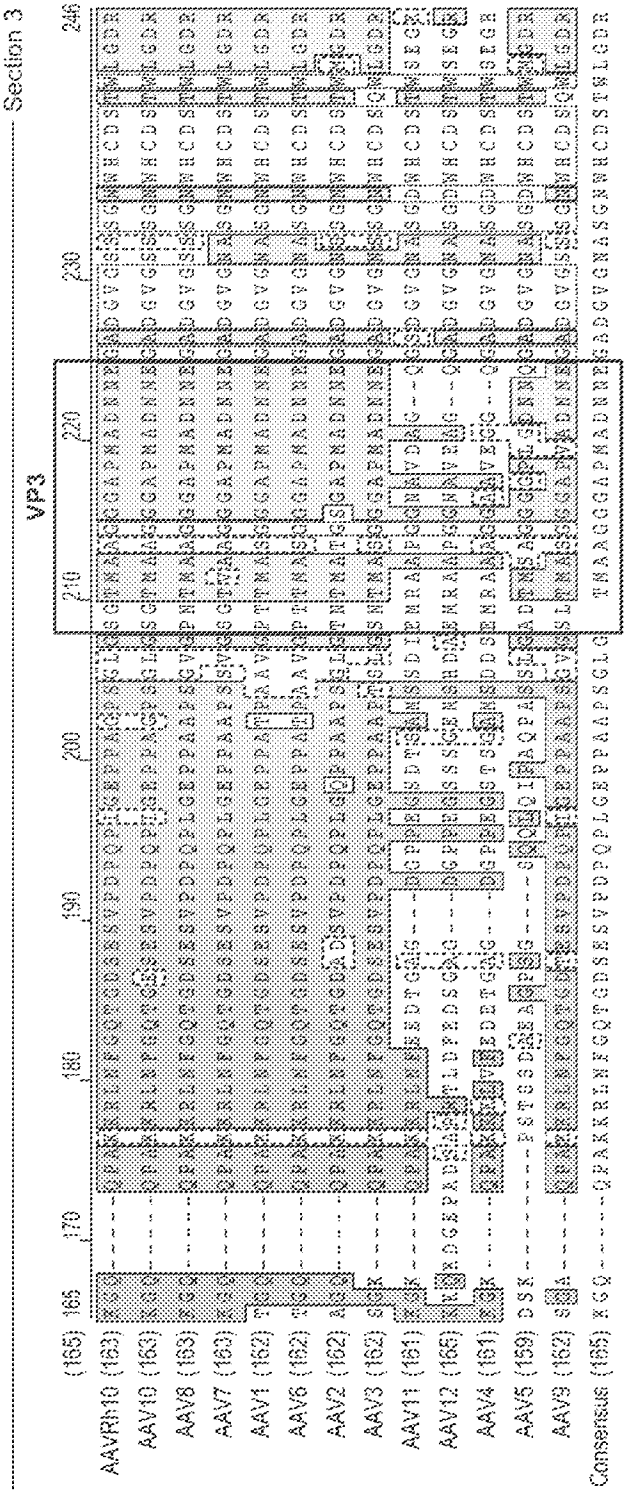

Section 3
VP3
165
170
180
190
200
210
220
230
246
AAVRh10 (163)
AAV10 (163)
AAV8 (163)
AAV7 (160)
AAV1 (162)
AAV6 (162)
AAV2 (162)
AAV3 (162)
AAV11 (161)
AAV12 (166)
AAV4 (161)
AAV5 (159)
AAV9 (162)
Consensus (165)

FIG. 5C

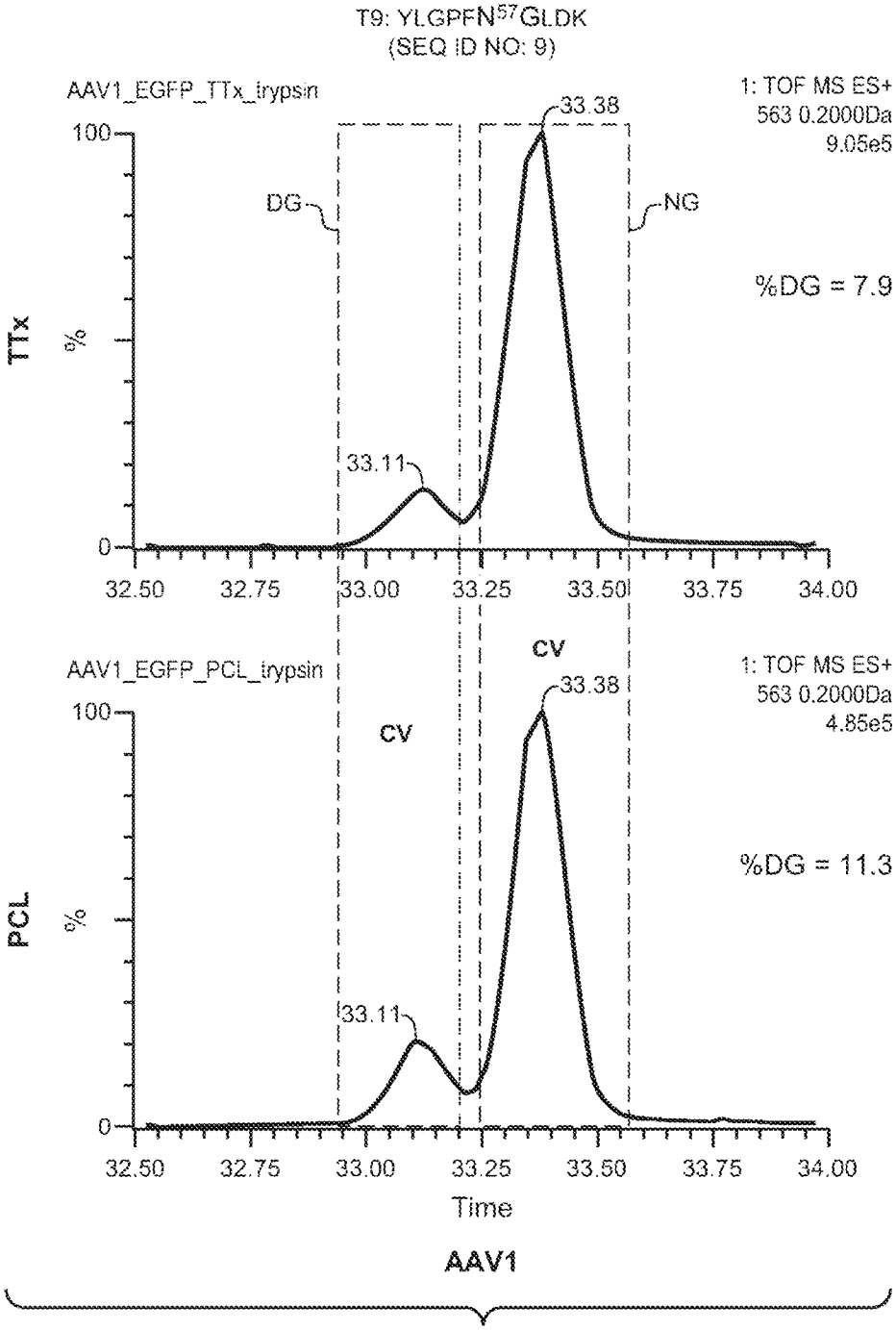

T9: YLGPFN57GLDK
(SEQ ID NO: 9)
AAV1_EGFP_TTx_trypsin
1: TOF MS ES+
563 0.2000Da
9.05e5
33.38
DG
NG
%DG = 7.9
TTx
%
100
0
33.11
32.50
32.75
33.00
33.25
33.50
33.75
34.00
AAV1_EGFP_PCL_trypsin
1: TOF MS ES+
563 0.2000Da
4.85e5
CV
CV
33.38
%DG = 11.3
PCL
%
100
0
33.11
32.50
32.75
33.00
33.25
33.50
33.75
34.00
Time
AAV1

FIG. 6A

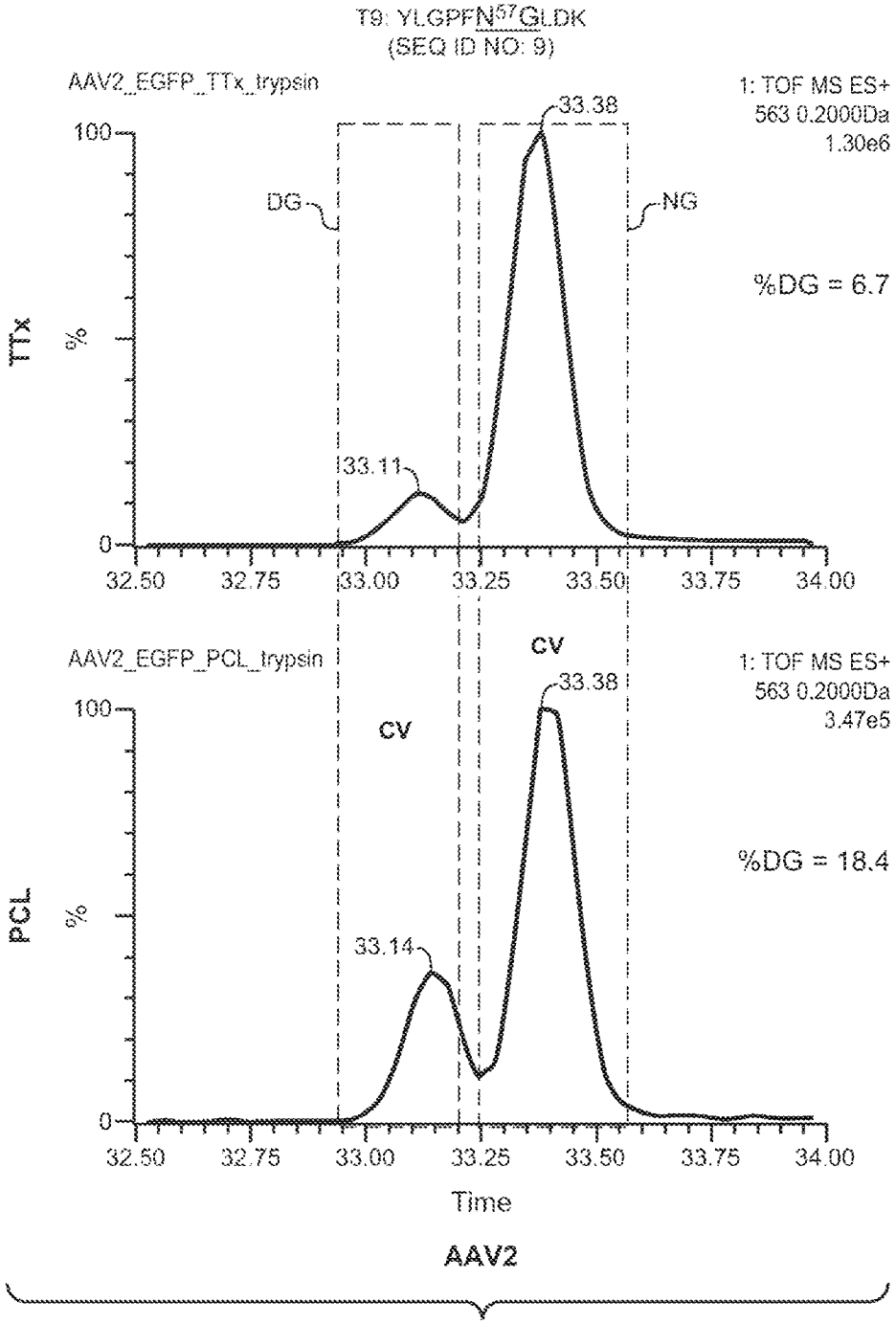

T9: YLGPFN57GLDK
(SEQ ID NO: 9)
AAV2_EGFP_TTx_trypsin
1: TOF MS ES+
563 0.2000Da
1.30e6
33.38
DG
NG
%DG = 6.7
TTx
%
33.11
100
0
32.50
32.75
33.00
33.25
33.50
33.75
34.00
AAV2_EGFP_PCL_trypsin
CV
CV
1: TOF MS ES+
563 0.2000Da
3.47e5
33.38
%DG = 18.4
PCL
%
33.14
Time
AAV2

FIG. 6B

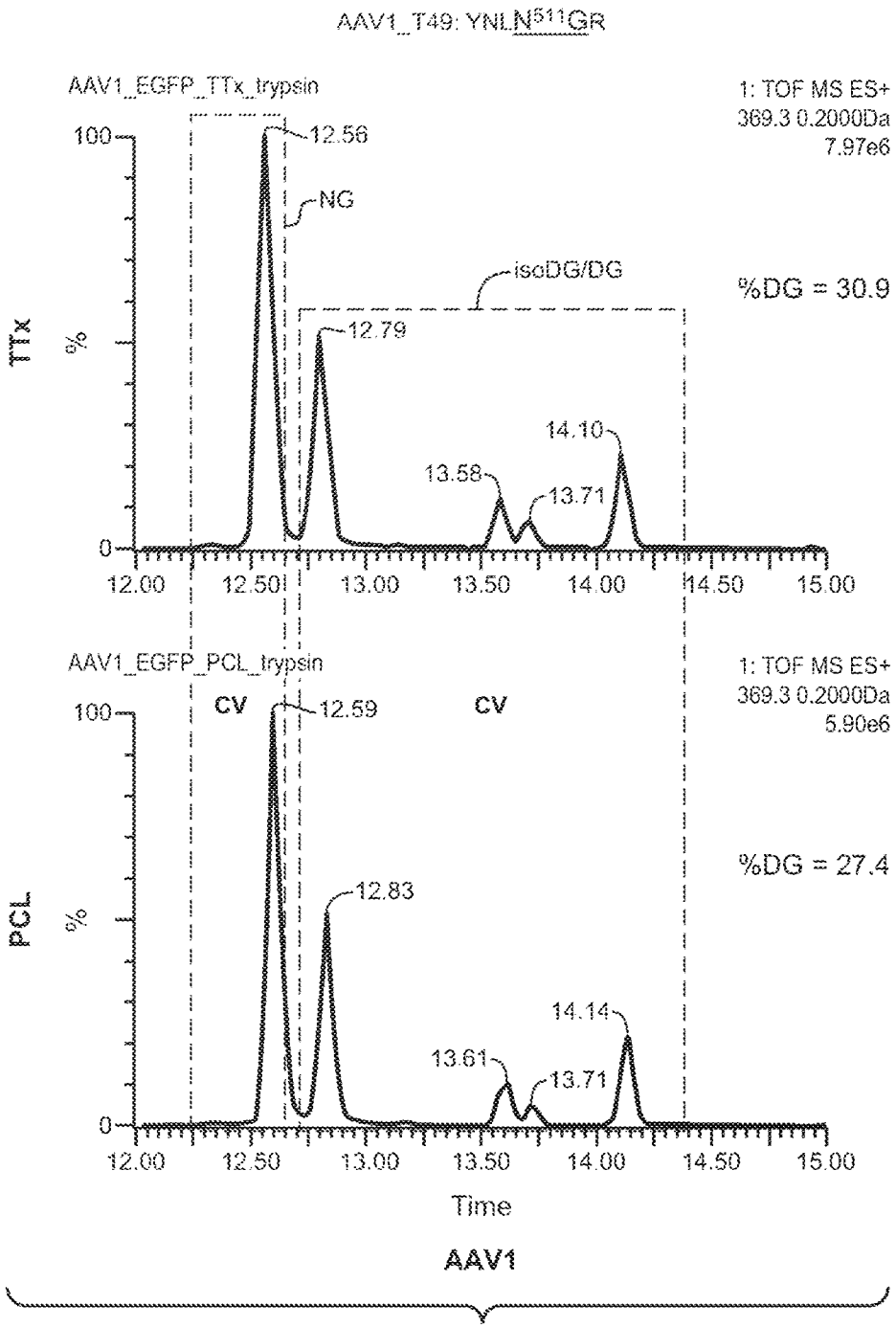

AAV1_T49: YNLN511GR
AAV1_EGFP_TTx_trypsin
1: TOF MS ES+
369.3 0.2000Da
7.97e6
100
12.56
NG
isoDG/DG
%DG = 30.9
TTx
%
12.79
14.10
13.58
13.71
0
12.00
12.50
13.00
13.50
14.00
14.50
15.00
AAV1_EGFP_PCL_trypsin
1: TOF MS ES+
369.3 0.2000Da
5.90e6
CV
12.59
CV
100
%DG = 27.4
PCL
%
12.83
14.14
13.61
13.71
0
12.00
12.50
13.00
13.50
14.00
14.50
15.00
Time
AAV1

FIG. 7A

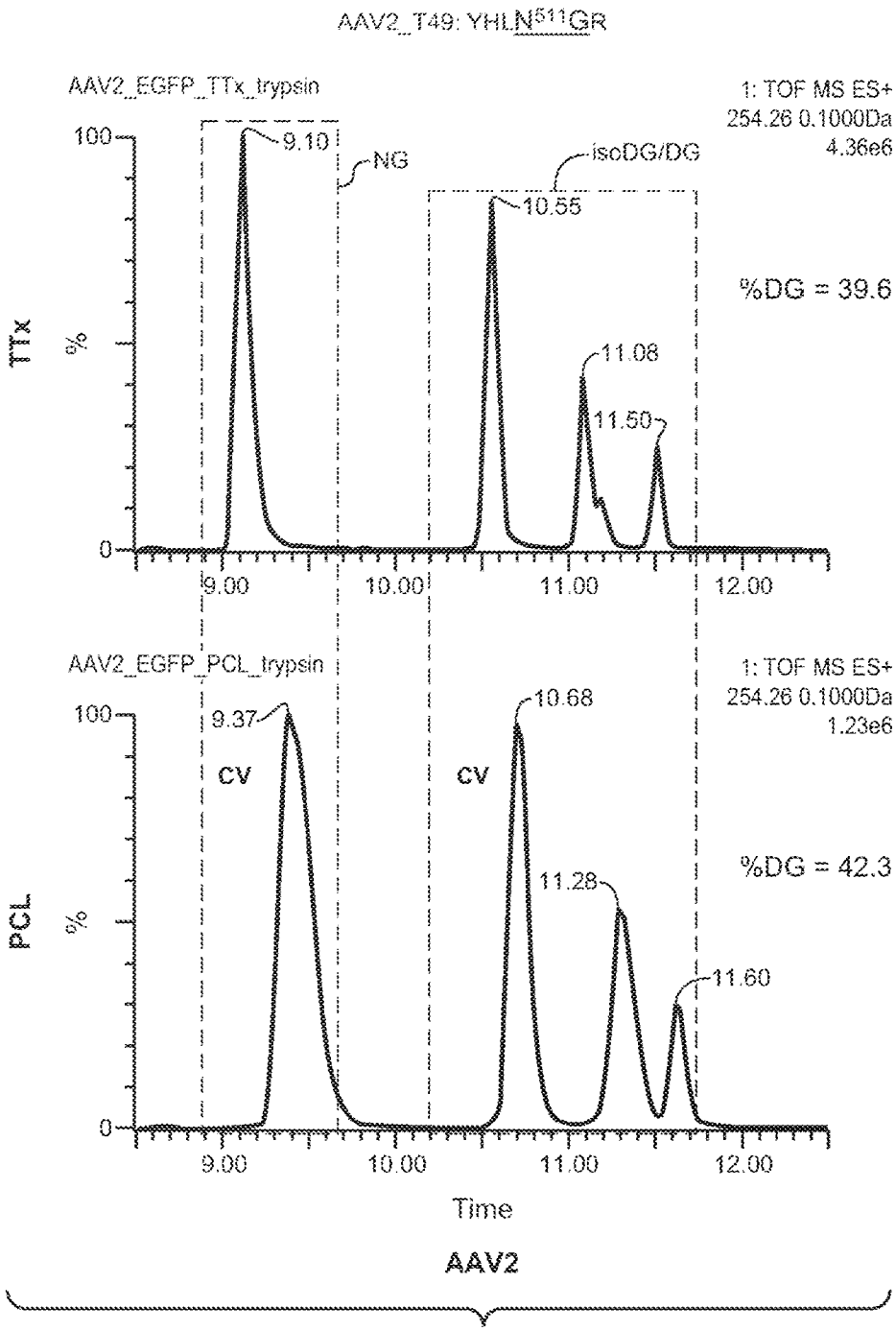

AAV2_T49: YHLN511GR
AAV2_EGFP_TTx_trypsin
1: TOF MS ES+
254.26 0.1000Da
4.36e6
100
9.10
NG
isoDG/DG
10.55
%DG = 39.6
11.08
11.50
TTx
%
0
9.00
10.00
11.00
12.00
AAV2_EGFP_PCL_trypsin
1: TOF MS ES+
254.26 0.1000Da
1.23e6
100
9.37
CV
CV
10.68
%DG = 42.3
11.28
11.60
PCL
%
0
9.00
10.00
11.00
12.00
Time
AAV2

FIG. 7B

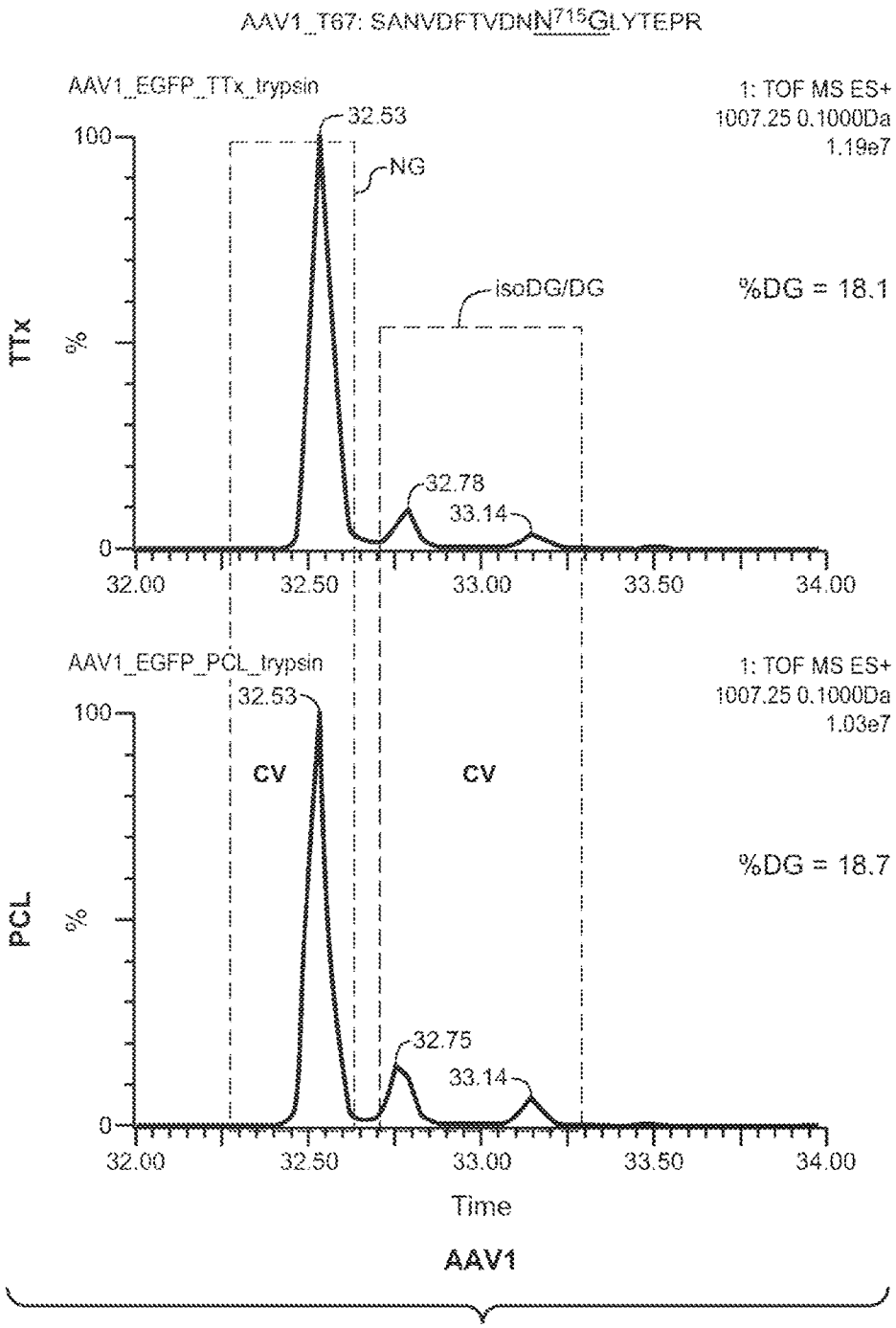

AAV1_T67: SANVDFTVDNN715GLYTEPR
AAV1_EGFP_TTx_trypsin
1: TOF MS ES+
1007.25 0.1000Da
1.19e7
32.53
NG
isoDG/DG
%DG = 18.1
TTx
%
100
0
32.78
33.14
32.00
32.50
33.00
33.50
34.00
AAV1_EGFP_PCL_trypsin
1: TOF MS ES+
1007.25 0.1000Da
1.03e7
32.53
CV
CV
%DG = 18.7
PCL
%
100
0
32.75
33.14
32.00
32.50
33.00
33.50
34.00
Time
AAV1

FIG. 8A

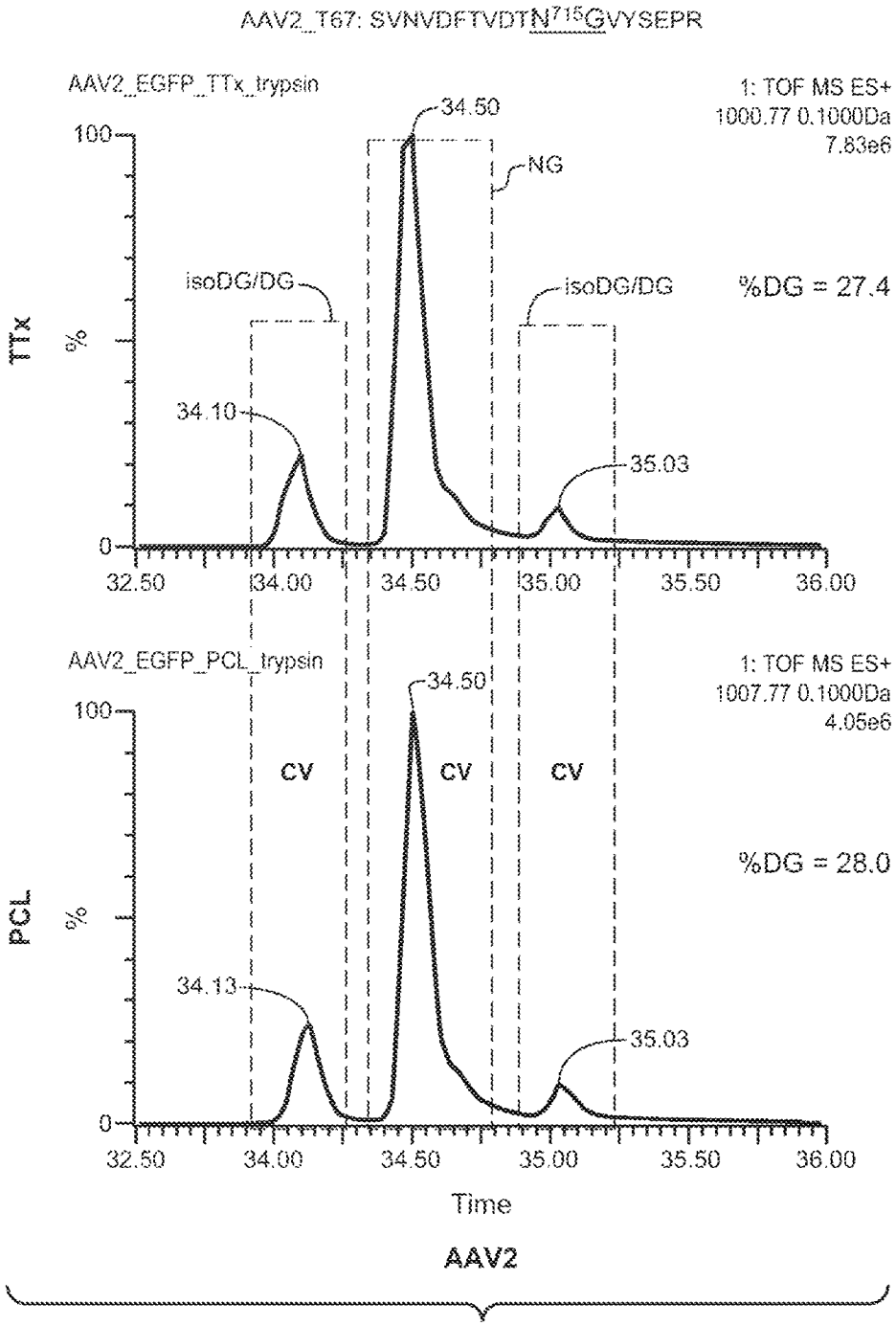
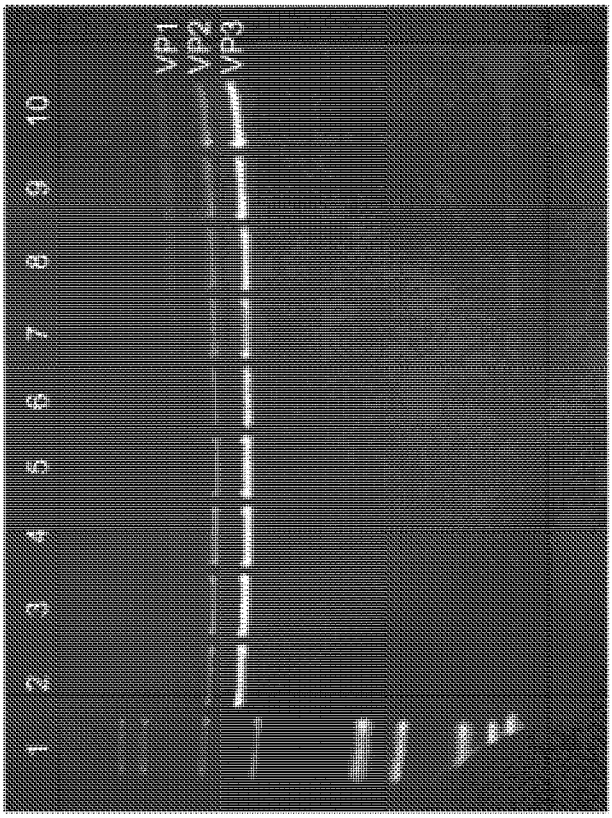

AAV2_T67: SVNVDFTVDTN715GVYSEPR
AAV2_EGFP_TTx_trypsin
1: TOF MS ES+
1000.77 0.1000Da
7.83e6
34.50
NG
isoDG/DG
isoDG/DG
%DG = 27.4
TTx
%
34.10
35.03
100
0
32.50
34.00
34.50
35.00
35.50
36.00
AAV2_EGFP_PCL_trypsin
1: TOF MS ES+
1007.77 0.1000Da
4.05e6
34.50
CV
CV
CV
%DG = 28.0
PCL
%
34.13
35.03
100
0
32.50
34.00
34.50
35.00
35.50
36.00
Time
AAV2

FIG. 8B



Deacetylation AAV5 Mutant Vectors

Lane	Sample
1	Mark12 Marker
2	deAC-AAV5 (S2G)/CBA-eGFP X17052C
3	deAC-AAV5 (S2L)/CBA-eGFP X17052D
4	deAC-AAV5 (S2GVP3)/CBA-eGFP X17058D
5	deAC-AAV5 (S2LVP3)/CBA-eGFP X17065A
6	deAC-AAV5 (S2PVP1VP3)/CBA-eGFP X17065B
7	deAC-AAV5 (S2GVP1VP3)/CBA-eGFP X17065C
8	deAC-AAV5 (S2PVP3)/CBA-eGFP X17065D
9	deAC-AAV5 (S2P)/CBA-eGFP X17065E
10	deAC-AAV5 (S2LVP1VP3)/CBA-eGFP X17072B

(~1E10 VGs/Lane)

FIG. 9

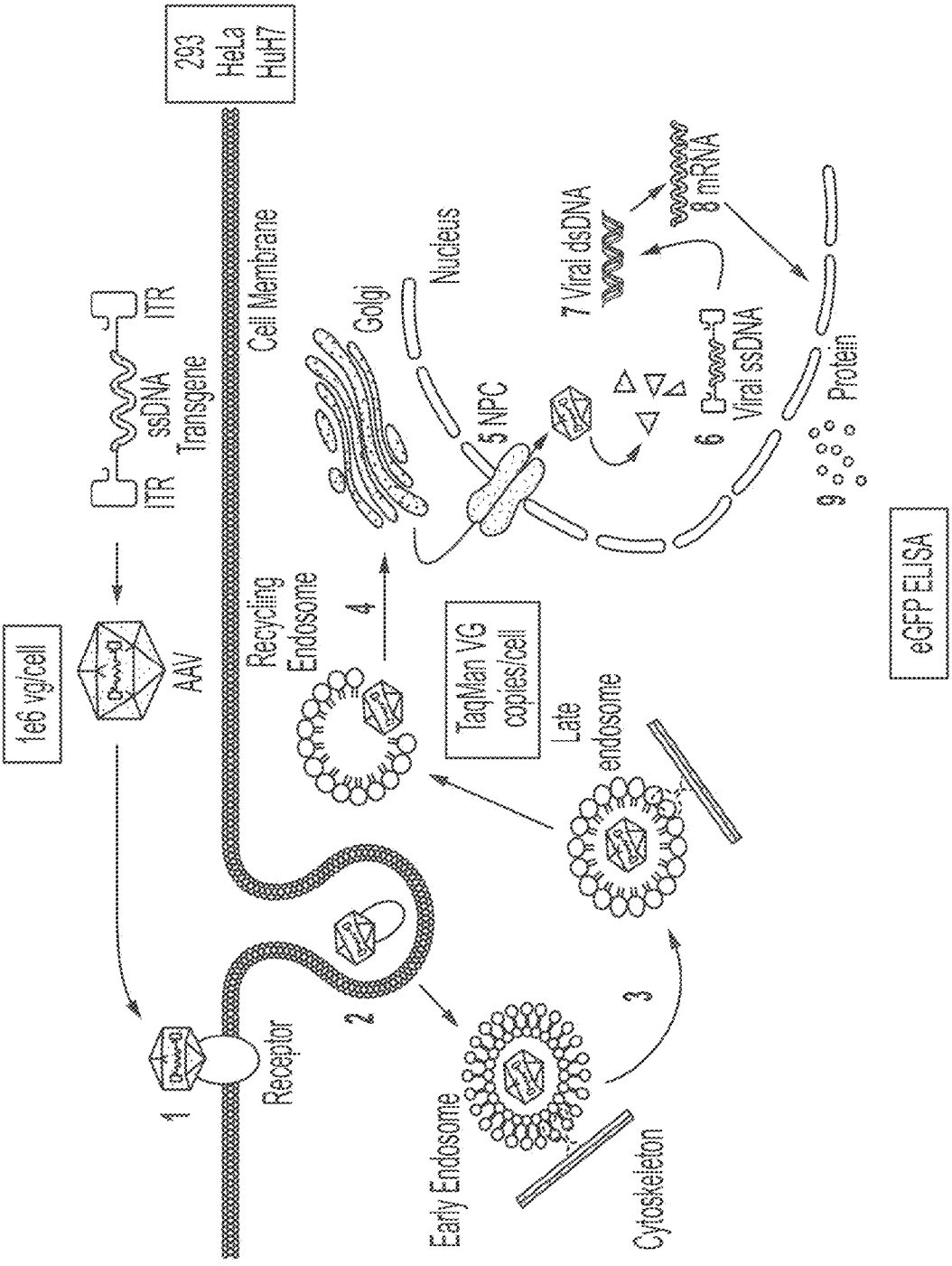

293
HeLa
HuH7
Cell Membrane
ITR
ssDNA
Transgene
ITR
1e6 vg/cell
AAV
Recycling
Endosome
4
Golgi
Nucleus
5 NPC
7 Viral dsDNA
8 mRNA
6 Viral ssDNA
9 Protein
TaqMan VG
copies/cell
Late
endosome
eGFP ELISA
1
Receptor
2
3
Early Endosome
Cytoskeleton

FIG. 10

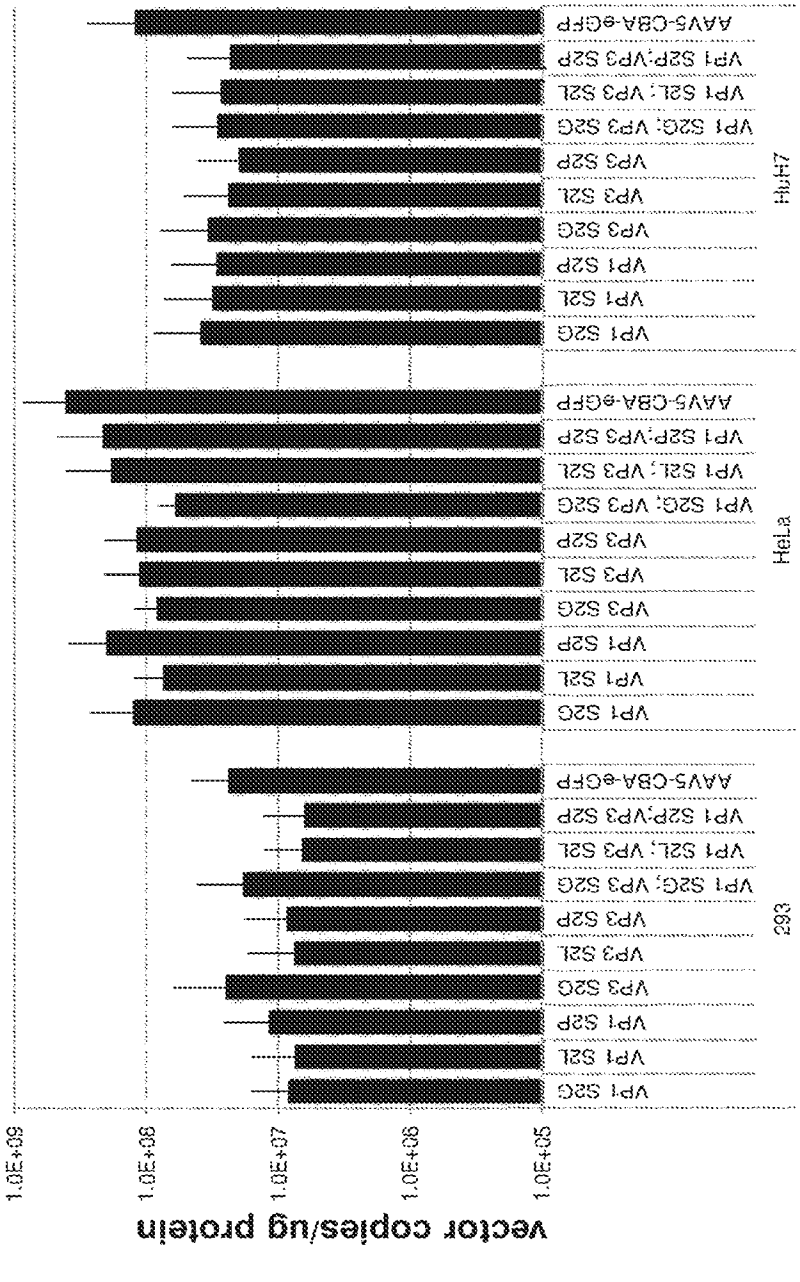

vector copies/ug protein
1.0E+09
1.0E+08
1.0E+07
1.0E+06
1.0E+05
293
HeLa
HuH7
VP1 S2G
VP1 S2L
VP1 S2P
VP3 S2G
VP3 S2L
VP3 S2P
VP1 S2G; VP3 S2G
VP1 S2L; VP3 S2L
VP1 S2P;VP3 S2P
AAV5-CBA-eGFP

FIG. 11

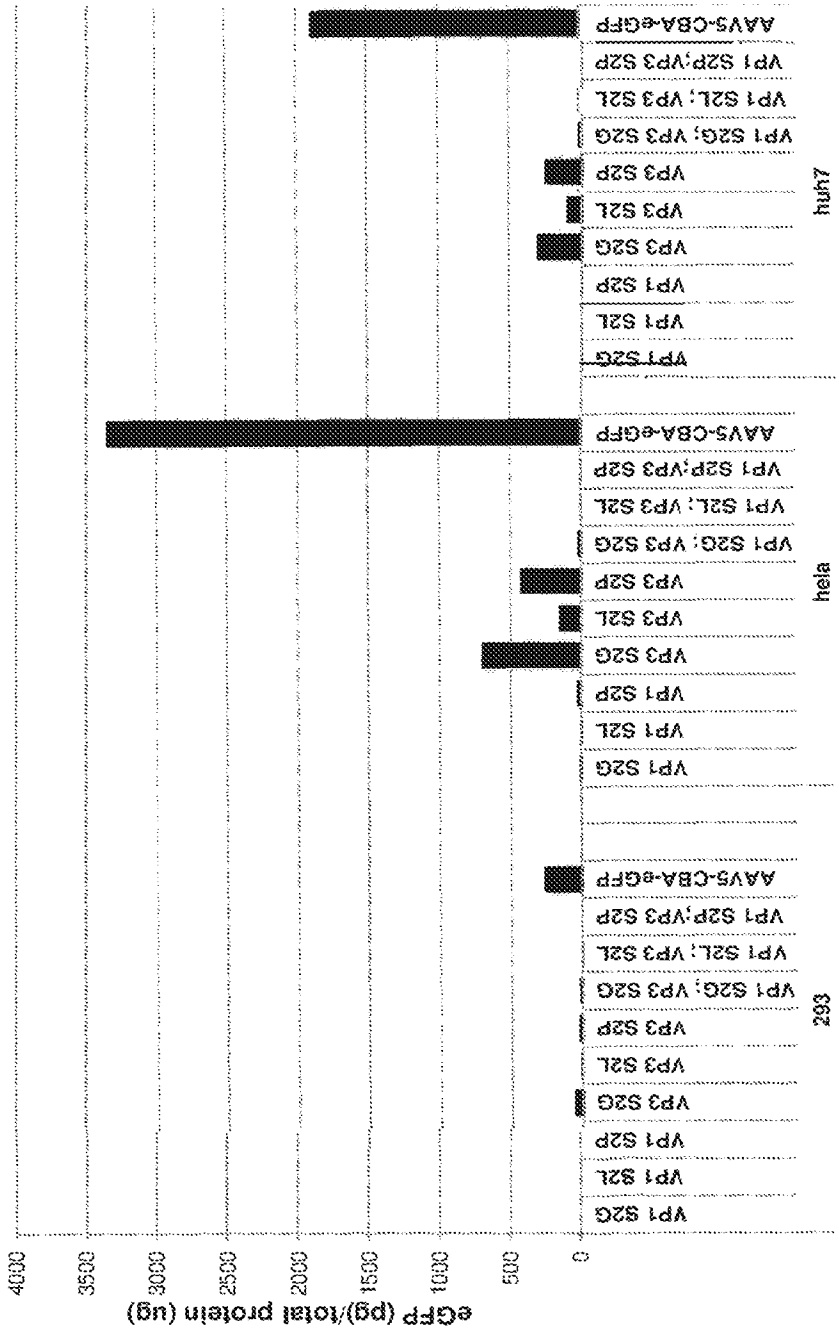

AAV5-CBA-eGFP
VP1 S2P;VP3 S2P
VP1 S2L; VP3 S2L
VP1 S2G; VP3 S2G
VP3 S2P
VP3 S2L
VP3 S2G
VP1 S2P
VP1 S2L
VP1 S2G
huh7
AAV5-CBA-eGFP
VP1 S2P;VP3 S2P
VP1 S2L; VP3 S2L
VP1 S2G; VP3 S2G
VP3 S2P
VP3 S2L
VP3 S2G
VP1 S2P
VP1 S2L
VP1 S2G
hela
AAV5-CBA-eGFP
VP1 S2P;VP3 S2P
VP1 S2L; VP3 S2L
VP1 S2G; VP3 S2G
VP3 S2P
VP3 S2L
VP3 S2G
VP1 S2P
VP1 S2L
VP1 S2G
293
4000
3500
3000
2500
2000
1500
1000
500
0
eGFP (pg)/total protein (ug)

FIG. 12

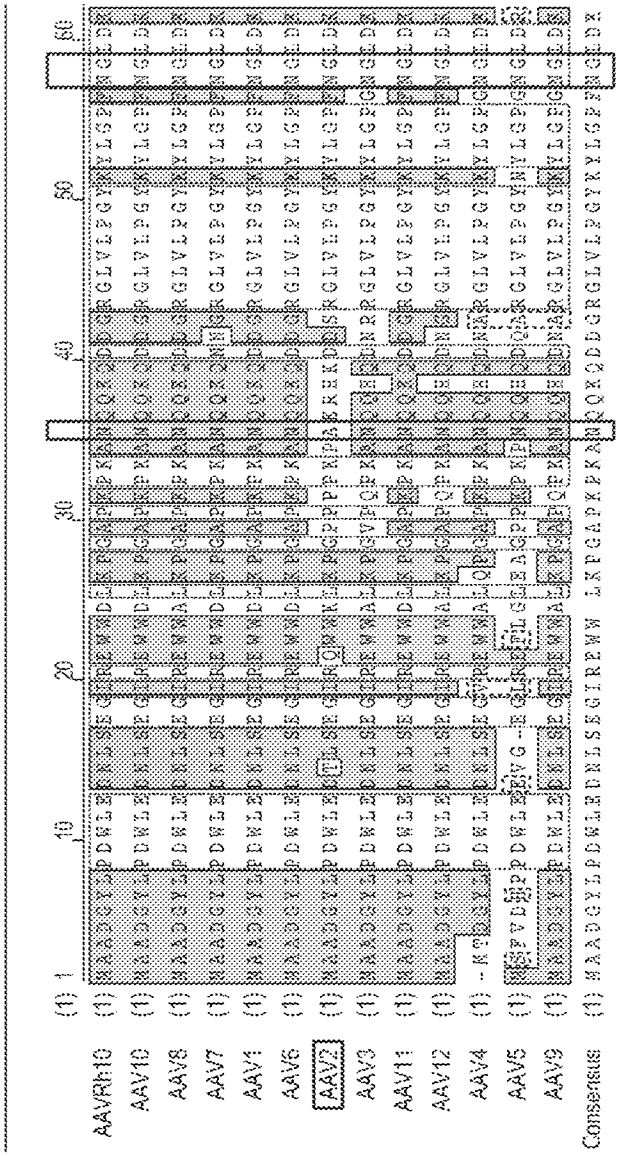

AAVRh10
AAV10
AAV8
AAV7
AAV1
AAV6
AAV2
AAV3
AAV11
AAV12
AAV4
AAV5
AAV9
Consensus

FIG. 13

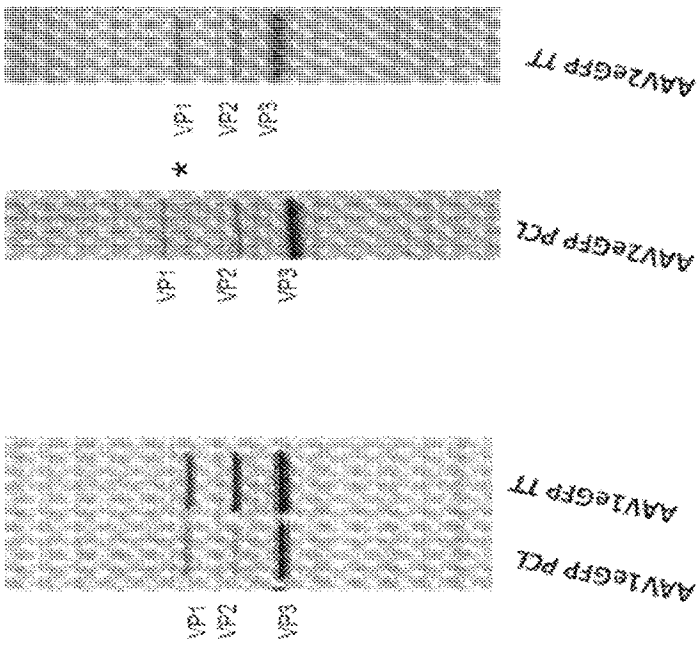

AAV2eGFP TT
VP1
VP2
VP3
*
AAV2eGFP PCL
VP1
VP2
VP3
AAV1eGFP TT
AAV1eGFP PCL
VP1
VP2
VP3

FIG. 14

Deamidation of N57(G)

	% Deamidation on N57	replicate
AAV2(A35N)	17.8	1
AAV2(G58D)	1.1	1
AAV2 control	5.7	2

AAV2(A35N) has been stored at 4C for 1 months

FIG. 15

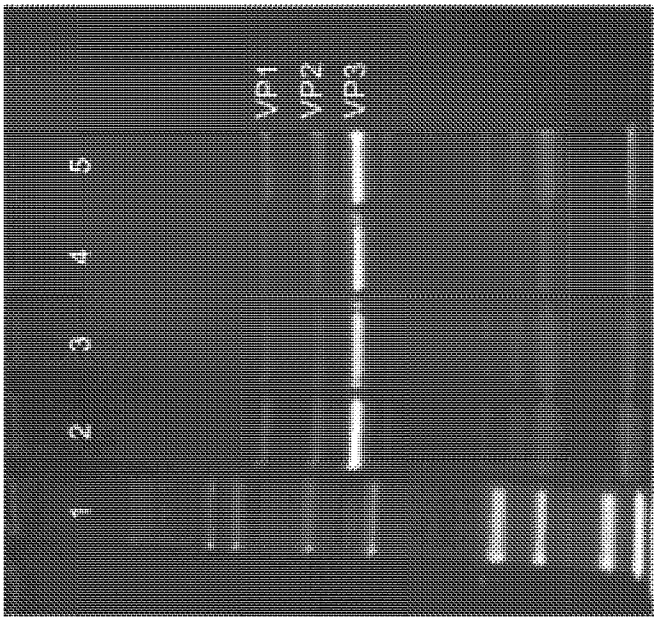

Deamidation AAV2 Mutant Vectors
Lane Sample
1 Mark12 Marker
2 deAMID-AAV2 (N57K)/CBA-eGFP X170798
3 deAMID-AAV2 (N57D)/CBA-eGFP X170799C
4 deAMID-AAV2 (N57Q)/CBA-eGFP X170799D
5 deAMID-AAV2 (A35N)/CBA-eGFP X170799E
(~5E10 VGs/Lane)
1
2
3
4
5
VP1
VP2
VP3

FIG. 16

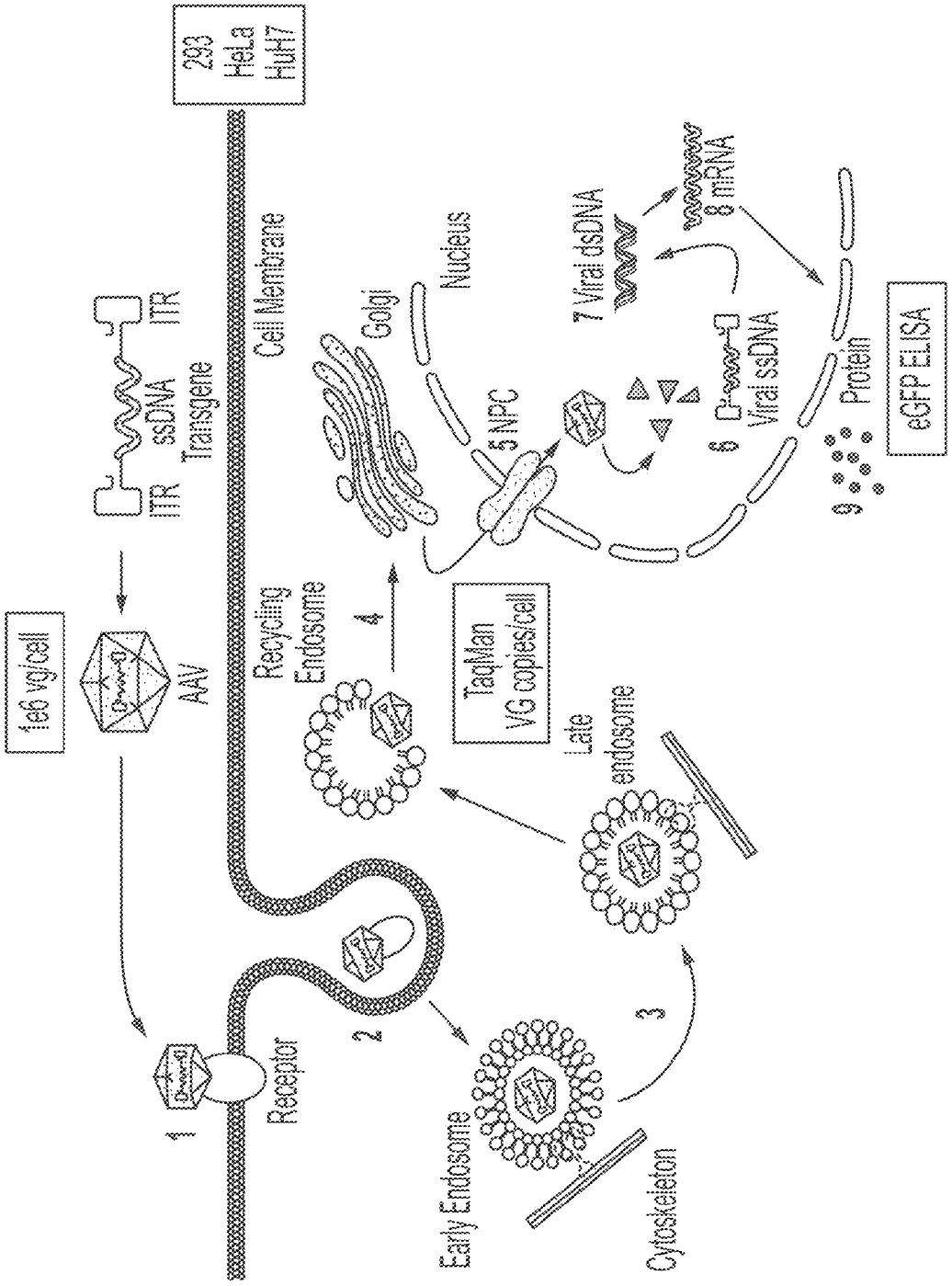

293
HeLa
HuH7
Cell Membrane
ITR
ssDNA
Transgene
ITR
1e6 vg/cell
AAV
Golgi
Nucleus
7 Viral dsDNA
8 mRNA
5 NPC
6
Viral ssDNA
9
Protein
eGFP ELISA
Recycling
Endosome
4
TaqMan
VG copies/cell
Late
endosome
1
Receptor
2
3
Early Endosome
Cytoskeleton

FIG. 17

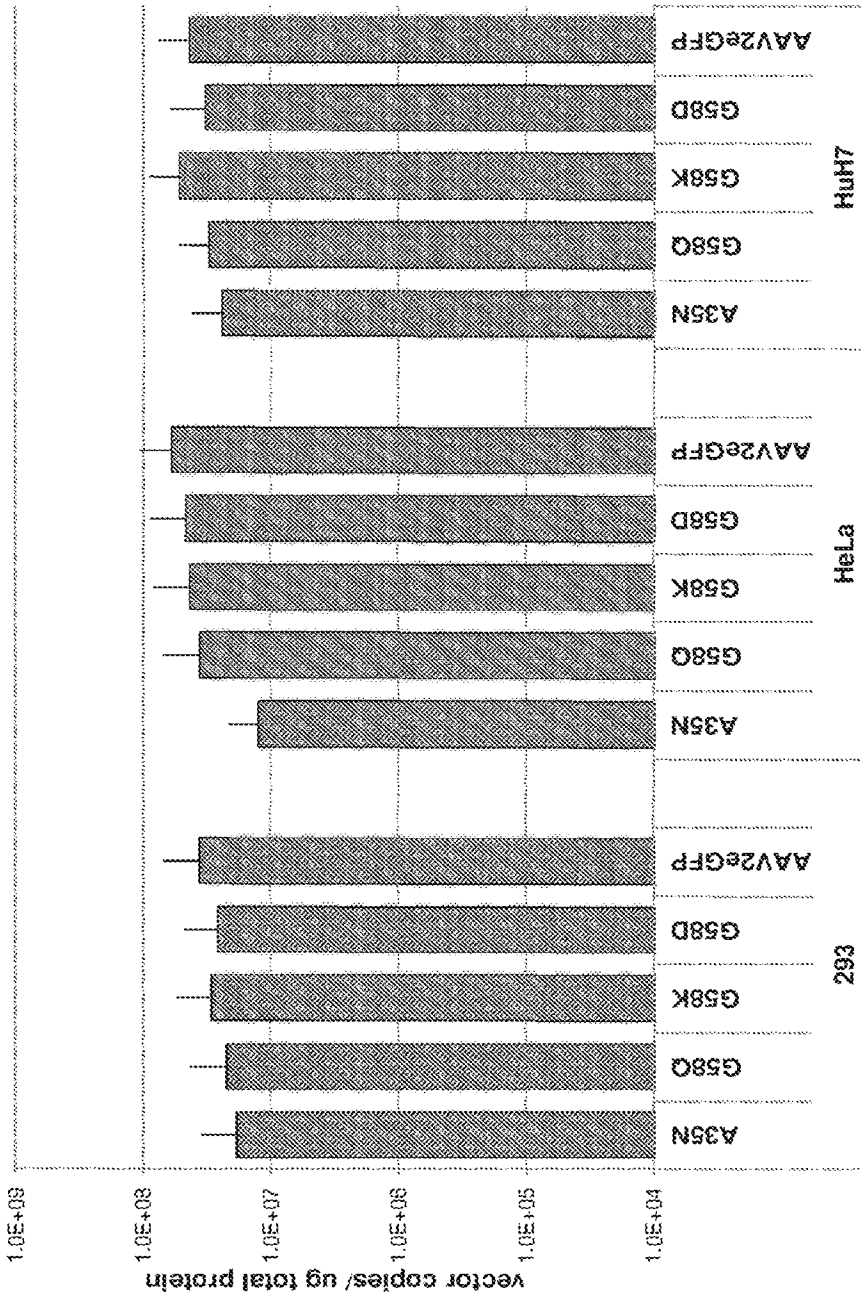

vector copies/ ug total protein
1.0E+09
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
293
HeLa
HuH7
A35N
G58Q
G58K
G58D
AAV2eGFP

FIG. 18

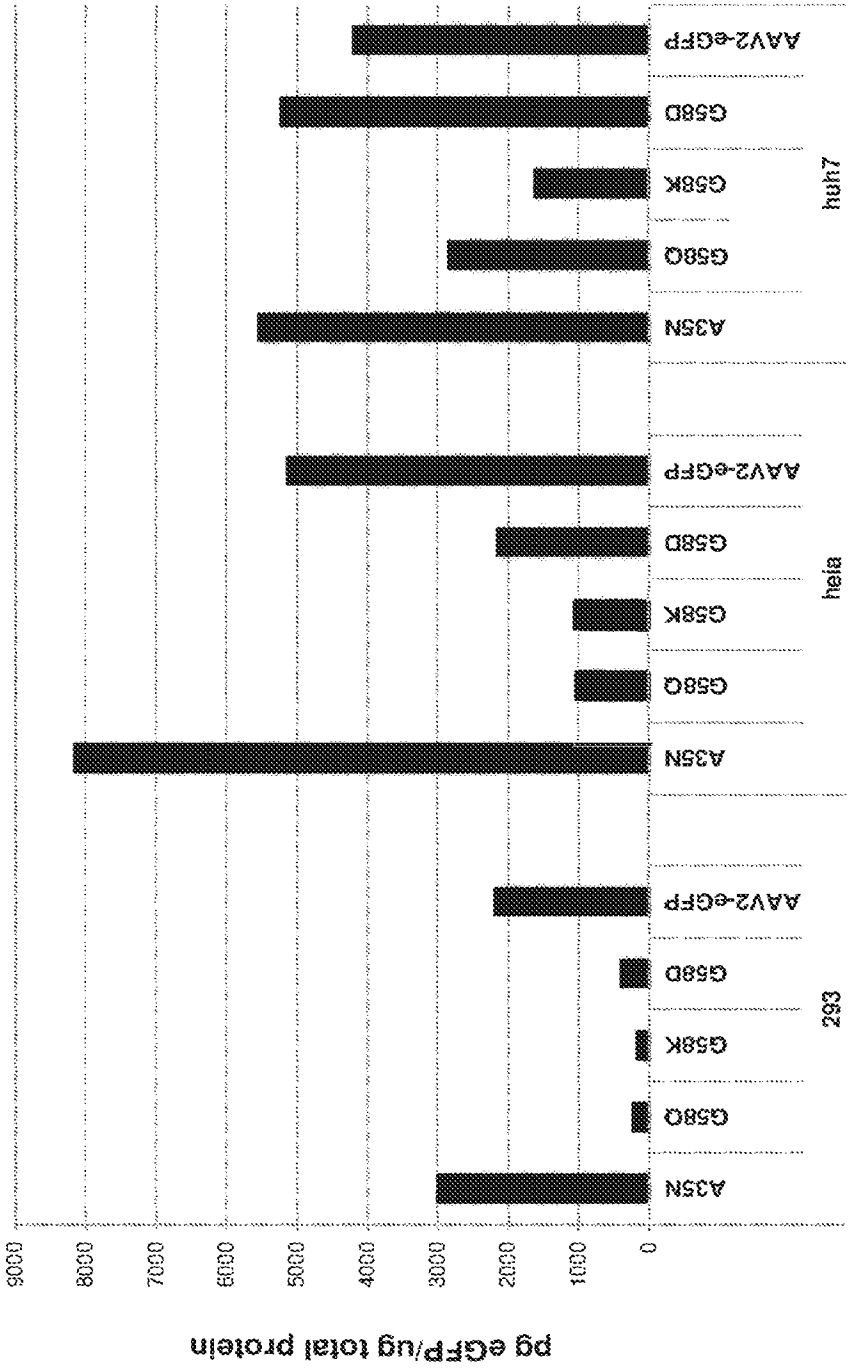

pg eGFP/ug total protein
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
293
A35N
G58Q
G58K
G58D
AAV2-eGFP
hela
A35N
G58Q
G58K
G58D
AAV2-eGFP
huh7
A35N
G58Q
G58K
G58D
AAV2-eGFP

FIG. 19

METHODS FOR DETECTING AAV

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. patent application Ser. No. 18/801,293, filed Aug. 12, 2024, which is a divisional of U.S. patent application Ser. No. 18/321,542 (U.S. Pat. No. 12,123,880), filed May 22, 2023, which is a divisional of U.S. patent application Ser. No. 16/325,653 (U.S. Pat. No. 11,698,377), which adopts the international filing date of Aug. 14, 2017, which is a National Phase application under 35 U.S.C. § 371 of International Application No. PCT/US2017/046814, filed Aug. 14, 2017, which claims the priority benefit of U.S. Provisional Application No. 62/375,314, filed Aug. 15, 2016, the disclosure of each of which is hereby incorporated by reference in its entirety.

REFERENCE TO AN ELECTRONIC SEQUENCE LISTING

The content of the electronic sequence listing (159792014103seglist.xml; Size: 56,199 bytes; and Date of Creation: Nov. 14, 2024) are herein incorporated by reference in their entirety.

FIELD OF THE INVENTION

The present invention relates to methods for serotyping and/or determining the heterogeneity of a viral particle (e.g., an adeno-associated virus (AAV) particle) using mass determination, e.g., by employing liquid chromatography/mass spectrometry (LC/MS) or liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS). In some aspects, the present invention relates to methods to improve the stability of AAV particles.

BACKGROUND OF THE INVENTION

Complete characterization of the viral capsid proteins of viral vectors (e.g., AAV vectors), including their sequence and post-translation modifications, is desired in gene therapy research and development since viral capsid proteins (VPs) are critical for viral infectivity.

Viral vector products such as recombinant Adeno-Associated Virus (rAAV) products are typically identified using molecular tools targeting the nucleic acid transgene. These methods may include polymerase chain reaction (PCR) targeting transgene-specific sequences and Restriction Fragment Length Polymorphism (RFLP) techniques. As rAAV technologies evolve, many facilities are beginning to investigate multiple AAV capsid serotypes encoding their therapeutic transgene in an effort to improve targeted tissue tropism.

Traditional molecular identification methods identify products containing unique transgenes but are unable to discern those that have differing AAV capsid serotypes. Currently, most AAV serotype identity tests are based on SDS-PAGE banding patterns, an antibody-based ELISA, or a Western blot assay. However, the banding patterns and antibodies are not specific enough to differentiate different AAV serotypes. Gel-LC/MS/MS has been reported as a capsid serotype identification method. However, this method involves multiple steps including SDS-PAGE, in-gel digestion, and LC/MS/MS and thus requires multiple days for the analysis while providing limited sequence coverage. Methods for identifying vectors such as rAAV vectors are of interest to gene therapy vectors (see, e.g., U.S. PG Pub. No. US20110275529). Thus, it would be useful to have improved methods of characterizing viral particles.

All references cited herein, including patent applications and publications, are incorporated by reference in their entirety.

BRIEF SUMMARY OF THE INVENTION

Using rAAV as an example, described herein is the use of LC/MS as an analytical tool to specifically identify different viral capsid serotypes (e.g., rAAV capsid serotypes). As part of viral characterization, LC/MS can be used to augment the molecular identification methods. This analytical combination can satisfy regulatory requirements by discerning both the identity of the product's therapeutic transgene and the identity of the capsid serotype. This method can be used e.g., as an AAV serotype identity test or to monitor viral capsid protein heterogeneity in recombinant AAV gene therapy development. It can also be used to confirm VP sequences in capsid engineering research. In addition, this technique can be used to study the impact of post translation modifications, such as N terminal acetylation of viral capsid proteins, on transfection potency and intracellular protein trafficking.

The methods described herein can also be used to design AAV particles for greater stability and/or improved transduction efficiency; for example by altering the amino acid residue at position 2 of VP1 and/or VP3 of the AAV capsid such that the amino acid at position 2 is acetylated to a higher extent compared to a wild type AAV capsid. In some embodiments, the methods can be used to design AAV particles with reduced transduction efficiency; for example by altering the amino acid residue at position 2 of VP1 and/or VP3 of the AAV capsid such that the amino acid at position 2 is deacetylated to a higher or lower extent compared to a wild type AAV capsid.

In some aspects, the invention provides a method to determine the serotype of a viral particle comprising a) denaturing the viral particle, b) subjecting the denatured viral particle to liquid chromatography/mass spectrometry (LC/MS), and c) determining the masses of one or more capsid proteins of the viral particle; wherein the specific combination of masses of the one or more capsid proteins are indicative of the virus serotype. In some embodiments, the calculated masses of the one or more capsid proteins are compared to the theoretical masses of the one or more capsid proteins of one or more virus serotypes.

In some aspects, the invention provides a method of determining the heterogeneity of a viral particle comprising a) denaturing the viral particle, b) subjecting the denatured viral particle to liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS), c) determining the masses of one or more capsid proteins of the viral particle, and d) comparing the masses of step c) with the theoretical masses of the one or more capsid proteins of the virus serotype; wherein a deviation of one or more of the masses of the one or more capsid proteins are indicative of the viral capsid heterogeneity. In some embodiments, the heterogeneity comprises one or more of mixed serotypes, variant capsids, capsid amino acid substitutions, truncated capsids, or modified capsids.

In some embodiments of the above aspects, the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography. In some embodiments, the viral particle comprises a viral vector encoding a heterologous transgene.

In some aspects, the invention provide a method to determine the serotype of a viral particle comprising a) denaturing the viral particle, b) subjecting the denatured viral particle to reduction and/or alkylation, c) subjecting the denatured viral particle to digestion to generate fragments of one or more capsid proteins of the viral particle, d) subjecting the fragments of the one or more capsid proteins to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), and e) determining the masses of fragments of the one or more capsid proteins of the viral particle; wherein the specific combination of masses of fragments of the one or more capsid proteins are indicative of the viral serotype. In some embodiments, the calculated masses of the fragments of the one or more capsid proteins are compared to the theoretical masses of fragments of the one or more capsid proteins of one or more viral serotypes.

In some aspects, the invention provides a method of determining the heterogeneity of a serotype of a viral particle comprising a) denaturing the viral particle, b) subjecting the denatured viral particle to reduction and/or alkylation, c) subjecting the denatured viral particle to digestion to generate fragments of one or more capsid proteins of the viral particle, d) subjecting the fragments of the one or more capsid proteins to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), e) determining the masses of fragments of the one or more capsid proteins of the viral particle, and f) comparing the masses of step e) with the theoretical masses of fragments of the one or more capsid proteins of the viral serotype; wherein a deviation of one or more of the masses of the one or more capsid proteins are indicative of the viral capsid heterogeneity. In some embodiments, the heterogeneity comprises one or more of mixed serotypes, variant capsids, capsid amino acid substitutions, truncated capsids, or modified capsids. In some embodiments, the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography.

As shown herein the methods can be performed in the absence of a gel separation step (e.g., sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)).

In some embodiments of the above aspects and embodiments, the viral particle comprises a viral vector encoding a heterologous transgene. In some embodiments, the viral particle belongs to a viral family selected from the group consisting of Adenoviridae, Parvoviridae, Retroviridae, Baculoviridae, and Herpesviridae. In some embodiments, the viral particle belongs to a viral genus selected from the group consisting of Atadenovirus, Aviadenovirus, Ichtadenovirus, Mastadenovirus, Siadenovirus, Ambidensovirus, Brevidensovirus, Hepandensovirus, Iteradensovirus, Penstyldensovirus, Amdoparvovirus, Aveparvovirus, Bocaparvovirus, Copiparvovirus, Dependoparvovirus, Erythroparvovirus, Protoparvovirus, Tetraparvovirus, Alpharetrovirus, Betaretrovirus, Deltaretrovirus, Epsilonretrovirus, Gammaretrovirus, Lentivirus, Spumavirus, Alphabaculovirus, Betabaculovirus, Deltabaculovirus, Gammabaculovirus, Iltovirus, Mardivirus, Simplexvirus, Varicellovirus, Cytomegalovirus, Muromegalovirus, Proboscivirus, Roseolovirus, Lymphocryptovirus, Macavirus, Percavirus, and Rhadinovirus.

In some aspects, the invention provides a method to determine the serotype of an adeno-associated virus (AAV) particle comprising a) denaturing the AAV particle, b) subjecting the denatured AAV particle to liquid chromatography/mass spectrometry (LC/MS), and c) determining the masses of VP1, VP2 and VP3 of the AAV particle; wherein the specific combination of masses of VP1, VP2 and VP3 are indicative of the AAV serotype. In some embodiments, the calculated masses of VP1, VP2 and VP3 are compared to the theoretical masses of VP1, VP2 and VP3 of one or more AAV serotypes.

In some aspects, the invention provides a method of determining the heterogeneity of an AAV particle comprising a) denaturing the AAV particle, b) subjecting the denatured AAV particle to liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS), c) determining the masses of VP1, VP2 and VP3 of the AAV particle, and d) comparing the masses of step c) with the theoretical masses of VP1, VP2 and VP3 of the AAV serotype; wherein a deviation of one or more of the masses of VP1, VP2 or VP3 are indicative of the AAV capsid heterogeneity. In some embodiments, the heterogeneity comprises one or more of mixed serotypes, variant capsids, capsid amino acid substitutions, truncated capsids, or modified capsids.

In some embodiments of the above aspects and embodiments, the AAV particle is denatured with acetic acid, guanidine hydrochloride and/or an organic solvent. In some embodiments, the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography. In some embodiments, the liquid chromatography is reverse phase liquid chromatography. In some embodiments, the reverse phase chromatography is a C4 or C8 reverse chromatography. In some embodiments, the chromatography uses a mobile phase A comprising formic acid in water. In some embodiments, the mobile phase A comprises about 0.1% formic acid. In some embodiments, the chromatography comprises a mobile phase B comprising formic acid in acetonitrile. In some embodiments, the mobile phase B comprises about 0.1% formic acid. In some embodiments, the proportion of mobile phase B in the chromatography increases over time. In some embodiments, the proportion of mobile phase B in the chromatography increases in a stepwise manner. In some embodiments, mobile phase B increases from about 10% to about 20%, from about 20% to about 30%, and from about 30% to about 38%. In some embodiments, mobile phase B increases from about 10% to about 20% in about 6 minutes, from about 20% to about 30% in about 10 minutes, and from about 30% to about 38% in about 40 minutes. In some embodiments, the liquid chromatography is ultra-performance liquid chromatography (UPLC).

In some embodiments of the above aspects and embodiments, the mass spectrometry comprises a capillary voltage of about 3.5 kV. In some embodiments, the mass spectrometry comprises a sampling cone voltage of about 45 V. In some embodiments, the mass spectrometry comprises assisted calibration. In some embodiments, sodium iodide is used as a calibrant.

In some embodiments of the above aspects and embodiments, the N-terminus of VP1 and/or VP3 is acetylated. In some embodiments, the AAV particle is a recombinant AAV (rAAV) particle. In some embodiments, the AAV particle comprises an AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an AAV LK03 capsid, an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid, an AAV DJ8 capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, or a mouse AAV capsid

rAAV2/HBoV1 (chimeric AAV/human bocavirus virus 1). In some embodiments, the AAV capsid comprises a tyrosine mutation or a heparin binding mutation. In some embodiments, the masses of VP1, VP2, and VP3 are compared to the theoretical masses of one or more of AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an AAV LK03 capsid, an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid, an AAV DJ8 capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, or a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV/human bocavirus virus 1), an AAV2HBKO capsid, an AAVPHP.B capsid or an AAVPHP.eB capsid.

In some embodiments of the above aspects and embodiments, the viral particle comprises an AAV1 ITR, an AAV2 ITR, an AAV3 ITR, an AAV4 ITR, an AAV5 ITR, an AAV6 ITR, an AAV7 ITR, an AAV8 ITR, an AAVrh8 ITR, an AAV9 ITR, an AAV10 ITR, an AAVrh10 ITR, an AAV11 ITR, or an AAV12 ITR. In some embodiments, the AAV particle comprises an AAV vector encoding a heterologous transgene.

In some aspects, the invention provides a method to determine the serotype of an adeno-associated virus (AAV) particle comprising a) denaturing the AAV particle, b) subjecting the denatured AAV particle to reduction and/or alkylation, c) subjecting the denatured AAV particle to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle, d) subjecting the fragments of VP1, VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), and e) determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle; wherein the specific combination of masses of fragments of VP1, VP2 and VP3 are indicative of the AAV serotype. In some embodiments, the calculated masses of the fragments of VP1, VP2 and/or VP3 are compared to the theoretical masses of fragments of VP1, VP2 and/or VP3 of one or more AAV serotypes.

In some aspects, the invention provides a method of determining the heterogeneity of a serotype of an AAV particle comprising a) denaturing the AAV particle, b) subjecting the denatured AAV particle to reduction and/or alkylation, c) subjecting the denatured AAV particle to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle, d) subjecting the fragments of VP1, VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), e) determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle, and f) comparing the masses of step e) with the theoretical masses of fragments of VP1, VP2 and VP3 of the AAV serotype; wherein a deviation of one or more of the masses of VP1, VP2 or VP3 are indicative of the AAV capsid heterogeneity. In some embodiments, the heterogeneity comprises one or more of mixed serotypes, variant capsids, capsid amino acid substitutions, truncated capsids, or modified capsids. In some embodiments, the reduction is by subjecting the AAV particle to dithiothreitol, beta-mercaptoethanol, or tris(2-carboxyethyl)phosphine (TCEP). In some embodiments, the alkylation is by subjecting the AAV particle to iodoacetic acid, iodoacetamide, or 4-vinylpyridine. In some embodiments, the digestion is an enzymatic digestion or a chemical digestion. In some embodiments, the enzymatic digestion is an endopeptidase digestion. In some embodiments, the enzymatic digestion is a trypsin digestion, a LysC digestion, an Asp-N digestion or a Glu-C digestion. In some embodiments, the chemical digestion is cyanogen bromide digestion or an acid digestion. In some embodiments, the AAV particle is denatured with acetic acid, guanidine hydrochloride and/or an organic solvent.

In some embodiments of the above aspects and embodiments, the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography. In some embodiments, the liquid chromatography is reverse phase liquid chromatography. In some embodiments, the reverse phase chromatography is a C18 reverse chromatography. In some embodiments, the chromatography uses a mobile phase A comprising formic acid in water. In some embodiments, the mobile phase A comprises about 0.1% formic acid. In some embodiments, the chromatography comprises a mobile phase B comprising formic acid in acetonitrile. In some embodiments, the mobile phase B comprises about 0.1% formic acid. In some embodiments, the proportion of mobile phase B in the chromatography increases over time. In some embodiments, mobile phase B increases from about 2% to about 60%. In some embodiments, mobile phase B increases from about 2% to about 60% in about 121 minutes. In some embodiments, the liquid chromatography is high-performance liquid chromatography (HPLC).

In some embodiments of the above aspects and embodiments, the mass spectrometry comprises a capillary voltage of about 3.5 kV. In some embodiments, the mass spectrometry comprises a sampling cone voltage of about 45 V. In some embodiments, the mass spectrometry comprises assisted calibration. In some embodiments, sodium iodide is used as a calibrant.

In some embodiments of the above aspects and embodiments, the N-terminus of VP1 and/or VP3 is acetylated. In some embodiments, the AAV particle is a recombinant AAV (rAAV) particle. In some embodiments, the AAV particle comprises an AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an AAV LK03 capsid, an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid, an AAV DJ8 capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, or a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV/human bocavirus virus 1). In some embodiments, the AAV capsid comprises a tyrosine mutation or a heparin binding mutation. In some embodiments, the masses of VP1, VP2, and VP3 are compared to the theoretical masses of one or more of AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an AAV LK03 capsid, an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid, an AAV DJ8 capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, or a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV/human bocavirus virus 1).

In some embodiments of the above aspects and embodiments, the viral particle comprises an AAV1 ITR, an AAV2 ITR, an AAV3 ITR, an AAV4 ITR, an AAV5 ITR, an AAV6 ITR, an AAV7 ITR, an AAV8 ITR, an AAVrh8 ITR, an

AAV9 ITR, an AAV10 ITR, an AAVrh10 ITR, an AAV11 ITR, or an AAV12 ITR. In some embodiments, the AAV particle comprises an AAV vector encoding a heterologous transgene.

In some embodiments, the invention provides a recombinant AAV (rAAV) particle comprising an amino acid substitution at amino acid residue 2 of VP1 and/or VP3; wherein the amino acid substitution at amino acid residue 2 of VP1 and/or VP3 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of VP1 and/or VP3 of the parent AAV particle. In some embodiments, the substitution results in a higher frequency of N-terminal acetylation or a lower frequency of N-terminal acetylation. In some embodiments, the rAAV particle comprises an amino acid substitution at amino acid residue 2 of VP1; wherein the amino acid substitution at amino acid residue 2 of VP1 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of VP1 of the parent AAV particle. In some embodiments, the rAAV particle comprises an amino acid substitution at amino acid residue 2 of VP3; wherein the amino acid substitution at amino acid residue 2 of VP3 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of VP3 of the parent AAV particle. In some embodiments, amino acid residue 2 is substituted with Cys, Ser, Thr, Val, Gly, Asn, Asp, Glu, Ile, Leu, Phe, Gln, Lys, Met, Pro or Tyr. In some embodiments, amino acid residue 2 is substituted with Ser, Asp or Glu.

In some embodiments of the above aspects and embodiments, the AAV particle comprises an AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV2/2-7m8, AAV DJ, an AAV DJ8 capsid, AAV2 N587A, AAV2 E548A, AAV2 N708A, AAV V708K, goat AAV, AAV1/AAV2 chimeric, bovine AAV, mouse AAV, rAAV2/HBoV1, AAV2HBKO, AAVPHP.B, or AAVPHP.eB serotype capsid. In some embodiments, the AAV capsid further comprises a tyrosine mutation or a heparin binding mutation. In some embodiments, the rAAV particle comprises a rAAV vector. In some embodiments, the rAAV vector comprises one or more AAV ITRs. In some embodiments, the rAAV vector comprises an AAV1 ITR, an AAV2 ITR, an AAV3 ITR, an AAV4 ITR, an AAV5 ITR, an AAV6 ITR, an AAV7 ITR, an AAV8 ITR, an AAVrh8 ITR, an AAV9 ITR, an AAV10 ITR, an AAVrh10 ITR, an AAV11 ITR, or an AAV12 ITR. In some embodiments, the AAV capsid and the AAV ITRs are derived from the same serotype. In some embodiments, the AAV capsid and the AAV ITRs are derived from different serotypes. In some embodiments, the AAV particle comprises an AAV vector encoding a heterologous transgene flanked by one or more AAV ITRs.

In some embodiments of the above aspects and embodiments, the rAAV vector is a self-complementary vector. In some embodiments, the rAAV vector comprises first nucleic acid sequence encoding the transgene and a second nucleic acid sequence encoding a complement of the transgene, wherein the first nucleic acid sequence can form intrastrand base pairs with the second nucleic acid sequence along most or all of its length. In some embodiments, the first nucleic acid sequence and the second nucleic acid sequence are linked by a mutated AAV ITR, wherein the mutated AAV ITR comprises a deletion of the D region and comprises a mutation of the terminal resolution sequence.

In some embodiments of the above aspects and embodiments, the rAAV particle is produced by transfecting a host cell with nucleic acid encoding the rAAV vector and nucleic acid encoding AAV rep and cap functions, and providing nucleic acid encoding AAV helper functions. In some embodiments, the AAV helper functions are provided by transfecting the host cell with nucleic acid encoding the AAV helper functions. In some embodiments, the AAV helper functions are provided by infecting the host cell with an AAV helper virus that provides the AAV helper functions. In some embodiments, the AAV helper virus is an adenovirus, a herpes simplex virus or a baculovirus. In some embodiments, the rAAV particle is produced by an AAV producer cell comprising nucleic acid encoding the rAAV vector and nucleic acid encoding AAV rep and cap functions, and providing nucleic acid encoding AAV helper functions. In some embodiments, the AAV producer cell comprises nucleic acid encoding AAV helper functions. In some embodiments, the AAV helper functions are provided by infecting the AAV producer cells with an AAV helper virus that provides the AAV helper functions. In some embodiments, the AAV helper virus is an adenovirus, a herpes simplex virus, or a baculovirus. In some embodiments, the AAV cap functions provide an amino acid substitution at amino acid residue 2 of VP1 and/or VP3, wherein the amino acid substitution at amino acid residue 2 of VP1 and/or VP3 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of VP1 and/or VP3 of the parent AAV particle.

In some aspects, the invention provides a pharmaceutical composition comprising the rAAV particle as described herein. In some aspects, the invention provides a kit comprising the rAAV particle or the pharmaceutical composition as described herein. In some aspects, the invention provides an article of manufacture comprising the rAAV particle or the pharmaceutical composition as described herein.

In some aspects, the invention provides as AAV capsid protein comprising an amino acid substitution at amino acid residue 2 of a parent AAV capsid protein; wherein the amino acid substitution at amino acid residue 2 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of the parent AAV capsid protein. In some embodiments, the substitution results in a higher frequency of N-terminal acetylation or a lower frequency of N-terminal acetylation. In some embodiments, the AAV capsid protein is VP1 or VP3. In some embodiments, amino acid residue 2 of the AAV capsid protein is substituted with Cys, Ser, Thr, Val, Gly, Asn, Asp, Glu, Ile, Leu, Phe, Gln, Lys, Met, Pro or Tyr. In some embodiments, amino acid residue 2 of the AAV capsid protein is substituted with Ser, Asp or Glu. In some embodiments, the amino acid substitution results in less deamidation of the AAV capsid.

In some embodiments of the above aspects and embodiments, the AAV particle comprises an AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV2/2-7m8, AAV DJ, an AAV DJ8 capsid, AAV2 N587A, AAV2 E548A, AAV2 N708A, AAV V708K, goat AAV, AAV1/AAV2 chimeric, bovine AAV, mouse AAV, rAAV2/HBoV1, AAV2HBKO, AAVPHP.B, or AAVPHP.eB serotype capsid. In some embodiments, the AAV capsid further comprises a tyrosine mutation or a heparin binding mutation.

In some aspects, the invention provides a method of improving stability of a rAAV particle comprising substituting amino acid residue 2 of VP1 and/or VP3 of a parent VP1 and/or VP3; wherein the substituting amino acid residue 2 alters N-terminal acetylation of VP1 and/or VP3, as compared to amino acid residue 2 of the parent VP1 and/or VP3. In some aspects, the invention provides a method of

improving assembly of rAAV particles in a cell comprising substituting amino acid residue 2 of VP1 and/or VP3 or a parental VP1 and/or VP3; wherein substituting amino acid at position 2 alters N-terminal acetylation of VP1 and/or VP3, as compared to amino acid residue 2 of the parent VP1 and/or VP3. In some aspects, the invention provides a method of improving the transduction of rAAV particles in a cell comprising substituting amino acid residue 2 of VP1 and/or VP3 or a parental VP1 and/or VP3; wherein substituting amino acid residue 2 alters N-terminal acetylation of VP1 and/or VP3, as compared to amino acid residue 2 of the parent VP1 and/or VP3. In some embodiments, the substituted amino acid results in a higher frequency of N-terminal acetylation or a lower frequency of N-terminal acetylation. In some embodiments, the amino acid substitution at amino acid residue 2 of VP1 is substituted. In some embodiments, the amino acid substitution at amino acid residue 2 of VP3 is substituted. In some embodiments, amino acid residue 2 is substituted with Cys, Ser, Thr, Val, Gly, Asn, Asp, Glu, Ile, Leu, Phe, Gln, Lys, Met, Pro or Tyr. In some embodiments, amino acid residue 2 is substituted with Ser, Asp or Glu. In some aspects, the invention provides a method of reducing the transduction of rAAV particles in a cell comprising substituting amino acid residue 2 of VP1 and/or VP3; wherein the substituted amino acid at position 2 alters N-terminal acetylation of VP1 and/or VP3, as compared to amino acid residue 2 of the parent VP1 and/or VP3.

In some embodiments of the above aspects and embodiments, the AAV particle comprises an AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV2/2-7m8, AAV DJ, an AAV DJ8 capsid, AAV2 N587A, AAV2 E548A, AAV2 N708A, AAV V708K, goat AAV, AAV1/AAV2 chimeric, bovine AAV, mouse AAV, rAAV2/HBoV1, AAV2HBKO, AAVPHP.B, or AAVPHP.eB serotype capsid. In some embodiments, the AAV capsid further comprises a tyrosine mutation or a heparin binding mutation. In some embodiments, the rAAV particle comprises a rAAV vector. In some embodiments, the rAAV vector comprises one or more AAV ITRs. In some embodiments, the rAAV vector comprises an AAV1 ITR, an AAV2 ITR, an AAV3 ITR, an AAV4 ITR, an AAV5 ITR, an AAV6 ITR, an AAV7 ITR, an AAV8 ITR, an AAVrh8 ITR, an AAV9 ITR, an AAV10 ITR, an AAVrh10 ITR, an AAV11 ITR, or an AAV12 ITR. In some embodiments, the AAV capsid and the AAV ITRs are derived from the same serotype. In some embodiments, the AAV capsid and the AAV ITRs are derived from different serotypes. In some embodiments, the AAV particle comprises an AAV vector encoding a heterologous transgene flanked by one or more AAV ITRs.

In some embodiments of the above aspects and embodiments, the rAAV vector is a self-complementary vector. In some embodiments, the rAAV vector comprises first nucleic acid sequence encoding the transgene and a second nucleic acid sequence encoding a complement of the transgene, wherein the first nucleic acid sequence can form intrastrand base pairs with the second nucleic acid sequence along most or all of its length. In some embodiments, the first nucleic acid sequence and the second nucleic acid sequence are linked by a mutated AAV ITR, wherein the mutated AAV ITR comprises a deletion of the D region and comprises a mutation of the terminal resolution sequence.

In some embodiments of the above aspects and embodiments, the rAAV particle is produced by transfecting a host cell with nucleic acid encoding the rAAV vector and nucleic acid encoding AAV rep and cap functions, and providing nucleic acid encoding AAV helper functions. In some embodiments, the AAV helper functions are provided by transfecting the host cell with nucleic acid encoding the AAV helper functions. In some embodiments, the AAV helper functions are provided by infecting the host cell with an AAV helper virus that provides the AAV helper functions. In some embodiments, the AAV helper virus is an adenovirus, a herpes simplex virus or a baculovirus. In some embodiments, the rAAV particle is produced by an AAV producer cell comprising nucleic acid encoding the rAAV vector and nucleic acid encoding AAV rep and cap functions, and providing nucleic acid encoding AAV helper functions. In some embodiments, the AAV producer cell comprises nucleic acid encoding AAV helper functions. In some embodiments, the AAV helper functions are provided by infecting the AAV producer cells with an AAV helper virus that provides the AAV helper functions. In some embodiments, the AAV helper virus is an adenovirus, a herpes simplex virus, or a baculovirus. In some embodiments, the AAV cap functions provide an amino acid substitution at amino acid residue 2 of VP1 and/or VP3, wherein the amino acid substitution at amino acid residue 2 of VP1 and/or VP3 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of VP1 and/or VP3 of the parent AAV particle.

In some aspects, the invention provides a recombinant AAV (rAAV) particle comprising one or more amino acid substitutions at amino acid residue A35, N57, G58, N382, G383, N511, G512, N715, or G716 of VP1 or VP3 of a parent particle, residue numbering based on VP1 of AAV2; wherein the one or more amino acid substitutions alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some embodiments, the one or more amino acid substitution is at amino acid residue A35, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3 and alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some embodiments, the one or more amino acid substitutions comprises a substitution with Asp at N57 of VP1, N382 of VP3, N511 of VP3, or N715 of VP3; and results in a higher frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some embodiments, the one or more amino acid substitutions comprise a N57K or a N57Q substitution and results in a lower frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some embodiments, the one or more amino acid substitution comprise a substitution with Asp at A35 of VP1 and results in a higher frequency of deamidation as compared to deamidation of VP1 of the parent AAV particle. In some embodiments, the one or more amino acid substitutions is at G58 of VP1, G383 of VP3, G512 of VP3, or G716 of VP3 and results in a lower frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some embodiments, the G58 of VP1 is substituted with Asp. In some embodiments, the rAAV particle is an AAV1 particle or an AAV2 particle.

In some aspects, the invention provides pharmaceutical compositions comprising AAV particles comprising one or more amino acid substitutions at amino acid residue A35, N57, G58, N382, G383, N511, G512, N715, or G716 of VP1 or VP3, residue numbering based on VP1 of AAV2; wherein the amino acid substitution alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some aspects, the invention provides kits comprising AAV particles or compositions comprising AAV

particles wherein the AAV particles comprise one or more amino acid substitutions at amino acid residue A35, N57, G58, N382, G383, N511, G512, N715, or G716 of VP1 or VP3, residue numbering based on VP1 of AAV2; wherein the amino acid substitution alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some aspects, the invention provides articles of manufacture comprising AAV particles or compositions comprising AAV particles wherein the AAV particles comprise one or more amino acid substitutions at amino acid residue A35, N57, G58, N382, G383, N511, G512, N715, or G716 of VP1 or VP3, residue numbering based on VP1 of AAV2; wherein the amino acid substitution alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some aspects, the invention provides an AAV capsid protein comprising an amino acid substitution of a parent AAV capsid protein; wherein the amino acid substitution alters deamidation of the capsid compared to the parent AAV capsid protein.

In some aspects, the invention provides a method of improving the stability of a rAAV particle comprising substituting one or more amino acid residues, wherein the one or more amino acid residues is A35, N57, G58, N382, G383, N511, G512, N715, or G716, residue numbering based on VP1 of AAV2; wherein the amino acid substitution alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some aspects, the invention provides a method of improving the assembly of rAAV particles in a cell comprising substituting one or more amino acid residues, wherein the one or more amino acid residues is A35, N57, G58, N382, G383, N511, G512, N715, or G716, residue numbering based on VP1 of AAV2; wherein the amino acid substitution alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some aspects, the invention provides a method of improving the transduction of rAAV particles in a cell comprising substituting one or more amino acid residues, wherein the one or more amino acid residues is A35, N57, G58, N382, G383, N511, G512, N715, or G716, residue numbering based on VP1 of AAV2; wherein the amino acid substitution alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some embodiments, the one or more amino acid substitutions is at A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3; wherein the amino acid substitution alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some embodiments, the parental Ala residue at position 35 of VP1 is substituted with Asn. In some embodiments, the parental Gly residue at position 58 of VP1 is substituted with Asp. In some embodiments, the rAAV particle is an AAV1 particle or an AAV2 particle.

In some embodiments, the invention provides a method of improving the stability, assembly and/or transduction efficiency of a rAAV particle comprising substituting one or more amino acid residues, wherein the one or more amino acid residues is A35, N57, G58, N382, G383, N511, G512, N715, or G716, residue numbering based on VP1 of AAV2; wherein the amino acid substitution alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle as described above, wherein the AAV particle comprises an AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV2/2-7m8, AAV DJ, an AAV DJ8 capsid, AAV2 N587A, AAV2 E548A, AAV2 N708A, AAV V708K, goat AAV, AAV1/AAV2 chimeric, bovine AAV, mouse AAV, or rAAV2/HBoV1 serotype capsid. In some embodiments, the AAV capsid further comprises a tyrosine mutation or a heparin binding mutation. In some embodiments, the rAAV particle comprises a rAAV vector. In some embodiments, the rAAV vector comprises one or more AAV ITRs. In some embodiments, the rAAV vector comprises an AAV1 ITR, an AAV2 ITR, an AAV3 ITR, an AAV4 ITR, an AAV5 ITR, an AAV6 ITR, an AAV7 ITR, an AAV8 ITR, an AAVrh8 ITR, an AAV9 ITR, an AAV10 ITR, an AAVrh10 ITR, an AAV11 ITR, or an AAV12 ITR. In some embodiments, the AAV capsid and the AAV ITRs are derived from the same serotype. In some embodiments, the AAV capsid and the AAV ITRs are derived from different serotypes. In some embodiments, the AAV particle comprises an AAV vector encoding a heterologous transgene flanked by one or more AAV ITRs.

In some embodiments of the above aspects and embodiments, the rAAV vector is a self-complementary vector. In some embodiments, the rAAV vector comprises first nucleic acid sequence encoding the transgene and a second nucleic acid sequence encoding a complement of the transgene, wherein the first nucleic acid sequence can form intrastrand base pairs with the second nucleic acid sequence along most or all of its length. In some embodiments, the first nucleic acid sequence and the second nucleic acid sequence are linked by a mutated AAV ITR, wherein the mutated AAV ITR comprises a deletion of the D region and comprises a mutation of the terminal resolution sequence.

In some embodiments of the above aspects and embodiments, the rAAV particle is produced by transfecting a host cell with nucleic acid encoding the rAAV vector and nucleic acid encoding AAV rep and cap functions, and providing nucleic acid encoding AAV helper functions. In some embodiments, the AAV helper functions are provided by transfecting the host cell with nucleic acid encoding the AAV helper functions. In some embodiments, the AAV helper functions are provided by infecting the host cell with an AAV helper virus that provides the AAV helper functions. In some embodiments, the AAV helper virus is an adenovirus, a herpes simplex virus or a baculovirus. In some embodiments, the rAAV particle is produced by an AAV producer cell comprising nucleic acid encoding the rAAV vector and nucleic acid encoding AAV rep and cap functions, and providing nucleic acid encoding AAV helper functions. In some embodiments, the AAV producer cell comprises nucleic acid encoding AAV helper functions. In some embodiments, the AAV helper functions are provided by infecting the AAV producer cells with an AAV helper virus that provides the AAV helper functions. In some embodiments, the AAV helper virus is an adenovirus, a herpes simplex virus, or a baculovirus. In some embodiments, the AAV cap functions provide an amino acid substitution of VP1 and/or VP3, wherein the amino acid substitution modulated deamidation of the capsid compared to the parent AAV particle.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. **1**A-D provide total ion Chromatograms of LC/MS of AAV2 VPs. FIG. **1**A: 10 cm long BEH C4 column with 1.7%/min gradient, FIG. **1**B: 10 cm long BEH C4 column with 0.5%/min gradicnt; FIG. **1**C: 15 cm long BEH C4 column with 0.5%/min gradient, FIG. **1**D: 15 cm long BEH C8 column with 0.5%/min gradient.

FIGS. **2**A-**2**B provide deconvoluted mass spectra from FIG. **1**D peak 1 (FIG. **2**A) and FIG. **1**D peak 2 (FIG. **2**B).

FIG. **3** provides the sequence coverage of AAV2 VP1 (SEQ ID NO:3): green, tryptic peptides, blue, Lys-C peptides, pink, Asp-N peptides.

FIGS. **4**A-**4**C provide MS/MS spectra of AAV2 VP N-terminal peptides. FIG. **4**A: VP1 N-terminal tryptic peptide A(Ac)ADGYLPDWLEDTLSEGIR (SEQ ID NO: 4), FIG. **4**B VP2 N-terminal Asp-N peptide APGKKRPVEHSPVEP (SEQ ID NO: 15). FIG. **4**C: VP-3 N-terminal Asp-N derived peptide A(Ac)TGSGAPM (SEQ ID NO: 5).

FIG. **5**A-**5**C provides the sequence alignment of 13 AAV serotypes black letter/white background: non-similar; blue letter/blue background: conservative; black letter/green background: block of similar; red letter/yellow background: identical; green letter/white background: weakly similar. AAVRh10 (SEQ ID NO: 17); AAV10 (SEQ ID NO: 18); AAV8 (SEQ ID NO: 19); AAV7 (SEQ ID NO: 20); AAV1 (SEQ ID NO: 21); AAV6 (SEQ ID NO: 22); AAV2 (SEQ ID NO: 23); AAV3 (SEQ ID NO: 24); AAV11 (SEQ ID NO: 25); AAV12 (SEQ ID NO: 26); AAV4 (SEQ ID NO: 27); AAV5 (SEQ ID NO: 28); AAV9 (SEQ ID NO: 29); Consensus (SEQ ID NO: 30).

FIGS. **6**A-**6**B show the results of LC/MS/MS analysis comparing the percentage of deamidation in AAV1 and AAV2 particles produced by the TTx and PCL methods. The T9 peptide YLGPFNGLDK (SEQ ID NO: 9) was used to monitor potential deamidation site N57 in both AAV1 and AAV2.

FIGS. **7**A-**7**B show the results of LC/MS/MS analysis comparing the percentage of deamidation in AAV1 and AAV2 particles produced by the TTx and PCL methods. The T49 peptides YNLNGR (SEQ ID NO: 11) and YHLNGR (SEQ ID NO: 12) were used to monitor potential deamidation site N511 in AAV1 and AAV2, respectively.

FIGS. **8**A-**8**B show the results of LC/MS/MS analysis comparing the percentage of deamidation in AAV1 and AAV2 particles produced by the TTx and PCL methods. The T67 peptides SANVDFTVDNNGLYTEPR (SEQ ID NO: 13) and SVNVDFTVDTNGVYSEPR (SEQ ID NO: 14) were used to monitor potential deamidation site N715 in AAV1 and AAV2, respectively.

FIG. **9** shows the results of SYPRO protein gel analysis of production and VP1:VP2:VP3 ratio of AAV5 deacetylated mutant variants.

FIG. **10** illustrates an in vitro transduction assay for testing transduction efficiency of AAV5 deacetylated variants.

FIG. **11** shows the efficiency of cell entry by the indicated AAV5 deacetylated variants or parental unmodified AAV5, as measured by vector genome copies/μg protein. Three cell lines were used: 293, HeLa, and HuH7.

FIG. **12** shows eGFP expression (as measured by ELISA) by cells transduced with the indicated AAV5 deacetylated variants as compared to transduction with parental unmodified AAV5. Three cell lines were used: 293, HeLa, and HuH7.

FIG. **13** provides the sequence alignment of 13 AAV serotypes, highlighting the conserved N57G58 deamidation site and the A35 residue in AAV2. AAVRh10 (SEQ ID NO: 31); AAV10 (SEQ ID NO: 31); AAV8 (SEQ ID NO: 32); AAV7 (SEQ ID NO: 33); AAV1 (SEQ ID NO: 31); AAV6 (SEQ ID NO: 31); AAV2 (SEQ ID NO: 34); AAV3 (SEQ ID NO: 35); AAV11 (SEQ ID NO: 31); AAV12 (SEQ ID NO: 36); AAV4 (SEQ ID NO: 37); AAV5 (SEQ ID NO: 38); AAV9 (SEQ ID NO: 39); Consensus (SEQ ID NO: 40).

FIG. **14** shows a protein gel of VP1, VP2, and VP3 capsid proteins from AAV1 or AAV2 particles produced by the PCL or TTx method. *highlights the truncated VP1 (tVP1) protein.

FIG. **15** shows the results of LC/MS analysis of deamidation of the indicated AAV2 mutants, as compared to control AAV2 capsids.

FIG. **16** shows the results of SYPRO protein gel analysis of production and VP1:VP2:VP3 ratio of AAV2 deamidation mutant variants.

FIG. **17** illustrates an in vitro transduction assay for testing transduction efficiency of AAV2 deamidation variants.

FIG. **18** shows the efficiency of cell entry by the indicated AAV2 deamidation variants or parental unmodified AAV2, as measured by vector genome copies/μg protein. Three cell lines were used: 293, HeLa, and HuH7.

FIG. **19** shows eGFP expression (as measured by ELISA) by cells transduced with the indicated AAV2 deamidation variants as compared to transduction with parental unmodified AAV2. Three cell lines were used: 293, HeLa, and HuH7.

DETAILED DESCRIPTION

In some aspects, the invention provides a method to determine the serotype of an adeno-associated virus (AAV) particle(s) comprising: a) denaturing the AAV particle, b) injecting the denatured AAV particle to liquid chromatography/mass spectrometry (LC/MS), and c) determining the masses of VP1, VP2 and VP3 of the AAV particle; wherein the specific combination of masses of VP1, VP2 and VP3 are indicative of the AAV serotype.

In other aspects, the invention provides a method of determining the heterogeneity of an AAV particle comprising: a) denaturing the AAV particle, b) injecting the denatured AAV particle to liquid chromatography/mass spectrometry (LC/MS), and c) determining the masses of VP1, VP2 and VP3 of the AAV particle, and comparing the masses of step c) with the theoretical masses of VP1, VP2 and VP3 of the AAV serotype; wherein a deviation of one or more of the masses of VP1, VP2 or VP3 are indicative of the AAV capsid heterogeneity.

In other aspects, the invention provides a method to determine the serotype of an adeno-associated virus (AAV) particle comprising a) denaturing the AAV particle, b) subjecting the denatured AAV particle to reduction and/or alkylation, c) injecting the denatured AAV particle to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle, d) subjecting the fragments of VP1, VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), and e) determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle; wherein the specific combination of masses of fragments of VP1, VP2 and VP3 are indicative of the AAV serotype.

In other aspects, the invention provides a method of determining the heterogeneity of an AAV particle of a serotype comprising: a) denaturing the AAV particle, b) subjecting the denatured AAV particle to reduction and/or alkylation, c) injecting the denatured AAV particle to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle, d) subjecting the fragments of VP1, VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), e) determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle, and f) comparing the masses of step e) with the theoretical

masses of fragments of VP1, VP2 and VP3 of the AAV serotype; wherein a deviation of one or more of the masses of VP1, VP2 or VP3 are indicative of the AAV capsid heterogeneity.

In some aspects, the invention provides a recombinant AAV (rAAV) particle comprising an amino acid substitution at amino acid residue 2 of VP1 and/or VP3; wherein the amino acid substitution at amino acid residue 2 of VP1 and/or VP3 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of VP1 and/or VP3 of the parent AAV particle.

In some aspects, the invention provides a method of improving the assembly of rAAV particles in a cell comprising substituting amino acid residue 2 of VP1 and/or VP3; wherein the substituted amino acid at position 2 is N-acetylated at a higher frequency than amino acid residue 2 of the parent VP1 and/or VP3. In some aspects, the invention provides a method of improving the transduction of rAAV particles in a cell comprising substituting amino acid residue 2 of VP1 and/or VP3; wherein the substituted amino acid at position 2 is N-acetylated at a higher frequency than amino acid residue 2 of the parent VP1 and/or VP3.

I. General Techniques

The techniques and procedures described or referenced herein are generally well understood and commonly employed using conventional methodology by those skilled in the art, such as, for example, the widely utilized methodologies described in *Molecular Cloning: A Laboratory Manual* (Sambrook et al., 4th ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2012); *Current Protocols in Molecular Biology* (F. M. Ausubel, et al. eds., 2003); the series Methods in Enzymology (Academic Press, Inc.); PCR 2: *A Practical Approach* (M. J. MacPherson, B. D. Hames and G. R. Taylor eds., 1995); *Antibodies, A Laboratory Manual* (Harlow and Lane, eds., 1988); *Culture of Animal Cells: A Manual of Basic Technique* and Specialized Applications (R. I. Freshney, 6th ed., J. Wiley and Sons, 2010); *Oligonucleotide Synthesis* (M. J. Gait, ed., 1984); Methods in Molecular Biology, Humana Press; *Cell Biology: A Laboratory Notebook* (J. E. Cellis, ed., Academic Press, 1998); *Introduction to Cell and Tissue Culture* (J. P. Mather and P. E. Roberts, Plenum Press, 1998); *Cell and Tissue Culture: Laboratory Procedures* (A. Doyle, J. B. Griffiths, and D. G. Newell, eds., J. Wiley and Sons, 1993-8); *Handbook of Experimental Immunology* (D. M. Weir and C. C. Blackwell, eds., 1996); *Gene Transfer Vectors for Mammalian Cells* (J. M. Miller and M. P. Calos, eds., 1987); *PCR: The Polymerase Chain Reaction*, (Mullis et al., eds., 1994); *Current Protocols in Immunology* (J. E. Coligan et al., eds., 1991); *Short Protocols in Molecular Biology* (Ausubel et al., eds., J. Wiley and Sons, 2002); *Immunobiology* (C. A. Janeway et al., 2004); *Antibodies* (P. Finch, 1997); *Antibodies: A Practical Approach* (D. Catty., ed., IRL Press, 1988-1989); *Monoclonal Antibodies: A Practical Approach* (P. Shepherd and C. Dean, eds., Oxford University Press, 2000); *Using Antibodies: A Laboratory Manual* (E. Harlow and D. Lane, Cold Spring Harbor Laboratory Press, 1999); *The Antibodies* (M. Zanetti and J. D. Capra, eds., Harwood Academic Publishers, 1995); and *Cancer: Principles and Practice of Oncology* (V. T. DeVita et al., eds., J. B. Lippincott Company, 2011).

II. Definitions

A "vector," as used herein, refers to a recombinant plasmid or virus that comprises a nucleic acid to be delivered into a host cell, either in vitro or in vivo.

The term "polynucleotide" or "nucleic acid" as used herein refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. Thus, this term includes, but is not limited to, single-, double- or multi-stranded DNA or RNA, genomic DNA, cDNA, DNA-RNA hybrids, or a polymer comprising purine and pyrimidine bases, or other natural, chemically or biochemically modified, non-natural, or derivatized nucleotide bases. The backbone of the nucleic acid can comprise sugars and phosphate groups (as may typically be found in RNA or DNA), or modified or substituted sugar or phosphate groups. Alternatively, the backbone of the nucleic acid can comprise a polymer of synthetic subunits such as phosphoramidates and thus can be an oligodeoxynucleoside phosphoramidate ($P\text{-}NH_2$) or a mixed phosphoramidate-phosphodiester oligomer. In addition, a double-stranded nucleic acid can be obtained from the single stranded polynucleotide product of chemical synthesis either by synthesizing the complementary strand and annealing the strands under appropriate conditions, or by synthesizing the complementary strand de novo using a DNA polymerase with an appropriate primer.

The terms "polypeptide" and "protein" are used interchangeably to refer to a polymer of amino acid residues, and are not limited to a minimum length. Such polymers of amino acid residues may contain natural or non-natural amino acid residues, and include, but are not limited to, peptides, oligopeptides, dimers, trimers, and multimers of amino acid residues. Both full-length proteins and fragments thereof are encompassed by the definition. The terms also include post-translational modifications of the polypeptide, for example, glycosylation, sialylation, acetylation, phosphorylation, and the like. Furthermore, for purposes of the present invention, a "polypeptide" refers to a protein which includes modifications, such as deletions, additions, and substitutions (generally conservative in nature), to the native sequence, as long as the protein maintains the desired activity. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutations of hosts which produce the proteins or errors due to PCR amplification.

A "recombinant viral vector" refers to a recombinant polynucleotide vector comprising one or more heterologous sequences (i.e., nucleic acid sequence not of viral origin). In the case of recombinant AAV vectors, the recombinant nucleic acid is flanked by at least one, e.g., two, inverted terminal repeat sequences (ITRs).

A "recombinant AAV vector (rAAV vector)" refers to a polynucleotide vector comprising one or more heterologous sequences (i.e., nucleic acid sequence not of AAV origin) that are flanked by at least one, e.g., two, AAV inverted terminal repeat sequences (ITRs). Such rAAV vectors can be replicated and packaged into infectious viral particles when present in a host cell that has been infected with a suitable helper virus (or that is expressing suitable helper functions) and that is expressing AAV rep and cap gene products (i.e. AAV Rep and Cap proteins). When a rAAV vector is incorporated into a larger polynucleotide (e.g., in a chromosome or in another vector such as a plasmid used for cloning or transfection), then the rAAV vector may be referred to as a "pro-vector" which can be "rescued" by replication and encapsidation in the presence of AAV packaging functions and suitable helper functions. A rAAV vector can be in any of a number of forms, including, but not limited to, plasmids, linear artificial chromosomes, complexed with lipids, encapsulated within liposomes, and, in embodiments, encapsidated in a viral particle, particularly an AAV particle.

A rAAV vector can be packaged into an AAV virus capsid to generate a "recombinant adeno-associated viral particle (rAAV particle)".

An "rAAV virus" or "rAAV viral particle" refers to a viral particle composed of at least one AAV capsid protein and an encapsidated rAAV vector genome.

A "parent AAV particle" and "parent AAV capsid protein" as used herein in the context of comparing N-acetylation and/or deamidation refers to an AAV particle or capsid protein into which amino acid modifications are introduced to modulate N-acetlylation and/or deamidation (e.g., an AAV particle/capsid protein that is the same as or similar to the AAV particle/capsid of the subject invention but does not comprise the mutations that modulate/alter N-aceytlation and/or deamidation as described herein). In some embodiments, the parent AAV particle is a recombinant AAV particle comprising a recombinant AAV genome. In some embodiments, the parent AAV capsid particle or parent AAV capsid protein comprises amino acid substitutions that affect other aspects of the AAV particle. For example, the parent AAV particle may comprise amino acid substitutions that affect the binding of AAV to its receptor, such as affecting binding of AAV2 to heparin sulfate proteoglycan (e.g. an AAV2 HBKO particle). An AAV2 HBKO particle can be mutated to introduce amino acid substitutions that modulate N-acetylation and/or deamidation. Such a mutated AAV particle may then be compared to the parent AAV2 HBKO particle in aspects of the invention as described herein. A parent AAV capsid protein may include a parent VP1 capsid protein, a parent VP2 capsid protein, or a VP3 capsid protein.

As used herein, the term "modulate" or "alter" in reference to a parent molecule means to change a feature of the parent molecule. For example, an AAV particle with altered N-acetylation may show increased or decreased N-acetylation compared to the parent AAV particle and an AAV particle with altered deamidation may show increased or decreased deamidation compared to the parent AAV particle.

"Heterologous" means derived from a genotypically distinct entity from that of the rest of the entity to which it is compared or into which it is introduced or incorporated. For example, a nucleic acid introduced by genetic engineering techniques into a different cell type is a heterologous nucleic acid (and, when expressed, can encode a heterologous polypeptide). Similarly, a cellular sequence (e.g., a gene or portion thereof) that is incorporated into a viral vector is a heterologous nucleotide sequence with respect to the vector.

The term "transgene" refers to a nucleic acid that is introduced into a cell and is capable of being transcribed into RNA and optionally, translated and/or expressed under appropriate conditions. In aspects, it confers a desired property to a cell into which it was introduced, or otherwise leads to a desired therapeutic or diagnostic outcome. In another aspect, it may be transcribed into a molecule that mediates RNA interference, such as siRNA.

The terms "genome particles (gp)," "genome equivalents," or "genome copies" as used in reference to a viral titer, refer to the number of virions containing the recombinant AAV DNA genome, regardless of infectivity or functionality. The number of genome particles in a particular vector preparation can be measured by procedures such as described in the Examples herein, or for example, in Clark et al. (1999) *Hum. Gene Ther.,* 10:1031-1039; Veldwijk et al. (2002) *Mol. Ther.,* 6:272-278.

The terms "infection unit (iu)," "infectious particle," or "replication unit," as used in reference to a viral titer, refer to the number of infectious and replication-competent recombinant AAV vector particles as measured by the infectious center assay, also known as replication center assay, as described, for example, in McLaughlin et al. (1988) *J. Virol.,* 62:1963-1973.

The term "transducing unit (tu)" as used in reference to a viral titer, refers to the number of infectious recombinant AAV vector particles that result in the production of a functional transgene product as measured in functional assays such as described in Examples herein, or for example, in Xiao et al. (1997) *Exp. Neurobiol.,* 144:113-124; or in Fisher et al. (1996) *J. Virol.,* 70:520-532 (LFU assay).

An "inverted terminal repeat" or "ITR" sequence is a term well understood in the art and refers to relatively short sequences found at the termini of viral genomes which are in opposite orientation.

An "AAV inverted terminal repeat (ITR)" sequence, a term well-understood in the art, is an approximately 145-nucleotide sequence that is present at both termini of the native single-stranded AAV genome. The outermost 125 nucleotides of the ITR can be present in either of two alternative orientations, leading to heterogeneity between different AAV genomes and between the two ends of a single AAV genome. The outermost 125 nucleotides also contains several shorter regions of self-complementarity (designated A, A', B, B', C, C' and D regions), allowing intrastrand base-pairing to occur within this portion of the ITR.

A "terminal resolution sequence" or "trs" is a sequence in the D region of the AAV ITR that is cleaved by AAV rep proteins during viral DNA replication. A mutant terminal resolution sequence is refractory to cleavage by AAV rep proteins. "AAV helper functions" refer to functions that allow AAV to be replicated and packaged by a host cell. AAV helper functions can be provided in any of a number of forms, including, but not limited to, helper virus or helper virus genes which aid in AAV replication and packaging. Other AAV helper functions are known in the art such as genotoxic agents.

"AAV helper functions" refer to functions that allow AAV to be replicated and packaged by a host cell. AAV helper functions can be provided in any of a number of forms, including, but not limited to, helper virus or helper virus genes which aid in AAV replication and packaging. Other AAV helper functions are known in the art such as genotoxic agents.

A "helper virus" for AAV refers to a virus that allows AAV (which is a defective parvovirus) to be replicated and packaged by a host cell. A number of such helper viruses have been identified, including adenoviruses, herpesviruses, poxviruses such as vaccinia, and baculovirus. The adenoviruses encompass a number of different subgroups, although Adenovirus type 5 of subgroup C (Ad5) is most commonly used. Numerous adenoviruses of human, non-human mammalian and avian origin are known and are available from depositories such as the ATCC. Viruses of the herpes family, which are also available from depositories such as ATCC, include, for example, herpes simplex viruses (HSV), Epstein-Barr viruses (EBV), cytomegaloviruses (CMV) and pseudorabies viruses (PRV). Baculoviruses available from depositories include *Autographa californica* nuclear polyhedrosis virus.

"Percent (%) sequence identity" with respect to a reference polypeptide or nucleic acid sequence is defined as the percentage of amino acid residues or nucleotides in a candidate sequence that are identical with the amino acid residues or nucleotides in the reference polypeptide or nucleic acid sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum

percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid or nucleic acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software programs, for example, those described in Current Protocols in Molecular Biology (Ausubel et al., eds., 1987), Supp. 30, section 7.7.18, Table 7.7.1, and including BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. A potential alignment program is ALIGN Plus (Scientific and Educational Software, Pennsylvania). Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For purposes herein, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows: 100 times the fraction X/Y, where X is the number of amino acid residues scored as identical matches by the sequence alignment program in that program's alignment of A and B, and where Y is the total number of amino acid residues in B. It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A. For purposes herein, the % nucleic acid sequence identity of a given nucleic acid sequence C to, with, or against a given nucleic acid sequence D (which can alternatively be phrased as a given nucleic acid sequence C that has or comprises a certain % nucleic acid sequence identity to, with, or against a given nucleic acid sequence D) is calculated as follows: 100 times the fraction W/Z, where W is the number of nucleotides scored as identical matches by the sequence alignment program in that program's alignment of C and D, and where Z is the total number of nucleotides in D. It will be appreciated that where the length of nucleic acid sequence C is not equal to the length of nucleic acid sequence D, the % nucleic acid sequence identity of C to D will not equal the % nucleic acid sequence identity of D to C.

An "isolated" molecule (e.g., nucleic acid or protein) or cell means it has been identified and separated and/or recovered from a component of its natural environment.

"Mass spectrometry" refers to the analytical chemistry technique of identifying an amount and/or type of a compound (e.g., a polypeptide) by measuring the mass-to-charge ratio and abundance of gas-phase ions. The term "mass spectrometry" may be used interchangeably herein.

"Heterogeneity" when used in reference to an AAV capsid refers to an AAV capsid characterized by one or more capsid polypeptides observed to deviate from a reference mass of a VP1, VP2, and/or VP3 polypeptide, or fragment thereof. A reference mass may include, without limitation, a theoretical, predicted, or expected mass of a VP1, VP2, and/or VP3 polypeptide, e.g., of a known AAV serotype. For example, an AAV capsid may be said to display heterogeneity if it demonstrates one or more of the following properties (without limitation): a mixed serotype, a variant capsid, a capsid amino acid substitution, a truncated capsid, or a modified capsid.

Reference to "about" a value or parameter herein includes (and describes) embodiments that are directed to that value or parameter per se. For example, description referring to "about X" includes description of "X."

As used herein, the singular form of the articles "a," "an," and "the" includes plural references unless indicated otherwise.

It is understood that aspects and embodiments of the invention described herein include "comprising," "consisting," and/or "consisting essentially of" aspects and embodiments.

III. Methods

Certain aspects of the present disclosure relate to methods of determining the serotype of a viral particle. Other aspects of the present disclosure relate to methods of determining the heterogeneity of a viral particle. As described below, the accurate masses of VP1, VP2 and VP3 of each AAV serotype are unique and can be used to identify or differentiate AAV capsid serotypes. These methods are based in part on the discovery described herein that direct LC/MS of different types of AAVs after denaturation may be used to monitor the protein sequence and post-translational modifications with accurate mass measurement in the intact protein level. Further, acetylations of N-termini of VP1 and VP3 may also be identified and/or monitored in different AAV serotypes. Based on these AAV results and the guidance provided herein, it is contemplated that such methods may readily be applied to profile a variety of viruses, e.g., the viral families, subfamilies, and genera of the present disclosure. The methods of the present disclosure may find use, e.g., in profile VPs to monitor VP expressions, posttranslational modifications, and truncations and to ensure product consistency during VLP production, to confirm site-direct mutagenesis or structural characterization for capsid protein engineering applications, and/or to monitor or detect heterogeneity of a viral particle or preparation.

In some embodiments, the methods include denaturing a viral particle. In some embodiments, a viral particle such as an AAV particle may be denatured using detergent, heat, high salt, or buffering with a low or high pH. In certain embodiments, an AAV particle may be denatured using acetic acid or guanidine hydrochloride. The skilled artisan will recognize that a variety of methods useful for promoting and/or monitoring protein denaturation are available in the art and may suitably select a denaturation method compatible with liquid chromatography/mass spectrometry. For example, if heat denaturation is used, care may be applied to avoid protein precipitation and reverse phase column clogging. Similarly, high salt denaturation may be coupled with a desalting step prior to LC/MS or LC/MS/MS. In other embodiments, high pH denaturation, low pH denaturation, or denaturation using organic solvents is used.

In some embodiments, the methods include subjecting a denatured viral particle of the present disclosure to liquid chromatography/mass spectrometry (LC/MS). As is known in the art, LC/MS utilizes liquid chromatography for physical separation of ions and mass spectrometry for generation of mass spectral data from the ions. Such mass spectral data may be used to determine, e.g., molecular weight or structure, identification of particles by mass, quantity, purity, and so forth. These data may represent properties of the detected ions such as signal strength (e.g., abundance) over time (e.g., retention time), or relative abundance over mass-to-charge ratio.

In some embodiments, liquid chromatography (e.g., used in LC/MS as described herein) is ultra-performance liquid chromatography (UPLC; the term "ultra high performance

liquid chromatography" or UHPLC may be used interchangeably herein). UPLC is known in the art as an LC technique that relies upon a column with reduced particle size (e.g., less than 2 μm) and increased flow velocity to improve chromatographic resolution, efficiency, peak capacity, and sensitivity (see, e.g., Plumb, R. et al. (2004) *Rapid Commun. Mass Spectrom.* 18:2331-2337). In some embodiments, UPLC refers to the use of a column with a particle size less than 2 μm in liquid chromatography. In some embodiments, UPLC refers to the use of a high linear solvent velocity (e.g., as observed when operating at 6000 psi or higher) in liquid chromatography. Exemplary UPLC machines are commercially available (e.g., the ACQUITY UPLC® from Waters; Milford, MA).

In some embodiments, mass spectrometry (e.g., used in LC/MS as described herein) may refer to electrospray ionization mass spectrometry (ESI-MS). ESI-MS is known in the art as a technique that uses electrical energy to analyze ions derived from a solution using mass spectrometry (see, e.g., Yamashita, M. and Fenn, J. B. (1984) *J. Phys. Chem.* 88:4451-4459). Ionic species (or neutral species that are ionized in solution or in gaseous phase) are transferred from a solution to a gaseous phase by dispersal in an aerosol of charged droplets, followed by solvent evaporation that reduces the size of the charged droplets and sample ion ejection from the charge droplets as the solution is passed through a small capillary with a voltage relative to ground (e.g., the wall of the surrounding chamber). In some embodiments, the capillary voltage is from about 2 kV to about 10 kV, or about 2.5 kV to about 6.0 kV. In certain embodiments, liquid chromatography (e.g., used in LC/MS as described herein) uses a capillary voltage of about 3.5 kV. In some embodiments the capillary voltage ranges from about 1 kV to about 10 kV. In other embodiments, mass spectrometry (e.g., used in LC/MS as described herein) may refer to matrix-assisted laser desorption/ionization (MALDI).

In some embodiments, mass spectrometry (e.g., used in LC/MS as described herein) uses a sampling cone and/or skimmer, through which ions may pass before entering the analyzer. In some embodiments, e.g., when applying voltage to the capillary as described above, the sample cone is held at a lower voltage than the capillary voltage. In certain embodiments, liquid chromatography (e.g., used in LC/MS as described herein) uses a sampling cone voltage of about 45 V. In some embodiments the sampling cone voltage ranges from about 0 V to about 200 V.

In some embodiments, mass spectrometry (e.g., used in LC/MS as described herein) uses assisted calibration. Calibration, when used in reference to mass spectrometry, may include the introduction of one or more compounds having a known mass (e.g., a standard) for the purpose of calibrating the instrument with respect to mass detection (e.g., m/z measurements). In some embodiments, assisted calibration may refer to using software to correlate a peak and/or position of a known standard (e.g., a calibrant) to a specific mass-to-charge (m/z) ratio. Once calibrated, the user may then perform mass spectrometry on a sample having one or more unknown compounds, or compounds present at an unknown concentration, within a certain degree of accuracy or error, and/or a desired level of reproducibility, e.g., as compared to a previous or known experimental condition. Various calibrants are known in the art, including without limitation sodium iodide, sodium cesium iodide, polyethylene glycol, and perfluorotributylamine. In certain embodiments, sodium iodide is used as a calibrant. In some embodiments the calibrants are Glu-1-fibrinopeptide B and leucine encephalin peptide to lock mass during LC/MS operation.

In some embodiments, the methods include subjecting a denatured viral particle of the present disclosure, or subjecting digested fragments of a denatured viral particle of the present disclosure, to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS). As is known in the art, LC/MS/MS (the term "liquid chromatography-tandem mass spectrometry" may be used interchangeably herein) utilizes liquid chromatography for physical separation of ions and mass spectrometry for generation of mass spectral data from the ions, where the mass spectrometry uses multiple stages of mass (e.g., m/z) separation, typically separated by a fragmentation step. For example, ions of interest within a range of m/z may be separated out in a first round of MS, fragmented, and then further separated based on individual m/z in a second round of MS. Ion fragmentation may include without limitation a technique such as collision-induced dissociation (CID), higher energy collision dissociation (HCD), electron-capture dissociation (ECD), or electron-transfer dissociation (ETD).

In some embodiments, the methods include subjecting a denatured viral particle of the present disclosure to reduction and/or alkylation. Means to reduce the viral particle include but are not limited to treatment with dithiothreitol, β-mercaptoethanol, or tris(2-carboxyethyl)phosphine (TCEP). Means to alkylate the viral particle include but are not limited to treating the AAV particle with iodoacetic acid, iodoacetamide, or 4-vinylpyridine.

In some embodiments, the methods include subjecting a denatured viral particle of the present disclosure to digestion, e.g., to generate fragments of VP1, VP2 and/or VP3 of an AAV particle. For example, a denatured AAV particle may be subjected to digestion to generate peptide fragments that may be analyzed, e.g., using LC for separation and MS/MS for analysis (see below for greater description). In some embodiments, the digestion is an enzymatic digestion. In some embodiments, the digestion uses chemical digestion such as CNBr treatment of instrument fragmentation (e.g., top down). In some embodiments, the digestion uses chemical digestion such as acid digestion.

In some embodiments, the enzymatic digestion is an endopeptidase digestion. An endopeptidase may include any peptidase that catalyzes the proteolysis of peptide bonds of non-terminal amino acids of a polypeptide. Known endopeptidases may include, without limitation, trypsin, chymotrypsin, AspN, Glu-C, LysC, pepsin, thermolysin, glutamyl endopeptidase, elastase, and neprilysin. In certain embodiments, the enzymatic digestion is a trypsin digestion or a LysC digestion.

In some embodiments, the liquid chromatography (e.g., used in LC/MS or LC/MS/MS as described herein) is reverse phase liquid chromatography (the terms "reversed phase liquid chromatography" or RPLC may be used interchangeably herein with reverse phase liquid chromatography). As is known in the art, reverse phase liquid chromatography may refer to a chromatographic separation using a hydrophobic stationary phase (e.g., a support or substrate such as a column) to adsorb hydrophobic molecules in a polar mobile phase. By decreasing the polarity of the mobile phase (e.g., by adding an organic solvent), one may achieve gradient separation of molecules by hydrophobicity, since more hydrophobic molecules will stay on the column in higher concentrations of organic solvent due to stronger hydrophobic interactions with the column. In some embodiment, separation is by capillary electrophoresis (CE), size exclusion chromatography (SEC), ion exchange chromatography (IEC) such as cation exchange chromatography, hydrophobic interaction chromatography (HIC), hydrophilic

interaction liquid chromatography (HILIC), but not limited to on-line LC/MS such as offline separation before MS; e.g., tips, columns; plates or cartridges.

Generally, a stationary phase suitable for reverse phase liquid chromatography (e.g., a hydrophobic moiety) may be coupled to a support including without limitation a column or resin packed with particles or beads (e.g., porous silica particles or polystyrene). A variety of hydrophobic stationary phases are known in the art, including without limitation hydrophobic alkyl chains, octyl or octadecyl silyl moieties, cyano moieties, and amino moieties. In some embodiments, the stationary phase may include a hydrophobic alkyl chain of a particular length, such as C4, C8, or C18. In certain embodiments, the reverse phase chromatography is a C4 or C8 reverse chromatography (e.g., reverse phase chromatography utilizing a C4 or C8 stationary phase). One of skill in the art may suitably select a stationary phase based on the molecule of interest (e.g., a denatured AAV particle or fragment thereof).

A variety of mobile phases suitable for reverse phase liquid chromatography are known in the art. As described above, a reverse phase liquid chromatography mobile phase may include a mixture of organic (e.g., hydrophobic) and aqueous (e.g., polar) solvents. Increasing the proportion of organic solvent increases its power to elute hydrophobic compounds from the stationary phase. Compound retention and/or selectivity may be altered, e.g., by changing the type or exposure of the stationary phase, adding polar reagents such as end capping reagents, altering the temperature, and/or altering mobile phase characteristics such as the proportion of organic solvent, pH, buffers, and the type of organic solvent used. In some embodiments, the polar component of the mobile phase may include without limitation water or an aqueous buffer. In some embodiments, the polar component of the mobile phase may include without limitation acetonitrile, methanol, ethanol, isopropyl alcohol, tetrahydrofuran (THF), and formic acid.

In some embodiments, two or more mobile phases may be used (e.g., mobile phase A, mobile phase B, etc.) in a gradient or proportion of interest. In certain embodiments, the chromatography uses a mobile phase A comprising formic acid in water. In certain embodiments, the mobile phase A comprises about 0.1% formic acid. In certain embodiments, the mobile phase A comprises about 0.1% to about 5% formic acid. In certain embodiments, the chromatography uses a mobile phase B comprising formic acid in acetonitrile. In certain embodiments, the mobile phase B comprises about 0.1% formic acid.

In some embodiments, the proportion of mobile phase B in the chromatography increases over time. For example, the proportion of mobile phase B in the chromatography may be increased in a stepwise manner. In certain embodiments, mobile phase B increases from about 10% to about 20%, from about 20% to about 30%, and from about 30% to about 38%. In other embodiments, mobile phase B increases from about 2% to about 60%. In other embodiments, mobile phase B increases from about 2% to about 100% from about 1 min to about 200 min In some embodiments, the remainder of the mobile phase is a second mobile phase of the present disclosure, e.g., mobile phase A. In certain embodiments, mobile phase B increases from about 10% to about 20% in about 6 minutes, from about 20% to about 30% in about 10 minutes, and from about 30% to about 38% in about 40 minutes. In other embodiments, mobile phase B increases from about 2% to about 60% in about 121 minutes. One of skill in the art may suitably adjust the mobile phase of interest and the gradient timing used based on the desired chromatographic separation and/or analyte of interest.

In some embodiments, the liquid chromatography is high-performance liquid chromatography (HPLC). HPLC is known in the art as a form of liquid chromatography in which a liquid solvent containing a sample is pressurized as it passes through a column containing solid phase. While traditional or low pressure LC may use gravity to pass a mobile phase through the solid phase, HPLC uses pumps to apply a pressure to the mobile phase and typically uses a solid phase with smaller particles to increase resolution. In some embodiments, the HPLC uses a pressure of between about 50 bar and about 350 bar. In some embodiments, reversed phase HPLC may be used to concentrate and/or desalt proteins (e.g., AAV capsid proteins) for MS analysis.

In some embodiments, one or more parameters including without limitation source voltage, capillary temperature, ESI voltage (if using ESI-MS), CID energy, and the number of MS/MS events may be adjusted, e.g., in LC/MS/MS as used herein, based on the findings described herein. In some embodiments, mass spectrometry (e.g., used in LC/MS/MS as described herein) uses a source voltage (e.g., capillary voltage) of about 2.5 kV. In some embodiments, mass spectrometry (e.g., used in LC/MS/MS as described herein) uses a capillary temperature of about 275° C. In some embodiments, the capillary temperature ranges from about 20° C. to about 400° C.

A variety of mass analyzers suitable for LC/MS and/or LC/MS/MS are known in the art, including without limitation time-of-flight (TOF) analyzers, quadrupole mass filters, quadrupole TOF (QTOF), and ion traps (e.g., a Fourier transform-based mass spectrometer or an Orbitrap). In Orbitrap, a barrel-like outer electrode at ground potential and a spindle-like central electrode are used to trap ions in trajectories rotating elliptically around the central electrode with oscillations along the central axis, confined by the balance of centrifugal and electrostatic forces. The use of such instruments employs a Fourier transform operation to convert a time domain signal (e.g., frequency) from detection of image current into a high resolution mass measurement (e.g., nano LC/MS/MS). Further descriptions and details may be found, e.g., in Scheltema, R. A. et al. (2014) *Mol. Cell Proteomics* 13:3698-3708; Perry, R. H. et al. (2008) *Mass. Spectrom. Rev.* 27:661-699; and Scigelova, M. et al. (2011) *Mol. Cell Proteomics* 10:M111.009431.

As described above, in some embodiments, the MS includes nano LC/MS/MS, e.g., using an Orbitrap mass analyzer. In some embodiments, the ion source may include a stacked-ring ion guide or S-lens. As is known in the art, an S-lens may be employed to focus the ion beam using radio frequency (RF), thereby increasing transmission of ions into the instrument. This may improve sensitivity (e.g., for low-intensity ions) and/or improve the scan rate. In certain embodiments, the S-lens RF level of the mass spectrometry is about 55%. In certain embodiments, the S-lens RF level of the mass spectrometry is about 20% to about 100%.

In some embodiments, masses of viral capsid proteins may be determined, e.g., based on LC/MS and/or LC/MS/MS data. In some embodiments, masses of VP1, VP2 and VP3 of an AAV particle, or of fragments of VP1, VP2 and VP3 of the AAV particle, may be determined, e.g., based on LC/MS and/or LC/MS/MS data. Various methods to determine protein mass and/or identity from MS data are known in the art. For example, peptide mass fingerprinting may be used to determine protein sequence based on MS data, or proteins may be identified based on MS/MS data related to one or more constituent peptides. When using tandem MS,

product ion scanning may be used to analyze m/z data related to one or more peptides of a protein of interest. Software known in the art may then be used, e.g., to match identified peaks to reference or known peaks, to group peaks into isotopomer envelopes, and so forth. Peptide mass values may be compared to a database of known peptide sequences. For example, Mascot may be used to match observed peptides with theoretical database peptides, e.g., resulting from application of a particular digest pattern to an in silico protein database. Other suitable software may include without limitation Proteome Discoverer, ProteinProspector, X!Tandem, Pepfinder, Bonics, or MassLynx™ (Waters). Other software suitable for various steps of MS data analysis may be found, e.g., at www.ms-utils.org/wiki/pmwiki.php/Main/SoftwareList.

In some embodiments, a determined or calculated mass of the present disclosure (e.g., the determined or calculated mass of VP1, VP2 and/or VP3 of the AAV particle) may be compared with a reference, e.g., a theoretical mass of a VP1, VP2, and/or VP3 of one or more AAV serotypes. A reference of the present disclosure may include a theoretical mass of a VP1, VP2, and/or VP3 of one or more of any of the AAV serotypes described herein. For example, in some embodiments, the masses of VP1, VP2, and/or VP3 are compared to the theoretical masses of one or more of an AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an AAV LK03 capsid (see U.S. Pat. No. 9,169,299), an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid (see U.S. Pat. No. 7,588,772), an AAV DJ8 capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, or a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV/human bocavirus virus 1), an AAV2HBKO capsid, an AAVPHP.B capsid or an AAVPHP.eB capsid. In some embodiments, a determined or calculated mass of the present disclosure (e.g., the determined or calculated mass of VP1, VP2 and/or VP3 of the AAV particle) may be compared with a theoretical mass of a VP1, VP2, and/or VP3 of the corresponding AAV serotype.

In some embodiments, the methods of the present disclosure may include determining the heterogeneity of an AAV particle. In some embodiments, a deviation of one or more of the masses of VP1, VP2 and/or VP3 (e.g., from a reference mass, such as a theoretical, predicted, or expected mass) is indicative of the AAV capsid heterogeneity. In some embodiments, heterogeneity may include one or more of the following, without limitation: mixed serotypes, variant capsids, capsid amino acid substitutions, truncated capsids, or modified capsids.

In some embodiments, the use of LC/MS and LC/MS/MS as described herein may be combined. In some embodiments, a method of determining the serotype of an AAV particle may include subjecting a denatured AAV particle to LC/MS (e.g., as described herein) and determining the masses of VP1, VP2 and VP3 of the AAV particle; as well as subjecting fragments of VP1, VP2 and/or VP3 to LC/MS/MS and determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle (the specific combination of masses of fragments of VP1, VP2 and VP3 are indicative of the AAV serotype). In some embodiments, a method of determining the heterogeneity of an AAV particle may include subjecting a denatured AAV particle to LC/MS (e.g., as described herein), determining the masses of VP1, VP2 and VP3 of the AAV particle, and comparing these masses with the theoretical masses of VP1, VP2 and VP3 of the AAV serotype; as well as subjecting fragments of VP1, VP2 and/or VP3 to LC/MS/MS, determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle, and comparing these masses with the theoretical masses of VP1, VP2 and VP3 of the AAV serotype (a deviation of one or more of the masses of VP1, VP2 or VP3 are indicative of the AAV capsid heterogeneity).

In some embodiments, an AAV particle of the present disclosure may be acetylated. For example, in some embodiments, the N-terminus of VP1 and/or VP3 is acetylated. As described in greater detail below, the amino acid at the 2nd position to the initiating methionine (iMet X) of an AAV capsid protein may be mutated in order to determine its effect on N-terminal (Nt-) acetylation, as well as the functional consequences of affecting Nt-acetylation on AAV particle trafficking, transduction, and/or post-translational modification (e.g., glycosylation, ubiquitination, and so forth). In some embodiments, the N-terminus of an AAV capsid protein (e.g., VP1 or VP3) may refer to the first amino acid after the initiating methionine, which in some cases may be removed by, e.g., a Met-aminopeptidase.

In some embodiments, an AAV particle of the present disclosure (e.g., a recombinant AAV or rAAV particle) comprises an amino acid substitution at amino acid residue 2 of VP1 and/or VP3. In some embodiments, the amino acid substitution at amino acid residue 2 of VP1 and/or VP3 leads to a VP1 and/or VP3 with a different frequency or proportion of N-terminal acetylation as compared to a reference (e.g., the parent AAV particle before the amino acid substitution, or an AAV particle with a different amino acid residue 2 of VP1 and/or VP3). In some embodiments, the amino acid substitution at amino acid residue 2 of VP1 and/or VP3 alters N-terminal acetylation as compared to N-terminal acetylation at amino acid residue 2 of VP1 and/or VP3 of the parent AAV particle. For example, in certain embodiments, the amino acid substitution at amino acid residue 2 of VP1 alters N-terminal acetylation as compared to N-terminal acetylation at amino acid residue 2 of VP1 of the parent AAV particle. In certain embodiments, the amino acid substitution at amino acid residue 2 of VP3 alters N-terminal acetylation as compared to N-terminal acetylation at amino acid residue 2 of VP3 of the parent AAV particle. In some embodiments, an amino acid substitution (e.g., an amino acid substitution at amino acid residue 2 of VP1 or VP3) that "alters" N-terminal acetylation results in a higher frequency of N-terminal acetylation or a lower frequency of N-terminal acetylation, e.g., as compared to a VP1 or VP3 without the substitution, such as the parental VP1 or VP3. The VP1 and/or VP3 may belong to any of the exemplary AAV serotypes described herein, including variants or hybrids thereof (e.g., bearing tyrosine mutation or heparin binding mutations). Exemplary assays for N-terminal acetylation include without limitation mass spectrometry, isotope labeling (e.g., with an isotope-labeled acetyl group or precursor thereof), Western blotting with an acetylation-specific antibody, and so forth. In some embodiments, amino acid residue 2 of the AAV capsid protein (e.g., VP1 or VP3) is substituted with Cys, Ser, Thr, Val, Gly, Asn, Asp, Glu, Ile, Leu, Phe, Gln, Lys, Met, Pro or Tyr. In some embodiments, the amino acid substitution results in less deamidation of the AAV capsid.

In some embodiments, an AAV particle of the present disclosure may be deamidated. For example, in some embodiments, N57 of VP1 and/or N382, N511, and/or N715 VP3 is deamidated. As described in greater detail below, an

amino acid selected from A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3 of an AAV capsid protein (e.g., VP1 or VP3) may be mutated in order to determine its effect on deamidation, as well as the functional consequences of affecting deamidation on AAV particle trafficking, transduction, and/or post-translational modification (e.g., glycosylation, ubiquitination, and so forth).

In some embodiments, an AAV particle of the present disclosure (e.g., a recombinant AAV or rAAV particle) comprises an amino acid substitution at one or more amino acid residues selected from A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, and G716 of VP3. In some embodiments, the amino acid substitution at A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, and/or G716 of VP3 leads to a VP1 and/or VP3 with a different frequency or proportion of deamidation as compared to a reference (e.g., the parent AAV particle before the amino acid substitution, or an AAV particle with a different corresponding amino acid residue 2). In some embodiments, an amino acid substitution (e.g., an amino acid substitution at A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3) that "alters" deamidation results in a higher frequency of deamidation or a lower frequency of deamidation, e.g., as compared to a VP1 or VP3 without the substitution, such as the parental VP1 or VP3. The VP1 and/or VP3 may belong to any of the exemplary AAV serotypes described herein, including variants or hybrids thereof (e.g., bearing tyrosine mutation or heparin binding mutations). Exemplary assays for deamidation include without limitation mass spectrometry, HPLC (see, e.g., the ISOQUANT® isoaspartate detection kit from Promega), and so forth. In some embodiments, N57 of VP1, N382 of VP3, N511 of VP3, and/or N715 of VP3 is substituted with Asp, and the amino acid substitution results in a higher frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In other embodiments, the amino acid substitution is N57K or N57Q, and the amino acid substitution results in a lower frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In yet other embodiments, G58 of VP1, G383 of VP3, G512 of VP3, and/or G716 of VP3 is substituted with an amino acid that is not Gly (e.g., Ala, Arg, Asn, Asp, Cys, Glu, Gln, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val), and the amino acid substitution results in a lower frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In yet other embodiments, A35 of VP1 is substituted with Asn and results in a higher frequency of deamidation as compared to deamidation of VP1 of a parent particle.

As used herein "N-acetylation" refers to a process whereby an acetyl group is covalently added to the amino group of the N-terminal amino acid of a protein. Typically, N-terminal acetyltransferases (NATs) transfer an acetyl group from acetyl-coenzyme A (Ac-CoA) to the α-amino group of the first amino acid residue of the protein.

As used here in, "deamidation" refers to a chemical reaction in which an amide functional group in the side chain of asparagine or glutamine is removed or converted to another functional group. For example, asparagine may be converted to aspartic acid or isoaspartic acid. In other examples, glutamine is converted to glutamic acid or pyroglutamic acid (5-oxoproline).

In some embodiments, the AAV particle is N-acetlyated to a higher extent compared to a parental AAV capsid protein. In some embodiments, the AAV particle comprises more than about any of 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% more N-acetyl groups compared to a parent AAV particle. In some embodiments, the AAV particle comprises between about any of 5%-10%, 10%-15%, 15%-20%, 20%-25%, 25%-30%, 30%-35%, 35%-40%, 40%-55%, 45%-50%, 50%-55%, 55%-60%, 60%-65%, 65%-70%, 70%-75%, 75%-80%, 80%-85%, 85%-90%, 90%-95%, 95%-100%, 5-25%, 25-50%, 50-75%, 75%-100%, 5-50% or 50%-100% more N-acetyl groups compared to a parent AAV particle. In some embodiments, the AAV particle comprises more than about any of 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 25-fold, 50-fold, 100-fold, 500-fold, or 1000-fold more N-acetyl groups compared to a parent AAV particle. In some embodiments, the AAV particle comprises between about any of 2-fold to 3-fold, 3-fold to 4-fold, 4-fold to 5-fold, 5-fold to 10-fold, 10-fold to 25-fold, 25-fold to 50-fold, 50-fold to 100-fold, 100-fold to 500-fold, 500-fold to 1000-fold, 2-fold to 10-fold, 10-fold to 100-fold, or 100-fold to 1000-fold more N-acetyl groups compared to a parent AAV particle.

In some embodiments, the AAV particle N-acetlyated to a lower extent compared to a parental AAV capsid protein. In some embodiments, the AAV particle comprises more than about any of 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% less N-acetyl groups compared to a parent AAV particle. In some embodiments, the AAV particle comprises between about any of 5%-10%, 10%-15%, 15%-20%, 20%-25%, 25%-30%, 30%-35%, 35%-40%, 40%-55%, 45%-50%, 50%-55%, 55%-60%, 60%-65%, 65%-70%, 70%-75%, 75%-80%, 80%-85%, 85%-90%, 90%-95%, 95%-100%, 5-25%, 25-50%, 50-75%, 75%-100%, 5-50% or 50%-100% less N-acetyl groups compared to a parent AAV particle. In some embodiments, the AAV particle comprises more than about any of 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 25-fold, 50-fold, 100-fold, 500-fold, or 1000-fold less N-acetyl groups compared to a parent AAV particle. In some embodiments, the AAV particle comprises between about any of 2-fold to 3-fold, 3-fold to 4-fold, 4-fold to 5-fold, 5-fold to 10-fold, 10-fold to 25-fold, 25-fold to 50-fold, 50-fold to 100-fold, 100-fold to 500-fold, 500-fold to 1000-fold, 2-fold to 10-fold, 10-fold to 100-fold, or 100-fold to 1000-fold less N-acetyl groups compared to a parent AAV particle.

In some embodiments, the AAV particle is deamidated to a higher extent compared to a parental AAV particle. In some embodiments, the AAV particle is more than about any of 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% more deamidated compared to a parent AAV particle. In some embodiments, the AAV particle is deamidated between about any of 5%-10%, 10%-15%, 15%-20%, 20%-25%, 25%-30%, 30%-35%, 35%-40%, 40%-55%, 45%-50%, 50%-55%, 55%-60%, 60%-65%, 65%-70%, 70%-75%, 75%-80%, 80%-85%, 85%-90%, 90%-95%, 95%-100%, 5-25%, 25-50%, 50-75%, 75%-100%, 5-50% or 50%-100% more than a parent AAV particle. In some embodiments, the AAV particle is deamidated more than about any of 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 25-fold, 50-fold, 100-fold, 500-fold, or 1000-fold compared to a parent AAV particle. In some embodiments, the AAV particle is deamidated between about any of 2-fold to 3-fold, 3-fold to 4-fold, 4-fold to 5-fold, 5-fold to 10-fold, 10-fold to 25-fold, 25-fold

to 50-fold, 50-fold to 100-fold, 100-fold to 500-fold, 500-fold to 1000-fold, 2-fold to 10-fold, 10-fold to 100-fold, or 100-fold to 1000-fold more than a parent AAV particle.

In some embodiments, a capsid protein of AAV is deamidated to a lower extent compared to a parental AAV capsid protein. In some embodiments, the AAV particle is more than about any of 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% less deamidated compared to a parent AAV particle. In some embodiments, the AAV particle is deamidated between about any of 5%-10%, 10%-15%, 15%-20%, 20%-25%, 25%-30%, 30%-35%, 35%-40%, 40%-55%, 45%-50%, 50%-55%, 55%-60%, 60%-65%, 65%-70%, 70%-75%, 75%-80%, 80%-85%, 85%-90%, 90%-95%, 95%-100%, 5-25%, 25-50%, 50-75%, 75%-100%, 5-50% or 50%-100% less than a parent AAV particle. In some embodiments, the AAV particle is deamidated more than about any of 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 25-fold, 50-fold, 100-fold, 500-fold, or 1000-fold less than a parent AAV particle. In some embodiments, the AAV particle is deamidated between about any of 2-fold to 3-fold, 3-fold to 4-fold, 4-fold to 5-fold, 5-fold to 10-fold, 10-fold to 25-fold, 25-fold to 50-fold, 50-fold to 100-fold, 100-fold to 500-fold, 500-fold to 1000-fold, 2-fold to 10-fold, 10-fold to 100-fold, or 100-fold to 1000-fold less than a parent AAV particle.

The invention provides any combination of N-acetlylation and deamidation. For example, the AAV capsid protein may be N-acetylated to a higher extent than a parent AAV capsid protein and deamidated to a higher extent than a parent AAV capsid protein, the AAV capsid protein may be N-acetylated to a higher extent than a parent AAV capsid protein and deamidated to the same extent than a parent AAV capsid protein, the AAV capsid protein may be N-acetylated to a higher extent than a parent AAV capsid protein and deamidated to a lower extent than a parent AAV capsid protein, the AAV capsid protein may be N-acetylated to the same extent than a parent AAV capsid protein and deamidated to a higher extent than a parent AAV capsid protein, the AAV capsid protein may be N-acetylated to the same extent than a parent AAV capsid protein and deamidated to the same extent than a parent AAV capsid protein, the AAV capsid protein may be N-acetylated to the same extent than a parent AAV capsid protein and deamidated to a lower extent than a parent AAV capsid protein, the AAV capsid protein may be N-acetylated to a lower extent than a parent AAV capsid protein and deamidated to a higher extent than a parent AAV capsid protein, the AAV capsid protein may be N-acetylated to a lower extent than a parent AAV capsid protein and deamidated to the same extent than a parent AAV capsid protein, or the AAV capsid protein may be N-acetylated to a lower extent than a parent AAV capsid protein and deamidated to a lower extent than a parent AAV capsid protein.

IV. Vectors

In certain aspects, the invention relates to viral particles, suitable for use in any of the methods described herein, which may comprise AAV vectors (e.g., rAAV vectors) or vectors derived from another virus. In some embodiments, the viral particle comprises a vector encoding a heterologous nucleic acid, e.g., a heterologous transgene. In some embodiments, the AAV particle comprises an AAV vector genome encoding a heterologous nucleic acid, e.g., a heterologous transgene.

The present invention contemplates the use of a recombinant viral genome for introduction of one or more nucleic acid sequences encoding a therapeutic polypeptide and/or nucleic acid for packaging into a rAAV viral particle. The recombinant viral genome may include any element to establish the expression of the therapeutic polypeptide and/or nucleic acid, for example, a promoter, an ITR of the present disclosure, a ribosome binding element, terminator, enhancer, selection marker, intron, polyA signal, and/or origin of replication.

In some embodiments, the heterologous nucleic acid encodes a therapeutic polypeptide. A therapeutic polypeptide may, e.g., supply a polypeptide and/or enzymatic activity that is absent or present at a reduced level in a cell or organism. Alternatively, a therapeutic polypeptide may supply a polypeptide and/or enzymatic activity that indirectly counteracts an imbalance in a cell or organism. For example, a therapeutic polypeptide for a disorder related to buildup of a metabolite caused by a deficiency in a metabolic enzyme or activity may supply a missing metabolic enzyme or activity, or it may supply an alternate metabolic enzyme or activity that leads to reduction of the metabolite. A therapeutic polypeptide may also be used to reduce the activity of a polypeptide (e.g., one that is overexpressed, activated by a gain-of-function mutation, or whose activity is otherwise misregulated) by acting, e.g., as a dominant-negative polypeptide.

The nucleic acids of the invention may encode polypeptides that are intracellular proteins, anchored in the cell membrane, remain within the cell, or are secreted by the cell transduced with the vectors of the invention. For polypeptides secreted by the cell that receives the vector; the polypeptide can be soluble (i.e., not attached to the cell). For example, soluble polypeptides are devoid of a transmembrane region and are secreted from the cell. Techniques to identify and remove nucleic acid sequences which encode transmembrane domains are known in the art.

The nucleic acids if the invention (e.g. the AAV vector genome) may comprise as a transgene, a nucleic acid encoding a protein or functional RNA that modulates or treats a CNS-associated disorder. The following is a non-limiting list of genes associated with CNS-associated disorders: neuronal apoptosis inhibitory protein (NAIP), nerve growth factor (NGF), glial-derived growth factor (GDNF), brain-derived growth factor (BDNF), ciliary neurotrophic factor (CNTF), tyrosine hydroxylase (TM, GTP-cyclohydrolase (GTPCH), aspartoacylase (ASPA), superoxide dismutase (SOD1), an anti-oxidant, an anti-angiogenic polypeptide, an anti-inflammatory polypeptide, and amino acid decorboxylase (AADC). For example, a useful transgene in the treatment of Parkinson's disease encodes TH, which is a rate limiting enzyme in the synthesis of dopamine. A transgene encoding GTPCII, which generates the TII cofactor tetrahydrobiopterin, may also be used in the treatment of Parkinson's disease. A transgene encoding GDNF or BDNF, or AADC, which facilitates conversion of L-Dopa to DA, may also be used for the treatment of Parkinson's disease. For the treatment of ALS, a useful transgene may encode: GDNF, BDNF or CNTF. Also for the treatment of ALS, a useful transgene may encode a functional RNA, e.g., shRNA, miRNA, that inhibits the expression of SOD1. For the treatment of ischemia a useful transgene may encode NAIP or NGF. A transgene encoding Beta-glucuronidase (GUS) may be useful for the treatment of certain lysosomal storage diseases (e.g., Mucopolysacharidosis type VII (MPS VII)). A transgene encoding a prodrug activation gene, e.g., HSV-Thymidine kinase which converts ganciclovir to a toxic nucleotide which disrupts DNA synthesis and leads to cell death, may be useful for treating certain cancers, e.g.,

when administered in combination with the prodrug. A transgene encoding an endogenous opioid, such a β-endorphin may be useful for treating pain. Examples of antioxidants include without limitation SOD1; SOD2; Catalase; Sirtuins 1, 3, 4, or 5; NRF2; PGC1a; GCL (catalytic subunit); GCL (modifier subunit); adiponectin; glutathione peroxidase 1; and neuroglobin. Examples of anti-angiogenic polypeptides include without limitation angiostatin, endostatin, PEDF, a soluble VEGF receptor, and a soluble PDGF receptor. Examples of anti-inflammatory polypeptides include without limitation IL-10, soluble IL17R, soluble TNF-R, TNF-R-Ig, an IL-1 inhibitor, and an IL18 inhibitor. Other examples of transgenes that may be used in the rAAV vectors of the invention will be apparent to the skilled artisan (See, e.g., Costantini L C, et al., *Gene Therapy* (2000) 7, 93-109).

In some embodiments, the heterologous nucleic acid encodes a therapeutic nucleic acid. In some embodiments, a therapeutic nucleic acid may include without limitation an siRNA, an shRNA, an RNAi, a miRNA, an antisense RNA, a ribozyme or a DNAzyme. As such, a therapeutic nucleic acid may encode an RNA that when transcribed from the nucleic acids of the vector can treat a disorder by interfering with translation or transcription of an abnormal or excess protein associated with a disorder of the invention. For example, the nucleic acids of the invention may encode for an RNA which treats a disorder by highly specific elimination or reduction of mRNA encoding the abnormal and/or excess proteins. Therapeutic RNA sequences include RNAi, small inhibitory RNA (siRNA), micro RNA (miRNA), and/or ribozymes (such as hammerhead and hairpin ribozymes) that can treat disorders by highly specific elimination or reduction of mRNA encoding the abnormal and/or excess proteins.

In some embodiments, the therapeutic polypeptide or therapeutic nucleic acid is used to treat a disorder of the CNS. Without wishing to be bound to theory, it is thought that a therapeutic polypeptide or therapeutic nucleic acid may be used to reduce or eliminate the expression and/or activity of a polypeptide whose gain-of-function has been associated with a disorder, or to enhance the expression and/or activity of a polypeptide to complement a deficiency that has been associated with a disorder (e.g., a mutation in a gene whose expression shows similar or related activity). Non-limiting examples of disorders of the invention that may be treated by a therapeutic polypeptide or therapeutic nucleic acid of the invention (exemplary genes that may be targeted or supplied are provided in parenthesis for each disorder) include stroke (e.g., caspase-3, Beclin1, Ask1, PAR1, HIF1α, PUMA, and/or any of the genes described in Fukuda, A. M. and Badaut, J. (2013) *Genes* (*Basel*) 4:435-456), Huntington's disease (mutant HTT), epilepsy (e.g., SCN1A, NMDAR, ADK, and/or any of the genes described in Boison, D. (2010) *Epilepsia* 51:1659-1668), Parkinson's disease (alpha-synuclein), Lou Gehrig's disease (also known as amyotrophic lateral sclerosis; SOD1), Alzheimer's disease (tau, amyloid precursor protein), corticobasal degeneration or CBD (tau), corticogasal ganglionic degeneration or CBGD (tau), frontotemporal dementia or FTD (tau), progressive supranuclear palsy or PSP (tau), multiple system atrophy or MSA (alpha-synuclein), cancer of the brain (e.g., a mutant or overexpressed oncogene implicated in brain cancer), and lysosomal storage diseases (LSD). Disorders of the invention may include those that involve large areas of the cortex, e.g., more than one functional area of the cortex, more than one lobe of the cortex, and/or the entire cortex. Other non-limiting examples of disorders of the invention that may be treated by a therapeutic polypeptide or therapeutic nucleic acid of the invention include traumatic brain injury, enzymatic dysfunction disorders, psychiatric disorders (including post-traumatic stress syndrome), neurodegenerative diseases, and cognitive disorders (including dementias, autism, and depression). Enzymatic dysfunction disorders include without limitation leukodystrophies (including Canavan's disease) and any of the lysosomal storage diseases described below.

In some embodiments, the therapeutic polypeptide or therapeutic nucleic acid is used to treat a lysosomal storage disease. As is commonly known in the art, lysosomal storage disease are rare, inherited metabolic disorders characterized by defects in lysosomal function. Such disorders are often caused by a deficiency in an enzyme required for proper mucopolysaccharide, glycoprotein, and/or lipid metabolism, leading to a pathological accumulation of lysosomally stored cellular materials. Non-limiting examples of lysosomal storage diseases of the invention that may be treated by a therapeutic polypeptide or therapeutic nucleic acid of the invention (exemplary genes that may be targeted or supplied are provided in parenthesis for each disorder) include Gaucher disease type 2 or type 3 (acid beta-glucosidase, GBA), GM1 gangliosidosis (beta-galactosidase-1, GLB1), Hunter disease (iduronate 2-sulfatase, IDS), Krabbe disease (galactosylceramidase, GALC), a mannosidosis disease (a mannosidase, such as alpha-D-mannosidase, MAN2B1), R mannosidosis disease (beta-mannosidase, MANBA), metachromatic leukodystrophy disease (pseudoarylsulfatase A, ARSA), mucolipidosisII/III disease (N-acetylglucosamine-1-phosphotransferase, GNPTAB), Niemann-Pick A discase (acid sphingomyclinasc, ASM), Nicmann-Pick C disease (Niemann-Pick C protein, NPC1), Pompe disease (acid alpha-1,4-glucosidase, GAA), Sandhoff disease (hexosaminidase beta subunit, HEXB), Sanfilippo A disease (N-sulfoglucosamine sulfohydrolase, MPS3A), Sanfilippo B disease (N-alpha-acetylglucosaminidase, NAGLU), Sanfilippo C disease (heparin acetyl-CoA:alpha-glucosaminidase N-acetyltransferase, MPS3C), Sanfilippo D disease (N-acetylglucosamine-6-sulfatase, GNS), Schindler disease (alpha-N-acetylgalactosaminidase, NAGA), Sly disease (beta-glucuronidase, GUSB), Tay-Sachs disease (hexosaminidase alpha subunit, HEXA), and Wolman disease (lysosomal acid lipase, LIPA).

Additional lysosomal storage diseases, as well as the defective enzyme associated with each disease, are listed in Table 1 below. In some embodiments, a disease listed in the table below is treated by a therapeutic polypeptide or therapeutic nucleic acid of the invention that complements or otherwise compensates for the corresponding enzymatic defect.

TABLE 1

Lysosomal storage disorders and associated defective enzymes.

Lysosomal storage disease	Defective enzyme
Aspartylglusoaminuria	Aspartylglucosaminidase
Fabry	Alpha-galactosidase A
Infantile Batten Disease (CNL1)	Palmitoyl protein thioesterase
Classic Late Infantile Batten Disease (CNL2)	Tripeptidyl peptidase
Juvenile Batten Disease (CNL3)	Lysosomal transmembrane protein
Batten, other forms (CNL4-CNL8)	multiple gene products
Cystinosis	Cysteine transporter
Farber	Acid ceramidase
Fucosidosis	Acid alpha-L-fucosidase
Galactosidosialidosis	Protective protein/cathepsin A

TABLE 1-continued

Lysosomal storage disorders and associated defective enzymes.

Lysosomal storage disease	Defective enzyme
Gaucher types 1, 2, and 3	Acid beta-glucosidase
GM1 gangliosidosis	Acid beta-galactosidase
Hunter	Iduronate-2-sulfatase
Hurler-Scheie	Alpha-L-iduronidase
Krabbe	Galactocerebrosidase
alpha-mannosidosis	Acid alpha-mannosidase
beta-mannosidosis	Acid beta-mannosidase
Maroteaux-Lamy	Arylsulfatase B
Metachromatic leukodystrophy	Arylsulfatase A
Morquio A	N-acetylgalactosamine-6-sulfate
Morquio B	Acid beta-galactosidase
Mucolipidosis II/III	N-acetylglucosamine-1-phosphotransferase
Niemann-Pick A, B	Acid sphingomyelinase
Niemann-Pick C	NPC-1
Pompe acid	alpha-glucosidase
Sandhoff	beta-hexosaminidase B
Sanfilippo A	Heparan N-sulfatase
Sanfilippo B	alpha-N-acetylglucosaminidase
Sanfilippo C	Acetyl-CoA:alpha-glucoasaminide N-acetyltransferase
Sanfilippo D	N-acetylglucosamine-6-sulfate
Schindler disease	alpha-N-acetylgalactosaminidase
Schindler-Kanzaki	alpha-N-acetylgalactosaminidase
Sialidosis	alpha-neuramidase
Sly	beta-glucuronidase
Tay-Sachs	beta-hexosaminidase A
Wolman	Acid lipase

In some embodiments, the heterologous nucleic acid is operably linked to a promoter. Exemplary promoters include, but are not limited to, the cytomegalovirus (CMV) immediate early promoter, the RSV LTR, the MoMLV LTR, the phosphoglycerate kinase-1 (PGK) promoter, a simian virus 40 (SV40) promoter and a CK6 promoter, a transthyretin promoter (TTR), a TK promoter, a tetracycline responsive promoter (TRE), an HBV promoter, an hAAT promoter, a LSP promoter, chimeric liver-specific promoters (LSPs), the E2F promoter, the telomerase (hTERT) promoter; the cytomegalovirus enhancer/chicken beta-actin/Rabbit β-globin promoter (CAG promoter; Niwa et al., *Gene,* 1991, 108(2):193-9) and the elongation factor 1-alpha promoter (EF1-alpha) promoter (Kim et al., *Gene,* 1990, 91(2):217-23 and Guo et al., *Gene Ther.,* 1996, 3(9):802-10). In some embodiments, the promoter comprises a human β-glucuronidase promoter or a cytomegalovirus enhancer linked to a chicken β-actin (CBA) promoter. The promoter can be a constitutive, inducible or repressible promoter. In some embodiments, the invention provides a recombinant vector comprising nucleic acid encoding a heterologous transgene of the present disclosure operably linked to a CBA promoter. Exemplary promoters and descriptions may be found, e.g., in U.S. PG Pub. 20140335054.

Examples of constitutive promoters include, without limitation, the retroviral Rous sarcoma virus (RSV) LTR promoter (optionally with the RSV enhancer), the cytomegalovirus (CMV) promoter (optionally with the CMV enhancer) [see, e.g., Boshart et al., Cell, 41:521-530 (1985)], the SV40 promoter, the dihydrofolate reductase promoter, the 13-actin promoter, the phosphoglycerol kinase (PGK) promoter, and the EF1a promoter [Invitrogen].

Inducible promoters allow regulation of gene expression and can be regulated by exogenously supplied compounds, environmental factors such as temperature, or the presence of a specific physiological state, e.g., acute phase, a particular differentiation state of the cell, or in replicating cells only. Inducible promoters and inducible systems are available from a variety of commercial sources, including, without limitation, Invitrogen, Clontech and Ariad. Many other systems have been described and can be readily selected by one of skill in the art. Examples of inducible promoters regulated by exogenously supplied promoters include the zinc-inducible sheep metallothionine (MT) promoter, the dexamethasone (Dex)-inducible mouse mammary tumor virus (MMTV) promoter, the T7 polymerase promoter system (WO 98/10088); the ecdysone insect promoter (No et al., *Proc. Natl. Acad. Sci. USA,* 93:3346-3351 (1996)), the tetracycline-repressible system (Gossen et al., *Proc. Natl. Acad. Sci. USA,* 89:5547-5551 (1992)), the tetracycline-inducible system (Gossen et al., *Science,* 268:1766-1769 (1995), see also Harvey et al., *Curr. Opin. Chem. Biol.,* 2:512-518 (1998)), the RU486-inducible system (Wang et al., *Nat. Biotech.,* 15:239-243 (1997) and Wang et al., *Gene Ther.,* 4:432-441 (1997)) and the rapamycin-inducible system (Magari et al., *J. Clin. Invest.,* 100:2865-2872 (1997)). Still other types of inducible promoters which may be useful in this context are those which are regulated by a specific physiological state, e.g., temperature, acute phase, a particular differentiation state of the cell, or in replicating cells only.

In another embodiment, the native promoter, or fragment thereof, for the transgene will be used. The native promoter can be used when it is desired that expression of the transgene should mimic the native expression. The native promoter may be used when expression of the transgene must be regulated temporally or developmentally, or in a tissue-specific manner, or in response to specific transcriptional stimuli. In a further embodiment, other native expression control elements, such as enhancer elements, polyadenylation sites or Kozak consensus sequences may also be used to mimic the native expression

In some embodiments, the regulatory sequences impart tissue-specific gene expression capabilities. In some cases, the tissue-specific regulatory sequences bind tissue-specific transcription factors that induce transcription in a tissue specific manner. Such tissue-specific regulatory sequences (e.g., promoters, enhancers, etc.) are well known in the art.

In some embodiments, the vector comprises an intron. For example, in some embodiments, the intron is a chimeric intron derived from chicken beta-actin and rabbit beta-globin. In some embodiments, the intron is a minute virus of mice (MVM) intron.

In some embodiments, the vector comprises a polyadenylation (polyA) sequence. Numerous examples of polyadenylation sequences are known in the art, such as a bovine growth hormone (BGH) Poly(A) sequence (see, e.g., accession number EF592533), an SV40 polyadenylation sequence, and an HSV TK pA polyadenylation sequence.

V. Viral Particles and Methods of Producing Viral Particles

Certain aspects of the present disclosure relate to recombinant viral particles (e.g., rAAV particles).

Based on the guidance provided herein, the techniques of the present disclosure may suitably be adapted by one of skill in the art for use with a variety of different viruses.

In some embodiments, the virus is of the family Adenoviridae, which includes non-enveloped viruses typically known as Adenoviruses. In some embodiments, the virus is of the genus Atadenovirus, Aviadenovirus, Ichtadenovirus, Mastadenovirus, or Siadenovirus.

In some embodiments, the virus is of the family Parvoviridae, which includes non-enveloped viruses such as AAV and Bocaparvovirus. In some embodiments, the virus is of

the subfamily Densovirinae. In some embodiments, the virus is of the genus Ambidensovirus, Brevidensovirus, Hepandensovirus, Iteradensovirus, or Penstyldensovirus. In some embodiments, the virus is of the subfamily Parvovirinae. In some embodiments, the virus is of the genus Amdoparvovirus, Aveparvovirus, Bocaparvovirus, Copiparvovirus, Dependoparvovirus, Erythroparvovirus, Protoparvovirus, or Tetraparvovirus.

In some embodiments, the virus is of the family Retroviridae, which includes enveloped viruses including lentivirus. In some embodiments, the virus is of the subfamily Orthoretrovirinae. In some embodiments, the virus is of the genus Alpharetrovirus, Betaretrovirus, Deltaretrovirus, Epsilonretrovirus, Gammaretrovirus, or Lentivirus. In some embodiments, the virus is of the subfamily Spumaretrovirinae. In some embodiments, the virus is of the genus Spumavirus.

In some embodiments, the virus is of the family Baculoviridae, which includes enveloped viruses including alphabaculovirus. In some embodiments, the virus is of the genus Alphabaculovirus, Betabaculovirus, Deltabaculovirus, or Gammabaculovirus.

In some embodiments, the virus is of the family Herpesviridae, which includes enveloped viruses such as the simplex viruses HSV-1 and HSV-2. In some embodiments, the virus is of the subfamily Alphaherpesvirinae. In some embodiments, the virus is of the genus Iltovirus, Mardivirus, Simplexvirus, or Varicellovirus. In some embodiments, the virus is of the subfamily Betaherpesvirinae. In some embodiments, the virus is of the genus Cytomegalovirus, Muromegalovirus, Proboscivirus, or Roseolovirus. In some embodiments, the virus is of the subfamily Gammaherpesvirinae. In some embodiments, the virus is of the genus Lymphocryptovirus, Macavirus, Percavirus, or Rhadinovirus.

In some embodiments, the virus is an AAV virus. In an AAV particle, a nucleic acid is encapsidated in the AAV particle. The AAV particle also comprises capsid proteins. In some embodiments, the nucleic acid comprises a heterologous nucleic acid and/or one or more of the following components, operatively linked in the direction of transcription, control sequences including transcription initiation and termination sequences, thereby forming an expression cassette.

In some embodiments, the viral particle comprises an AAV ITR sequence. For example, an expression cassette may be flanked on the 5' and 3' end by at least one functional AAV ITR sequence. By "functional AAV ITR sequences" it is meant that the ITR sequences function as intended for the rescue, replication and packaging of the AAV virion. See Davidson et al., *PNAS,* 2000, 97(7)3428-32; Passini et al., *J. Virol.,* 2003, 77(12):7034-40; and Pechan et al., *Gene Ther.,* 2009, 16:10-16, all of which are incorporated herein in their entirety by reference. For practicing some aspects of the invention, the recombinant vectors comprise at least all of the sequences of AAV essential for encapsidation and the physical structures for infection by the rAAV. AAV ITRs for use in the vectors of the invention need not have a wild-type nucleotide sequence (e.g., as described in Kotin, *Hum. Gene Ther.,* 1994, 5:793-801), and may be altered by the insertion, deletion or substitution of nucleotides or the AAV ITRs may be derived from any of several AAV serotypes. More than 40 serotypes of AAV are currently known, and new serotypes and variants of existing serotypes continue to be identified. See Gao et al., *PNAS,* 2002, 99(18): 11854-6; Gao et al., *PNAS,* 2003, 100(10):6081-6; and Bossis et al., *J. Virol.,* 2003, 77(12):6799-810. Use of any AAV serotype is considered within the scope of the present invention. In some embodiments, a rAAV vector is a vector derived from an AAV serotype, including without limitation, AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV DJ, AAV DJ8, a goat AAV, bovine AAV, or mouse AAV ITRs or the like. In some embodiments, the nucleic acid in the AAV (e.g., an rAAV vector) comprises an ITR of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV DJ, AAV DJ8, a goat AAV, bovine AAV, or mouse AAV ITRs or the like. In some embodiments, the AAV particle comprises an AAV vector encoding a heterologous transgene flanked by one or more AAV ITRs.

In some embodiments, a rAAV particle comprises an encapsulation protein selected from AAV1, AAV2, AAV3, AAV4, AAV5, AAV6 (e.g., a wild-type AAV6 capsid, or a variant AAV6 capsid such as ShH10, as described in U.S. PG Pub. 2012/0164106), AAV7, AAV8, AAVrh8, AAVrh8R, AAV9 (e.g., a wild-type AAV9 capsid, or a modified AAV9 capsid as described in U.S. PG Pub. 2013/0323226), AAV10, AAVrh10, AAV11, AAV12, a tyrosine capsid mutant, a heparin binding capsid mutant, an AAV2R471A capsid, an AAVAAV2/2-7m8 capsid, an AAV LK03 capsid, an AAV DJ capsid (e.g., an AAV-DJ/8 capsid, an AAV-DJ/9 capsid, or any other of the capsids described in U.S. PG Pub. 2012/0066783), AAV2 N587A capsid, AAV2 E548A capsid, AAV2 N708A capsid, AAV V708K capsid, goat AAV capsid, AAV1/AAV2 chimeric capsid, bovine AAV capsid, mouse AAV capsid, rAAV2/HBoV1 capsid, an AAV2HBKO capsid, an AAVPHP.B capsid or an AAVPHP.eB capsid, or an AAV capsid described in U.S. Pat. No. 8,283,151 or International Publication No. WO/2003/042397. In further embodiments, a rAAV particle comprises capsid proteins of an AAV serotype from Clades A-F.

Certain aspects of the present disclosure relate to an AAV (e.g., a rAAV) capsid protein comprising an amino acid substitution at amino acid residue 2. In some embodiments, the amino acid substitution at amino acid residue 2 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of the parent AAV capsid protein. As described herein, the amino acid at the 2^{nd} position to the initiating methionine (iMet X) of an AAV capsid protein may be examined for effects on N-terminal acetylation, trafficking, transduction, and/or other post-translational modification(s) (e.g., glycosylation, ubiquitination, and so forth). Any assay described herein for examining acetylation, or a functional consequence thereof related to AAV particles, may be used to assess N-terminal acetylation. In some embodiments, amino acid residue 2 of the AAV capsid protein (e.g., VP1 or VP3) is substituted with Cys, Ser, Thr, Val, Gly, Asn, Asp, Glu, Ile, Leu, Phe, Gln, Lys, Met, Pro or Tyr. In some embodiments, the amino acid substitution results in less deamidation of the AAV capsid.

Other aspects of the present disclosure relate to an AAV (e.g., a rAAV) capsid protein comprising an amino acid substitution that alters deamidation. In some embodiments, an amino acid substitution (e.g., an amino acid substitution at A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3) that "alters" deamidation results in a higher frequency of deamidation or a lower frequency of deamidation, e.g., as compared to a VP1 or VP3 without the substitution, such as the parental VP1 or VP3. As described herein, a potential deamidation site of an AAV capsid protein (e.g., VP1 or VP3) may be examined for effects on deami-

dation, trafficking, transduction, and/or other post-translational modification(s) (e.g., glycosylation, ubiquitination, and so forth). Any assay described herein for examining deamidation, or a functional consequence thereof related to AAV particles, may be used to assess deamidation.

Several potential deamidation sites are described herein. In some embodiments, an amino acid substitution that alters deamidation is selected from A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3. For example, in some embodiments, N57 of VP1, N382 of VP3, N511 of VP3, and/or N715 of VP3 is substituted with Asp, and the amino acid substitution results in a higher frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In other embodiments, the amino acid substitution is N57K or N57Q, and the amino acid substitution results in a lower frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In yet other embodiments, G58 of VP1, G383 of VP3, G512 of VP3, and/or G716 of VP3 is substituted with an amino acid that is not Gly (e.g., Ala, Arg, Asn, Asp, Cys, Glu, Gln, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val), and the amino acid substitution results in a lower frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle.

In some embodiments, the AAV capsid protein is VP1, VP2, or VP3. The AAV particle may comprise any of the exemplary AAV capsid serotypes described herein, such as AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV2/2-7m8, AAV DJ, AAV DJ8, AAV2 N587A, AAV2 E548A, AAV2 N708A, AAV V708K, goat AAV, AAV1/AAV2 chimeric, bovine AAV, mouse AAV, or rAAV2/HBoV1. The AAV capsid protein may further comprise any of the capsid protein mutations described herein, such as tyrosine and/or heparin binding mutations.

Other aspects of the present disclosure relate to methods of improving the stability of a rAAV particle. In some embodiments, the methods include substituting amino acid residue 2 of VP1 and/or VP3, e.g., as described herein. For example, in some embodiments, amino acid residue 2 of VP1 is substituted. In other embodiments, amino acid residue 2 of VP3 is substituted. In some embodiments, the substituted amino acid at position 2 is N-acetylated at a higher frequency than amino acid residue 2 of the parent VP1 and/or VP3, e.g., as described herein. In some embodiments, substituting amino acid residue 2 of VP1 and/or VP3 improves the stability of a rAAV particle by at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 100%. In some embodiments, the stability of a rAAV particle with a substituted amino acid at position 2 may be compared to a wild-type or parental AAV capsid, e.g., of the same serotype. For example, in some embodiments, substituting amino acid residue 2 of VP1 and/or VP3 improves the stability of a rAAV particle by any one of about 10% to about 100%, about 20% to about 100%, about 30% to about 100%, about 40% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 10% to about 90%, about 20% to about 90%, about 30% to about 90%, about 40% to about 90%, about 50% to about 90%, about 60% to about 90%, about 70% to about 90%, about 80% to about 90%, about 10% to about 80%, about 20% to about 80%, about 30% to about 80%, about 40% to about 80%, about 50% to about 80%, about 60% to about 80%, about 70% to about 80%, about 10% to about 70%, about 20% to about 70%, about 30% to about 70%, about 40% to about 70%, about 50% to about 70%, about 60% to about 70%, about 10% to about 60%, about 20% to about 60%, about 30% to about 60%, about 40% to about 60%, about 50% to about 60%, about 10% to about 50%, about 20% to about 50%, about 30% to about 50%, about 40% to about 50%, about 10% to about 40%, about 20% to about 40%, about 30% to about 40%, about 10% to about 30%, about 20% to about 30%, or about 10% to about 20%, e.g., as compared to stability of a rAAV particle comprising a wild-type capsid. AAV particle stability may be measured using various assays known in the art, including without limitation differential scanning fluorescence (DSF), differential scanning calorimetry (DSC), other thermal denaturation assays, susceptibility to proteolysis, imaging or structural analysis to observe denaturation (e.g., using electron microscopy), transduction efficiency or another functional assay on AAV particle compositions kept for a designated time interval at a particular temperature (e.g., room temperature, or 4° C., for thermal stability) or treated at a particular pH (e.g., pH stability), and the like.

Other aspects of the present disclosure relate to methods of improving the assembly of a rAAV particle. In some embodiments, the methods include substituting amino acid residue 2 of VP1 and/or VP3, e.g., as described herein. For example, in some embodiments, amino acid residue 2 of VP1 is substituted. In other embodiments, amino acid residue 2 of VP3 is substituted. In some embodiments, the substituted amino acid at position 2 is N-acetylated at a higher frequency than amino acid residue 2 of the parent VP1 and/or VP3, e.g., as described herein. In some embodiments, substituting amino acid residue 2 of VP1 and/or VP3 improves the assembly of a rAAV particle by at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 100%. In some embodiments, the assembly of a rAAV particle with a substituted amino acid at position 2 may be compared to a wild-type or parental AAV capsid, e.g., of the same serotype. For example, in some embodiments, substituting amino acid residue 2 of VP1 and/or VP3 improves the assembly of a rAAV particle by any one of about 10% to about 100%, about 20% to about 100%, about 30% to about 100%, about 40% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 10% to about 90%, about 20% to about 90%, about 30% to about 90%, about 40% to about 90%, about 50% to about 90%, about 60% to about 90%, about 70% to about 90%, about 80% to about 90%, about 10% to about 80%, about 20% to about 80%, about 30% to about 80%, about 40% to about 80%, about 50% to about 80%, about 60% to about 80%, about 70% to about 80%, about 10% to about 70%, about 20% to about 70%, about 30% to about 70%, about 40% to about 70%, about 50% to about 70%, about 60% to about 70%, about 10% to about 60%, about 20% to about 60%, about 30% to about 60%, about 40% to about 60%, about 50% to about 60%, about 10% to about 50%, about 20% to about 50%, about 30% to about

50%, about 40% to about 50%, about 10% to about 40%, about 20% to about 40%, about 30% to about 40%, about 10% to about 30%, about 20% to about 30%, or about 10% to about 20%, e.g., as compared to assembly of a rAAV particle comprising a wild-type capsid. AAV particle assembly may be measured using various assays known in the art, including without limitation, measuring particle production amount and/or rate, quantifying capsid production (e.g., after purification using any of the methods described herein), assaying production of complete vectors vs. empty capsids, measuring transduction efficiency, imaging or structural analysis to observe particle formation (e.g., using electron microscopy), production of AAV capsid proteins (e.g., as assayed by Western blotting), and the like.

Other aspects of the present disclosure relate to methods of improving the transduction of a rAAV particle. In some embodiments, the methods include substituting amino acid residue 2 of VP1 and/or VP3, e.g., as described herein. For example, in some embodiments, amino acid residue 2 of VP1 is substituted. In other embodiments, amino acid residue 2 of VP3 is substituted. In some embodiments, the substituted amino acid at position 2 is N-acetylated at a higher frequency than amino acid residue 2 of the parent VP1 and/or VP3, e.g., as described herein. In some embodiments, substituting amino acid residue 2 of VP1 and/or VP3 improves the transduction of a rAAV particle by at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 100%. In some embodiments, the transduction of a rAAV particle with a substituted amino acid at position 2 may be compared to a wild-type or parental AAV capsid, e.g., of the same serotype. For example, in some embodiments, substituting amino acid residue 2 of VP1 and/or VP3 improves the transduction of a rAAV particle by any one of about 10% to about 100%, about 20% to about 100%, about 30% to about 100%, about 40% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 10% to about 90%, about 20% to about 90%, about 30% to about 90%, about 40% to about 90%, about 50% to about 90%, about 60% to about 90%, about 70% to about 90%, about 80% to about 90%, about 10% to about 80%, about 20% to about 80%, about 30% to about 80%, about 40% to about 80%, about 50% to about 80%, about 60% to about 80%, about 70% to about 80%, about 10% to about 70%, about 20% to about 70%, about 30% to about 70%, about 40% to about 70%, about 50% to about 70%, about 60% to about 70%, about 10% to about 60%, about 20% to about 60%, about 30% to about 60%, about 40% to about 60%, about 50% to about 60%, about 10% to about 50%, about 20% to about 50%, about 30% to about 50%, about 40% to about 50%, about 10% to about 40%, about 20% to about 40%, about 30% to about 40%, about 10% to about 30%, about 20% to about 30%, or about 10% to about 20%, e.g., as compared to transduction of a rAAV particle comprising a wild-type capsid. AAV particle transduction may be measured using various assays known in the art, including without limitation, the transduction efficiency assays described herein. In some embodiments, the invention provide methods of reducing the transduction of a rAAV particle; for example, by substituting amino acid residue 2 of VP1 and/or VP3.

Other aspects of the present disclosure relate to methods of improving the stability of a rAAV particle. In some embodiments, the methods include substituting an amino acid of VP1 and/or VP3 that alters deamidation, e.g., as described herein. For example, in some embodiments, A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3 is substituted. In some embodiments, the substituted amino acid is deamidated at a higher frequency than the amino acid residue of the parent VP1 and/or VP3, e.g., as described herein. In some embodiments, substituting A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, and/or G716 of VP3 improves the stability of a rAAV particle by at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 100%. In some embodiments, the stability of a rAAV particle with a substituted A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3 may be compared to a wild-type or parental AAV capsid, e.g., of the same serotype. For example, in some embodiments, substituting A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, and/or G716 of VP3 improves the stability of a rAAV particle by any one of about 10% to about 100%, about 20% to about 100%, about 30% to about 100%, about 40% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 10% to about 90%, about 20% to about 90%, about 30% to about 90%, about 40% to about 90%, about 50% to about 90%, about 60% to about 90%, about 70% to about 90%, about 80% to about 90%, about 10% to about 80%, about 20% to about 80%, about 30% to about 80%, about 40% to about 80%, about 50% to about 80%, about 60% to about 80%, about 70% to about 80%, about 10% to about 70%, about 20% to about 70%, about 30% to about 70%, about 40% to about 70%, about 50% to about 70%, about 60% to about 70%, about 10% to about 60%, about 20% to about 60%, about 30% to about 60%, about 40% to about 60%, about 50% to about 60%, about 10% to about 50%, about 20% to about 50%, about 30% to about 50%, about 40% to about 50%, about 10% to about 40%, about 20% to about 40%, about 30% to about 40%, about 10% to about 30%, about 20% to about 30%, or about 10% to about 20%, e.g., as compared to stability of a rAAV particle comprising a wild-type capsid. AAV particle stability may be measured using various assays known in the art, including without limitation differential scanning fluorescence (DSF), differential scanning calorimetry (DSC), other thermal denaturation assays, susceptibility to proteolysis, imaging or structural analysis to observe denaturation (e.g., using electron microscopy), transduction efficiency or another functional assay on AAV particle compositions kept for a designated time interval at a particular temperature (e.g., room temperature, or 4° C., for thermal stability) or treated at a particular pH (e.g., pH stability), and the like.

Other aspects of the present disclosure relate to methods of improving the assembly of a rAAV particle. In some embodiments, the methods include substituting an amino acid of VP1 and/or VP3 that alters deamidation, e.g., as described herein. For example, in some embodiments, A35

of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3 is substituted. In some embodiments, the substituted amino acid is deamidated at a higher frequency than the amino acid residue of the parent VP1 and/or VP3, e.g., as described herein. In some embodiments, substituting A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, and/or G716 of VP3 improves the assembly of a rAAV particle by at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 100%. In some embodiments, the stability of a rAAV particle with a substituted A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3 may be compared to a wild-type or parental AAV capsid, e.g., of the same serotype. For example, in some embodiments, substituting A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, and/or G716 of VP3 improves the assembly of a rAAV particle by any one of about 10% to about 100%, about 20% to about 100%, about 30% to about 100%, about 40% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 10% to about 90%, about 20% to about 90%, about 30% to about 90%, about 40% to about 90%, about 50% to about 90%, about 60% to about 90%, about 70% to about 90%, about 80% to about 90%, about 10% to about 80%, about 20% to about 80%, about 30% to about 80%, about 40% to about 80%, about 50% to about 80%, about 60% to about 80%, about 70% to about 80%, about 10% to about 70%, about 20% to about 70%, about 30% to about 70%, about 40% to about 70%, about 50% to about 70%, about 60% to about 70%, about 10% to about 60%, about 20% to about 60%, about 30% to about 60%, about 40% to about 60%, about 50% to about 60%, about 10% to about 50%, about 20% to about 50%, about 30% to about 50%, about 40% to about 50%, about 10% to about 40%, about 20% to about 40%, about 30% to about 40%, about 10% to about 30%, about 20% to about 30%, or about 10% to about 20%, e.g., as compared to assembly of a rAAV particle comprising a wild-type capsid. AAV particle assembly may be measured using various assays known in the art, including without limitation, measuring particle production amount and/or rate, quantifying capsid production (e.g., after purification using any of the methods described herein), assaying production of complete vectors vs. empty capsids, measuring transduction efficiency, imaging or structural analysis to observe particle formation (e.g., using electron microscopy), production of AAV capsid proteins (e.g., as assayed by Western blotting), and the like.

Other aspects of the present disclosure relate to methods of improving the transduction of a rAAV particle. In some embodiments, the methods include substituting an amino acid of VP1 and/or VP3 that alters deamidation, e.g., as described herein. For example, in some embodiments, A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3 is substituted. In some embodiments, the substituted amino acid is deamidated at a higher frequency than the amino acid residue of the parent VP1 and/or VP3, e.g., as described herein. In some embodiments, substituting A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, and/or G716 of VP3 improves the transduction of a rAAV particle by at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 100%. In some embodiments, the stability of a rAAV particle with a substituted A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3 may be compared to a wild-type or parental AAV capsid, e.g., of the same serotype. For example, in some embodiments, substituting A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, and/or G716 of VP3 improves the transduction of a rAAV particle by any one of about 10% to about 100%, about 20% to about 100%, about 30% to about 100%, about 40% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 10% to about 90%, about 20% to about 90%, about 30% to about 90%, about 40% to about 90%, about 50% to about 90%, about 60% to about 90%, about 70% to about 90%, about 80% to about 90%, about 10% to about 80%, about 20% to about 80%, about 30% to about 80%, about 40% to about 80%, about 50% to about 80%, about 60% to about 80%, about 70% to about 80%, about 10% to about 70%, about 20% to about 70%, about 30% to about 70%, about 40% to about 70%, about 50% to about 70%, about 60% to about 70%, about 10% to about 60%, about 20% to about 60%, about 30% to about 60%, about 40% to about 60%, about 50% to about 60%, about 10% to about 50%, about 20% to about 50%, about 30% to about 50%, about 40% to about 50%, about 10% to about 40%, about 20% to about 40%, about 30% to about 40%, about 10% to about 30%, about 20% to about 30%, or about 10% to about 20%, e.g., as compared to transduction of a rAAV particle comprising a wild-type capsid. AAV particle transduction may be measured using various assays known in the art, including without limitation, the transduction efficiency assays described herein.

In some aspects, the invention provides viral particles comprising a recombinant self-complementing genome (e.g., a self-complementary or self-complimenting rAAV vector). AAV viral particles with self-complementing vector genomes and methods of use of self-complementing AAV genomes are described in U.S. Pat. Nos. 6,596,535; 7,125,717; 7,465,583; 7,785,888; 7,790,154; 7,846,729; 8,093,054; and 8,361,457; and Wang Z., et al., (2003) *Gene Ther* 10:2105-2111, each of which are incorporated herein by reference in its entirety. A rAAV comprising a self-complementing genome will quickly form a double stranded DNA molecule by virtue of its partially complementing sequences (e.g., complementing coding and non-coding strands of a heterologous nucleic acid). In some embodiments, the vector comprises a first nucleic acid sequence encoding a heterologous nucleic acid and a second nucleic acid sequence encoding a complement of the nucleic acid, where the first nucleic acid sequence can form intrastrand base pairs with the second nucleic acid sequence along most or all of its length.

In some embodiments, the first heterologous nucleic acid sequence and a second heterologous nucleic acid sequence are linked by a mutated ITR (e.g., the right ITR). In some

embodiments, the ITR comprises the polynucleotide sequence 5'-CACTCCCTCTCTGCGCGCTCGCTCGCT-CACTGAGGCC GGGCGACCAAAGGTCGCC-CACGCCCGGGCTTTGCCCGGGCG-3' (SEQ ID NO:8). The mutated ITR comprises a deletion of the D region comprising the terminal resolution sequence. As a result, on replicating an AAV viral genome, the rep proteins will not cleave the viral genome at the mutated ITR and as such, a recombinant viral genome comprising the following in 5' to 3' order will be packaged in a viral capsid: an AAV ITR, the first heterologous polynucleotide sequence including regulatory sequences, the mutated AAV ITR, the second heterologous polynucleotide in reverse orientation to the first heterologous polynucleotide and a third AAV ITR.

Different AAV serotypes are used to optimize transduction of particular target cells or to target specific cell types within a particular target tissue (e.g., a diseased tissue). A rAAV particle can comprise viral proteins and viral nucleic acids of the same serotype or a mixed serotype. For example, a rAAV particle may contain one or more ITRs and capsid derived from the same AAV serotype, or a rAAV particle may contain one or more ITRs derived from a different AAV serotype than capsid of the rAAV particle.

In some embodiments, the AAV capsid comprises a mutation, e.g., the capsid comprises a mutant capsid protein. In some embodiments, the mutation is a tyrosine mutation or a heparin binding mutation. In some embodiments, a mutant capsid protein maintains the ability to form an AAV capsid. In some embodiments, the rAAV particle comprises an AAV2 or AAV5 tyrosine mutant capsid (see, e.g., Zhong L. et al., (2008) *Proc Nal Acad Sci USA* 105(22):7827-7832), such as a mutation in Y444 or Y730 (numbering according to AAV2). In further embodiments, the rAAV particle comprises capsid proteins of an AAV serotype from Clades A-F (Gao, et al., *J. Virol.* 2004, 78(12):6381).

In some embodiments, a capsid protein comprises one or more amino acid substitutions at one or more positions that interact with a heparin sulfate proteoglycan or at one or more positions corresponding to amino acids 484, 487, 527, 532, 585 or 588, numbering based on VP1 numbering of AAV2. Heparan sulfate proteoglycan (HSPG) is known in the art to act as the cellular receptor for AAV2 particles (Summerford, C. and Samulski, R. J. (1998) *J. Virol.* 72(2):1438-45). Binding between an AAV2 particle and HSPG at the cell membrane serves to attach the particle to the cell. Other cell surface proteins such as fibroblast growth factor receptor and αvβ5 integrin may also facilitate cellular infection. After binding, an AAV2 particle may enter the cell through mechanisms including receptor mediated endocytosis via clathrin-coated pits. An AAV2 particle may be released from an endocytic vesicle upon endosomal acidification. This allows the AAV2 particle to travel to the perinuclear region and then the cell nucleus. AAV3 particles are also known to bind heparin (Rabinowitz, J. E., et al. (2002) *J. Virol.* 76(2):791-801).

The binding between AAV2 capsid proteins and HSPG is known to occur via electrostatic interactions between basic AAV2 capsid protein residues and negatively charged glycosaminoglycan residues (Opie, S R et al., (2003) *J. Virol.* 77:6995-7006; Kern, A et al., (2003) *J. Virol.* 77:11072-11081). Specific capsid residues implicated in these interactions include R484, R487, K527, K532, R585, and R588. Mutations in these residues have been shown to reduce AAV2 binding to Hela cells and heparin itself (Opie, S R et al., (2003) *J. Virol.* 77:6995-7006; Kern, A et al., (2003) *J. Virol.* 77:11072-11081; WO 2004/027019 A2, U.S. Pat. No. 7,629,322). Further, without wishing to be bound to theory, it is thought that amino acid substitution(s) at one or more of the residues corresponding to amino acids 484, 487, 527, 532, 585 or 588, numbering based on VP1 numbering of AAV2 may modulate the transduction properties of AAV capsid types that do not bind to HSPG, or may modulate the transduction properties of AAV capsid types independent from their ability to bind HSPG. In some embodiments, the one or more amino acid substitutions comprises a substitution at position R484, R487, K527, K532, R585 and/or R588 of VP1, VP2 and/or VP3, numbering based on VP1 of AAV2.

In some embodiments, the one or more amino acid substitutions reduce binding of the rAAV particle to the heparin sulfate proteoglycan by about at least 10%, about at least 25%, about at least 50%, about at least 75%, or about at least 100%. In some embodiments, the one or more amino acid substitutions reduce binding of the rAAV particle to the heparin sulfate proteoglycan by about at least 10%, about at least 15%, about at least 20%, about at least 25%, about at least 30%, about at least 35%, about at least 40%, about at least 45%, about at least 50%, about at least 55%, about at least 60%, about at least 65%, about at least 70%, about at least 75%, about at least 80%, about at least 85%, about at least 90%, about at least 95%, or about at least 100% (as compared to binding of a rAAV particle comprising a wild-type capsid). In some embodiments, the one or more amino acid substitutions reduce binding of the rAAV particle to the heparin sulfate proteoglycan by any one of about 10% to about 100%, about 20% to about 100%, about 30% to about 100%, about 40% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 10% to about 90%, about 20% to about 90%, about 30% to about 90%, about 40% to about 90%, about 50% to about 90%, about 60% to about 90%, about 70% to about 90%, about 80% to about 90%, about 10% to about 80%, about 20% to about 80%, about 30% to about 80%, about 40% to about 80%, about 50% to about 80%, about 60% to about 80%, about 70% to about 80%, about 10% to about 70%, about 20% to about 70%, about 30% to about 70%, about 40% to about 70%, about 50% to about 70%, about 60% to about 70%, about 10% to about 60%, about 20% to about 60%, about 30% to about 60%, about 40% to about 60%, about 50% to about 60%, about 10% to about 50%, about 20% to about 50%, about 30% to about 50%, about 40% to about 50%, about 10% to about 40%, about 20% to about 40%, about 30% to about 40%, about 10% to about 30%, about 20% to about 30%, or about 10% to about 20%, (as compared to binding of a rAAV particle comprising a wild-type capsid). In some embodiments, the one or more amino acid substitutions results in no detectable binding of the rAAV particle to the heparin sulfate proteoglycan compared to binding of a wild-type rAAV particle. Means to measure binding of AAV particles to HSPG are known in the art; e.g., binding to a heparin sulfate chromatography media or binding to a cell known to express HSPG on its surface. For example, see Opie, S R et al., (2003) *J. Virol.* 77:6995-7006 and Kern, A et al., (2003) *J. Virol.* 77:11072-11081. In some embodiments, the one or more amino acid substitutions improve the transduction efficiency of the rAAV particle to a cell (e.g., a cell in the eye or CNS) by about at least 10%, about at least 15%, about at least 20%, about at least 25%, about at least 30%, about at least 35%, about at least 40%, about at least 45%, about at least 50%, about at least 55%, about at least 60%, about at least 65%, about at least 70%, about at least 75%, about at least 80%, about at least 85%, about at least 90%, about at least 95%, or about at least

100% (as compared to transduction efficiency of a rAAV particle comprising a wild-type capsid). In some embodiments, the one or more amino acid substitutions improve the transduction efficiency of the rAAV particle to a cell (e.g., a cell in the eye or CNS) by any one of about 10% to about 100%, about 20% to about 100%, about 30% to about 100%, about 40% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 10% to about 90%, about 20% to about 90%, about 30% to about 90%, about 40% to about 90%, about 50% to about 90%, about 60% to about 90%, about 70% to about 90%, about 80% to about 90%, about 10% to about 80%, about 20% to about 80%, about 30% to about 80%, about 40% to about 80%, about 50% to about 80%, about 60% to about 80%, about 70% to about 80%, about 10% to about 70%, about 20% to about 70%, about 30% to about 70%, about 40% to about 70%, about 50% to about 70%, about 60% to about 70%, about 10% to about 60%, about 20% to about 60%, about 30% to about 60%, about 40% to about 60%, about 50% to about 60%, about 10% to about 50%, about 20% to about 50%, about 30% to about 50%, about 40% to about 50%, about 10% to about 40%, about 20% to about 40%, about 30% to about 40%, about 10% to about 30%, about 20% to about 30%, or about 10% to about 20%, (as compared to transduction efficiency of a rAAV particle comprising a wild-type capsid). Means to measure transduction efficiency of AAV particles to a cell (e.g., a cell in culture or part of a tissue) are known in the art. For example, a population of cells (e.g., in culture or part of a tissue) may be infected with a concentration of rAAV particles containing a vector that, when expressed in the cells, produces an assayable reporter (e.g., GFP fluorescence, sFLT production, etc.).

AAV Capsid Proteins

In some aspects, the invention provides an AAV capsid protein comprising an amino acid substitution at amino acid residue 2; wherein the amino acid substitution at amino acid residue 2 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of the parent AAV capsid protein. In some embodiments, the AAV capsid protein is VP1 or VP3. In some embodiments, amino acid residue 2 of the AAV capsid protein (e.g., VP1 or VP3) is substituted with Cys, Ser, Thr, Val, Gly, Asn, Asp, Glu, Ile, Leu, Phe, Gln, Lys, Met, Pro or Tyr. In some embodiments, the amino acid substitution results in less deamidation of the AAV capsid protein. Non-limiting examples of AAV capsid proteins of the invention include VP1 and/or VP3 of any of the following AAV serotypes: AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV2/2-7m8, AAV DJ, AAV DJ8, AAV2 N587A, AAV2 E548A, AAV2 N708A, AAV V708K, goat AAV, AAV1/AAV2 chimeric, bovine AAV, mouse AAV, or rAAV2/HBoV1 serotype capsid. In some embodiments, the AAV capsid further comprises a tyrosine mutation or a heparin binding mutation.

Production of AAV Particles

Numerous methods are known in the art for production of rAAV vectors, including transfection, stable cell line production, and infectious hybrid virus production systems which include adenovirus-AAV hybrids, herpesvirus-AAV hybrids (Conway, J E et al., (1997) *J. Virology* 71(11):8780-8789) and baculovirus-AAV hybrids (Urabe, M. et al., (2002) *Human Gene Therapy* 13(16):1935-1943; *Kotin, R.* (2011) *Hum Mol Genet.* 20(R1): R2-R6). rAAV production cultures for the production of rAAV viral particles all require; 1) suitable host cells, 2) suitable helper virus function, 3) AAV rep and cap genes and gene products; 4) a nucleic acid (such as a therapeutic nucleic acid) flanked by at least one AAV ITR sequences (e.g., an AAV genome encoding GNPTAB); and 5) suitable media and media components to support rAAV production. In some embodiments, the suitable host cell is a primate host cell. In some embodiments, the suitable host cell is a human-derived cell lines such as HeLa, A549, 293, or Perc.6 cells. In some embodiments, the suitable helper virus function is provided by wild-type or mutant adenovirus (such as temperature sensitive adenovirus), herpes virus (HSV), baculovirus, or a plasmid construct providing helper functions. In some embodiments, the AAV rep and cap gene products may be from any AAV serotype. In general, but not obligatory, the AAV rep gene product is of the same serotype as the ITRs of the rAAV vector genome as long as the rep gene products may function to replicated and package the rAAV genome. Suitable media known in the art may be used for the production of rAAV vectors. These media include, without limitation, media produced by Hyclone Laboratories and JRH including Modified Eagle Medium (MEM), Dulbecco's Modified Eagle Medium (DMEM), custom formulations such as those described in U.S. Pat. No. 6,566,118, and Sf-900 II SFM media as described in U.S. Pat. No. 6,723,551, each of which is incorporated herein by reference in its entirety, particularly with respect to custom media formulations for use in production of recombinant AAV vectors. In some embodiments, the AAV helper functions are provided by adenovirus or HSV. In some embodiments, the AAV helper functions are provided by baculovirus and the host cell is an insect cell (e.g., *Spodoptera frugiperda* (Sf9) cells). In some embodiments, the AAV cap functions provide an amino acid substitution at amino acid residue 2 of VP1 and/or VP3, wherein the amino acid substitution at amino acid residue 2 of VP1 and/or VP3 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of VP1 and/or VP3 of the parent AAV particle. In some embodiments, amino acid residue 2 of the AAV capsid protein (e.g., VP1 or VP3) is substituted with Cys, Ser, Thr, Val, Gly, Asn, Asp, Glu, Ile, Leu, Phe, Gln, Lys, Met, Pro or Tyr. In some embodiments, the amino acid substitution results in less deamidation of the AAV capsid.

One method for producing rAAV particles is the triple transfection method. Briefly, a plasmid containing a rep gene and a capsid gene, along with a helper adenoviral plasmid, may be transfected (e.g., using the calcium phosphate method) into a cell line (e.g., HEK-293 cells), and virus may be collected and optionally purified. As such, in some embodiments, the rAAV particle was produced by triple transfection of a nucleic acid encoding the rAAV vector, a nucleic acid encoding AAV rep and cap, and a nucleic acid encoding AAV helper virus functions into a host cell, wherein the transfection of the nucleic acids to the host cells generates a host cell capable of producing rAAV particles.

In some embodiments, rAAV particles may be produced by a producer cell line method (see Martin et al., (2013) *Human Gene Therapy Methods* 24:253-269; U.S. PG Pub. No. US2004/0224411; and Liu, X. L. et al. (1999) *Gene Ther.* 6:293-299). Briefly, a cell line (e.g., a HeLa, 293, A549, or Perc.6 cell line) may be stably transfected with a plasmid containing a rep gene, a capsid gene, and a vector genome comprising a promoter-heterologous nucleic acid sequence (e.g., GNPTAB). Cell lines may be screened to select a lead clone for rAAV production, which may then be expanded to a production bioreactor and infected with a helper virus (e.g., an adenovirus or HSV) to initiate rAAV

production. Virus may subsequently be harvested, adenovirus may be inactivated (e.g., by heat) and/or removed, and the rAAV particles may be purified. As such, in some embodiments, the rAAV particle was produced by a producer cell line comprising one or more of nucleic acid encoding the rAAV vector, a nucleic acid encoding AAV rep and cap, and a nucleic acid encoding AAV helper virus functions. As described herein, the producer cell line method may be advantageous for the production of rAAV particles with an oversized genome, as compared to the triple transfection method.

In some embodiments, the nucleic acid encoding AAV rep and cap genes and/or the rAAV genome are stably maintained in the producer cell line. In some embodiments, nucleic acid encoding AAV rep and cap genes and/or the rAAV genome is introduced on one or more plasmids into a cell line to generate a producer cell line. In some embodiments, the AAV rep, AAV cap, and rAAV genome are introduced into a cell on the same plasmid. In other embodiments, the AAV rep, AAV cap, and rAAV genome are introduced into a cell on different plasmids. In some embodiments, a cell line stably transfected with a plasmid maintains the plasmid for multiple passages of the cell line (e.g., 5, 10, 20, 30, 40, 50 or more than 50 passages of the cell). For example, the plasmid(s) may replicate as the cell replicates, or the plasmid(s) may integrate into the cell genome. A variety of sequences that enable a plasmid to replicate autonomously in a cell (e.g., a human cell) have been identified (see, e.g., Krysan, P. J. et al. (1989) *Mol. Cell Biol.* 9:1026-1033). In some embodiments, the plasmid(s) may contain a selectable marker (e.g., an antibiotic resistance marker) that allows for selection of cells maintaining the plasmid. Selectable markers commonly used in mammalian cells include without limitation blasticidin, G418, hygromycin B, zeocin, puromycin, and derivatives thereof. Methods for introducing nucleic acids into a cell are known in the art and include without limitation viral transduction, cationic transfection (e.g., using a cationic polymer such as DEAE-dextran or a cationic lipid such as lipofectamine), calcium phosphate transfection, microinjection, particle bombardment, electroporation, and nanoparticle transfection (for more details, see e.g., Kim, T. K. and Eberwine, J. H. (2010) *Anal. Bioanal. Chem.* 397:3173-3178).

In some embodiments, the nucleic acid encoding AAV rep and cap genes and/or the rAAV genome are stably integrated into the genome of the producer cell line. In some embodiments, nucleic acid encoding AAV rep and cap genes and/or the rAAV genome is introduced on one or more plasmids into a cell line to generate a producer cell line. In some embodiments, the AAV rep, AAV cap, and rAAV genome are introduced into a cell on the same plasmid. In other embodiments, the AAV rep, AAV cap, and rAAV genome are introduced into a cell on different plasmids. In some embodiments, the plasmid(s) may contain a selectable marker (e.g., an antibiotic resistance marker) that allows for selection of cells maintaining the plasmid. Methods for stable integration of nucleic acids into a variety of host cell lines are known in the art. For example, repeated selection (e.g., through use of a selectable marker) may be used to select for cells that have integrated a nucleic acid containing a selectable marker (and AAV cap and rep genes and/or a rAAV genome). In other embodiments, nucleic acids may be integrated in a site-specific manner into a cell line to generate a producer cell line. Several site-specific recombination systems are known in the art, such as FLP/FRT (see, e.g., O'Gorman, S. et al. (1991) *Science* 251:1351-1355), Cre/loxP (see, e.g., Sauer, B. and Henderson, N. (1988) *Proc. Natl. Acad. Sci.* 85:5166-5170), and phi C31-att (see, e.g., Groth, A. C. et al. (2000) *Proc. Natl. Acad. Sci.* 97:5995-6000).

In some embodiments, the producer cell line is derived from a primate cell line (e.g., a non-human primate cell line, such as a Vero or FRhL-2 cell line). In some embodiments, the cell line is derived from a human cell line. In some embodiments, the producer cell line is derived from HeLa, 293, A549, or PERC.6® (Crucell) cells. For example, prior to introduction and/or stable maintenance/integration of nucleic acid encoding AAV rep and cap genes and/or the oversized rAAV genome into a cell line to generate a producer cell line, the cell line is a HeLa, 293, A549, or PERC.6® (Crucell) cell line, or a derivative thereof.

In some embodiments, the producer cell line is adapted for growth in suspension. As is known in the art, anchorage-dependent cells are typically not able to grow in suspension without a substrate, such as microcarrier beads. Adapting a cell line to grow in suspension may include, for example, growing the cell line in a spinner culture with a stirring paddle, using a culture medium that lacks calcium and magnesium ions to prevent clumping (and optionally an antifoaming agent), using a culture vessel coated with a siliconizing compound, and selecting cells in the culture (rather than in large clumps or on the sides of the vessel) at each passage. For further description, see, e.g., ATCC frequently asked questions document (available on the world wide web at atcc.org/Global/FAQs/9/1/Adapting %20a %20monolayer %20cell %20line % 20 to %20suspension-40.aspx) and references cited therein.

Suitable AAV production culture media of the present invention may be supplemented with serum or serum-derived recombinant proteins at a level of 0.5%-20% (v/v or w/v). Alternatively, as is known in the art, AAV vectors may be produced in serum-free conditions which may also be referred to as media with no animal-derived products. One of ordinary skill in the art may appreciate that commercial or custom media designed to support production of AAV vectors may also be supplemented with one or more cell culture components know in the art, including without limitation glucose, vitamins, amino acids, and or growth factors, in order to increase the titer of AAV in production cultures.

AAV production cultures can be grown under a variety of conditions (over a wide temperature range, for varying lengths of time, and the like) suitable to the particular host cell being utilized. As is known in the art, AAV production cultures include attachment-dependent cultures which can be cultured in suitable attachment-dependent vessels such as, for example, roller bottles, hollow fiber filters, microcarriers, and packed-bed or fluidized-bed bioreactors. AAV vector production cultures may also include suspension-adapted host cells such as HeLa, 293, and SF-9 cells which can be cultured in a variety of ways including, for example, spinner flasks, stirred tank bioreactors, and disposable systems such as the Wave bag system.

AAV vector particles of the invention may be harvested from AAV production cultures by lysis of the host cells of the production culture or by harvest of the spent media from the production culture, provided the cells are cultured under conditions known in the art to cause release of AAV particles into the media from intact cells, as described more fully in U.S. Pat. No. 6,566,118). Suitable methods of lysing cells are also known in the art and include for example multiple freeze/thaw cycles, sonication, microfluidization, and treatment with chemicals, such as detergents and/or proteases.

In a further embodiment, the AAV particles are purified. The term "purified" as used herein includes a preparation of

AAV particles devoid of at least some of the other components that may also be present where the AAV particles naturally occur or are initially prepared from. Thus, for example, isolated AAV particles may be prepared using a purification technique to enrich it from a source mixture, such as a culture lysate or production culture supernatant. Enrichment can be measured in a variety of ways, such as, for example, by the proportion of DNase-resistant particles (DRPs) or genome copies (gc) present in a solution, or by infectivity, or it can be measured in relation to a second, potentially interfering substance present in the source mixture, such as contaminants, including production culture contaminants or in-process contaminants, including helper virus, media components, and the like.

In some embodiments, the AAV production culture harvest is clarified to remove host cell debris. In some embodiments, the production culture harvest is clarified by filtration through a series of depth filters including, for example, a grade DOHC Millipore Millistak+ HC Pod Filter, a grade A1HC Millipore Millistak+ HC Pod Filter, and a 0.2 μm Filter Opticap XL1O Millipore Express SHC Hydrophilic Membrane filter. Clarification can also be achieved by a variety of other standard techniques known in the art, such as, centrifugation or filtration through any cellulose acetate filter of 0.2 μm or greater pore size known in the art.

In some embodiments, the AAV production culture harvest is further treated with Benzonase® to digest any high molecular weight DNA present in the production culture. In some embodiments, the Benzonase® digestion is performed under standard conditions known in the art including, for example, a final concentration of 1-2.5 units/ml of Benzonase® at a temperature ranging from ambient to 37° C. for a period of 30 minutes to several hours.

AAV particles may be isolated or purified using one or more of the following purification steps: equilibrium centrifugation; flow-through anionic exchange filtration; tangential flow filtration (TFF) for concentrating the AAV particles; AAV capture by apatite chromatography; heat inactivation of helper virus; AAV capture by hydrophobic interaction chromatography; buffer exchange by size exclusion chromatography (SEC); nanofiltration; and AAV capture by anionic exchange chromatography, cationic exchange chromatography, or affinity chromatography. These steps may be used alone, in various combinations, or in different orders. In some embodiments, the method comprises all the steps in the order as described below. Methods to purify AAV particles are found, for example, in Xiao et al., (1998) *Journal of Virology* 72:2224-2232; U.S. Pat. Nos. 6,989,264 and 8,137,948; and WO 2010/148143.

Pharmaceutical Compositions

In some embodiments, an AAV particle of the present disclosure (e.g., a rAAV particle) is in a pharmaceutical composition. The pharmaceutical compositions may be suitable for any mode of administration described herein or known in the art. In some embodiments, the pharmaceutical composition comprises rAAV particles modified to improve the stability and/or improve the transduction efficiency of rAAV particles; for example, for use in substituting the amino acid residue at position 2 of VP1 and/or VP3 to improve acetylation of rAAV capsid proteins. In some embodiments, the pharmaceutical composition comprises rAAV particles modified to modulate the stability and/or the transduction efficiency of rAAV particles (e.g., increase stability and/or transduction efficiency or decrease stability and/or transduction efficiency); for example, for use in substituting the amino acid residues that modulate deamidation (e.g., increase deamidation or decrease deamidation).

In some embodiments, the rAAV particle is in a pharmaceutical composition comprising a pharmaceutically acceptable excipient. As is well known in the art, pharmaceutically acceptable excipients are relatively inert substances that facilitate administration of a pharmacologically effective substance and can be supplied as liquid solutions or suspensions, as emulsions, or as solid forms suitable for dissolution or suspension in liquid prior to use. For example, an excipient can give form or consistency, or act as a diluent. Suitable excipients include but are not limited to stabilizing agents, wetting and emulsifying agents, salts for varying osmolarity, encapsulating agents, pH buffering substances, and buffers. Such excipients include any pharmaceutical agent suitable for direct delivery to the eye which may be administered without undue toxicity. Pharmaceutically acceptable excipients include, but are not limited to, sorbitol, any of the various TWEEN compounds, and liquids such as water, saline, glycerol and ethanol. Pharmaceutically acceptable salts can be included therein, for example, mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. A thorough discussion of pharmaceutically acceptable excipients is available in REMINGTON'S PHARMACEUTICAL SCIENCES (Mack Pub. Co., N.J. 1991). In some embodiments, the pharmaceutical composition comprising a rAAV particle described herein and a pharmaceutically acceptable carrier is suitable for administration to human. Such carriers are well known in the art (see, e.g., Remington's Pharmaceutical Sciences, 15th Edition, pp. 1035-1038 and 1570-1580).

Such pharmaceutically acceptable carriers can be sterile liquids, such as water and oil, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, and the like. Saline solutions and aqueous dextrose, polyethylene glycol (PEG) and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. The pharmaceutical composition may further comprise additional ingredients, for example preservatives, buffers, tonicity agents, antioxidants and stabilizers, nonionic wetting or clarifying agents, viscosity-increasing agents, and the like. The pharmaceutical compositions described herein can be packaged in single unit dosages or in multidosage forms. The compositions are generally formulated as sterile and substantially isotonic solution.

Kits and Articles of Manufacture

The present invention also provides kits or articles of manufacture comprising any of the rAAV particles and/or pharmaceutical compositions of the present disclosure. The kits or articles of manufacture may comprise any of the rAAV particles or rAAV particle compositions of the invention. In some embodiments the kits are used to improve the stability and/or improve the transduction efficiency of rAAV particles; for example, for use in substituting the amino acid residue at position 2 of VP1 and/or VP3 to improve acetylation of rAAV capsid proteins. In some embodiments the kits are used to modulate the stability and/or the transduction efficiency of rAAV particles (e.g., increase stability and/or transduction efficiency or decrease stability and/or transduction efficiency); for example, for use in substituting the amino acid residues that modulate deamidation (e.g., increase deamidation or decrease deamidation).

In some embodiments, the kits or articles of manufacture further include instructions for administration of a composition of rAAV particles. The kits or articles of manufacture described herein may further include other materials desir-

able from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for performing any methods described herein. Suitable packaging materials may also be included and may be any packaging materials known in the art, including, for example, vials (such as sealed vials), vessels, ampules, bottles, jars, flexible packaging (e.g., sealed Mylar or plastic bags), and the like. These articles of manufacture may further be sterilized and/or sealed.

In some embodiments, the kits or articles of manufacture further contain one or more of the buffers and/or pharmaceutically acceptable excipients described herein (e.g., as described in REMINGTON'S PHARMACEUTICAL SCIENCES (Mack Pub. Co., N.J. 1991). In some embodiments, the kits or articles of manufacture include one or more pharmaceutically acceptable excipients, carriers, solutions, and/or additional ingredients described herein. The kits or articles of manufacture described herein can be packaged in single unit dosages or in multidosage forms. The contents of the kits or articles of manufacture are generally formulated as sterile and can be lyophilized or provided as a substantially isotonic solution.

EXAMPLES

The invention will be more fully understood by reference to the following examples. They should not, however, be construed as limiting the scope of the invention. It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

Example 1: Direct LC/MS and LC/MS/MS for Complete Characterization of Recombinant AAV Viral Capsid Protein

Recombinant adeno-associated viruses (rAAVs) have become popular gene therapy vectors due to their nonpathogenic nature, ability to infect both dividing and non-dividing cells and long term gene expression. Currently, AAV-based gene therapies are used in clinical trials for numerous disease targets, such as muscular dystrophy, hemophilia, Parkinson's disease, Leber's congenital amaurosis and macular degeneration.

AAV is a small and nonenveloped parvovirus with a single stranded DNA genome encapsulated in an icosahedra shell. Each capsid includes sixty copies of three viral capsid proteins VP1 (87 kDa), VP2 (73 kDa) and VP3 (62 kDa) in an approximately 1:1:10 ratio. The three viral capsid proteins are expressed from the same open reading frame by using alternative splicing and an atypical start codon and thus have overlapping sequences. VP1 has ~137 additional N-terminal amino acid residues compared to VP3 while VP2 has ~65 additional N-terminal amino acid residues compared to VP3. At least 13 AAV serotypes and ~150 gene sequences have been isolated from human and non-human primate tissues; AAV serotypes differ in the amino acid sequence of viral capsid proteins and their corresponding cellular receptors and co-receptors for targeting.

The AAV capsid, in addition to protecting the genome inside, plays an important role in mediating receptor binding, escape of virus from endosome, and transport of viral DNA into nucleus in the viral infection cycle, thus directly impacting viral infectivity. It has been shown that the VP1 N-terminus contains a phospholipase PLA2 domain (a.a. 52-97) which is critical in endosomal escaping of virus [1-3]. N-termini of VP1 and VP2 also contain three basic amino acid clusters as nuclear localization signals. These sequences are highly conserved among different AAV serotypes. Mutations of these amino acids have been shown to reduce or abolish infectivity completely [4]. In addition, each AAV serotype has corresponding sequence-specific receptors and co-receptors. For example, heparin sulfate proteoglycan was identified as a major receptor of AAV2 and several other co-receptors, including aVP5 integrin, fibroblast growth factor receptor 1, and hepatocyte growth receptor have been identified [5-8]. Mutation analysis of AAV2 capsid proteins has identified a group of basic amino acids (Arginine484, 487, 585, and Lysine532) as a heparin-binding motif which contributes to the heparin and HeLa cell binding [9]. NGR domain in AAV2 was identified as an integrin a5(31 binding domain which is essential for viral cell entry [10]. In summary, viral capsid protein sequences are important in cellular targeting and trafficking in the viral infection cycle. Since different production conditions may cause different expression levels of viral capsid proteins, post-translational modifications, and truncations, the viral capsid proteins need to be characterized and monitored to ensure the product consistency in gene therapy development programs.

Traditionally, SDS-PAGE has been used to characterize the AAV viral capsid proteins, providing rough molecular weight information such as 87 kDa, 73 kDa and 62 kDa. No sequence information was obtained from Edman sequencing, possibly due to the blocked N-termini of viral capsid proteins, except VP2. Although X-ray structures of multiple AAVs have been solved, only the VP3 region sequence was observed in the crystal structures. Fifteen N-terminal amino acid residues of VP3 were still missing in the X-ray structure, possibly due to its intrinsic disorder [11-13]. It is possible that the lack of information of VP1 and VP2 N-terminal regions in the atomic structure might be due to low stoichiometry of VP1 and VP2 in the capsid. In addition, N-termini of VP1 and VP2 are buried inside the capsid and are not accessible to antibodies in the native state as reported in the some literature [3, 14, 15]. Conventionally, a Gel-LC/MS method (SDS-PAGE, in-gel tryptic digestion and LC/MS/MS) was used in characterization of VPs [16-18]. However, N-termini of VP1, VP2 and VP3 have not been confirmed using this approach, since this method failed to obtain 100% sequence coverage of VPs due to the limited recovery of peptide from gel.

Direct analysis using MALDI-TOF MS was reported for several virus capsid proteins including tobacco mosaic virus U2 after dissociation with organic acid [19]. Direct peptide mapping after amide hydrogen exchange and mass spectrometry have been used to study the pH-induced structural changes in the capsid of brome mosaic virus (BMV) [20]. Since AAVs are nonenveloped viruses containing only capsid proteins and genome, AAVs capsids could be directly analyzed by RP-LC/MS of proteins and LC/MS/MS of peptide mapping to achieve 100% sequence coverage after capsid dissociation without SDS-PAGE separation. The DNA fragments could elute in the void volume and thus have no interference on protein/peptide detection by LC/MS. In order to investigate these methods, direct LC/MS of different types of AAVs after denaturation was used to monitor the protein sequence and post-translational modifications of AAV capsid proteins. As described herein, N-termini of VP1, VP2 and VP3 of AAVs have been confirmed by mass spectrometry. Acetylations of N-termini of VP1 and

VP3 were also identified in the different serotype of AAVs. Direct LC/MS/MS peptide mapping of AAVs has also been developed to provide sequence coverage of VP1, VP2 and VP3 and confirm the N-termini acetylation of VP1 and VP3.

Methods

Materials and Reagents

Dithiothreitol (DTT), 4-vinylpyridine, ultra-pure formic acid, acetic acid, guanidine-HCl, Tris-HCl and Tris base were purchased from Sigma Chemicals (St. Louis, MO). Amicon ultra-4 filters were purchased from Millipore (Billerica, MA). The porcine sequencing grade trypsin was purchased from Promega (Milwaukee, WI). Endoproteinase Lys-C and Asp-N were purchased from Roche (Germany). Slide-A-Lyzer cassettes with 10,000 MWCO were purchased from Pierce (Rockford, IL).

Vector Production and Purification

AAV vectors were produced using the transient triple transfection method as previously described (Xiao, 1998 #123). Briefly, HEK293 cells were transfected using polyethyleneimine, PEI, and a 1:1:1 ratio of three plasmids (ITR vector, AAV rep/cap and Ad helper plasmid). The vector plasmid contains the vector genome CBA-EGFP and ITR sequences from AAV2. EGFP expression is driven by the CMV enhancer chicken beta actin hybrid promoter (CBA) as described (Miyazaki, 1989 #124) (Niwa, 1991 #125). The AAV rep/cap helpers contained rep sequences from AAV2 and serotype specific capsid sequences with the nomenclature, rep2/cap2, rep2/cap5, rep2/cap7 etc. The pAd helper used was pHelper (Stratagene/Agilent Technologies, Santa Clara, CA). Purification of AAV was performed as described by Qu et al. (2007, *J. Virol. Methods* 140:183-192).

LC/MS Intact Protein Analysis

The AAV virions were concentrated with an Amicon ultra-4 filter (10 kDa MWCO) and denatured with 10% acetic acid followed by direct analysis in an Acquity UPLC-Xevo® QTOF MS instrument (Waters, Milford, MA). The separations were performed with a UPLC BEH C4 or C8 column (1.7 μm, 2.1 mm i.d.) at a 0.25 ml/min flow rate. Mobile phase A was 0.1% formic acid in water while mobile phase B was 0.1% formic acid in acetonitrile. The final gradient was as follows: from 10% B to 20% B for 6 minutes, from 20% B to 30% B in 10 min, then from 30% to 38% B for 40 minutes. For MS the capillary voltage and sampling cone voltage were set at 3.5 kV and 45 V respectively. The mass spectra were acquired in the sensitivity mode with m/z range of 500-4000. Assisted calibration with sodium iodide as calibrant was performed for mass calibration. MaxEnt1 in Masslynx software was used for protein deconvolution.

Enzymatic Digestions of AAV2 VPs

The concentrated AAV2 virions were denatured with 6 M Guanidine-HCl, 0.1 M Tris at pH 8.5. The proteins were reduced with 30 mM DTT at 55° C. for 1 hour in darkness and alkylated with 0.07% 4-vinylpyridine at room temperature for 2 hours. The reactions were quenched by the addition of 1M DTT. The samples were dialyzed with Slide-A-Lyzer cassettes (10,000 MWCO) against 25 mM Tris buffer at pH 8.5 for ~18 hours. After dialysis, the samples were split into three aliquots. Each aliquot was digested with trypsin at 1:25 or Lys-C at 1:50 or Asp-N at 1:100 enzyme: protein ratio (wt/wt) for 18 hours at 37° C., respectively.

LC/MS/MS Peptide Mapping

Nano LC/MS/MS was performed in using a NanoAcquity HPLC system (Waters, Milford, MA) in conjunction with an Orbitrap Velos mass spectrometer (Thermo-Fisher Scientific, Waltham, MA) using home packed nanoLC column (75 μm×10 mm) with Magic C18 with packing material (5 μm, Bruker, Billerica, MA) at a 300 nl/min flow rate. The mobile phases A and B were 0.1% formic acid in water and acetonitrile, respectively. The gradient was from 2% B to 60% B in 121 min.

The source parameters for velos were as follows: source voltage: 2.5 kv, capillary temperature 275° C.; S-lens RF level: 55%. Data were acquired using the top-ten data dependent method with accurate ms at 60,000 resolution and 10 MS/MS in ion trap. Mascot was used for database searching against AAV2 viral capsid protein sequences. MS tolerance of 10 ppm and ms/ms tolerance of 0.8 Da were used for the database search.

UPLC/MS/MS Peptide Mapping

The protein digests were also analyzed by UPLC/MS/MS in Acquity UPLC-Xevo qTOF MS. A BEH300 C18 column (2.1×150 mm) was used for separation in the mobile phases with 0.1% formic acid in water/acetontitrile gradient at a flow rate 0.25 ml/min. The mass spectra were acquired in the positive MSe mode in the mass range of 200-2000.

Results

AAV Denaturation Method

AAVs can be denatured through a number of methods using detergent, heat, high salt, or buffer with low or high pHs. Heat denaturation can lead to protein precipitation and as a result reverse phase columns are easily clogged and over pressurized. Denaturation with high salt requires an additional desalting step before LC/MS analysis. Denaturing with 10% acetic acid was used for the LC/MS intact protein analysis, as it allowed for clean mass spectrum. For peptide mapping, either 0.1% RapiGest or 6 M Guanidine HCl can be used as a denaturing reagent.

Intact Protein Analysis Method Development

Initial intact protein analysis of AAV2 was performed using an UPLC BEH C4 column at fast gradient. Under this condition, only one single peak in the total ion chromatogram was observed, with a mass corresponding to VP3 (FIG. **1**A). Without wishing to be bound by theory, it is thought that the absence of VP1 and VP2 is possibly due to low stoichiometry of VP1 and VP2 or suppression of VP1 and VP2 signals by VP3 if all VPs co-elute. Increasing injection or column length, using a shallower gradient, and using alternative columns have been attempted in order to detect VP1 and VP2. Higher loading (1.7 μg) with a shallower gradient at 0.5% B/min resulted in a shoulder peak on the left (FIG. **1**B). The increase in column length from 10 cm to 15 cm did not enhance the separation of the shoulder peak (FIG. **1**C). However, the shoulder peak was further separated from the main peak using a BEH C8 column, with improved signal intensities observed (FIG. **1**D).

As a result, the VP1 and VP2 masses were obtained in this shoulder peak at the signal intensities shown in FIG. **2**A. The masses of VP1 and VP3 correspond to a.a. 2-735(acetylation) and a.a. 204-735(acetylation), respectively (FIGS. **2**A&**2**B). No acetylation was observed in VP2 (a.a.139-735). In addition, a minor peak with a smaller mass than VP3 was observed, with a mass corresponding to amino acid sequence 212-735 with one acetylation (FIG. **2**B). These data are consistent with DNA sequences since VP3 contains two ATG initiation codons in AAV2: ATGGCTACAGGCAGTGGCGCACCAATGGCAGAC (SEQ ID NO:1), resulting in two possible N termini (underlined): MATGSGAPMAD (SEQ ID NO:2). The N-terminal methionine residues were not present in both VP1 and VP3 as measured by intact protein analysis. The acetylation of

VP1 and VP3 is not a method-induced artifact (denaturation of AAV by 10% acetic acid) since acetylation of VP1 and VP3 is also observed in an AAV preparation using an alternative denature method without acetic acid. The intact protein data also confirmed that no glycosylation was present in the viral capsid proteins, even though several N-linked consensus sequences are present [16].

LC/MS/MS Peptide Mapping

To further confirm the N-termini and acetylation observed in the intact protein analysis, peptide mapping was performed using multiple enzymes and analyzed using multiple instruments. Various sample preparation methods, including denaturation methods and desalting steps, have been evaluated. The final digestion method, including denaturation with 6M guanidine HCl, reduction and alkylation with 4-vinylpyridine, and dialysis using slide-A-lyzer followed by enzymatic digestion, created clean peptide mapping with low artificial modifications during the digestion process. As low as 5 μg starting material was tested, yielding complete sequence coverage using nano LC/MS/MS and UPLC/MS/MS.

Mascot search of tryptic digests from nano LC/MS/MS alone yielded 78% sequence coverage with an ion score 13 cut off as shown in FIG. **3**. The two large missing tryptic peptides, T27 and T38 (boxed) from nano LC/MS/MS were found in the LC/MS in Xevo TOF MS with BEH C18 column (FIG. **3**). In addition, most of the T27 and T38 peptide sequences were further confirmed by nano LC/MS/MS of Asp-N digests as shown in Italics in FIG. **3**. The complete N-terminal and C-terminal peptides were covered by Lys-C digests as underlined in FIG. **3**. Therefore, 100% sequence coverage of VP1 was achieved through multiple enzyme digestions and two LC/MS/MS methods.

LC/MS/MS confirmed the N- and C-termini of VP1, VP2 and VP3 and N-terminal acetylation of VP1 and VP3 observed in the intact protein analysis. FIGS. **4**A-**4**C show the MS/MS spectra of the VP1 N-terminal tryptic peptide A(Ac)ADGYLPDWLEDTLSEGIR (SEQ ID NO:4) (FIG. **4**A), VP2 N-terminal Asp-N derived peptide (APGKKRPVEHSPVEP) (SEQ ID NO:15) (FIG. **4**B), and VP3 N-terminal Asp-N peptide A(Ac)TGSGAPM (SEQ ID NO:5) (FIG. **4**C). MS/MS has confirmed the location of acetylation at the N-terminal alanine residues in both VP1 and VP3 peptides. The presence of unmodified y18 and y17 ions, and all detected b ions with 42 Da mass shift in FIG. **4**A indicates the 42 da-modification is located in N-terminal of VP1. Similarly, the presence of unmodified y3 to y8 ions in FIG. **4**C confirmed the location of acetylation at the N-terminal alanine residue.

Comparison of AAV VP N-Termini

In addition to AAV2, AAV1, AAV5, AAV7, AAV9 and AAV Rh10 have also been analyzed by intact protein analysis. The theoretical and predicted masses of VPs in AAVs are shown in Table 2.

TABLE 2

Theoretical Mass vs Experimental Mass for AAV VPs

Serotype	Isoform	Predicted amino acid sequence	Actual amino acid sequence	Theoretical Ms.(Da)	Experimental Ms.(Da)
AAV1	VP1	1-736	2(ac)-736	81286	81291
	VP2	138-736	139-736	66093	66098
	VP3	203-736	204(ac)-736	59517	59520

TABLE 2-continued

Theoretical Mass vs Experimental Mass for AAV VPs

Serotype	Isoform	Predicted amino acid sequence	Actual amino acid sequence	Theoretical Ms.(Da)	Experimental Ms.(Da)
AAV2	VP1	1-735	2(ac)-735	81856	81856
	VP2	138-735	139-735	66488	66488
	VP3	203-735	204(ac)-735	59974	59974
AAV5	VP1	1-724	2(ac)-724	80336	80336
	VP2	137-724	138-724	65283	65284
	VP3	193-724	194(ac)-724	59463	59463
AAV7	VP1	1-737	2(ac)-737	81564	81567
	VP2	138-737	139-737	66372	66374
	VP3	204-737	213(ac)-737	59101	59103
AAV9	VP1	1-736	2(ac)-736	81291	81288
	VP2	138-736	139-736	66210	66209
	VP3	203-736	204(ac)-736	59733	59733
AAVRh10	VP1	1-738	2(ac)-738	81455	81455
	VP2	138-738	139-738	66253	66252
	VP3	204-738	205(ac)-738	59634	59634

N-termini, as well as their posttranslational modifications, are highly conserved among the AAV serotypes analyzed, even though AAV5 is reported as the most diverse AAV serotype sequence, as shown in the sequence alignments in FIG. **5**. In 11 out of 13 AAV serotypes, the N-termini of VP1 share an identical 13 amino acid residue sequence (MAADGYLPDWLED) (SEQ ID NO:6) while all 13 AAV serotypes have identical TAP . . . N-terminal sequences in VP2 (FIG. **5**). LC/MS of AAV2 indicated that T is missing in VP2 at protein level. The N-termini of VP3 are the most diverse among the three viral capsid proteins, with 8 out of 13 AAV serotypes sharing a MA . . . N-terminal sequence. Similar to AAV2, AAV1 and AAV Rh10 also have two ATG initiation codons with the first one as predominant N-terminal based on LC/MS intact protein analysis. Interestingly, though AAV7 has two potential initiation codons (GTGGCTGCAGGCGGTGGCGCACCAATGGCAGACAATAAC . . .) (SEQ ID NO:7), the second initiation codon (ATG) was favorable based on the intact protein analysis: the VP3' with 213(ac)-737 was a predominant peak while VP3 with 203(ac)-737 was a minor peak.

Conclusions

Applications of LC/MS Intact Protein Analysis and LC/MS/MS Peptide Mapping of AAV VPs in Gene Therapy Research and Development

These results demonstrate that direct LC/MS of different types of AAVs after denaturation was proved to be a simple and effective way to monitor the protein sequence and post-translational modifications with accurate mass measurement in the intact protein level. N-termini of VP1, VP2 and VP3 of AAVs were confirmed by mass spectrometry. Acetylations of N-termini of VP1 and VP3 were also identified in different serotypes of AAVs. Direct LC/MS/MS peptide mapping of AAVs was developed, provided 100% sequence coverage of VP1, VP2 and VP3, and confirmed the N-termini acetylation of VPs. The theoretical masses of predicted sequences of 13 AAV serotypes based on sequence alignment and intact protein analysis of several AAV serotypes are shown in Table 3.

TABLE 3

Predicted Sequences and Masses

	Predicted VP1 sequence	Mass(Da)	Predicted VP2 sequence	Mass(Da)	Predicted VP3 sequence	Mass(Da)
AAV1	2(ac)-736	81286	139-736	66093	204(ac)-736	59517
AAV2	2(ac)-735	81856	139-735	66488	204(ac)-735	59974
AAV3	2(ac)-736	81571	139-736	66319	204(ac)-736	59849
AAV4	2(ac)-734	80550	138-734	65626	198(ac)-734	59529
AAV5	2(ac)-724	80336	138-724	65283	194(ac)-724	59463
AAV6	2(ac)-736	81322	139-736	66096	204(ac)-736	59519
AAV7	2(ac)-737	81564	139-737	66372	213(ac)-737	59101
AAV8	2(ac)-738	81667	139-738	66519	205(ac)-738	59805
AAV9	2(ac)-736	81291	139-736	66210	204(ac)-736	59733
AAV10	2(ac)-738	81477	139-738	66271	205(ac)-738	59638
AAV11	2(ac)-733	80987	139-733	65794	198(ac)-733	59696
AAV12	2(ac)-742	82106	139-742	66905	207(ac)-742	59846
AAVRh10	2(ac)-738	81455	139-738	66253	205(ac)-738	59634

The accurate masses of VP1, VP2 and VP3 of each serotype are unique and therefore intact protein analysis can be used as an identity test to differentiate AAV capsid serotypes. Tables 4-6 show the mass differences of VPs among 13 common AAV serotypes. Shown in regular font are delta masses larger than 10, with delta masses less than 10 bolded.

TABLE 4

Mass Differences of VP1 Among 13 AAV Isotypes

	AAV1											
AAV2	570	AAV2										
AAV3	285	285	AAV3									
AAV4	736	1306	1021	AAV4								
AAV5	950	1520	1235	215	AAV5							
AAV6	36	534	249	772	987	AAV6						
AAV7	277	292	**8**	1013	1228	241	AAV7					
AAV8	381	189	96	1117	1332	345	104	AAV8				
AAV9	**5**	585	280	741	955	31	272	376	AAV9			
AAV10	191	379	94	927	1142	155	86	190	186	AAV10		
AAV11	299	869	584	436	651	335	577	681	304	490	AAV11	
AAV12	820	250	535	1555	1770	784	542	439	815	629	1119	AAV12
AAVRh10	169	401	116	905	1119	133	109	212	164	22	468	651

TABLE 5

Mass Differences of VP2 among 13 AAV Isotypes

	AAV1											
AAV2	395	AAV2										
AAV3	226	169	AAV3									
AAV4	467	862	693	AAV4								
AAV5	810	1205	1036	343	AAV5							
AAV6	**2**	392	224	470	812	AAV6						
AAV7	278	116	52	746	1088	276	AAV7					
AAV8	425	31	199	893	1235	423	147	AAV8				
AAV9	117	278	109	584	927	115	161	308	AAV9			
AAV10	177	217	49	645	987	175	101	248	60	AAV10		
AAV11	299	694	525	168	511	301	578	725	416	476	AAV11	
AAV12	812	417	586	1279	1622	810	533	386	695	635	1111	AAV12
AAVRh10	160	235	66	627	970	157	119	266	43	18	459	652

TABLE 6

Mass Differences of VP3 among 13 AAV Isotypes

	AAV1											
AAV2	457	AAV2										
AAV3	332	125	AAV3									
AAV4	12	445	320	AAV4								
AAV5	54	511	386	66	AAV5							
AAV6	**2**	455	330	**10**	56	AAV6						
AAV7	1416	673	748	428	362	418	AAV7					
AAV8	288	169	44	276	342	286	704	AAV8				
AAV9	216	241	116	204	270	214	632	72	AAV9			
AAV10	121	336	211	109	175	119	537	167	95	AAV10		
AAV11	179	278	153	167	233	177	595	109	37	58	AAV11	
AAV12	329	128	**3**	317	383	327	745	41	113	208	150	AAV12
AAVRh10	117	340	215	105	171	115	533	171	99	**4**	62	212

No masses within 10 Da of all three VPs between two isotypes are observed. Even though both VP2 and VP3 have only a 2 Da difference between AAV1 and AAV6, the mass difference of VP1 between AAV1 and AAV6 is 36, significant enough to be distinguished by an accurate mass measurement. Therefore, intact protein measurement of VP1, VP2 and VP3 is highly specific as an identity test.

These results demonstrate that intact protein analysis and LC/MS/MS can be used to profile VPs to monitor VP expressions, posttranslational modifications, and truncations and to ensure product consistency during VLP production. These two analyses can also be used to confirm site-direct mutagenesis or structural characterization for capsid protein engineering applications.

Example 2: The Role of N Terminal Acetylation of AAV Capsid Proteins

Chemical modifications of cellular proteins are a common means of controlling their functions (Arnesen, T. (2006) *Virology* 353(2): 283-293). N-terminal acetylation (Nt-acetylation), which involves the transfer of an acetyl group from acetyl coenzyme A to the α-amino group of the first amino acid residue of a protein (Brown, J. L. and Roberts, W. K. (1976) *J Biol Chem* 251: 1009-1014; Arnesen, T. et al. (2009) *Proc Natl Acad Sci USA* 106: 8157-8162), is among the most abundant of protein modifications. Unlike most other protein modifications, Nt-acetylation is irreversible; it occurs mainly during the synthesis of the protein, catalyzed by N-terminal acetyltransferases (NATs) associated with ribosomes (Gautschi, M. et al. (2003) *Mol Cell Biol* 23: 7403-7414; Pestana, A. and Pitot, H. C. (1975) *Biochemistry* 14: 1404-1412; Polevoda, B. et al. (2003) *J Biol Chem* 278: 30686-97). There are several distinct NATs in eukaryotes—NatA-NatF—each composed of one or more subunits and each acetylating a specific subgroup of N-termini depending on the amino acid sequence of the first few amino acids (Jornvall, H. (1975) *J Theor Biol* 55: 1-12; Persson, B. et al. (1985) *Eur J Biochem* 152: 523-527).

Experimental data indicate that proteins with acetylated N-termini are more stable in vivo than non-acetylated proteins; i.e., Nt-acetylation protects proteins from degradation (Hershko, A. et al. (1984) *Proc Natl Acad Sci USA* 81: 7021-7025). One explanation for this might be the discovery in 2004 that another N-terminal modification, ubiquitination, which involves direct attachment of the small protein ubiquitin to the N-terminal amino acid residue, promotes the subsequent degradation of the protein (Ben Saadon, R. et al. (2004) *J Biol Chem* 279: 41414-41421). Conversely, the Nt-acetylation signals can also be part of a quality control mechanism to degrade unfolded or misfolded proteins and to regulate in vivo protein stoichiometries (Hwang, C. S. et al. (2010) *Science* 327: 973-977).

A systematic analysis of the predicted N-terminal processing of cytosolic proteins, versus those destined to be sorted to the secretory pathway, revealed that the cytosolic proteins were profoundly biased in favor of processing, but there is an equal and opposite bias against such modification for secretory proteins (Forte, G. M. A. et al. (2011) *PLoS Biology,* 4 May 2011 Volume 9). Mutations in secretory signal sequences that lead to their acetylation result in mis-sorting to the cytosol in a manner that is dependent upon the N-terminal processing machinery. Hence N-terminal acetylation represents an early determining step in the cellular sorting of nascent polypeptides that represent an extra layer of stringency in order to ensure that proteins destined to remain in the cytosol actually reside in the cytosol. The eukaryotic cell comprises several distinct compartments, called organelles, required to perform specific functions. The proteins in these compartments are synthesized in the cytoplasm and so require complex sorting mechanisms to ensure their delivery to the appropriate organelle. Proteins are modified by acetylation of their amino terminus at a very early stage in their synthesis. There is a profound difference between the likelihood of such a modification on cytoplasmic proteins and on those destined for one of the major organelles, the endoplasmic reticulum (ER): whereas cytoplasmic proteins are typically acetylated, those bound for the ER are largely unmodified. Moreover, when specific ER proteins are engineered to induce their acetylation their targeting to the ER was inhibited (Forte, G. M. A. et al. (2011) *PLoS Biology,* 4 May 2011 Volume 9).

The contractile proteins actin and tropomyosin have been shown to require NatB-mediated Nt-acetylation for proper function, specifically involving actin-tropomyosin binding and actomyosin regulation (Coulton, A. T. et al. (2010) *J Cell Sci* 123: 3235-3243; Polevoda, B. et al. (2003) *J Biol Chem* 278: 30686-97). Thus Nt acetylation of AAV capsid proteins may have importance in the transduction potential of rAAV vectors. If AAV vectors fail to gain entry into the nucleus, they consequently fail to transduce cells. The role of actin filaments and FKBP52 (FK506-binding protein p52) in the translocation of AAV capsids from the endosome to the nucleus is well defined (Zhao, W. et al. (2006) *Virology* 353(2): 283-293). Importantly, Nt-acetylation is essential for the functioning of actin filaments by modulating protein-protein interactions (Coulton, A. T. et al. (2010) *J Cell Sci* 123: 3235-3243; Polevoda, B. et al. (2003) *J Biol Chem* 278: 30686-97).

Though N-terminal acetylation of proteins is a widely known phenomenon, the biological significance of Nt-acetylation on AAV capsid proteins is not well understood. The predicted N-termini of VP1 and VP3 based on DNA sequencing are both methionine followed by alanine. It has been reported that removal of N-terminal methionine by Met-aminopeptidases frequently leads to Nt-acetylation of the resulting N-terminal alanine, valine, serine, threonine, and cysteine residues and that the acetylation of the N-terminus acts as a potential degradation signal [21]. Ubiquitination of viral capsid proteins was suggested as a potential signal for processing of the capsid at the time of virion disassembly [22]. The link between N-acetylation of VP1 and VP3 and viral capsid degradation and uncoating before the nuclear entry is further investigated.

To understand the functional implications of N-terminal acetylation with regard to AAV capsid proteins, site-directed mutagenesis of VP3 N-terminal initiation codons is used to generate AAV mutants.

Methods

AAV capsid proteins are generated with differing amino acids at the 2nd position to the initiating methionine (iMet X) to determine if Nt-acetylation is inhibited or reduced, and the functional consequences are then measured. The ability of the capsid proteins to be trafficked intra-cellularly and/or to acquire post translational modifications such as glycosylation is assessed, and whether this ability affects the infectivity of the assembled AAV particle is subsequently determined. In addition, the impact of acetylation on ubiquitination/degradation and targeting to the lysosome, ER, Golgi, or inner nuclear membrane is determined.

For example, to assay trafficking or targeting, AAV particles with capsid proteins having a mutated 2nd position (e.g., iMet X) are fluorescently labeled and used to infect cells (e.g., HeLa cells). These AAV particles are assayed for one or more of: time of viral particle uptake, colocalization of AAV particles with specific compartmental markers (e.g., Golgi, ER, or lysosomal proteins or other markers), nuclear accumulation (e.g., as assayed by colocalization with a nuclear marker or stain), and/or sensitivity of trafficking to specific inhibitors of early endosomal escape (such as bafilomycin A or ammonium chloride), as compared to fluorescently labeled wild-type AAV particles used to infect the same cell line (see, e.g., Bartlett, J. S. et al. (2000) *J. Virol.* 74:2777-2785 for a description of such assays).

To assay infectivity, AAV particles with capsid proteins having a mutated 2nd position (e.g., iMet X) are used to infect cells (e.g., HeLa cells), and their transduction efficiency is compared to wild-type AAV particles (e.g., having the same AAV serotype and infecting the same type of cells).

To assay glycosylation, AAV particles with capsid proteins having a mutated 2nd position (e.g., iMet X) are used to infect cells (e.g., HeLa cells). AAV particles from infected cells are subjected to one or more assays including without limitation chemical detection of glycosylation (e.g., applying a commercially available digoxigenin (DIG) glycan detection and/or fluorescent glycoprotein detection kit on denatured and electrophoretically separated capsid proteins) and mass spectrometry (e.g., FT-ICR MS), as compared to wild-type AAV particles used to infect the same cell line (see, e.g., Murray, S. et al. (2006) *J. Virol.* 80:6171-6176 for a description of such assays).

To assay ubiquitination, AAV particles with capsid proteins having a mutated 2nd position are used to infect cells (e.g., HeLa cells). AAV particles are immunoprecipitated from infected cells with an anti-capsid antibody, then subjected to Western blotting with an anti-ubiquitin antibody and compared to wild-type AAV particles used to infect cells in the same manner. Mutant AAV particles may also be used in in vitro ubiquitination assays, as compared to wild-type AAV particles (see, e.g., Yan, Z. et al. (2002) *J. Virol.* 76:2043-2053).

Example 3: The Role of Deamidation of AAV2 Capsid Proteins

Sequence analysis of the AAV2 capsid protein revealed potential deamidation sites, as underlined in the following amino acid sequence:

(SEQ ID NO: 3)

MAADGYLPDWLEDTLSEGIRQWWKLKPGPPPPKPAERHKDDSRGLVLPGY

K***YLGPFNGLDKGEPVNEADAAALEHDKAYDRQLDSGDNPYLKYNHADA***EF

QERLKEDTSFGGNLGRAVFQAKKRVLEPLGLVEEPVKTAPGKKRPVEHSP

VEPDSSSGTGKAGQQPARKRLNFGQTGDADSVPDPQPLGQPPAAPSGLGT

NTMATGSGAPMADNNEGADGVGNSSGNWHCDSTWMGDRVITTSTRTWALP

TYNNHLYKQISSQSGASNDNHYFGYSTPWGYFDFNRFHCHFSPRDWQRLI

NNNWGFRPKRLNFKLFNIQVKEVTQNDGTTTIANNLTSTVQVFTDSEYQL

PYVLGSAHQGCLPPFPADVFMVPQYGYLTLNNGSQAVGRSSFYCLEYFPS

QMLRTGNNFTFSYTFEDVPFHSSYAHSQSLDRLMNPLIDQYLYYLSRTNT

PSGTTTQSRLQFSQAGASDIRDQSRNWLPGPCYRQQRVSKTSADNNNSEY

SWTGATKYHLNGRDSLVNPGPAMASHKDDEEKFFPQSGVLIFGKQGSEKT

NVDIEKVMITDEEEIRTTNPVATEQYGSVSTNLQRGNRQAATADVNTQGV

LPGMVWQDRDVYLQGPIWAKIPHTDGHFHPSPLMGGFGLKHPPPQILIKN

TPVPANPSTTFSAAKFASFITQYSTGQVSVEIEWELQKENSKRWNPEIQY

TSNYNKSVNVDFTVDTNGVYSEPRPIGTRYLTRNL.

In particular, a potential deamidation site is found at N57/G58 in the phospholipase A2 domain (Ca++ binding site), as bolded and italicized in the above sequence. The following experiments were aimed at exploring whether deamidation at N57 can lead to reduced potency and/or truncation of AAV2, as well as whether different AAV production methods may have different effects on deamidation. For example, the producer cell line method (see Martin et al., (2013) *Human Gene Therapy Methods* 24:253-269; U.S. PG Pub. No. US2004/0224411; and Liu, X. L. et al. (1999) *Gene Ther.* 6:293-299) may induce a higher level of deamidation at N57, as compared to the triple transfection method. According to the crystal structure of AAV2, N57 is not shown; however, N382 and N511 are partially exposed, and N715 is fully exposed.

Methods

Enzymatic Digestions of AAV1 and AAV2 VPs

10 μg of each AAV1-EGFP or AAV2-EGFP material (generated from triple transfection as well as producer cell line process) were concentrated using Amicon filters (10 kDa MWCO), denatured with 6 M Guanidine-HCl, 50 mM Tris at pH 8.5. The proteins were reduced with 5 mM DTT at 60° C. for 30 minutes in darkness, alkylated with 15 mM iodoacetamide at room temperature for 30 minutes, and then buffer exchanged into 25 mM Tris pH 7.1 for digestion using Bio-Spin® 6 Tris micro-columns. After buffer exchange, the samples were split into two aliquots. Each aliquot was

digested with trypsin at 1:25 or Asp-N at 1:50 enzyme: protein ratio (wt/wt) for 2 hours at 37° C., respectively.

UPLC/MS/MS Peptide Mapping

The protein digests were also analyzed by UPLC/MS/MS in Acquity UPLC-Xevo qTOF MS. BEH300 C18 column (2.1×150 mm) was used for separation in the mobile phases with 0.1% formic acid in water/acetontitrile gradient at a flow rate 0.25 ml/min. The mass spectra were acquired in the positive MSe resolution mode in the mass range of 50-2000.

Determination of Deamidation Levels in AAV VPs

The extracted ion chromatograms (XIC) of peptides containing NG sites (T9, T49, and T67 in AA1 and AAV2 VP) and their corresponding deamidated species were used for calculation of deamidation levels.

In order to compare AAV vectors produced by the triple transfection (TTx) and producer cell line (PCL) methods, AAV1 or AAV2 tagged with EGFP was produced using the TTx or PCL method. Truncated VP1 (tVP1) was found to be present in AAV2-EGFP produced by PCL, but not in the AAV2-EGFP produced by TTx. AAV1-EGFP was not found to have tVP1, regardless of the production method. The in vitro potency of AAV2 produced by the PCL method was also found to be reduced, as compared to AAV2 produced by TTx. Mutant N57K and N57Q AAV2 particles were also found to have reduced potency and disrupted Ca++ binding.

The following table provides the tryptic peptides that were analyzed to examine each potential deamidation site, as well as the corresponding residue.

TABLE 7

Tryptic peptides containing NG sites

Peptide (NG sequence underlined)	Residue
YLGPF**NG**LDK (SEQ ID NO: 9)	N57
EVTQNDGTTTIANNLTSTVQVFTDSEYQLPYVLGSAHQGC LPPFPADVFMVPQYGYLTLN**NG**SQAVGRSSFYCLEYFPSQ MLR (SEQ ID NO: 10)	N382
YNL**NG**R (AAV1) (SEQ ID NO: 11)	N511
YHL**NG**R (AAV2) (SEQ ID NO: 12)	N511
SANVDFTVDN**NG**LYTEPR (AAV1) (SEQ ID NO: 13)	N715
SVNVDFTVDT**NG**VYSEPR (AAV2) (SEQ ID NO: 14)	N715

As shown in Table 7, the T9 peptide YLGPFNGLDK (SEQ ID NO: 9) was used to monitor N57, the T38 peptide EVTQNDGTTTIANNLTSTVQVFTDSEYQLPYVLG-SAHQGCLPPFPADVFMVPQYGYLTL NNG-SQAVGRSSFYCLEYFPSQMLR (SEQ ID NO: 10) was used to monitor N382, the T49 peptides YNLNGR (SEQ ID NO: 11) and YHLNGR (SEQ ID NO: 12) were used to monitor N511 in AAV1 or AAV2 (respectively), and the T67 peptides SANVDFTVDNNGLYTEPR (SEQ ID NO: 13) and SVNVDFTVDTNGVYSEPR (SEQ ID NO: 14) were used to monitor N715 in AAV1 or AAV2 (respectively).

LC/MS/MS analysis was used to compare the percentage of deamidation in AAV1 and AAV2 particles produced by the TTx and PCL methods. The results from the T9 peptide are shown in FIGS. **6**A & **6**B. The results from the T49 peptide are shown in FIGS. **7**A & **7**B. The results from the T67 peptide are shown in FIGS. **8**A & **8**B. These results are summarized in Table 8. The T38 peptide was not detected due to its size.

TABLE 8

Summary of LC/MS/MS results

		% Deamidation		
		N57	N511	N715
AAV1	TTx	7.9	30.9	18.1
	PCL	11.3	27.4	18.7
AAV2	TTx	6.7	39.6	27.4
	PCL	18.4	42.3	28.0

In particular, AAV2 produced by PCL showed nearly a 3-fold increase in deamidation as compared to AAV2 produced by TTx. These results suggest that deamidation decreases AAV potency, as the in vitro potency of AAV2 produced by PCL is reduced.

Conclusions

Taken together, Examples 1-3 demonstrate methods for analyzing intact proteins of viral particles (e.g., AAV capsid proteins) using LC/MS. Molecular weights were measured accurately, and these techniques may be also used to assess N-termini and/or modifications of viral capsid proteins. Moreover, these methods are adaptable as capsid serotype identity assays useful in gene therapy, e.g., as an analytical platform. These results further establish a correlation between capsid protein structure (e.g., truncations, deamidation, etc.) and potency, suggesting that point mutations at key sites may be used to engineer more effective vectors.

Example 4: Elucidating the Role of N Terminal Acetylation of AAV Capsid Proteins

As discussed above, the N-termini of AAV capsid proteins are highly conserved across serotypes (FIG. **5**). The techniques described in Example 1 allow for interrogation of VP expression and posttranslational modifications. The role and biological significance of N-terminal acetylation of AAV capsid proteins was next examined.

Results

To elucidate the potential role of deacetylation of AAV capsid proteins, AAV5 deacetylation variants were tested. An AAV5 particle expressing eGFP under the CBA promoter (AAV5-CBA-Egfp) was compared to AAV5 variants with the amino acid adjacent to the initiating methionine (iMET) mutated for VP1 and VP3 (deAC-AAV5-CBA-eGFPs). Three amino acids predicted to have a low likelihood of acetylation by NatA, NatC, or NatD were chosen for generating variants: Gly, Leu, and Pro, as illustrated in Table 9 below.

TABLE 9

N-terminal acetylation frequency

N-term aa	Transferase	NT-AC FREQUENCY
MET-ALA Normally found in VP1 & VP3	NatA	High
MET-SER Normally found in VP1 & VP3 for AAV5	NatA	High
AAV variants		
MET-GLY	NatA	Low
MET-LEU	NatC	Low
MET-PRO	NatD/other	Low

The following AAV5 deacetylated (deAC) mutants were generated:

S2GVP1—Ser changed to Gly at position 2 in AAV5VP1
S2LVP1—Ser changed to Len at position 2 in AAV5VP1
S2PVP1—Ser changed to Pro at position 2 in AAV5VP1
S2GVP3—Ser changed to Gly at position 2 in AAV5VP3
S2LVP3—Ser changed to Len at position 2 in AAV5VP3
S2PVP3—Ser changed to Pro at position 2 in AAV5VP3
S2PVP1/VP3—Ser changed to Pro at position 2 in both AAV5 VP1 and VP3
S2GVP1/VP3—Ser changed to Gly at position 2 in both AAV5 VP1 and VP3
S2LVP1/VP3—Ser changed to Len at position 2 in both AAV5 VP1 and VP3

These variants were generated using the TTX method as described above. All AAV5 variants showed good productivity, with yields greater than 10^{13} total VG. All AAV5 variants also showed the expected VP1:VP2:VP3 protein ratio by SYPRO protein gel analysis (FIG. **9**). Next, LC/MS was used to confirm that all AAV5 variants had decreased acetylation, as shown in Table 10.

TABLE 10

LC/MS analysis of AAV5 variant acetylation

	mutants	VP1 Theo.	VP1 Exp.	Δmass (VP1)	VP2 Theo.	VP2 Exp.	Δmass (VP2)	VP3 Theo.	VP3 Exp.	Δmass (VP3)	note
1	deAC-AAV5 (S2GVP1)/ CBA-eGFP	80234	nd		65283	65293	10	59463	59472	9	VP1 not detectable
2	deAC-AAV5 (S2LVP1)/ CBA-eGFP	80346	80501	181	65283	65292	9	59463	59471	8	VP1 incorrect
3	deAC-AAV5 (S2GVP3)/ CBA-eGFP	80234	nd		65253	65261	8	59391	59398	7	confirmed
4	deAC-AAV5 (S2LVP3)/ CBA-eGFP	80336	80363	27	65309	65309	0	59447	59620	173	VP3 incorrect
5	deAC-AAV5 (S2PVP1VP3)/ CBA-eGFP	80314	80324	10	65293	65300	7	59431	59438	7	confirmed
6	deAC-AAV5 (S2GVP1VP3)/ CBA-eGFP	80234	80243	9	65253	65261	8	59391	59398	7	confirmed
7	deAC-AAV5 (S2PVP3)/ CBA-eGFP	80336	80346	10	65293	65292	1	59431	59430	1	confirmed
8	deAC-AAV5 (S2PVP1)/ CBA-eGFP	80314	80313	1	65283	65291	8	59463	59470	7	confirmed
9	deAC-AAV5 (S2L VP1VP3)/ CBA-eGFP	80346	nd		65309	65318	9	59447	59629	182	VP3 incorrect

nd = not determined

These LC/MS analyses confirmed that AAV5 variants were deacetylated. The variants S2LVP1, S2LVP3, and S2LVP1/VP3 all showed increased mass (increased from 173 to 182) in VP1 and VP3 proteins, suggesting that changing the second N-terminal amino acid to a leucine in VP1 or VP3 alters the protein, resulting in an increase in mass.

Next, AAV5 variants were assayed in an in vitro transduction assay using eGFP as a reporter gene (FIG. **10**). The assay was designed to evaluate transduction by AAV5 deacetylated mutant variants at 10^6 multiplicity of infection (MOI), comparing each variant to the parental, unmodified AAV5 particle. Three cell lines were used: 293, HuH7, and HeLa cells. Following infection, cells were assayed to determine vector genome copy number (vg/μg cellular protein) and eGFP expression (by ELISA). Vector genome copy number (vg/μg protein) represents the efficiency at which the AAV5 variant enters the cell, and eGFP represents the efficiency of capsid intracellular trafficking, since transgene expression requires the capsid/vector DNA to efficiently traffic to the nucleus (FIG. **10**). Vector genomes were quantified by TaqMan analysis.

FIG. **11** shows that, based on vector genome analyses, AAV5 deacetylated mutant vectors infected all three test cell lines at similar, but reduced, levels as compared to the parental unmodified AAV5 particles. FIG. **12** shows that AAV5 deacetylated mutant vectors all resulted in reduced eGFP expression in all three cell lines, as compared to transduction with parental unmodified AAV5.

Conclusions

As predicted, no acetylation was observed in N-terminal Ser to Pro/Leu/Gly mutant variants when examined by LC/MS. AAV5 deAC variants showed robust vector production, and AAV5 deAC variants infected cells at levels comparahle to parental AAV5. However, functional protein levels in cells infected with deAC variants were greatly reduced when compared to the parental AAV5. These data suggest that tropism is minimally affected by a lack of N-terminal deacetylation in VP1/VP3, but downstream processing (e.g., trafficking and/or degradation) is significantly affected. Since the variants tested demonstrated reduced in vitro activity, one of skill in the art may appreciate that variants characterized by reduced or eliminated acetylation could be employed, inter alia, when decreased levels of transduction are desirable.

Example 5: Assessment of Deamidation of AAV Capsid Proteins

Examples 1 and 3 demonstrate techniques that allow the interrogation of post-translational modifications of AAV capsid proteins and explore the role of deamidation of the AAV2 capsid. The following Example tested whether deamidation reduces potency and/or induces truncation of capsid proteins, and whether different manufacturing processes can induce different levels of deamidation.

Methods

AAV particles were generated and deamidation status assayed as described in Example 3.

Results

As described in Example 3, a potential deamidation site is found at N57/G58 in the phospholipase A2 domain (Ca++ binding site) in VP1 of the AAV2 capsid. The N57/G58 motif is conserved across AAV serotypes (FIG. **13**). Example 3 showed that AAV2 produced by PCL exhibited nearly a 3-fold increase in deamidation as compared to AAV2 produced by TTx (see FIGS. **6**A & **6**B and Table 8).

In examining VP1, VP2, and VP3 production by protein gels, a truncated VP1 protein (tVP1) was detected only in AAV2 capsid proteins produced by the PCL method (FIG. **14**).

A series of AAV2 deamidation mutants was generated next. These mutants targeted the Gly residue in the canonical NG sequence. Mutations targeting the A35 residue (see FIG. **13**), the N-terminal amino acid for tVP1 were also generated, as shown in Table 11. The pAF277 and pAF279 mutants bearing multiple mutations did not package.

TABLE 11

Deamidation mutants

Name	mutation	avg drp/cell
pAF274	G58K	4.54E+03
pAF275	G58D	5.00E+03
pAF276	G58Q	5.41E+03
pAF277	G58, 383, 512, 716K	1.2
pAF278	A35N	6.89E+03
pAF279	A35N, G58, 383, 512, 765K	2.2
293	—	0.9
PIM45	Control	6.28E+03

K = positive charge (basic)
D = negative charge (acidic)
Q = polar

Deamidation of variants were next analyzed by LC/MS as described in Example 3 above. The AAV2A35N and AAV2G58D variants had altered deamidation as compared to the parental AAV2 (FIG. **15**). In particular, the AAV2A35N mutant had increased deamidation (17.8%) as compared to parental AAV2 (5.7%). The AAV2G58D variant had reduced deamidation (1.1%) as compared to parental AAV2. SYPRO protein gel analysis demonstrated that the AAV2 deamidation mutants exhibited the correct VP1:VP2:VP2 ratio (FIG. **16**).

Next, AAV2 deamidation variants were assayed in an in vitro transduction assay using eGFP as a reporter gene (FIG. **17**). The assay was designed to evaluate transduction by AAV2 deamidation mutant variants at 10^6 multiplicity of infection (MOI), comparing each variant to the parental, unmodified AAV2 particle. Three cell lines were used: 293, HuH7, and HeLa cells. Following infection, cells were assayed to determine vector genome copy number (vg/μg cellular protein) and eGFP expression (by ELISA). Vector genome copy number (vg/μg protein) represents the efficiency at which the AAV2 variant enters the cell, and eGFP represents the efficiency of capsid intracellular trafficking, since transgene expression requires the capsid/vector DNA to efficiently traffic to the nucleus (FIG. **17**). Vector genomes were quantified by TaqMan analysis.

Vector genome analysis indicated that AAV2 deamidation mutant variants infected all cell lines tested at levels comparable to that of parental AAV2 vectors (FIG. **18**). Importantly, the AAV2A35N variant was found to be more potent than the parental AAV2 vector for transduction in all three cell lines (FIG. **19**). The AAV2G58D variant was found to be more potent than the parental AAV2 vector in HuH7 cells (FIG. **19**).

Conclusions

In summary, AAV2 deamidation mutant vectors infect cells at levels comparable to the parent AAV2 particles (e.g., comparable vg/μg cellular protein). However, based on analysis of eGFP levels in transduced cells, the AAV2A35N variant had higher potency than the parental AAV2 in all cell lines tested, and the AAV2G58D variant had higher potency than the parental AAV2 in HuH7 cells (a liver-derived cell line). These results suggest that the A35N mutation may be effective in increasing vector potency for transducing many cell types, and that the G58D mutation may also be effective in increasing potency in certain cell types, e.g., liver cells.

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Sequences

All polypeptide sequences are presented N-terminal to C-terminal unless otherwise noted. All nucleic sequences are presented 5' to 3' unless otherwise noted.

Nucleotide sequence of potential AAV2 VP3 initiation codons (ATG codons underlined)
(SEQ ID NO: 1)
ATGGCTACAGGCAGTGGCGCACCAATGGCAGAC

-continued

Polypeptide sequence corresponding to potential AAV2 VP3 initiation codons (methionines underlined)
(SEQ ID NO: 2)
MATGSGAPMAD

AAV2 VP1 polypeptide sequence
(SEQ ID NO: 3)
MAADGYLPDWLEDTLSEGIRQWWKLKPGPPPPKPAERHKDDSRGLVLPGY
KYLGPFNGLDKGEPVNEADAAALEHDKAYDRQLDSGDNPYLKYNHADAEF
QERLKEDTSFGGNLGRAVFQAKKRVLEPLGLVEEPVKTAPGKKRPVEHSP
VEPDSSSGTGKAGQQPARKRLNFGQTGDADSVPDPQPLGQPPAAPSGLGT
NTMATGSGAPMADNNEGADGVGNSSGNWHCDSTWMGDRVITTSTRTWALP
TYNNHLYKQISSQSGASNDNHYFGYSTPWGYFDFNRFHCHFSPRDWQRLI
NNNWGFRPKRLNFKLFNIQVKEVTQNDGITTIANNLTSTVQVFTDSEYQL
PYVLGSAHQGCLPPFPADVFMVPQYGYLTLNNGSQAVGRSSFYCLEYFPS
QMLRTGNNFTFSYTFEDVPFHSSYAHSQSLDRLMNPLIDQYLYYLSRTNT
PSGTTTQSRLQFSQAGASDIRDQSRNWLPGPCYRQQRVSKTSADNNNSEY
SWTGATKYHLNGRDSLVNPGPAMASHKDDEEKFFPQSGVLIFGKQGSEKT
NVDIEKVMITDEEEIRTTNPVATEQYGSVSTNLQRGNRQAATADVNTQGV
LPGMVWQDRDVYLQGPIWAKIPHTDGHFHPSPLMGGFGLKHPPPQILIKN
TPVPANPSTTFSAAKFASFITQYSTGQVSVEIEWELQKENSKRWNPEIQY
TSNYNKSVNVDFTVDINGVYSEPRPIGTRYLTRNL

VP1 N-terminal tryptic peptide (N-terminal alanine is acetylated)
(SEQ ID NO: 4)
AADGYLPDWLEDTLSEGIR

VP3 N-terminal Asp-N peptide (N-terminal alanine is acetylated)
(SEQ ID NO: 5)
ATGSGAPM

Common VP1 N-terminal sequence
(SEQ ID NO: 6)
MAADGYLPDWLED

Nucleotide sequence of potential AAV7 VP3 initiation codons (start codons underlined)
(SEQ ID NO: 7)
GTGGCTGCAGGCGGTGGCGCACCAATGGCAGACAATAAC

Nucleotide sequence of mutated ITR
(SEQ ID NO: 8)
CACTCCCTCTCTGCGCGCTCGCTCGCTCACTGAGGCCGGGCGACCAAAGG
TCGCCCACGCCCGGGCTTTGCCCGGGCG

SEQUENCE LISTING

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                        organism = Adeno-associated virus 2
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HSSYAHSQSL DRLMNPLIDQ YLYYLSRTNT PSGTTTQSRL QFSQAGASDI RDQSRNWLPG  480
PCYRQQRVSK TSADNNNSEY SWTGATKYHL NGRDSLVNPG PAMASHKDDE EKFFPQSGVL  540
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LPGMVWQDRD VYLQGPIWAK IPHTDGHFHP SPLMGGFGLK HPPPQILIKN TPVPANPSTT  660
FSAAKFASFI TQYSTGQVSV EIEWELQKEN SKRWNPEIQY TSNYNKSVNV DFTVDTNGVY  720
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AKKRVLEPLG LVEEGAKTAP GKKRPVEPSP QRSPDSSTGI GKKGQQPAKK RLNFGQTGDS  180
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ESVPDPQPLG EPPAAPSGVG PNTMAAGGGA PMADNNEGAD GVGSSSGNWH CDSTWLGDR   239

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DFEDSGAGDG PPEGSSSGEM SHDAEMRAAP GGNAVEAGQG ADGVGNASGD WHCDSTWSEG 240
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KKRVLEPFGL VEEGAKTAPT GKRIDDHFPK RKKARTEEDS KPSTSSDAEA GPSGSQQLQI  180
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AKKRVLEPLG LVEEGAKTAP GKKRPVEXSP XXQXXPDSSS GIGKKGQXXX XXQPAKKRLN  180
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TWLGDR                                                             246

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K                                                                  61

SEQ ID NO: 33          moltype = AA  length = 61
FEATURE                Location/Qualifiers
REGION                 1..61
                       note = Synthetic Construct
source                 1..61
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K                                                                  61

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FEATURE                Location/Qualifiers
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source                  1..61
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K                                                                  61

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K                                                                  61

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K                                                                  61

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FEATURE                 Location/Qualifiers
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source                  1..61
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K                                                                  61
```

What is claimed is:

1. A method of detecting post-translational modifications of one or more viral proteins (VPs) in a preparation of adeno-associated virus (AAV) particles, the method comprising

a) denaturing the AAV particles;

b) subjecting the denatured AAV particles to liquid chromatography/mass spectrometry (LC/MS) intact protein analysis;

c) determining the masses of the one or more VPs; and

d) determining any deviation of the determined masses of the one or more VPs from the theoretical masses of corresponding VPs that have not undergone post-translational modifications to detect a deviation in the compared masses,

wherein the VPs comprise VP1, VP2 and VP3 capsid proteins, and wherein the method is performed in the absence of a gel separation step.

2. The method of claim 1, wherein the post-translational modifications are selected from the group consisting of acetylation, deacetylation, deamidation, glycosylation, truncation and ubiquitination.

3. The method of claim 2, wherein the post-translational modification is N-terminal acetylation.

4. The method of claim 1, wherein the AAV particles are denatured using detergent, heat, high salt or buffer with low or high pHs.

5. The method of claim 1, wherein the liquid chromatography is reverse phase chromatography.

6. The method of claim 5, wherein the reverse phase chromatography is C8 reverse phase chromatography.

7. The method of claim 1, further comprising determining the sequence of one or more VPs that has undergone post-translational modifications.

8. The method of claim 7, wherein the post-translational modifications are selected from the group consisting of acetylation, deacetylation, deamidation, glycosylation, truncation and ubiquitination.

9. The method of claim 8, wherein the post-translational modification is N-terminal acetylation.

10. The method of claim 7, wherein the sequences of VP1, VP2 and VP3 are determined.

11. A method of determining the heterogeneity of viral particles in a preparation of adeno-associated virus (AAV) particles comprising VP1, VP2 and VP3 capsid proteins, the method comprising

a) denaturing the AAV particles;

b) subjecting the denatured AAV particles to liquid chromatography/mass spectrometry (LC/MS) intact protein analysis, thereby separating the peaks of the VP1, VP2 and VP3 capsid proteins;

c) deconvoluting the peaks of the VP1, VP2 and VP3 capsid proteins; and

d) determining the masses of one or more of the VP1, VP2 and VP3 capsid proteins and additional capsid proteins within one or more of the deconvoluted peaks,

wherein the method is performed in the absence of a gel separation step.

12. The method of claim 11, wherein the additional capsid proteins within one or more of the deconvoluted peaks are variant capsids.

13. The method of claim 11, wherein the additional capsid proteins within one or more of the deconvoluted peaks are capsid amino acid substitutions.

14. The method of claim 11, wherein the additional capsid proteins within one or more of the deconvoluted peaks are truncated capsids.

15. The method of claim 11, wherein the additional capsid proteins within one or more of the deconvoluted peaks are modified capsids.

16. The method of claim 15, wherein the modifications of the modified capsids are selected from the group consisting of acetylation, deacetylation, deamidation, glycosylation, truncation and ubiquitination.

17. The method of claim 16, wherein the modification is N-terminal acetylation.

18. The method of claim 11, wherein the liquid chromatography is reverse phase chromatography.

19. The method of claim 18, wherein the reverse phase chromatography is C8 reverse phase chromatography.

20. A method of preparing a pharmaceutical composition of adeno-associated virus (AAV) particles, the method comprising:

monitoring AAV particles for consistency and/or identity;

wherein the AAV particles comprise viral proteins (VPs) comprising VP1, VP2 and VP3 capsid proteins of an AAV particle capsid,

wherein the AAV particle is monitored for consistency and/or identity by:

a) extracting an aliquot of an AAV particle preparation;

b) denaturing the AAV particles;

c) subjecting the denatured AAV particles to liquid chromatography/mass spectrometry (LC/MS) intact protein analysis;

d) determining the masses of one or more VPs of the AAV particles; and

e) comparing the determined masses of the one or more VPs to theoretical masses of corresponding VPs, wherein the theoretical masses of corresponding VPs are those VPs of known AAV serotypes and/or those that have not undergone undesired post-translational modifications; and

f) determining if there is any deviation of the determined masses of the one or more VPs from the theoretical masses of the corresponding VPs;

wherein the determination of any deviation of the determined masses of the one or more VPs from the theoretical masses of corresponding VPs thereby monitors the AAV particles for consistency and/or identity;

wherein the monitoring for consistency and/or identity is performed in the absence of a gel separation step; and

wherein if less than an undesirable amount of deviation is determined during the monitoring for consistency and/or identity, the AAV particles are combined with one or more pharmaceutically acceptable excipients to form the pharmaceutical composition.

21. The method of claim 20, wherein the monitoring of the AAV particles for consistency and/or identity includes determining the serotype of the AAV particles based on the comparison of the determined masses of the VPs to the theoretical masses of the corresponding VPs.

22. The method of claim 20, wherein a determination of any actual deviation in masses reflects heterogeneity in the AAV particle preparation.

23. The method of claim 22, wherein the heterogeneity in the AAV particle preparation is due to mixed AAV capsid serotypes, variant AAV capsid proteins, AAV capsid protein amino acid substitutions, truncated AAV capsid proteins or modified AAV capsid proteins.

24. The method of claim 21, wherein the undesired post-translational modifications are selected from the group consisting of acetylation, deacetylation, deamidation, glycosylation, truncation and ubiquitination.

25. The method of claim **21**, wherein the AAV particles are denatured using detergent, heat, high salt or buffer with low or high pHs.

26. The method of claim **21**, wherein the liquid chromatography is reverse phase chromatography.

27. The method of claim **26**, wherein the reverse phase chromatography is C8 reverse phase chromatography.

* * * * *

Exhibit T

AUC Insights - Assessing the quality of adeno-associated virus gene therapy vectors by sedimentation velocity analysis

By Dr. Elmar Spies, Beckman Coulter Life Sciences

Introduction

Since the first gene therapy study in the late 1980s, there has been an ongoing interest in gene therapy strategies. According to Ginn et al. (1) about 2600 gene therapy clinical trials are being completed, ongoing or approved in 2018. The largest number of trials is concerned with cancer, followed by monogenic and infectious diseases (Table 1). In general, gene therapies can be divided into nonviral or viral delivery systems. Nonviral systems are usually characterized by an undirected and inefficient delivery and only transient expression; on the contrary, they show a larger packaging capacity and a better biosafety profile than viral gene therapies (1). However, the great advantage of viral systems lies in their high delivery efficiency and the exploitation of the viral biology (being able to infect host cells and to exploit their replication machinery). Among the known viral delivery systems, adenoviruses are used most often, followed by retroviruses

and adeno-associated viruses (AAV). Interestingly, AAV show an increase in interest in recent years in contrast to other virus types (1). One reason might be the fact that AAV is less immunogenic than other viruses (2).

Gene Therapy Clinical Trials	
Cancer Diseases	65%
Monogenetic Diseases	11.1%
Infectious Diseases	7%
Cardiovasular Diseases	6.9%
Others	10%
Tab. 1: Distribution of completed, ongoing or approved clinical trails by disease category 2018, in % (1)	

Adeno-Associated Viruses

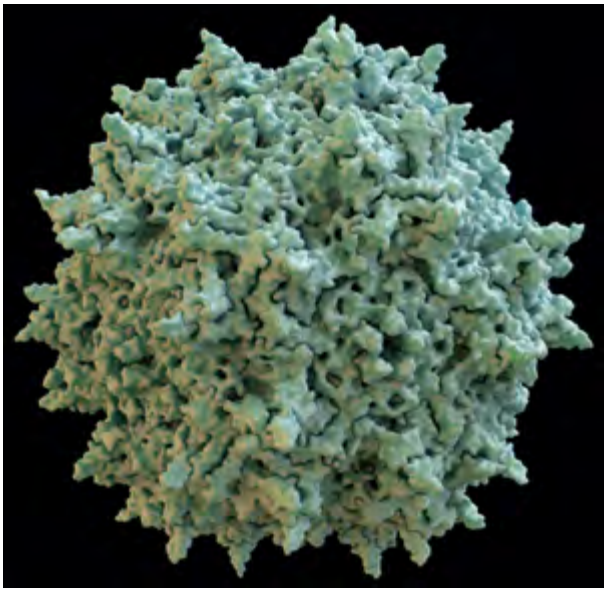
AAV is a nonpathogenic, nonenveloped parvovirus with the size of approx. 22 nm that is not able to replicate without the assistance of a helper virus (adeno- or herpes simplex virus) (2). The virus capsid can carry a transgene of about 5kb (including viral ITR sequences) without significant reduction in viral production yields (2). A promoter, the gene of interest and a terminator are cloned between both ITR sequences. After transduction, the transgene is present as an episome in the nucleus from where the expression of the gene of interest takes place. The episomal DNA limits the risk of integration but it might be diluted out after several cell cycles (3). Several AAV serotypes are used as gene therapy vectors. These serotypes show a specific tissue-tropism thus limit their use to certain organs. Efforts are made to optimize the capsid and therefore modulate the viral tropism to certain cell types (4, 5).

Example 1: Quality control of rAAV vectors by AUC

Due to its popularity and increased usage, it is of major interest to assess the quality of clinical-grade rAAV vectors before administration.

Burnham and colleagues (6) showed that sedimentation velocity analytical ultracentrifugation (AUC) experiments are able to characterize the quality of viral vector productions (ratio between empty and filled capsids). A single AUC experiment provides

insights into the composition of the encapsulated DNA, the success of the purification, the presence of aggregates and the ratio between empty, partially filled and filled viral particles. The AAV samples are measured at 260 nm at a speed of 20,000 rpm (6). A peak at 63S corresponds to empty vector capsids whereas a peak at 93S shows the proportion of filled capsids. There are also peaks between 63S and 93S visible, which might be partially filled capsids, and finally larger peaks (larger than 93S) appeared that could be aggregated viral particles. Furthermore, the scientists tried to assess if the AUC is able to discriminate between transgenes of different sizes. Firstly, they used a transgene of 3370 nucleotides in length that yields an s-value of about 92 after sedimentation velocity whereas a transgene of 4200 nucleotides results in a peak with an s-value of 101. Taken together, the AUC is a valuable tool to analyze rAAV vectors notwithstanding the composition and length of the transgene or the viral serotype.



3D CG rendered image of Adeno-Associated Virus (AAV) Capsid

Example 2: Sedimentation Velocity analysis with exceptional resolution proves to be the gold standard for interpreting orthogonal techniques

In the following example, Wang et. al. have investigated the potential of anion exchange chromatography (AEX) for the determination of empty and full AAV capsids and also for other populations in the sample like partially loaded capsids (7). They also mentioned transmission electron microscopy (TEM) and a number of other techniques like ELISA in combination with qPCR as possible methods to verify the quality of AAVs. However, they acknowledge that the AUC is the gold standard in the detection and characterization of AAV particles. TEM is not a quantitative method, giving rather qualitative information on the tested AAV batch. ELISA/qPCR and CDMS are shown to not be accurate enough to provide proper resolution. Finally, they directly compared AEX and AUC: AUC showed a much higher resolution as compared to AEX – as evident in the baseline separation between empty and full capsids in the AUC experiment. The comparable AEX experiment

shows the empty capsids as an overlapped shoulder peak of the main filled-capsid peak. The AUC experiment even revealed a small population of what might be fragmented genome capsids. This species was not visible in AEX (Figure 1).

In contrast to AUC, every chromatographic technique uses a matrix, which particles have to pass through. Thus, not only can particles interact with the matrix, but also dilution effects might dissolve aggregated AAV particles and finally might falsify the true picture of a virus batch. AUC as a matrix-free in-method allows the characterization in a near-native environment.

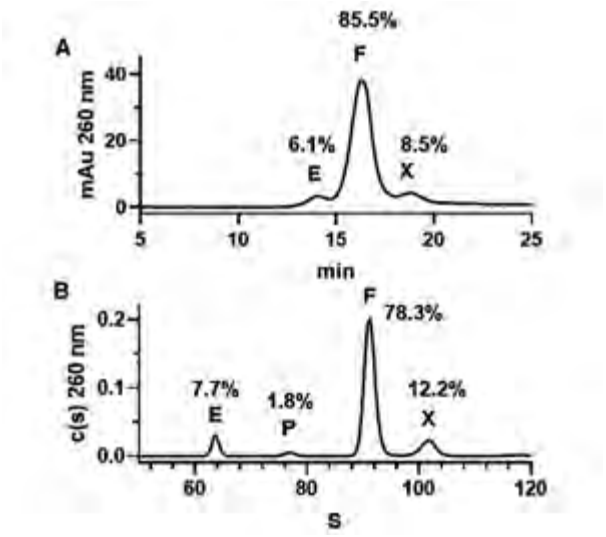


Figure 1: Characterization of AAV empty and full capsids by AEX and AUC (reproduced with permission from Wang et al. (7)) Comparison of the AEX chromatogram (A) and AUC sedimentation coefficient distribution (B) of an affinity-purified AAV6.2 sample. E, empty capsid; F, full capsid; P, capsid with fragmented genome; X, unknown species. The peak percents labeled on the plots are area percents determined at UV 260 nm, without response factor corrections.

Optima AUC

- First-principle technique that does not depend on a matrix and does not require standards
- Samples are analyzed in their native state with almost no buffer restrictions
- One experiment reveals information about shape, diameter, mass, stoichiometry, purity, formulation heterogeneity, aggregation, association and conformation of a protein or protein complex
- Optical systems
  - Rayleigh Interface
  - UV absorbance
- Sample volume:
  Max. Volume for 2-sector centerpieces: 450µl
  Max. volume for 6-channel equilibrium center-pieces: 120µl
- Wavelength range: 190-800 nm
- Molecular weight range:
  10^2 Da (i.e. Peptides/Oligosaccharides) -10^8 Da (i.e. Viruses/Organelles)
- Concentration range:
  UV absorption: 0.005 – 1-2 mg/ml Lutenizing
  Hormone Interference: 0.025 – 4-5 mg/ml BSA

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Exhibit U

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(54) **METHODS FOR ANALYZING AAV CAPSID PROTEINS**

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CPC ***G01N 33/6848*** (2013.01); ***C07K 14/005*** (2013.01); *C12N 2750/14122* (2013.01); *G01N 2030/8831* (2013.01)

(57) **ABSTRACT**

Provided are methods to characterize the VP1, VP2 and VPS capsid proteins in an adeno-associated virus (AAV) particle using liquid chromatography mass spectrometry, and/or ultraviolet (UV)-visible spectroscopy. The methods generally include the steps of (a) subjecting an AAV particle to liquid chromatography to denature and then separate the VP1, VP2 and VPS capsid proteins, and (b) subjecting the separated VP1, VP2 and VPS capsid proteins produced in step (a) to UV and mass spectrometry to determine the ratio and masses of the VP1, VP2 and VPS capsid proteins in the AAV particle. In another aspect, the disclosure provides an AAV composition comprising a post-translation modification. The disclosure also provides methods for characterizing the purity of AAV compositions using liquid chromatography mass spectrometry.

Specification includes a Sequence Listing.

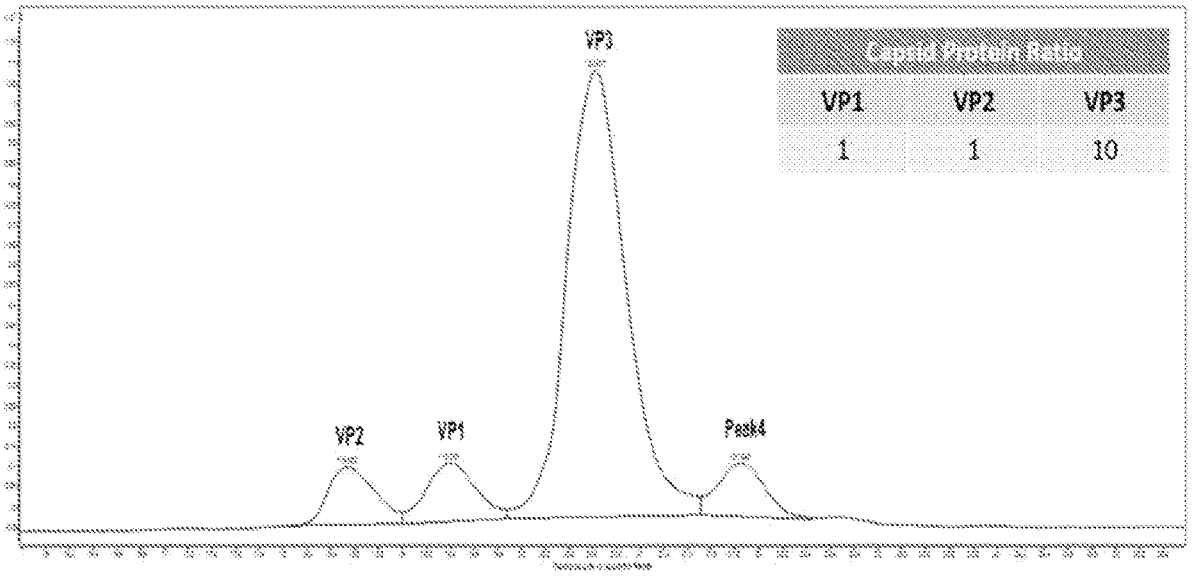


Fig. 1

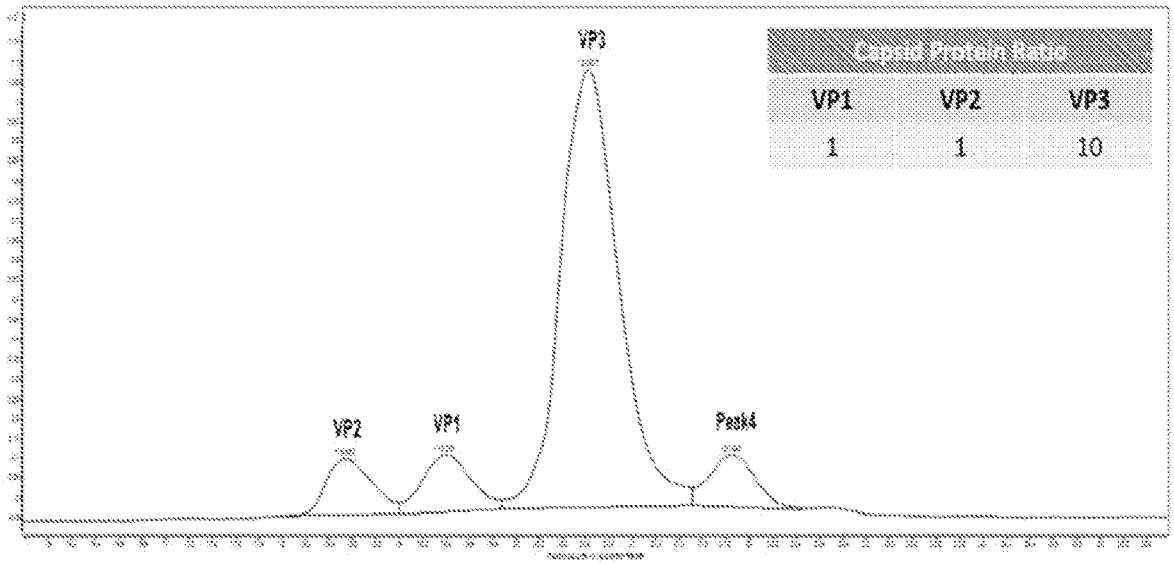

VP3
VP2
VP1
Peak4
Capsid Protein Ratio
VP1 VP2 VP3
1 1 10

Fig. 2

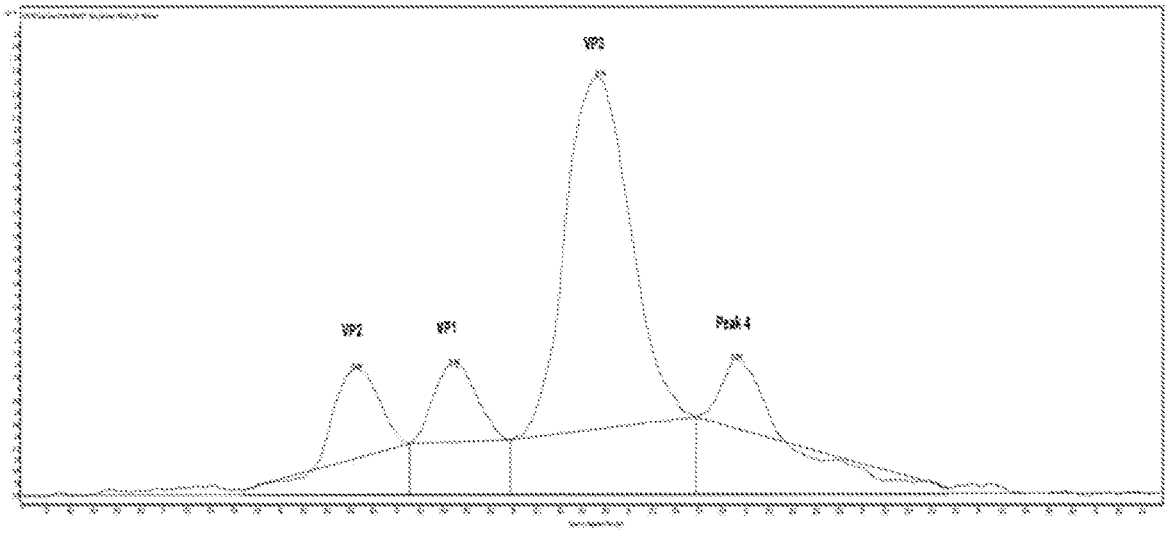

VP3
VP2
VP1
Peak4

FIG. 3A

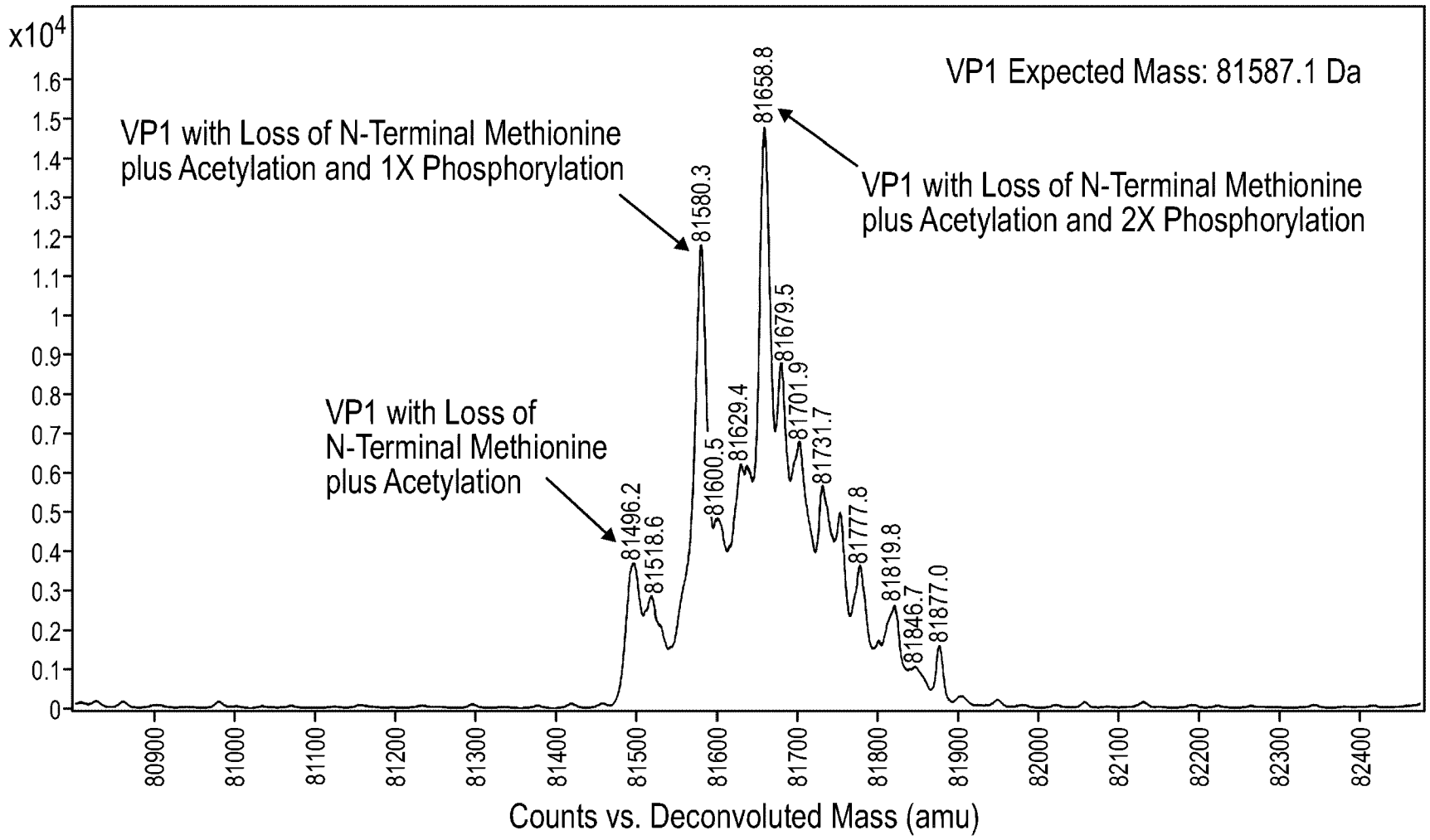


FIG. 3B

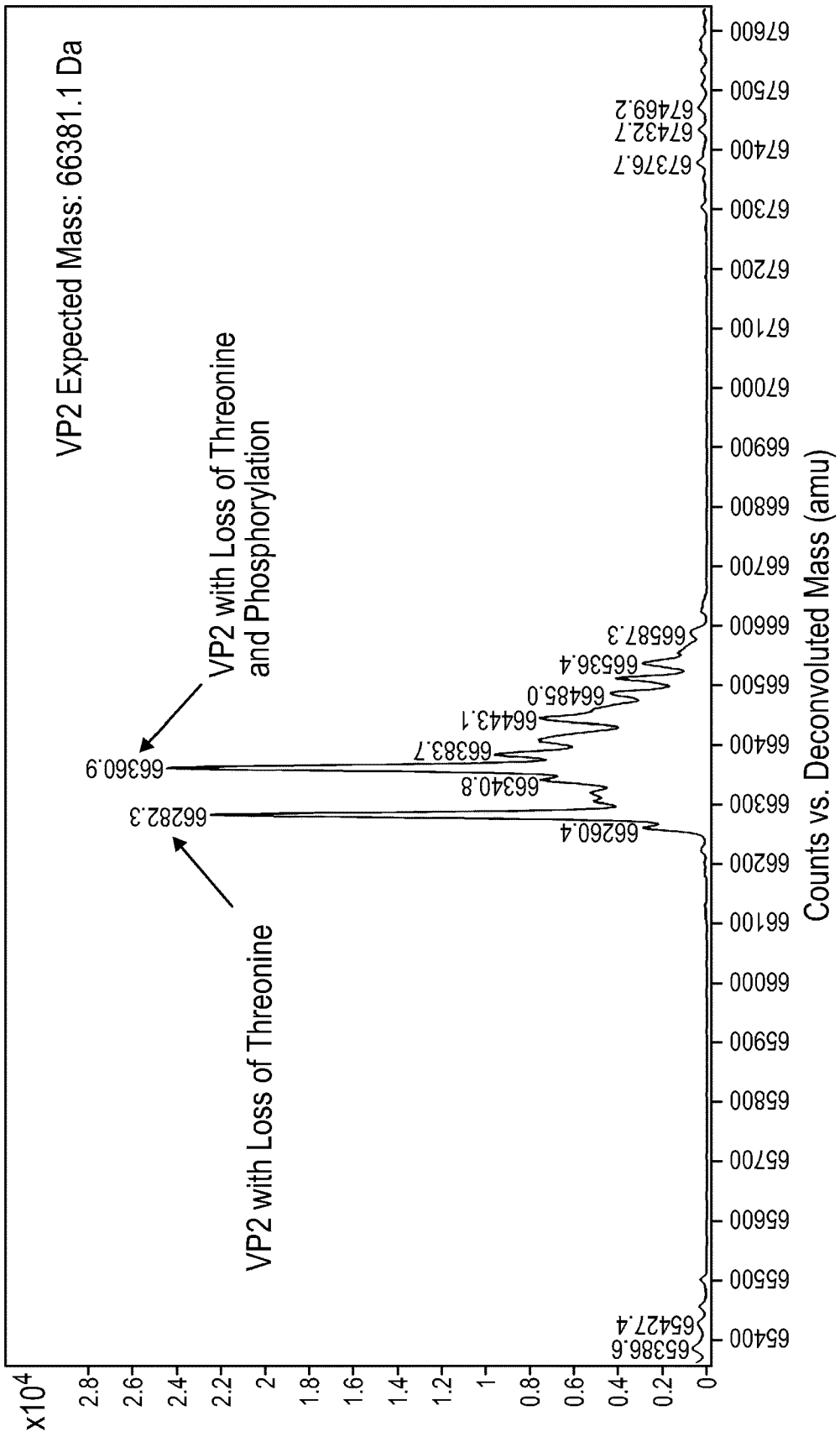

VP2 Expected Mass: 66381.1 Da
VP2 with Loss of Threonine and Phosphorylation
VP2 with Loss of Threonine
66360.9
66282.3
67469.2
67432.7
67376.7
66587.3
66536.4
66485.0
66443.1
66383.7
66340.8
66260.4
65386.6
65427.4
Counts vs. Deconvoluted Mass (amu)
x10^4

Fig. 3C

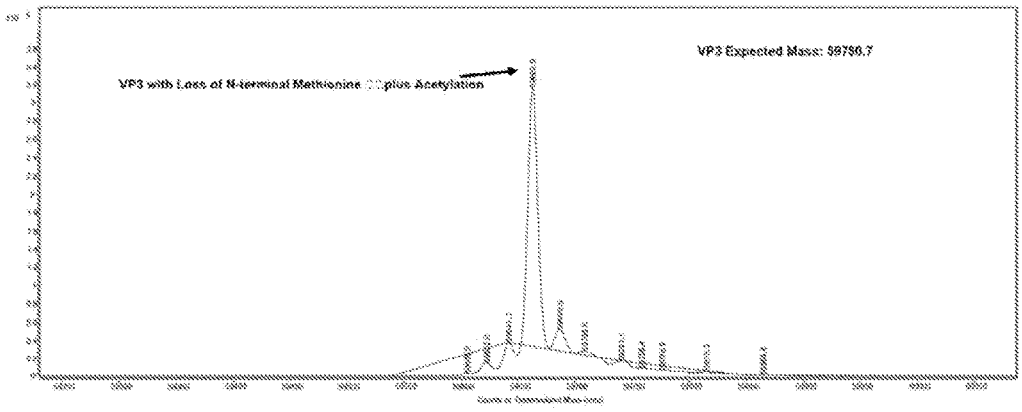

VP3 Expected Mass: 59756.7
VP3 with Loss of N-terminal Methionine plus Acetylation

Fig. 3D

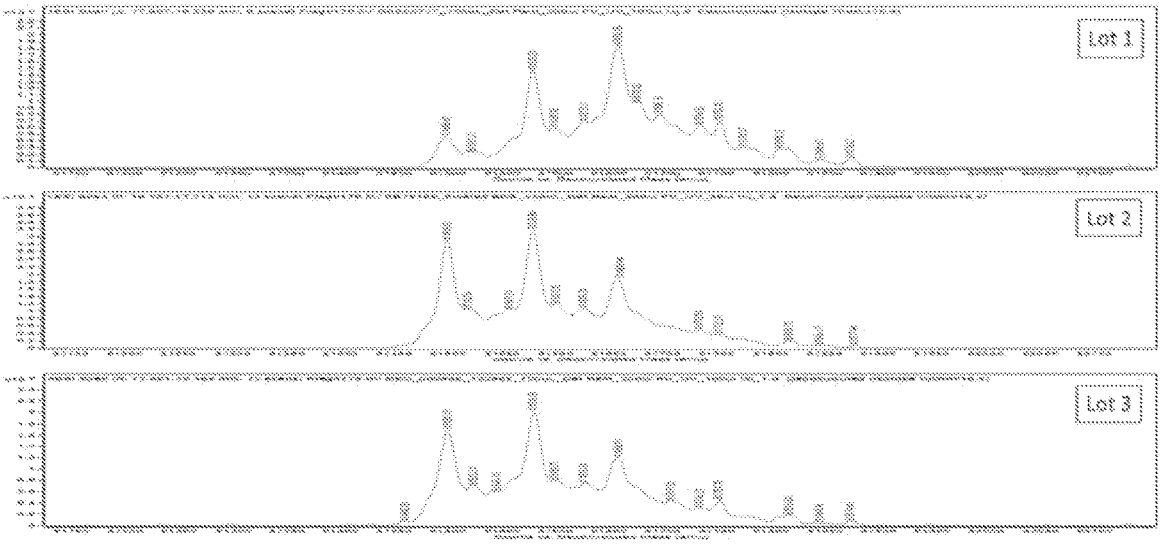

Lot 1
Lot 2
Lot 3

Fig. 3E

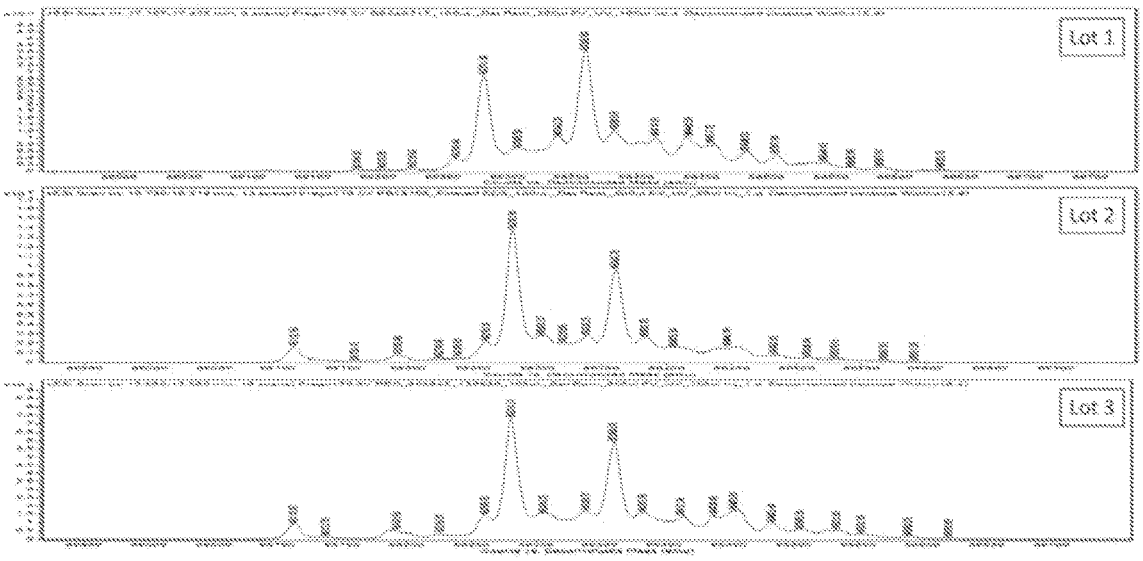


Fig. 3F

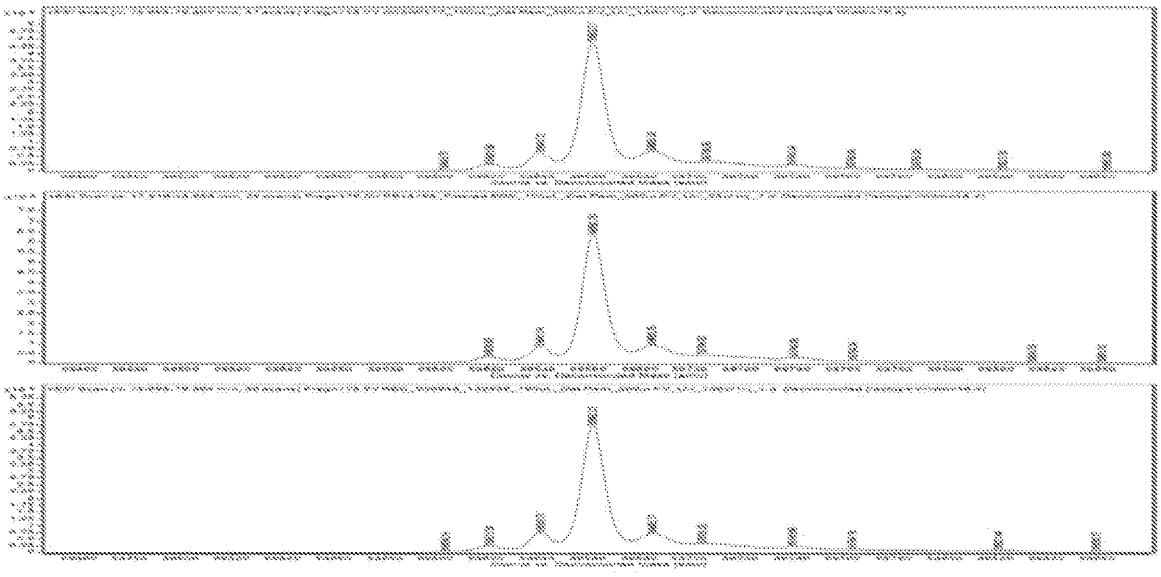
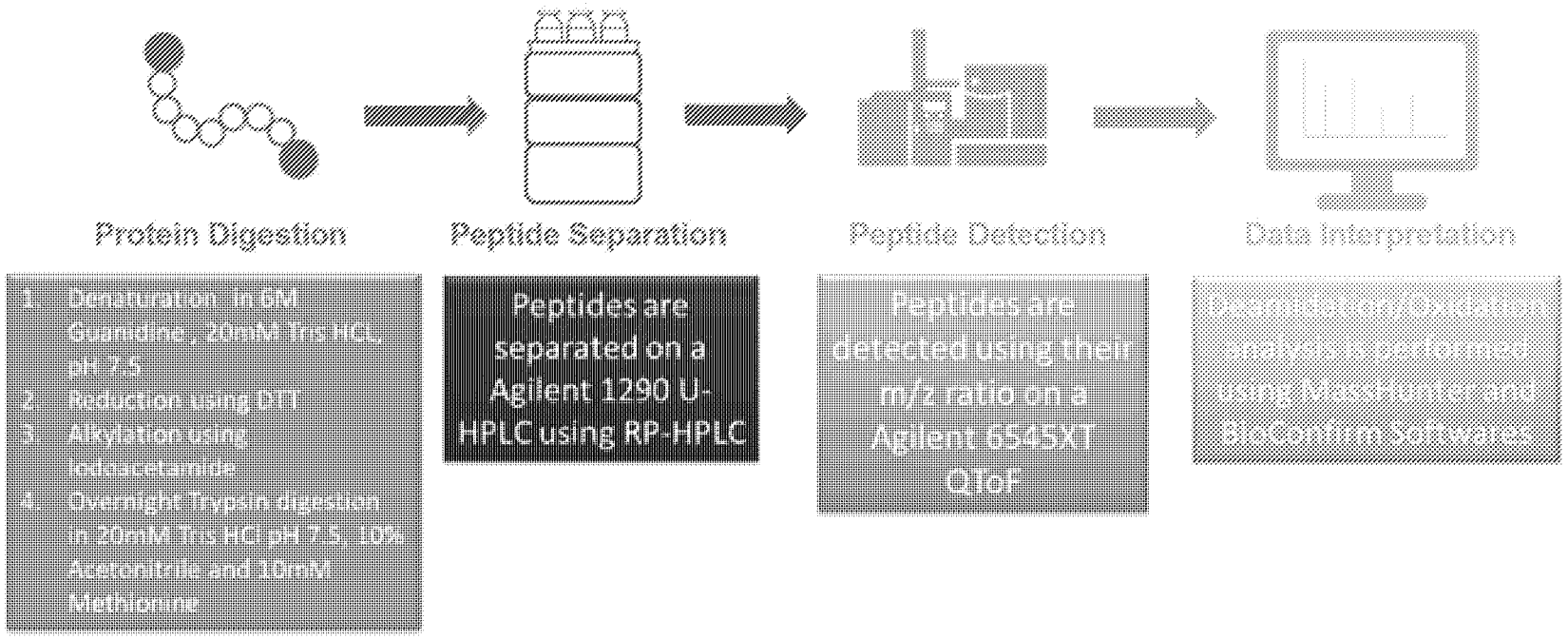
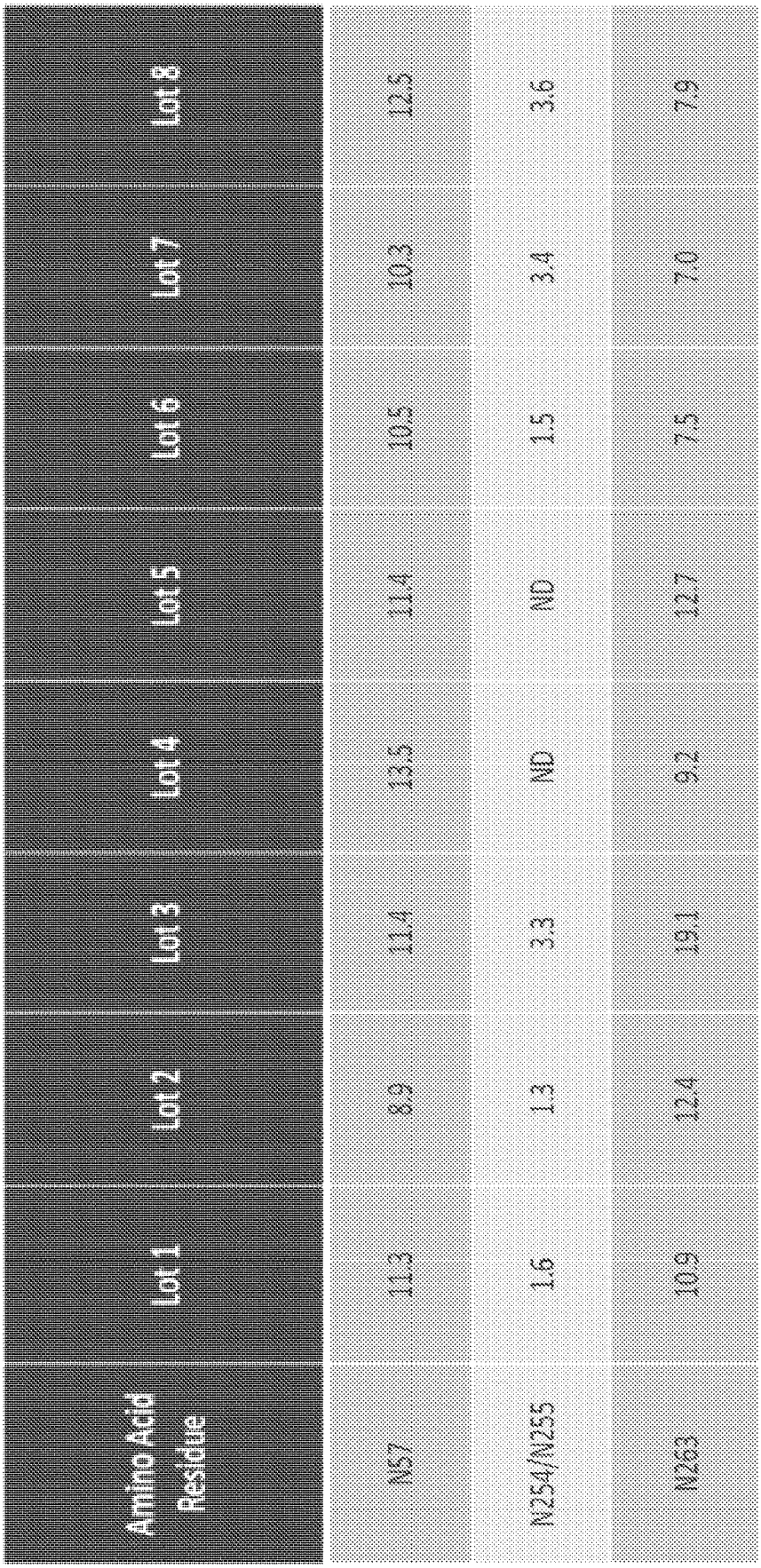


Fig. 4


Protein Digestion
Peptide Separation
Peptide Detection
Data Interpretation
1 Denaturation in 6M Guanidine, 20mM Tris HCl, pH 7.5
2 Reduction using DTT
3 Alkylation using Iodoacetamide
4 Overnight Trypsin digestion in 20mM Tris HCl pH 7.5 +10% Acetonitrile and 10mM Methionine
Peptides are separated on a Agilent 1290 U-HPLC using RP-HPLC
Peptides are detected using their m/z ratio on a Agilent 6545XT Q ToF
Peptides identified/validated & analysis is performed using MassHunter and BioConfirm Softwares.



Amino Acid Residue	Lot 1	Lot 2	Lot 3	Lot 4	Lot 5	Lot 6	Lot 7	Lot 8
N57	11.3	8.9	11.4	13.5	11.4	10.5	10.3	12.3
N254/N255	1.6	1.3	3.3	ND	ND	1.5	3.4	3.6
N263	10.9	12.4	19.1	9.2	12.7	7.5	7.0	7.9

Fig. 5

Fig. 6

Amino Acid Residue	Lot 1	Lot 2	Lot 3	Lot 4	Lot 5	Lot 6	Lot 7	Lot 8
M437	0.5	0.4	0.5	0.3	0.5	0.4	0.4	0.4
M473	ND	0.6	ND	0.8	ND	ND	0.8	0.7
M526	ND	0.8	0.6	0.4	0.6	2.1	0.8	0.6
M544	1.1	1.1	0.9	0.8	0.9	0.8	1.1	0.8
M580	ND	0.7	ND	0.9	1.0	0.9	0.9	1.0
M637	0.7	0.8	0.9	0.7	0.9	0.8	0.9	0.8

METHODS FOR ANALYZING AAV CAPSID PROTEINS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application No. 62/956,681 filed Jan. 3, 2020, U.S. Provisional Application No. 63/073,188 filed Sep. 1, 2020, and U.S. Provisional Application No. 63/119,909 filed Dec. 1, 2020, the contents of each of which are incorporated herein by reference in their entireties.

REFERENCE TO SEQ REFERENCE TO SEQUENCE LISTING SUBMITTED ELECTRONICALLY VIA EFS-WEB

[0002] The content of the electronically submitted sequence listing in ASCII text file (Name:
[0003] 4140 0600003 Seqlisting ST25; Size: 1,978 bytes; and Date of Creation: November 21, 2022) is herein incorporated by reference in its entirety.

TECHNICAL FIELD

[0004] The disclosure relates to methods for characterizing the VP1, VP2 and VP3 capsid proteins in an adeno-associated virus (AAV) particle and the purity of an AAV composition using liquid chromatography and mass spectrometry.

BACKGROUND

[0005] Adeno-Associated viruses (AAVs) are quickly becoming one of the most widely used vehicles for delivering gene therapy. The excellent safety profile along with the high efficiency of transduction of a broad range of target tissues has made AAV the most widely used platform for gene therapy. AAV is a small virus belonging to the Parvoviridae family. The virus is composed of a non-enveloped icosahedral capsid containing a linear single stranded DNA genome of about 4.7 kilobases. AAV is commonly expressed recombinantly in suitable host cells. However, recombinant AAV may be contaminated by proteins from the host cell lysate.

[0006] The AAV capsid includes a mixture of VP1, VP2 and VP3 proteins, which are produced from a single viral Cap gene by alternative splicing and translation, and which self-assemble to form the capsid. AAV capsid proteins play a critical role in viral infectivity, tissue tropism, and potency, and the ability to fully characterize the mass and ratios of capsid proteins is becoming increasingly important for the commercial manufacturing of AAV for gene therapy.

[0007] In particular, the stoichiometry of the VPs is crucial for infectivity of viral vectors. For example, high levels of VP3 capsid was negatively associated with poor transduction efficiency and reduced potency even when the VP1/VP2 ratio was not in balance. (Gene Therapy, volume 25, pages 415-424 (2018)). Since the ratio of structural proteins VP1, VP2, and VP3 from manufacture may fluctuate in a wide range, e.g., 1:1:5 to 1:1:20 (Biotechnol Adv., 26(1):73-88 (2008)), the accurate measurement of the ratio among the three capsid proteins is important in the AAV vector quality control. However, the current methods attempted to measure the masses of capsid proteins but failed to determine the stoichiometry of each of the VPs (WO 2018/035059). Thus, robust methods for a more accurate characterization of ratios and modifications of AAV capsid proteins and the purity of rAAV compositions is needed in the gene therapy industry.

SUMMARY

[0008] The disclosure provides methods to characterize the VP1, VP2 and VP3 capsid proteins in an adeno-associated virus (AAV) particle using liquid chromatography and mass spectrometry. The methods disclosed herein are used to determine the ratio of VP1, VP2 and VP3 capsid proteins in AAV particle, and/or the masses of one or more of the VP1, VP2 and VP3 capsid proteins.

[0009] In some aspects, the present disclosure provides a method to determine the ratio of VP1,

[0010] VP2 and VP3 capsid proteins in an adeno-associated virus (AAV) particle. The method includes the steps of subjecting the AAV particle to a liquid chromatography at about 70° C. to about 90° C., wherein the masses and ratio of VP1, VP2 and VP3 capsid proteins is determined by mass spectrometry and/or ultraviolet (UV)-visible spectroscopy. In some aspects, the individual masses of the capsid proteins are measured by mass spectrometry. In some aspects, the capsids on the AAV particle is denatured into the individual VP1, VP2 and VP3 proteins in the column of the liquid chromatography. In some aspects, the capsid proteins are separated by the liquid chromatography.

[0011] In some aspects, the method further includes determining the masses of one or more of the VP1, VP2 and VP3 capsid proteins in the AAV particle using mass spectrometry.

[0012] In some aspects, the relative amounts of the VP1, VP2 and VP3 capsid proteins are determined by analyzing the ultraviolet (UV) chromatogram of the VP1, VP2 and VP3 capsid proteins. In some aspects the liquid chromatography is reverse phase liquid chromatography. In some aspects, the AAV particle is AAVrh74.

[0013] In some aspects, the chromatography uses a first mobile phase including trifluoroacetic acid in water. In some aspects, the chromatography uses a second mobile phase including trifluoroacetic acid in the mixture of acetonitrile and water. In some aspects, the percentage of the second mobile phase, in a combination of the first mobile phase and the second mobile phase, in the chromatography is increased over time.

[0014] In some aspects, the mass spectrometry comprises a fragmentor voltage of about 125-350

[0015] V.

[0016] Deamidation is one of the common Post-Translational modifications (PTM) observed in proteins that is known to have a significant impact on the activity and stability of proteins. Deamidation is usually caused by the hydrolysis of the amide side chain of Asparagine to form a mixture of aspartic and isoaspartic acid. In some aspects, the deamidation is the hydrolysis reaction of cytosine into uracil, releasing ammonia in the process. This can occur in vitro through the use of bisulfite, which deaminates cytosine, but not 5-methylcytosine. In some aspects, deamination of 5-methylcytosine results in thymine and ammonia. In some aspects, glutamine residues also undergo deamidation to form a mixture of glutamic and isoglutamic acid, however glutamine residues are significantly less susceptible to deamidation as compared to Asparagine. In some aspects, deamination of guanine results in the formation of xanthine. In some aspects, deamidation of adenine results in the

formation of hypoxanthine. Deamidation of capsid proteins can impact the stability and activity of the AAV formulations.

[0017] In some aspects of the disclosure, mass spectrometry is used to study post-translational modifications like deamidation. In some aspects, the protein can be denatured using reagents like Guanidine and Urea. The denatured protein is reduced using 1,4-Dithiothreitol (DTT) or Tris(2-carboxyehtyl)phosphine (TECP) to break the disulfide linkages. The reduced disulfide linkages are then alkylated using Iodoacetamide. The denaturation and alkylation steps are carried out to ensure that the protein is unfolded and therefore completely accessible to the proteases. The denatured and reduced protein is then digested using one of several proteases like Trypsin. The digested peptides are separated on a HPLC/UPLC using RP-HPLC. Separated peptides are then detected using their m/z ratios on the Mass Spectrometer, typically a Q-ToF or an Orbitrap. Using appropriate software and databases the peptides are identified. Deamidation is identified as an increase of approximately 1 Da as compared to the theoretical value of the peptide.

[0018] In some aspects, the method further includes determining post translational modification of at least one of VP1, VP2 and VP3 capsid proteins. In some aspects, the method further includes post translational phosphorylation or acetylation of at least one of VP1, VP2 and VP3 capsid proteins.

[0019] The disclosure also provides a method of characterizing host cell proteins in an AAV composition, comprising immunoprecipitating viral capsid proteins from the composition; digesting residual host cell proteins; and analyzing the digested proteins with liquid chromatography quadrupole time of flight mass spectrometry (LC-QTOF-MS) to identify host cell proteins.

[0020] In some aspects, the immunoprecipitation comprises incubating the AAV composition with an anti-AAV VP1 antibody, an anti-AAV VP2 antibody, an anti-AAV VP3 antibody, or combinations thereof

[0021] In some aspects, the method further comprises analyzing the digested host cell proteins with iterative MS/MS.

[0022] In some aspects, the digestion is done in solution. In some aspects, the digestion is performed at a temperature of about 60° C. to about 80° C. In some aspects the digestion is performed at about 70° C.

[0023] In some aspects, the method further comprises spiking the AAV composition with a known amount of at least one known protein standard. In some aspects, the at least one known protein standard is a human or bovine protein standard. In some aspects, the method further comprises quantifying the amount of the digested host cell proteins relative to the at least one protein standard.

[0024] In some aspects, the liquid chromatography is a reverse phase liquid chromatography. In some aspects, the reverse phase liquid chromatography is performed using a C18 column, a C8 column, or a C4 column. In some aspects, the liquid chromatography is performed using a C8 column. In some aspects, the column comprises particles of about 1.2-3.5 μm. In some aspects, the column comprises particles of about 1.7 μm or about 1.8 μm. In some aspects, the column is from about 50 mm to about 300 mm long and has an internal diameter of from about 1 mm to about 4.6 mm. In some aspects, the column is about 150 mm long and has an internal diameter of about 2.1 mm.

[0025] In some aspects, the liquid chromatography is performed at about 40° C. to about 50° C. In some aspects, the liquid chromatography is performed at about 45° C.

[0026] In some aspects, the liquid chromatography comprises a first mobile phase that comprises formic acid. In some aspects, the first mobile phase comprises from about 0.05% to about 0.15% formic acid by volume. In some aspects, the first mobile phase comprises about 0.1% formic acid by volume.

[0027] In some aspects, the liquid chromatography comprises a second mobile phase that comprises formic acid in a mixture of acetonitrile and water. In some aspects, the second mobile phase comprises from about 0.05% to about 0.15% formic acid by volume. In some aspects, the second mobile phase comprises about 0.1% formic acid by volume. In some aspects, the second mobile phase comprises about 80-95% acetonitrile by volume. In some aspects, the second mobile phase comprises about 90% acetonitrile by volume and about 10% water by volume.

[0028] In some aspects, the percentage of the second mobile phase, as compared to a combination of the first mobile phase and the second mobile phase, in the liquid chromatography is increased over time. In some aspects, the percentage of the second mobile phase is increased from about 2% to about 50%. In some aspects, the percentage of the second mobile phase is increased from about 2% to about 50% by volume over about 120 minutes. In some aspects, the percentage of the second mobile phase is subsequently increased to 100% by volume over about 25 minutes. In some aspects, the percentage of the second mobile phase is subsequently maintained at 100% by volume for about one minute. In some aspects, the second mobile phase is subsequently decreased to about 2% by volume over about 4 minutes. In some aspects, the percentage of the second mobile phase is subsequently increased to 100% by volume over about 5 minutes. In some aspects, the percentage of the second mobile phase is subsequently maintained at 100% by volume for about 3 minutes. In some aspects, the second mobile phase is subsequently decreased to about 2% by volume over about 2 minutes.

[0029] In some aspects, the mass spectrometry is performed using a fragmentor voltage of about 125-350 V. In some aspects, the mass spectrometry is performed using a fragmentor voltage of about 135V. In some aspects, the mass spectrometry is performed using a capillary voltage of about 3-6 kV. In some aspects, the mass spectrometry is performed using a capillary voltage of about 4 kV.

[0030] Some aspects of the disclosure are directed to a recombinant AAV (rAAV) comprising a heterogeneous group of capsid proteins that contain a subpopulation with an amino acid modification. In some aspects, the modification is deamidation or oxidation.

[0031] In some aspects, the heterogeneous group comprises a deamidated asparagine (N) at one or more of N57, N255, N256, and N263 of AAV. rh74 or the equivalent residues of AAV1, AAV2, AAV3, AAV4, AAVS, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, AAV 13, or AAVrh10, as measured by mass spectrometry and/or ultraviolet (UV)-visible spectroscopy. In another embodiment, the heterogeneous group comprises a deamidated asparagine (N) within a peptide sequence of any one of SEQ ID Nos: 1-5, or its equivalent peptide sequence of other AAV serotype.

[0032] In some aspects, the heterogeneous group comprises less than 70%, 60%, 50%, 40%, 30%, 20%, 15%, 10% or 5% of capsid proteins with deamidation at N57 of AAV. rh74 capsid. In some aspects, the heterogeneous group comprises less than 15% of capsid proteins with deamidation at N57 of AAV. rh74 capsid. In some aspects, the heterogeneous group comprises less than 70%, 60%, 50%, 40%, 30%, 20%, 15%, 10%, or 5% of capsid proteins with deamidation at N254 and/or N255 of AAV. rh74 capsid. In some aspects, the heterogeneous group comprises less than 70%, 60%, 50%, 40%, 30%, 20%, 10% or 5% of capsid proteins with deamidation at N263.

[0033] In some aspects, the heterogeneous group comprises an oxidized methionine at one or more of M437, M473, M526, M544, M560, and M637 of AAV. Rh74 or the equivalent residues of AAV1, AAV2, AAV3, AAV4, AAVS, AAV6, AAV7, AAV8, AAV9, AAV10, CAAV11, AAV12, AAV 13, or AAVrh10, as measured by mass spectrometry and/or ultraviolet (UV)-visible spectroscopy. In some aspects, the heterogeneous group comprises less than 30%, 20%, 10%, 5%, or 1% of capsid proteins with oxidation at M437. In some aspects, the heterogeneous group comprises less than 30%, 20%, 10%, 5%, or 1% of capsid proteins with oxidation at M473. In some aspects, the heterogeneous group comprises less than 30%, 20%, 10%, 5%, or 3% of capsid proteins with oxidation at M526. In some aspects, the heterogeneous group comprises less than 30%, 20%, 10%, 5%, or 2% of capsid proteins with oxidation at M544. In some aspects, the heterogeneous group comprises less than 30%, 20%, 10%, 5%, or 2% of capsid proteins with oxidation at M560. In some aspects, the heterogeneous group comprises less than 30%, 20%, 10%, 5%, or 1% of capsid proteins with oxidation at M637.

[0034] Those skilled in the art will be aware that the invention described herein is subject to variations and modifications other than those specifically described. It is to be understood that the invention described herein includes all such variations and modifications. The invention also includes all such steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of the steps or features.

BRIEF DESCRIPTION OF THE FIGURES

[0035] The following figures form part of the present specification and are included to further illustrate aspects of the present invention.

[0036] FIG. **1** depicts a UV chromatogram for the AAVrh74 capsid proteins, with integrations confirming the capsid protein ratio.

[0037] FIG. **2** depicts a total ion chromatogram for the AAVrh74 capsid proteins.

[0038] FIG. **3** shows deconvoluted MS spectra for VP1 (FIG. **3**A), VP2 (FIG. **3**B) and VP3 (FIG. **3**C) capsid proteins respectively, confirming the intact masses of all three capsid proteins and detection of post-translation modifications of the capsid proteins. FIG. **3**D shows the deconvoluted MS spectra for VP1 in multiple samples. FIG. **3**E shows the deconvoluted MS spectra for VP2 in multiple samples. FIG. **3**F shows the deconvoluted MS spectra for VP3 in multiple samples.

[0039] FIG. **4** shows the process of deamidation analysis with Tris-HCl as the buffer.

[0040] FIG. **5** shows the deamidation results for AAV.rh74 with Tris-HCl buffer.

[0041] FIG. **6** shows the oxidation results for AAV.rh74 with Tris-HCl buffer.

DETAILED DESCRIPTION

[0042] Provided are methods to characterize the VP1, VP2 and VP3 capsid proteins in an adeno-associated virus (AAV) particle using liquid chromatography, mass spectrometry, or ultraviolet (UV)-visible spectroscopy. In some aspects, methods are provided for determining the ratio of VP1, VP2 and VP3 capsid proteins in AAV particle, and/or the masses of one or more of the VP1, VP2 and VP3 capsid proteins. The disclosure also provides methods to characterize the purity of rAAV compositions using liquid chromatography and mass spectrometry.

[0043] Definitions

[0044] For convenience, before further description of the present invention, certain terms used in the specification, examples and appended claims are collected here. These definitions should be read in light of the remainder of the disclosure and understood as by a person of skill in the art. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by a person of ordinary skill in the art. The terms used throughout this specification are defined as follows, unless otherwise limited in specific instances.

[0045] The articles "a," "an" and "the" are used to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article.

[0046] As used herein, "about" is used to indicate that a value includes the inherent variation of error for the device, the method being employed to determine the value, or the variation that exists among the study subjects. In some aspects, "about" indicates that deviations of 5% to 10% above (e.g., up to 5% to 10% above) and 5% to 10% below (e.g., up to 5% to 10% below) a given value or range remain within the intended meaning of the recited value or range.

[0047] The term "AAV" or "adeno-associated virus" refers to a Dependoparvovirus within the

[0048] Parvoviridae genus of viruses. Herein, AAV can refer to a wild-type virus, or an AAV derived from a naturally occurring wild-type virus, e.g., an AAV derived from a rAAV genome packaged into a capsid derived from capsid proteins encoded by a naturally occurring cap gene and/or a rAAV genome packaged into a capsid derived from capsid proteins encoded by a non-natural capsid cap gene, for example, AAVrh.74.

[0049] The AAV can be any serotype, for example AAV1, AAV2, AAV3, AAV4, AAV5, AAV6,

[0050] AAV7, AAV8, AAV9, AAV-10, AAV-11, AAV-12, AAV-13, AAV rh.10, AAV rh.74, or variants and derivatives thereof In some aspects, the rAAV is of the serotype AAVrh. 74. Production of pseudotyped rAAV is disclosed in, for example, WO 01/83692, which is incorporated by reference in its entirety. Other types of rAAV variants, for example rAAV with capsid mutations, are also contemplated. See, for example, Marsic et al., Molecular Therapy, 22(11): 1900-1909 (2014).

[0051] As used herein, the term "AAV particle," "AAV vector," "AAV virion," "AAV viral particle," or "AAV vector particle" is used to refer to a viral particle composed of an AAV capsid and an encapsidated AAV genome. The AAV particle, in some aspects, comprises a heterologous

polynucleotide (i.e. a polynucleotide other than a wild-type AAV genome such as a transgene to be delivered to a mammalian cell). Production of AAV viral particles, in some aspects, includes production of AAV vector, as such a vector is contained within an AAV vector particle.

[0052] For example, a wild-type (wt) AAV virus particle comprising a linear, single-stranded

[0053] AAV nucleic acid genome associated with an AAV capsid protein coat. The AAV virion can be either a single-stranded (ss) AAV or self-complementary (SC) AAV. In some aspects, a single-stranded AAV nucleic acid molecules of either complementary sense, e.g., "sense" or "antisense" strands, can be packaged into an AAV virion and both strands are equally infectious.

[0054] The term "recombinant AAV," or "rAAV" is defined herein as an infectious, replication-defective virus composed of an AAV protein shell, encapsulating a heterologous nucleotide sequence of interest which is flanked on both sides by AAV ITRs. A rAAV, in some aspects, is produced in a suitable host cell which has an AAV vector, AAV helper functions and accessory functions introduced therein. In this manner, the host cell is capable of encoding AAV polypeptides that are required for packaging the AAV vector (containing a recombinant nucleotide sequence of interest) into infectious recombinant virion particles for subsequent gene delivery.

[0055] As used herein, the term "capsid protein" refers to a protein that forms the coat or shell of a virus. The term "AAV capsid protein" refers to the protein that forms the coat of an adeno-associated virus (AAV), which is composed of a total of 60 subunits; each subunit is an amino acid sequence, e.g., viral protein 1 (VP1), VP2 or VP3.

[0056] As used herein, the term "liquid chromatography (LC)" refers to a technique used to separate, identify, and quantitate components in a mixture. In column liquid chromatography, the liquid mobile phase passes through the column and components of the mobile phase interact with the solid stationary phase. The composition of the mobile phase can be changed during a separation run to alter the strengths of interactions of the compounds of interest. As the mobile phase continues to flow through the column, the eluent is typically collected in fractions while monitoring the concentrations of the compounds eluted from the column over time to produce an elution curve, or chromatogram.

[0057] As used herein, the term "stationary phase" refers to the substance that stays fixed in the column. The most commonly used stationary phase columns are carbon chain-bonded silica, phenyl-bonded silica, and cyano-bonded silica. In some aspects, the stationary phase may include a hydrophobic alkyl chain of a particular length, such as C4, C8, or C18. In some aspects, the reverse phase chromatography is a C8 reverse chromatography (e.g., reverse phase chromatography utilizing a C8 stationary phase).

[0058] As used herein, the term "mobile phase" refers to water, solvents, or mixtures of water and solvents that are used to elute compounds from columns. The most common mobile phase solvents include but are not limited to acetonitrile, methanol, tertrahydrofuran, ethanol, or isopropyl alcohol. In some aspects, two mobile phases are used. For example, a first mobile phase and a second mobile phase can be mixed in situ to obtain a solvent used for eluting materials from the column. In some aspects, the volume ratio of the second mobile phase to the first mobile phase is in a gradient that increases during the elution step.

[0059] As used herein, the term "mass spectrometry" or "MS" refers to an analytical technique that measures the mass-to-charge (m/z) ratio of ions to identify and quantify molecules in simple and complex mixtures. MS technology generally includes: (1) ionizing the compounds to form charged compounds; and (2) detecting the mass-to-charge ratio of the charged compounds and calculating the molecular weight. The compounds may be ionized and detected by any suitable means. A "mass spectrometer" generally includes an ionizer, a mass analyzer, and an ion detector. In general, one or more molecules of interest are ionized, and the ions are subsequently introduced into a mass spectrometric instrument where, due to a combination of magnetic and electric fields, the ions follow a path in space that is dependent upon mass ("m") and charge ("z"). In some mass spectrometry methods, ions may be separated from one another using time-of-flight (TOF), an orbitrap, a Fourier transform ion cyclotron resonance spectrometer, a quadrupole or an ion trap, for example, and then detected using an ion detector.

[0060] As used herein, the term "ultraviolet—visible spectroscopy," "ultraviolet—visible spectrophotometry," "UV—Vis," or "UV/Vis" refers to an absorption spectroscopy or a reflectance spectroscopy that is used to determine the optical properties (transmittance, reflectance and absorbance) of liquids and solids. In some aspects, the ultraviolet—visible spectroscopy is used to characterize the capsid proteins of AAV particles.

[0061] As used herein, the term "total ion chromatogram (TIC)" refers to a type of chromatogram created by summing up the intensities of all the mass spectral peaks belonging to the same scan.

[0062] As used herein, the term "AAVrh74" refers to an AAV particle having AAVrh74 VP1,

[0063] VP2 and VP3 capsid proteins or variants thereof. An exemplary AAVrh74 VP1 capsid protein sequence is set forth in SEQ ID NO:4 of U.S. Pat. No. 9,909,142, which is hereby incorporated by reference in its entirety. Exemplary variants of AAVrh74 VP1 capsid proteins are also set forth in U.S. Pat. No. 9,909,142.

[0064] As used herein, the term "subpopulation" of VP proteins refers to a group of VP proteins which has at least one defined characteristic in common and which consists of at least one group member to less than all members of the reference group, unless otherwise specified. For example, a "subpopulation" of VP1 proteins may be at least one VP1 protein and less than all VP1 proteins in an assembled AAV capsid, unless otherwise specified. A "subpopulation" of VP3 proteins may be one VP3 protein to less than all VP3 proteins in an assembled AAV capsid, unless otherwise specified. For example, VP1 proteins may be a subpopulation of VP proteins; VP2 proteins may be a separate subpopulation of VP proteins, and VP3 are yet a further subpopulation of VP proteins in an assembled AAV capsid. In another example, VP1, VP2 and VP3 proteins may contain subpopulations having different modifications, e.g., at least one, two, three or four highly deamidated asparagines, e.g., at asparagine—glycine pairs.

Characterization of AAV VP1, VP2, and VP3 Capsid Proteins

[0065] In some aspects, the present disclosure provides a method to characterize VP1, VP2 and VP3 capsid proteins in an adeno-associated virus (AAV) particle, which com-

prises subjecting the AAV particle to a liquid chromatography at about 70° C. to about 90° C., wherein the masses and ratio of VP1, VP2 and VP3 capsid proteins are determined by mass spectrometry and/or ultraviolet (UV)-visible spectroscopy. In some aspects, the masses and ratio of VP1, VP2 and VP3 capsid proteins are determined by mass spectrometry and ultraviolet (UV)-visible spectroscopy. In some aspects, the capsids on the AAV particle is denatured into the individual VP1, VP2 and VP3 proteins in the column of the liquid chromatography. In some aspects, the capsid proteins are separated by the liquid chromatography. In some aspects, the method includes (a) subjecting the AAV particle to liquid chromatography to separate the VP1, VP2 and VP3 capsid proteins; and (b) subjecting the separated VP1, VP2 and VP3 capsid proteins produced in step (a) to mass spectrometry and/or ultraviolet-visible spectroscopy to determine the relative amounts of the VP1, VP2 and VP3 capsid proteins, thereby determining the ratio of VP1, VP2 and VP3 capsid proteins in the AAV particle. In some aspects, the liquid chromatography is performed at about 70° C. to about 90° C. In some aspects, the liquid chromatography is performed at about 70° C., 74° C., 76° C., 78° C., 80° C., 82° C., 84° C., 86° C., 88° C., or 90° C. In some aspects, the liquid chromatography is performed at about 80° C.

[0066] In some aspects, the method further includes determining the masses of one or more of the VP1, VP2 and VP3 capsid proteins of the AAV particle.

[0067] In some aspects, the relative amounts of the VP1, VP2 and VP3 capsid proteins are determined by comparing the total ion chromatogram (TIC) of the VP1, VP2 and VP3 capsid proteins.

[0068] In some aspects, the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography. In some aspects, the liquid chromatography is reverse phase liquid chromatography.

[0069] In some aspects, the reverse phase chromatography is performed using a C18 column, a C8 column, or a C4 column. In some aspects, the liquid chromatography is performed using a C8 column.

[0070] In some aspects, the stationary phase of the reverse phase liquid chromatography is comprised within a chromatography column that is about 50-300 mm long and has an internal diameter of about 1-4.6 mm. In some aspects, the column is a BEH column. In some aspects, the column has an internal diameter of 1, 2.1, 3, or 4.6 mm. In some aspects, the column has a length of 50, 75, 100, 150, or 300 mm. In some aspects, the column size is 1 mm×50 mm, 2.1 mm×50 mm, 3 mm×50 mm , 4.6 mm×50 mm, 1 mm×75 mm, 2.1 mm×75 mm, 3 mm×75 mm , 4.6 mm×75 mm, 1 mm×100 mm, 2.1 mm×100 mm, 3 mm×100 mm , 4.6 mm×100 mm, 1 mm×150 mm, 2.1 mm×150 mm, 3 mm×150 mm, 4.6 mm×150 mm, 1 mm×300 mm, 2.1 mm×300 mm, 3 mm×300 mm, or 4.6 mm×300 mm. In some aspects, the column size is 1.6×50 mm, 1.6×60 mm, 1.6×70 mm, 1.6×80 mm, 1.6×90 mm, 1.6×100 mm, 1.6×110 mm, 1.6×120 mm, 1.6×130 mm, 1.6×140 mm, 1.6×150 mm, 1.7×50 mm, 1.7×60, 1.7×70 mm, 1.7×80 mm, 1.7×90 mm, 1.7×100 mm, 1.7×110 mm, 1.7×120 mm, 1.7×130 mm, 1.7×140 mm, 1.7×150 mm, 1.8×50 mm, 1.8×60, 1.8×70 mm, 1.8×80 mm, 1.8×90 mm, 1.8×100 mm, 1.8×110 mm, 1.8×120 mm, 1.8×130 mm, 1.8×140 mm, 1.8×150 mm, 1.9×50 mm, 1.9×60 mm, 1.9×70 mm, 1.9×80 mm, 1.9×90 mm, 1.9×100 mm, 1.9×110 mm, 1.9×120 mm, 1.9×130 mm, 1.9×140 mm, 1.9×150 mm, 2.0×50 mm, 2.0×60 mm, 2.0×70 mm, 2.0×80 mm, 2.0×90 mm, 2.0×100 mm, 2.0×110 mm, 2.0×120 mm, 2.0×130 mm, 2.0×140 mm, 2.0×150 mm, 2.1×50 mm, 2.1×60 mm, 2.1×70 mm, 2.1×80 mm, 2.1×90 mm, 2.1×100 mm, 2.1×110 mm, 2.1×120 mm, 2.1×130 mm, 2.1×140 mm, 2.1×150 mm, 2.2×50 mm, 2.2×60 mm, 2.2×70 mm, 2.2×80 mm, 2.2×90 mm, 2.2×100 mm, 2.2×110 mm, 2.2×120 mm, 2.2×130 mm, 2.2×140 mm, 2.2×150 mm, 2.3×50 mm, 2.3×60 mm, 2.3×70 mm, 2.3×80 mm, 2.3×90 mm, 2.3×100 mm, 2.3×110 mm, 2.3×120 mm, 2.3×130 mm, 2.3×140 mm, 2.3×150 mm, 2.4×50 mm, 2.4×60 mm, 2.4×70 mm, 2.4×80 mm, 2.4×90 mm, 2.4×100 mm, 2.4×110 mm, 2.4×120 mm, 2.4×130 mm, 2.4×140 mm, 2.4×150 mm, 2.5×50 mm, 2.5×60, 2.5×70 mm, 2.5×80 mm, 2.5×90 mm, 2.5×100 mm, 2.5×110 mm, 2.5×120 mm, 2.5×130 mm, 2.5×140 mm, 2.5×150 mm, 2.6×50 mm, 2.6×60 mm, 2.6×70 mm, 2.6×80 mm, 2.6×90 mm, 2.6×100 mm, 2.6×110 mm, 2.6×120 mm, 2.6×130 mm, 2.6×140 mm, or 2.6×150 mm. In some aspects, the stationary phase of the reverse phase liquid chromatography is comprised within a chromatography column that is about 100 mm long and has an internal diameter of about 2.1 mm.

[0071] In some aspects, the stationary phase of the reverse phase liquid chromatography comprises particles sized between about 1.2 μm-2.5 μm. In another aspects, the stationary phase of the reverse phase liquid chromatography comprises particles sized at about 1.7 μm, 1.8 μm or 2.1 μm. In some aspects, the particle size is about 1.2 μm, 1.3 μm, 1.4 μm, 1.5 μm, 1.6 μm, 1.7 μm, 1.8 μm, 1.9 μm, 2.0 μm, 2.1 μm, 2.2 μm, 2.3 μm, 2.4 μm, or 2.5 μm. In some aspects, the stationary phase of the reverse phase liquid chromatography is comprised of particles of about 1.7 μm.

[0072] In some aspects, the chromatography uses a first mobile phase including fluoro-substituted acetic acid in water. The fluoro-substituted acetic acids include monofluoroacetic acid, difluoroacetic acid, and trifluoroacetic acid. In some aspects, the chromatography uses a first mobile phase including trifluoroacetic acid in water.

[0073] In some aspects, the first mobile phase includes from about 0.05 to about 0.15% of fluoro-substituted acetic acid by volume. In some aspects, the first mobile phase comprises about 0.05%, 0.06%, 0.07%, 0.08%, 0.09%, 0.1%, 0.11%, 0.12%, 0.13%, 0.14%, 0.15%, 0.16%, 0.17%, 0.18%, 0.19%, or 0.2% of fluoro-substituted acetic acid by volume. In some aspects, the first mobile phase comprises about 0.05% or 0.1% of fluoro-substituted acetic acid by volume. In some aspects, the first mobile phase includes about 0.1% of fluoro-substituted acetic acid by volume. In some aspects, the fluoro-substituted acetic acid is trifluoroacetic acid. In some aspects, the first mobile phase includes about 0.1% of trifluoroacetic acid by volume.

[0074] In some aspects, the chromatography uses a second mobile phase including fluoro-substituted acetic acid in acetonitrile. In some aspects, the chromatography uses a second mobile phase including trifluoroacetic acid in acetonitrile. In some aspects, the chromatography uses a second mobile phase including fluoro-substituted acetic acid in the mixture of acetonitrile and water. In some aspects, the chromatography uses a second mobile phase including trifluoroacetic acid in the mixture of acetonitrile and water.

[0075] In some aspects, the second mobile phase includes about 0.05-0.2% of fluoro-substituted acetic acid by volume. In some aspects, the second mobile phase includes about 0.05-0.15% of fluoro-substituted acetic acid by volume. In

some aspects, the second mobile phase includes about 0.05%, 0.06%, 0.07%, 0.08%, 0.09%, 0.1%, 0.11%, 0.12%, 0.13%, 0.14%, 0.15%, 0.16%, 0.17%, 0.18%, 0.19%, or 0.2% of fluoro-substituted acetic acid by volume. In some aspects, the second mobile phase comprises about 0.05% or 0.1% of fluoro-substituted acetic acid by volume. In some aspects, the second mobile phase includes about 0.1% fluoro-substituted acetic acid. In some aspects, the fluoro-substituted acetic acid is trifluoroacetic acid. In some aspects, the second mobile phase includes about 0.1% trifluoroacetic acid.

[0076] In some aspects, the second mobile phase includes about 75-95% of acetonitrile by volume.

[0077] In some aspects, the second mobile phase includes about 75%, 80%, 85%, 90%, or 95% of acetonitrile by volume. In some aspects, the second mobile phase includes about 90% acetonitrile and 10% water by volume.

[0078] In some aspects, the percentage of the second mobile phase, in the combination of the first mobile phase and the second mobile phase, in the chromatography is increased over time. In some aspects, the percentage of the second mobile phase is increased from about 10% to about 40% by volume. In some aspects, the percentage of the second mobile phase is increased from about 10% to about 45% by volume. In some aspects, the percentage of the second mobile phase is increased from about 10% to about 100% by volume. In some aspects, the percentage of the second mobile phase is increased from about 10% to about 45% in about 30-40 minutes. In some aspects, the percentage of the second mobile phase is increased from about 10% to about 45% in about 35 minutes. In some aspects, the percentage of the second mobile phase is increased from about 10% to about 100% by volume in about 30-50 minutes. In some aspects, the percentage of the second mobile phase is increased from about 10% to about 100% by volume in about 36 minutes.

[0079] In some aspects, the percentage of the second mobile phase is increased from about 10% to about 40% by volume in about 5-10 minutes, from about 40% to about 45% in about 25-35 minutes. In some aspects, the percentage of the second mobile phase is increased from about 45% to about 100% in about 0.5-2 minutes. In some aspects, the percentage of the second mobile phase is decreased from about 100% to about 10% in about 0.5-2 minutes.

[0080] In some aspects, the percentage of the second mobile phase is increased from about 10% to about 40% by volume in about 6 minutes, from about 40% to about 45% in about 29 minutes. In some aspects, the percentage of the second mobile phase is increased from about 45% to about 100% in about 1 minute. In some aspects, the percentage of the second mobile phase is decreased from about 100% to about 10% in about 1 minute.

[0081] In some aspects, the liquid chromatography is high-pressure liquid chromatography

[0082] (HPLC). In some aspects, the liquid chromatography is ultra-high pressure liquid chromatography (UHPLC).

[0083] In some aspects, the mass spectrometry may use any ionization modes, particularly those modes suitable for analyzing biological molecules including, but not limited to, direct infusion-mass spectrometry, electrospray ionization (ESI)-MS, desorption electrospray ionization (DESI)-MS, direct analysis in real-time (DART)-MS, atmospheric pressure chemical ionization (APCI)-MS, electron impact (EI) or chemical ionization (CI), matrix-assisted laser desorption/ionization (MALDI)-MS, and Atmospheric Pressure Ionization-Electrospray (API-ES). In some aspects, the mass spectrometry uses API-ES ionization mode.

[0084] In some aspects, the mass spectrometry scans signals over a range of 400-16000 m/z. In some aspects, the mass spectrometry scans signals over a range of 700-13700 m/z.

[0085] In some aspects, the scan type of the mass spectrometry is positive polarity. In some aspects, the data acquisition time of the mass spectrometry is about 10-35 minutes. In some aspects, the data acquisition time of the mass spectrometry is about 17-28 minutes.

[0086] In some aspects, the nozzle voltage of the mass spectrometry is about 400-600 V. In some aspects, the nozzle voltage of the mass spectrometry is about 500 V. In some aspects, the skimmer voltage of the mass spectrometry is about 60-70 V. In some aspects, the skimmer voltage of the mass spectrometry is about 65 V. In some aspects, the difference between the nozzle and skimmer voltage is about 400-450 V. In some aspects, the difference between the nozzle and skimmer voltage is about 435 V.

[0087] In some aspects, the drying gas temperature of the mass spectrometry is about 200-350° C.

[0088] In some aspects, the drying gas temperature of the mass spectrometry is about 300° C. In some aspects, the drying gas flow rate of the mass spectrometry is about 5-13 L/min. In some aspects, the drying gas flow rate of the mass spectrometry is about 13 L/min.

[0089] In some aspects, the mass spectrometry uses a capillary voltage of about 3-6 kV. In some aspects, the mass spectrometry uses a capillary voltage of about 3, 4, 5, or 6 kV. In some aspects, the mass spectrometry uses a capillary voltage of about 5 kV.

[0090] In some aspects, the mass spectrometry uses a fragmentor voltage of about 125-350 V. In some aspects, the mass spectrometry uses a fragmentor voltage of about 125, 130, 135, 145, 155, 160, 165, 175, 185, 190, 195, 200, 205, 210, 215, 220, 225, 230, 235, 240, 245, 250, 255, 260, 265, 270, 275, 280, 285, 290, 295, 300, 305, 310, 315, 320, 325, 330, 335, 340, 345, or 350V. In some aspects, the mass spectrometry uses a fragmentor voltage of about 175 V.

[0091] In some aspects, the AAV particle is AAV1, AAV2, AAV3, AAV4, AAVS, AAV6, AAV7,

[0092] AAV8, AAV9, AAV10, AAV11, AAV12, AAV13, AAVrh10, AAVrh74 or any naturally occurring, recombinant, or synthetic AAV particles. In some aspects, the AAV particle is a recombinant AAV (rAAV) particle. In some aspects, the AAV particle is AAVrh74.

[0093] In some above aspects, the disclosure further includes determining post translational modification of at least one of VP1, VP2 and VP3 capsid proteins. In some aspects, the disclosure further includes determining post translational glycosylation, sialylation, acetylation, loss of amino acid, amidation, phosphorylation, formylation, hydroxylation, methylation, and/or sulfation of at least one of VP1, VP2 and VP3 capsid proteins. The post translational modification comprises one or more of loss of N-terminal methionine, loss of threonine, phosphorylation, and acetylation.

[0094] In some aspects, the disclosure includes determining removal of N-terminal methionine in

[0095] VP1, VP2, or VP3 capsid proteins. In some aspects, the disclosure includes determining removal of N-terminal methionine in VP1 or VP3 capsid proteins. In some aspects,

the disclosure includes determining N-terminal acetylation after removal of N-terminal methionine in VP1, VP2, or VP3 capsid proteins. In some aspects, the disclosure includes determining N-terminal acetylation after removal of N-terminal methionine in VP1 or VP3 capsid proteins.

[0096] In some aspects, the disclosure provides a method to characterize capsid proteins of an AAV particle at least based in part on the ratio of VP1, VP2 and VP3 capsid proteins and/or the masses of one or more of the VP1, VP2 and VP3 capsid proteins in an AAV particle.

[0097] In some aspects, the disclosure provides a method to determine the serotype of an AAV particle at least based in part on the ratio of VP1, VP2 and VP3 capsid proteins in an AAV particle and/or the masses of one or more of the VP1, VP2 and VP3 capsid proteins, wherein the ratio of VP1, VP2 and VP3 capsid proteins and the masses of one or more of the VP1, VP2 and VP3 capsid proteins are determined by the methods disclosed herein.

[0098] Mass Spectrometry is an analytical technique for protein characterization. In some aspects, a method for the characterization of the AAVrh74 capsid protein ratio along with the intact mass for all three capsid proteins by liquid chromatography and mass spectrometry is provided. In some aspects, the AAVrh74 capsid is denatured on-column into the individual capsid proteins VP1, VP2, and VP3. The denaturation is achieved by heating the column compartment to 80° C. (3,4). The capsid proteins are then baseline resolved on a Waters BEH C8 column with the help of trifluoroacetic acid, as an ion-pairing agent in the mobile phases (5). The denatured proteins are first analyzed in the UV to achieve the capsid ratio and then in the mass spectrometer to obtain the intact mass for the individual proteins.

[0099] Deamidation is a common Post-Translational Modification resulting in the conversion of an asparagine residue to a mixture of isoaspartate and aspartate. Deamidation of glutamine residues also occurs, but at a much slower rate. Oxidation is also a common Post-Translational Modification which a result of the reaction of proteins with a variety of free radicals and reactive oxygen species. Methionine Oxidation is most common, however oxidation of several other amino acid residues like cysteine and tryptophan have also been observed. Deamidation/Oxidation are also common degradation pathways for proteins occurring during manufacturing and storage. Deamidation can have an impact on the activity and stability of proteins. Oxidation can cause conformational changes in proteins and therefore impact protein activity and stability. Oxidation can also impact the immunogenicity of proteins. Therefore, Critical Quality Attributes (CQAs) for proteins needs to be monitored carefully on the post-translation modifications.

[0100] The current method with ammonium bicarbonate generated false signals or overestimated the deamidation in AAV capsid protein (Table 7). Here, the disclosure provides a method to more accurate measure post-translation modification on the capsid proteins with Tris-HCl. In some aspects, the LC MS method uses a buffer comprising Tris-HCl. In some aspects, the buffer comprises acetonitrile. In some aspects, the buffer comprises methionine. In some aspects, the buffer comprises Tris-HCl at 5mM to 50 mM, 5%-20% acetonitrile, and methionine at 1 mM to 50 mM. In some aspects, the buffer comprises Tris-HCl at 20 mM, 5%-10% acetonitrile, and methionine at 10 mM.

[0101] In some aspects, the post-translation modification comprises deamidation at one or more of N263, N514, N57, N502, N254, and N94 of AAV8 or its equivalent residue at AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV9, AAV10, AAV11, AAV12, AAV 13, AAVrh10, or AAVrh74. In some aspects, the post-translation modification comprises deamidation at one or more of N57, N255, N256, and N263 of AAV. Rh74. In some aspects, the post-translation modification comprises oxidation at one or more of M437, M473, M526, M544, M560, and M637 of AAV. Rh74.

[0102] AAV Compositions

[0103] Some aspects of the disclosure are directed to a recombinant AAV (rAAV) comprising a heterogeneous group of capsid proteins that contain a subpopulation with an amino acid modification. In some aspects, the modification can be deamidation, acetylation, isomerization, phosphorylation, or oxidation. In some aspects, the modification is deamidation or oxidation.

[0104] In some aspects, the rAAV capsid can contain subpopulations of VP1, VP2 and VP3 having at least 1, at least 2, at least 3, at least 4, at least 5 to at least about 25 deamidated amino acid residues, of which at least about 1% to about 10%, at least about 10% to about 25%, at least about 25% to about 50%, at least about 50% to about 70%, at least about 70% to about 100%, at least about 75% to about 100%, at least about 80% to about 100% or at least about 90% to about 100% are deamidated as compared to the encoded amino acid sequence of the VP proteins. In some aspects, the majority of these can be N residues. In some aspects, Q residues can be deamidated.

[0105] In some aspects, the disclosure provides an AAV composition comprising an AAV capsid that comprises deamidation at one or more of N57, N255, N256, and N263 of AAV. Rh74, or the equivalent residues of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, AAV 13, or AAVrh10 as measured by mass spectrometry and/or ultraviolet (UV)-visible spectroscopy. In some aspects, the deamidation is measured by any of the methods disclosed herein.

[0106] In some aspects, the heterogeneous group comprises less than about 80%, less than about 78%, less than about 76%, less than about 74%, less than about 72%, less than about 70%, less than about 68%, less than about 66%, less than about 64%, less than about 62%, less than about 60%, less than about 58%, less than about 56%, less than about 54%, less than about 52%, less than about 50%, less than about 48%, less than about 46%, less than about 44%, less than about 42%, less than about 40%, less than about 38%, less than about 36%, less than about 34%, less than about 32%, less than about 30%, less than about 28%, less than about 26%, less than about 24%, less than about 22%, less than about 20%, less than about 18%, less than about 16%, less than about 15%, less than about 14%, less than about 13%, less than about 12%, less than about 11%, or less than about 10% of capsid proteins with deamidation at N57 of AAV. rh74 capsid.

[0107] In some aspects, wherein the heterogeneous group comprises less than about 25%, less than about 24%, less than about 23%, less than about 22%, less than about 21%, less than about 20%, less than about 19%, less than about 18%, less than about 17%, less than about 16%, less than about 15%, less than about 14%, less than about 13%, less

than about 12%, less than about 11%, or less than about 10% of capsid proteins with deamidation at N57 of AAV. rh74 capsid.

[0108] In some aspects, the heterogeneous group comprises less than about 80%, less than about 78%, less than about 76%, less than about 74%, less than about 72%, less than about 70%, less than about 68%, less than about 66%, less than about 64%, less than about 62%, less than about 60%, less than about 58%, less than about 56%, less than about 54%, less than about 52%, less than about 50%, less than about 48%, less than about 46%, less than about 44%, less than about 42%, less than about 40%, less than about 38%, less than about 36%, less than about 34%, less than about 32%, less than about 30%, less than about 28%, less than about 26%, less than about 24%, less than about 22%, less than about 20%, less than about 18%, less than about 16%, less than about 15%, less than about 14%, less than about 13%, less than about 12%, less than about 11%, or less than about 10%, less than about 8%, less than about 7%, less than about 6%, less than about 5%, less than about 4%, less than about 3%, less than about 2%, or less than about 1% of capsid proteins with deamidation at N254 and/or N255 of AAV. rh74 capsid.

[0109] In some aspects, the heterogeneous group comprises less than about 80%, less than about 78%, less than about 76%, less than about 74%, less than about 72%, less than about 70%, less than about 68%, less than about 66%, less than about 64%, less than about 62%, less than about 60%, less than about 58%, less than about 56%, less than about 54%, less than about 52%, less than about 50%, less than about 48%, less than about 46%, less than about 44%, less than about 42%, less than about 40%, less than about 38%, less than about 36%, less than about 34%, less than about 32%, less than about 30%, less than about 28%, less than about 26%, less than about 24%, less than about 22%, less than about 20%, less than about 18%, less than about 16%, less than about 15%, less than about 14%, less than about 13%, less than about 12%, less than about 11%, or less than about 10% of capsid proteins with deamidation at N263.

[0110] In some aspects, the AAV composition comprises an AAV capsid that comprises oxidation at one or more of M437, M473, M526, M544, M560, and M637 of AAV. Rh74 or the equivalent residues of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, AAV 13, or AAVrh10 as measured by mass spectrometry and/or ultraviolet (UV)-visible spectroscopy. In some aspects, the deamidation is measured by any of the methods disclosed herein.

[0111] In some aspects, the heterogeneous group comprises less than about 40%, less than about 48%, less than about 46%, less than about 44%, less than about 42%, less than about 40%, less than about 38%, less than about 36%, less than about 34%, less than about 32%, less than about 30%, less than about 28%, less than about 26%, less than about 24%, less than about 22%, less than about 20%, less than about 18%, less than about 16%, less than about 14%, less than about 12%, less than about 10%, less than about 9%, less than about 8%, less than about 7%, less than about 6%, less than about 5%, less than about 4%, less than about 3%, less than about 2%, less than about 1%, less than about 0.9%, less than about 0.8%, less than about 0.7%, less than about 0.6%, or less than 0.5% of capsid proteins with oxidation at M437.

[0112] In some aspects, the heterogeneous group comprises less than about 40%, less than about 48%, less than about 46%, less than about 44%, less than about 42%, less than about 40%, less than about 38%, less than about 36%, less than about 34%, less than about 32%, less than about 30%, less than about 28%, less than about 26%, less than about 24%, less than about 22%, less than about 20%, less than about 18%, less than about 16%, less than about 14%, less than about 12%, less than about 10%, less than about 9%, less than about 8%, less than about 7%, less than about 6%, less than about 5%, less than about 4%, less than about 3%, less than about 2%, less than about 1%, less than about 0.9%, less than about 0.8%, less than about 0.7%, less than about 0.6%, or less than 0.5% of capsid proteins with oxidation at M473.

[0113] In some aspects, the heterogeneous group comprises less than about 40%, less than about 48%, less than about 46%, less than about 44%, less than about 42%, less than about 40%, less than about 38%, less than about 36%, less than about 34%, less than about 32%, less than about 30%, less than about 28%, less than about 26%, less than about 24%, less than about 22%, less than about 20%, less than about 18%, less than about 16%, less than about 14%, less than about 12%, less than about 10%, less than about 9%, less than about 8%, less than about 7%, less than about 6%, less than about 5%, less than about 4%, less than about 3%, less than about 2%, less than about 1%, less than about 0.9%, less than about 0.8%, less than about 0.7%, less than about 0.6%, or less than 0.5% of capsid proteins with oxidation at M526.

[0114] In some aspects, the heterogeneous group comprises less than about 40%, less than about 48%, less than about 46%, less than about 44%, less than about 42%, less than about 40%, less than about 38%, less than about 36%, less than about 34%, less than about 32%, less than about 30%, less than about 28%, less than about 26%, less than about 24%, less than about 22%, less than about 20%, less than about 18%, less than about 16%, less than about 14%, less than about 12%, less than about 10%, less than about 9%, less than about 8%, less than about 7%, less than about 6%, less than about 5%, less than about 4%, less than about 3%, less than about 2%, less than about 1%, less than about 0.9%, less than about 0.8%, less than about 0.7%, less than about 0.6%, or less than 0.5% of capsid proteins with oxidation at M544.

[0115] In some aspects, the heterogeneous group comprises less than about 40%, less than about 48%, less than about 46%, less than about 44%, less than about 42%, less than about 40%, less than about 38%, less than about 36%, less than about 34%, less than about 32%, less than about 30%, less than about 28%, less than about 26%, less than about 24%, less than about 22%, less than about 20%, less than about 18%, less than about 16%, less than about 14%, less than about 12%, less than about 10%, less than about 9%, less than about 8%, less than about 7%, less than about 6%, less than about 5%, less than about 4%, less than about 3%, less than about 2%, less than about 1%, less than about 0.9%, less than about 0.8%, less than about 0.7%, less than about 0.6%, or less than 0.5% of capsid proteins with oxidation at M560.

[0116] In some aspects, the heterogeneous group comprises less than about 40%, less than about 48%, less than about 46%, less than about 44%, less than about 42%, less than about 40%, less than about 38%, less than about 36%,

less than about 34%, less than about 32%, less than about 30%, less than about 28%, less than about 26%, less than about 24%, less than about 22%, less than about 20%, less than about 18%, less than about 16%, less than about 14%, less than about 12%, less than about 10%, less than about 9%, less than about 8%, less than about 7%, less than about 6%, less than about 5%, less than about 4%, less than about 3%, less than about 2%, less than about 1%, less than about 0.9%, less than about 0.8%, less than about 0.7%, less than about 0.6%, or less than 0.5% of capsid proteins with oxidation at M637.

[0117] Characterization of Host Cell Proteins in an AAV Composition

[0118] In some aspects, the disclosure provides a method of characterizing host cell proteins in an AAV composition, such as an AAV based gene therapy drug product. In some aspects, the method of characterizing host cell protein sin an AAV composition comprises immunoprecipitating viral capsid proteins from the compositions, digesting residual host cell proteins, and analyzing the digested proteins with liquid chromatography quadrupole time of flight mass spectrometry (LC-QTOF-MS) to identify host cell proteins. As used herein, the term "residual host cell proteins" or "residual proteins" means the protein remaining in solution after immunoprecipitation. In some aspects, the method further comprises analyzing the digested host cell proteins with iterative MS/MS.

[0119] In some aspects, immunoprecipitation comprises incubating the AAV composition with an

[0120] VP antibody. In some aspects, the VP antibody comprises an anti-AAV VP1 antibody, an anti-AAV VP2 antibody, an anti-AAV VP3 antibody, or combinations thereof. In some aspects, the antibody can be anti-Adeno-associated virus (AAV), VP1/VP2/VP3 from American Research Products, Inc. (Catalog #:03-61058)

[0121] In some aspects, the residual host cell proteins are digested in solution. In some aspects, the digestion is rapid digestion. In some aspects, rapid digestion is performed at about 60° C. to about 80° C. In some aspects, rapid digestion is performed at about 60° C., about 61° C., about 62° C., about 63° C., about 64° C., about 65° C., about 66° C., about 67° C., about 68° C., about 69° C., about 70° C., about 71° C., about 72° C., about 73° C., about 74° C., about 75° C., about 76° C., about 77° C., about 78° C., about 79° C., or about 80° C. In some aspects, the rapid digestion is performed at about 70° C.

[0122] In some aspects, the AAV composition is spiked with a known amount of at least one known protein standard. In some aspects, the at least one known protein standard is a human or bovine protein standard. In some aspects, the method further comprises quantifying the amount of the residual host cell proteins relative to the at least one known protein standard.

[0123] In some aspects, the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography. In some aspects, the liquid chromatography is reverse phase liquid chromatography.

[0124] In some aspects, the liquid chromatography is performed at about 35° C. to about 55° C. In some aspects, the liquid chromatography is performed at about 35° C., about 36° C., about 37° C., about 38° C., about 39° C., about 40° C., about 41° C., about 42° C., about 43° C., about 44° C., about 45° C., about 46° C., about 47° C., about 48° C., about 49° C., about 50° C., about 51° C., about 52° C., about 53° C., about 54° C., or about 55° C. In some aspects, the liquid chromatography is performed at about 45° C.

[0125] In some aspects, the reverse phase chromatography is performed using a C18 column, a C8 column, or a C4 column. In some aspects, the liquid chromatography is performed using a C8 column.

[0126] In some aspects, the stationary phase of the reverse phase liquid chromatography is comprised within a chromatography column that is about 50-300 mm long and has an internal diameter of about 1-4.6 mm. In some aspects, the column is a BEH column. In some aspects, the column has an internal diameter of 1, 2.1, 3, or 4.6 mm. In some aspects, the column has a length of 50, 75, 100, 150, or 300 mm. In some aspects, the column size is 1 mm×50 mm, 2.1 mm×50 mm, 3 mm×50 mm , 4.6 mm×50 mm, 1 mm×75 mm, 2.1 mm×75 mm, 3 mm×75 mm , 4.6 mm×75 mm, 1 mm×100 mm, 2.1 mm×100 mm, 3 mm×100 mm , 4.6 mm×100 mm, 1 mm×150 mm, 2.1 mm×150 mm, 3 mm×150 mm, 4.6 mm×150 mm, 1 mm×300 mm, 2.1 mm×300 mm, 3 mm×300 mm, or 4.6 mm×300 mm. In some aspects, the column size is 1.6×50 mm, 1.6×60 mm, 1.6×70 mm, 1.6×80 mm, 1.6×90 mm, 1.6×100 mm, 1.6×110 mm, 1.6×120 mm, 1.6×130 mm, 1.6×140 mm, 1.6×150 mm, 1.7×50 mm, 1.7×60, 1.7×70 mm, 1.7×80 mm, 1.7×90 mm, 1.7×100 mm, 1.7×110 mm, 1.7×120 mm, 1.7×130 mm, 1.7×140 mm, 1.7×150 mm, 1.8×50 mm, 1.8×60, 1.8×70 mm, 1.8×80 mm, 1.8×90 mm, 1.8×100 mm, 1.8×110 mm, 1.8×120 mm, 1.8×130 mm, 1.8×140 mm, 1.8×150 mm, 1.9×50 mm, 1.9×60 mm, 1.9×70 mm, 1.9×80 mm, 1.9×90 mm, 1.9×100 mm, 1.9×110 mm, 1.9×120 mm, 1.9×130 mm, 1.9×140 mm, 1.9×150 mm, 2.0×50 mm, 2.0×60 mm, 2.0×70 mm, 2.0×80 mm, 2.0×90 mm, 2.0×100 mm, 2.0×110 mm, 2.0×120 mm, 2.0×130 mm, 2.0×140 mm, 2.0×150 mm, 2.1×50 mm, 2.1×60 mm, 2.1×70 mm, 2.1×80 mm, 2.1×90 mm, 2.1×100 mm, 2.1×110 mm, 2.1×120 mm, 2.1×130 mm, 2.1×140 mm, 2.1×150 mm, 2.2×50 mm, 2.2×60 mm, 2.2×70 mm, 2.2×80 mm, 2.2×90 mm, 2.2×100 mm, 2.2×110 mm, 2.2×120 mm, 2.2×130 mm, 2.2×140 mm, 2.2×150 mm, 2.3×50 mm, 2.3×60 mm, 2.3×70 mm, 2.3×80 mm, 2.3×90 mm, 2.3×100 mm, 2.3×110 mm, 2.3×120 mm, 2.3×130 mm, 2.3×140 mm, 2.3×150 mm, 2.4×50 mm, 2.4×60 mm, 2.4×70 mm, 2.4×80 mm, 2.4×90 mm, 2.4×100 mm, 2.4×110 mm, 2.4×120 mm, 2.4×130 mm, 2.4×140 mm, 2.4×150 mm, 2.5×50 mm, 2.5×60, 2.5×70 mm, 2.5×80 mm, 2.5×90 mm, 2.5×100 mm, 2.5×110 mm, 2.5×120 mm, 2.5×130 mm, 2.5×140 mm, 2.5×150 mm, 2.6×50 mm, 2.6×60 mm, 2.6×70 mm, 2.6×80 mm, 2.6×90 mm, 2.6×100 mm, 2.6×110 mm, 2.6×120 mm, 2.6×130 mm, 2.6×140 mm, or 2.6×150 mm. In some aspects, the stationary phase of the reverse phase liquid chromatography is comprised within a chromatography column that is about 150 mm long and has an internal diameter of about 2.1 mm.

[0127] In some aspects, the stationary phase of the reverse phase liquid chromatography comprises particles sized between about 1.2 μm-2.5 μm. In some aspects, the stationary phase of the reverse phase liquid chromatography comprises particles sized at about 1.7 μm, 1.8 μm or 2.1 μm. In some aspects, the particle size is about 1.2 μm, 1.3 μm, 1.4 μm, 1.5 μm, 1.6 μm, 1.7 μm, 1.8 μm, 1.9 μm, 2.0 μm, 2.1 μm, 2.2 μm, 2.3 μm, 2.4 μm, or 2.5 μm. In some aspects, the stationary phase of the reverse phase liquid chromatography is comprised of particles of about 1.7 μm.

[0128] In some aspects, the chromatography uses a first mobile phase including fluoro-substituted acetic acid in water. The fluoro-substituted acetic acids include monofluoroacetic acid, difluoroacetic acid, and trifluoroacetic acid. In some aspects, the chromatography uses a first mobile phase including trifluoroacetic acid in water.

[0129] In some aspects, the chromatography uses a first mobile phase including formic acid.

[0130] In some aspects, the first mobile phase includes from about 0.05 to about 0.15% of formic acid by volume. In some aspects, the first mobile phase comprises about 0.05%, 0.06%, 0.07%, 0.08%, 0.09%, 0.1%, 0.11%, 0.12%, 0.13%, 0.14%, 0.15%, 0.16%, 0.17%, 0.18%, 0.19%, or 0.2% of formic acid by volume. In some aspects, the first mobile phase comprises about 0.05% or 0.1% of formic acid by volume. In some aspects, the first mobile phase includes about 0.1% of formic acid by volume.

[0131] In some aspects, the chromatography uses a second mobile phase including fluoro-substituted acetic acid in acetonitrile. In some aspects, the chromatography uses a second mobile phase including trifluoroacetic acid in acetonitrile. In some aspects, the chromatography uses a second mobile phase including fluoro-substituted acetic acid in the mixture of acetonitrile and water. In some aspects, the chromatography uses a second mobile phase including trifluoroacetic acid in the mixture of acetonitrile and water.

[0132] In some aspects, the chromatography uses a second mobile phase including formic acid in acetonitrile. In some aspects, the chromatography uses a second mobile phase including formic acid in the mixture of acetonitrile and water.

[0133] In some aspects, the second mobile phase includes about 0.05-0.2% of formic acid by volume. In some aspects, the second mobile phase includes about 0.05-0.15% of formic acid by volume. In some aspects, the second mobile phase includes about 0.05%, 0.06%, 0.07%, 0.08%, 0.09%, 0.1%, 0.11%, 0.12%, 0.13%, 0.14%, 0.15%, 0.16%, 0.17%, 0.18%, 0.19%, or 0.2% of formic acid by volume. In some aspects, the second mobile phase comprises about 0.05% or 0.1% of formic acid by volume. In some aspects, the second mobile phase includes about 0.1% formic acid by volume.

[0134] In some aspects, the second mobile phase includes about 75-95% of acetonitrile by volume.

[0135] In some aspects, the second mobile phase includes about 75%, 80%, 85%, 90%, or 95% of acetonitrile by volume. In some aspects, the second mobile phase includes about 90% acetonitrile and about 10% water by volume.

[0136] In some aspects, the percentage of the second mobile phase, in the combination of the first mobile phase and the second mobile phase, in the chromatography is increased over time. In some aspects, the percentage of the second mobile phase is increased from about 2% to about 50% by volume. In some aspects, the percentage of the second mobile phase is increased from about 50% to about 100% by volume. In some aspects, the percentage of the second mobile phase is increased from about 2% to about 50% in about 110-130 minutes. In some aspects, the percentage of the second mobile phase is increased from about 2% to about 50% in about 120 minutes. In some aspects, the percentage of the second mobile phase is increased from about 50% to about 100% by volume in about 20-30 minutes. In some aspects, the percentage of the second mobile phase is increased from about 50% to about 100% by volume in about 25 minutes. In some aspects, the percentage of the second mobile phase is subsequently increased to 100% by volume over about 5 minutes. In some aspects, the percentage of the second mobile phase is subsequently maintained at 100% by volume for about 3 minutes. In some aspects, the second mobile phase is subsequently decreased to about 2% by volume over about 2 minutes.

[0137] In some aspects, the percentage of the second mobile phase is maintained at about 100% by volume for about 0.5-1.5 minutes. In some aspects, the percentage of the second mobile phase is maintained at 100% by volume for about 1 minute.

[0138] In some aspects, the percentage of the second mobile phase is decreased from about 100% to about 2% in about 1-10 minutes. In some aspects, the percentage of the second mobile phase is decreased from about 100% to about 2% in about 4 minutes.

[0139] In some aspects, the liquid chromatography is high-pressure liquid chromatography

[0140] (HPLC). In some aspects, the liquid chromatography is ultra-high pressure liquid chromatography (UHPLC).

[0141] In some aspects, the mass spectrometry may use any ionization modes, particularly those modes suitable for analyzing biological molecules including, but not limited to, direct infusion-mass spectrometry, electrospray ionization (ESI)-MS, desorption electrospray ionization (DESI)-MS, direct analysis in real-time (DART)-MS, atmospheric pressure chemical ionization (APCI)-MS, electron impact (EI) or chemical ionization (CI), matrix-assisted laser desorption/ionization (MALDI)-MS, and Atmospheric Pressure Ionization-Electrospray (API-ES). In some aspects, the mass spectrometry uses API-ES ionization mode.

[0142] In some aspects, the mass spectrometry scans signals over a range of 40-5000 m/z. In some aspects, the mass spectrometry scans signals over a range of 50-3000 m/z. In some aspects, the mass spectrometry scans signals over a range of 300-3000 m/z.

[0143] In some aspects, the scan type of the mass spectrometry is positive polarity. In some aspects, the data acquisition time of the mass spectrometry is about 1-130 minutes. In some aspects, the data acquisition time of the mass spectrometry is about 2-120 minutes.

[0144] In some aspects, the nozzle voltage of the mass spectrometry is about 400-600 V. In some aspects, the nozzle voltage of the mass spectrometry is about 500 V. In some aspects, the skimmer voltage of the mass spectrometry is about 60-70 V. In some aspects, the skimmer voltage of the mass spectrometry is about 65 V. In some aspects, the difference between the nozzle and skimmer voltage is about 400-450 V. In some aspects, the difference between the nozzle and skimmer voltage is about 435 V.

[0145] In some aspects, the drying gas temperature of the mass spectrometry is about 200-375° C.

[0146] In some aspects, the drying gas temperature of the mass spectrometry is about 325° C. In some aspects, the drying gas flow rate of the mass spectrometry is about 5-13 L/min. In some aspects, the drying gas flow rate of the mass spectrometry is about 12 L/min.

[0147] In some aspects, the mass spectrometry uses a capillary voltage of about 3-6 kV. In some aspects, the mass spectrometry uses a capillary voltage of about 3, 4, 5, or 6 kV. In some aspects, the mass spectrometry uses a capillary voltage of about 5 kV.

[0148] In some aspects, the mass spectrometry uses a fragmentor voltage of about 125-350 V. In some aspects, the

mass spectrometry uses a fragmentor voltage of about 125, 130, 135, 145, 155, 160, 165, 175, 185, 190, 195, 200, 205, 210, 215, 220, 225, 230, 235, 240, 245, 250, 255, 260, 265, 270, 275, 280, 285, 290, 295, 300, 305, 310, 315, 320, 325, 330, 335, 340, 345, or 350V. In some aspects, the mass spectrometry uses a fragmentor voltage of about 135 V.

[0149] Although the subject matter has been described in considerable detail with reference to certain aspects thereof, other aspects are possible. As such, the spirit and scope of the appended claims should not be limited to the description of the specific aspects contained therein.

EXAMPLE

[0150] The disclosure will now be illustrated with working examples, and which is intended to illustrate the working of disclosure and not intended to restrict any limitations on the scope of the present disclosure. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this disclosure belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice of the disclosed methods and compositions, the exemplary methods, devices and materials are described herein.

Example 1

General Methods and Equipment

[0151] 1.1 Reagents

[0152] LCMS water, acetonitrile, and trifluoroacetic acid were obtained from Fisher Scientific and ammonium bicarbonate was obtained from Sigma Aldrich. Table 1 provides reagents used in liquid chromatography and solution preparation.

TABLE 1

Reagents used for liquid chromatography

Reagent Name	CAS Number	Required Grade, Purity or Concentration	Suggested Source/ Catalog Number
LCMS Water	7732-18-5	LCMS	Fisher/W6
Acetonitrile	67-63-0	LCMS	Fisher/A955
Trifluoroacetic Acid	76-05-1	LCMS	Fisher/A11610X1AMP
Ammonium Bicarbonate	1066-33-7	BioUltra	Sigma Aldrich/09830

[0153] 1.2 Equipment

[0154] The following equipment was used to perform the examples disclosed herein.

[0155] a) Waters ACQUITY UPLC BEH C8 Column, 2.1×100 mm, 1.7 μm; Part no. 186002878

[0156] b) Pierce Detergent Removal Spin Columns, 0.5 mL; Catalog # 87777

[0157] c) Suitable analytical balance

[0158] d) Automatic pipette

[0159] e) Class A volumetric glassware

[0160] f) HPLC vials and caps

[0161] g) Spatula and weigh boat

[0162] h) Agilent 1290 Infinity II UHPLC System

[0163] i) Agilent 6545XT AdvanceBio Quadrupole Time of Flight Mass Spectrometer (Q-ToF)

Example 2

Solution Preparation

[0164] 2.1 Preparation of 100 mM ammonium bicarbonate

[0165] 0.395±0.01 grams of ammonium bicarbonate was weighed in a 50 mL Falcon® tube.

[0166] Using a measuring cylinder, 50 mL of LCMS water was transferred to the tube, and a vortex mixer was used to thoroughly dissolve ammonium bicarbonate to obtain 100 mM ammonium bicarbonate solution. The solution is stable for one month at 2-8° C.

[0167] 2.2 Preparation of the First Mobile Phase (0.1% Trifluoroacetic Acid in Water)

[0168] Using a measuring cylinder, 1 L of LCMS water was transferred to a 1 L bottle. Using a pipette, 1000 μL of trifluoroacetic acid was transferred to the bottle. Trifluoroacetic acid and water were mixed well for 5 minutes to obtain the first mobile phase. The first mobile phase is stable for up to one month at ambient conditions.

[0169] 2.3 Preparation of the second mobile phase (0.1% Trifluoroacetic Acid in 90% Acetonitrile, and 10% Water)

[0170] 900 mL of acetonitrile was added to a 1 L measuring cylinder. LCMS water was added to the 1 L measuring cylinder to make 1 L solution. The solution was transferred to a 1 L bottle. Using a pipette, 1000 μL of trifluoroacetic Acid was transferred to the bottle. Trifluoroacetic acid and the solution were mixed well for 5 minutes to obtain the second mobile phase. The second mobile phase is stable for up to one month at ambient conditions.

Example 3

Sample Preparation

[0171] The bottom closure of the spin columns was removed and the cap of the spin columns was loosened. The columns were placed into a 2 mL collection tube and were centrifuged at 1500 X g for 1 minute. When using fixed angle rotors, a mark was placed on the side of the column where the compacted resin was slanted upward. Column was then placed in the centrifuge with the mark facing outward for all subsequent steps.

[0172] 400 μL of the 100 mM ammonium bicarbonate solution was added to the column and the column was centrifuged at 1500×g for 1 minute. This step was repeated two more times, and after each step, the flow-through was discarded. The column was placed into a new 2 mL collection tube. 5μg of the sample was slowly applied to the top of the compacted resin bed and incubated for 2 minutes at room temperature. The column was centrifuged at 1500×g for 2 minutes and the polymer free sample was collected. The sample volume was then made up to 100 μL with 100 mM Ammonium Bicarbonate and then transferred into a HPLC vial.

Example 4

Characterization of the VP1, VP2 and VP3 capsid proteins in an AAV particle

[0173] This example describes the methods of determining the ratio of VP1, VP2 and VP3 capsid proteins in an AAV particle, and the masses of the VP1, VP2 and VP3 capsid proteins.

[0174] Here, an AAV particle was denatured and separated to the VP1, VP2 and VP3 capsid proteins in liquid chromatography. The separated VP1, VP2 and VP3 capsid proteins were first subjected to UV to determine the ratio of VP1, VP2 and VP3 capsid proteins in the AAV particle, and then to mass spectrometry to obtain the mass of each of the VP1, VP2 and VP3 capsid proteins.

[0175] 4.1 LC Operating Conditions

[0176] The AAV VP1, VP2 and VP3 capsid proteins separations were performed on an ACQUITY UPLC® system using an ACQUITY UPLC® BEH 1.7 μm, 2.1×100 mm, C8 analytical column (Part no. 186002878). Mobile phases used were:

[0177] First mobile phase (A): 0.1% trifluoroacetic acid in water; and

[0178] Second mobile phase (B): 0.1% trifluoroacetic acid in 90% acetonitrile and 10% water.

[0179] The column temperature was maintained at about 80° C., and separation was achieved by using mobile phase B increasing from 10% to 40%, and from 40% to 45% at a flow rate of 0.4 mL/minute, followed by flushing with 100% mobile phase B for 1 minute and re-equilibrating with the starting mobile phase composition (10% mobile phase B) for another five minutes.

[0180] The LC operation conditions are listed in Table 2.

TABLE 2

LC Operation Conditions

Parameter	Setting		
Mobile Phase A	0.1% Trifluoroacetic Acid in water		
Mobile Phase B	0.1% Trifluoroacetic Acid in 90% Acetonitrile		
Flow Rate	0.4 mL/minute		
Column	Waters ACQUITY UPLC BEH C8 Column, 2.1 × 100 mm, 1.7 μm; Part no. 186002878		
Column Temperature	80° C.		
Autosampler Temperature	5° C.		
Injector Volume	100 μL		
Detector Wavelength	280 nm		
Acquisition Time	37 minutes		
Post time	5 minutes		
Gradient Program	Time (minutes)	% A	% B
	0.0	90	10
	3.0	90	10
	6.0	60	40
	35.0	55	45
	36.0	0	100
	37.0	90	10

[0181] 4.2 Mass Spectrometer (MS) Operating Conditions

[0182] Mass spectroscopy were performed using Agilent 6545XT AdvanceBio Quadrupole Time of Flight Mass Spectrometer (Q-ToF), using API-ES ionization in a survey scan in the range of m/z values 700-13700 m/z. The capillary voltage, nozzle voltage, fragmentor voltage, and skimmer voltage were set at 5 kV, 500 V, 175 V, and 65 V, respectively. The drying gas temperature and drying gas flow were set at 300° C. and 13 L/min, respectively.

[0183] The mass spectrometer operating conditions are listed in Table 3.

TABLE 3

Mass Spectrometer (MS) Operating Conditions

Parameter	Setting
Ionization Mode	API-ES
Mass Range	700-13700 m/z
Scan Type	Positive polarity
Data Acquisition Time	17-28 minutes
Capillary Voltage	5000 V
Nozzle Voltage	500 V
Fragmentor	175 V
Skimmer	65 V
OctopoleRFPeak	750
Nebulizer Pressure	40 psig
Drying Gas Temperature	300° C.
Drying Gas Flow	13 L/min
Sheath Gas Temperature	275° C.
Sheath Gas Flow	12 L/min
MS Scan Rate	0.50 spectra/second
Data Storage	Profile and Centroid

Note:
MS parameters are for the Agilent 6545XT QToF and may need to be modified depending on the instrument used.

[0184] 4.3 Analysis and Results

[0185] The capsid proteins were first denatured by heating the column compartment to 80° C. The three capsid proteins VP1, VP2 and VP3 were then baseline separated by using the Waters UPLC BEH C8 column (Part no. 186002878), eluted by the combination of a first mobile phase including 0.1% trifluoroacetic acid in water and a second mobile phase including 0.1% trifluoroacetic acid in the mixture of acetonitrile and water, wherein the percentage of the second mobile phase increases over time. The use of 0.1% trifluoroacetic acid as an ion-pairing agent in the mobile phases helps the baseline resolution.

[0186] The VP1, VP2 and VP3 capsid proteins separated in the liquid chromatography were first subjected to UV to determine the relative amounts and then to mass spectrometry to determine the masses of the VP1, VP2 and VP3 capsid proteins. The stoichiometry around 1:1:10 for VP1/VP2/VP3 was obtained by baseline integration of the UV chromatogram as shown in FIG. **1** and Table 4.

TABLE 4

AAVrh74 Sample	VP1	VP2	VP3
Sample 1	1.0	0.9	9.5
Sample 2	1.0	1.7	7.9
Sample 3	1.0	1.4	9.7

[0187] Peaks for the three capsid proteins were then deconvoluted using the parameters listed in

[0188] Table 5. The total ion chromatogram, and deconvoluted spectra for all three peaks are shown in FIG. **2** and FIGS. **3**A-**3**C. The three major masses detected under the VP1 peak (Theoretical Mass 81587 Da) were 81496 Da, 81578 Da and 81658 Da. The 81496 Da peak represents the VP1 protein with a loss of the N-terminal methionine and single acetylation modification. The other two peaks with a mass shift of +80 Da represents phosphorylation. Deconvolution of the VP2 peak (Theoretical Mass 66381 Da) showed two major masses: 66282 Da and 66360 Da. The 66282 Da peak represents the VP2 protein with a loss of threonine and the 66360 Da peak matches a single phosphorylation with a mass shift of +80. The VP3 peak (theoretical mass 59750 Da) showed a single major mass of 59662 Da which matches

the mass of the VP3 protein with a loss of the N-terminal methionine and a single acetylation.

TABLE 5

Deconvolution Parameters

Parameter	Setting
Deconvolution Algorithm	Maximum Entropy
Mass Range	45000-90000 Daltons
Mass Step	0.1 Daltons
m/z Range	700.00-3000.00
Baseline Subtraction Factor	7.00
Isotope Width	Automatic
Peak Signal to Noise	30.0
Maximum Number of Peaks	100
Calculate Average Mass	90% of Peak Height
Minimum Consecutive	5
Minimum Protein Fit Score	8

[0189] FIG. **3** shows detection of post-translation modification of VP1, VP2, and VP3. Table 6 shows the intact mass analysis of AAV.rh74 capsid proteins.

TABLE 6

Mass Analysis

Capsid Protein	Theoretical Mass (Da)	Mass Observed (Da)	Species Identification
VP1	81586.4	81499.4	Loss of Methionine plus Acetylation
		81579.0	Loss of Methionine plus Acetylation and 1X Phosphorylation
		81660.6	Loss of Methionine plus Acetylation and 2X Phosphorylation
VP2	66380.5	66280.9	Loss of Threonine
		66360.7	Loss of Threonine and 1X Phosphorylation

TABLE 6-continued

Mass Analysis

Capsid Protein	Theoretical Mass (Da)	Mass Observed (Da)	Species Identification
VP3	59750.2	59663.3	Loss of Methionine plus Acetylation

Example 5

Characterization of Deamidation of AAVrh74 using LCMS

[0190] Extensive deamidation in capsid proteins can be was determined by Mass Spectrometry, with which the sites of deamidation in the capsid proteins and also the levels of deamidation at these sites can be determined. To measure the AAV capsid deamidation, the capsid proteins were denatured and reduced at 90° C. for 10 minutes in the presence of 2M Guanidine Hydrochloride and 10 mM DTT. After cooling down the samples to room temperature, 30 mM Iodoacetamide was added for alkylation and incubated in dark at room temperature for 30 minutes. Alkylation was then quenched by addition of 1 mL of DTT. 20 mM Ammonium Bicarbonate was added to the samples to dilute down the Guanidine Hydrochloride to 200 mM. Samples were then digested using Trypsin in a 1:20 Enzyme:Protein ratio and incubated overnight at 37° C. After overnight incubation the digestion was quenched by adding to Trifluoroacetic Acid to a final concentration of 0.5% and the samples were analyzed on Thermo UltiMate 3000 RSLC system coupled to a Q Exactive HF with a NanoFlex source.

[0191] Table 7 shows major deamidation sites identified and the levels of deamidation (i.e., deamidation percentage) at these sites. The data for AAV8 in Table 7 is disclosed from a prior publication (Molecular Therapy, Volume 26 No 12, Pages 2848 — 2962 (2018)).

TABLE 7

Deamidation analysis results for AAV capsids

Peptide Sequence	Sequence Location in VP1	Amino Acid Residue	AAV8 (Ammonium Bicarbonate as buffer)	AAVrh74 (Ammonium Bicarbonate as buffer)	AAVrh74 (Tris HCl as buffer)
QISNGTSGGSTNDNT YFGYSTPWGYFDFNR (SEQ ID NO: 1)	A (260-289)	N263	100	89	ND
YHLNGR (SEQ ID NO: 2)	A (511-516)	N514	92	94	22
YLGPFNGLDK (SEQ ID NO: 3)	A (52-61)	N57	67	74	26
VSTTLSQNNNSNF AWTGATK (SEQ ID NO: 4)	A (491-510)	N502	66	36	3
TWALPTYNNHLYK (SEQ ID NO: 5)	A (247-259)	N254	21	24	2
YNHADAEFQER (SEQ ID NO: 6)	A (93-103)	N94	11	12	ND

[0192] Two buffers (ammonium bicarbonate and Tris-HC1) were used separately to measure the deamidation status of AAV.rh74.

[0193] For ammonium bicarbonate, samples were denatured by performing a buffer exchange into 100 mM Ammonium Bicarbonate. The denatured samples were reduced by addition of 10 mM DTT and incubated at 37° C. for 45 minutes. Alkylation was then performed by addition of Iodoacetamide in the samples to a final concentration of 30 mM. The denatured, reduced and alkylated samples were then exchanged back into 100 mM Ammonium Bicarbonate using a 10 kDa Amicon Ultra filter. Samples were then digested using Trypsin and incubated overnight at 37° C. Using this sample preparation in which the digestion was performed in Ammonium Bicarbonate, similar levels of deamidation were obtained for AAVrh74 as seen in Table 7.

[0194] For Tris-HCl, a 60 µg sample aliquot was buffer-exchanged into 4 M guanidine and 200 mM Tris pH 7.5 using an Amicon 10 K centrifugal filter to remove the sample matrix and concentrate the protein. The guanidine concentration was adjusted to 6 M and DTT (10 mM) was added to the 60 µg aliquot. The reaction mixture was incubated at 56° C. for 45 minutes, then cooled to room temperature. Iodoacetamide (30 mM) was added, with incubation at room temperature in the dark for 60 minutes. Tris buffer (100 mM, pH=7.5) was then added to dilute the guanidine HCl concentration to 0.6 M. Trypsin/Lys-C (60 µg) was added to 60 µg of the reduced and alkylated sample (enzyme:protein ratio —1:1 (w:w)). Methionine was added to 10 mM in the digestion to minimize artefactual oxidations. The digestion was carried out overnight (17 hours) at 37° C. TFA (1%) was then added prior to LC-MS/MS analysis.

[0195] As shown in Table 7, with Tris-HCl as buffer, the deamidation status was significantly lower as compared to those with Ammonium Bicarbonate.

[0196] To further optimize the methods with Tris-HCl, eight separate AAV capsid samples were measured according to the flow chart in FIG. **4**. The samples were first denatured by performing a buffer exchange into 6M Guanidine Hydrochloride, 20 mM Tris-HCl, pH 7.5. Samples were then reduced by adding DTT to a final concentration of 10 mM and incubated at 37° C. for 45 minutes. Alkylation was performed by adding Iodoacetamide to a final concentration of 30 mM and incubated in the dark at room temperature for 1 hour. The samples were again buffer exchanged into 20 mM Tris-HCl, pH 7.5 using 10 kDa Amicon Ultra filters. Acetonitrile was then added to the samples to a final concentration of 10% and Methionine was also added to a final concentration of 10 mM. Samples were digested overnight at 37° C. using Trypsin. Peptides are then separated on an Agilent 1290 U-HPLC using RP-HPLC. The separated peptides are then detected using an Agilent 6545XT QToF and deamidation analysis is performed using the MassHunter and Bioconfirm softwares. The deamidation status is shown in FIG. **5**, and the oxidation status is shown in FIG. **6**. Out of the 52 Asparagine residues present, no deamidation was observed at 48 residues (Total Asparagine residues in VP1: 56). Out of the 39 Glutamine residues present, no deamidation was observed at all 39 residues (Total Glutamine residues in VP1: 48). Using 018 Labeled water a small amount of the deamidation at N57 was shown to be a sample preparation related artifact. No Oxidation was detected at the 5 remaining Methionine residues (Total Methionine residues in VP1: 11). Out of the 14 Tryptophan residues detected, no oxidation was observed at all 14 residues (Total Tryptophan residues in VP1: 15).

[0197] Digestion in Ammonium Bicarbonate can increase deamidation artifacts significantly due to the increase in the pH over time. Therefore, the higher levels of deamidation observed could be deamidation artifacts generated during sample preparation. Tris HCl based digestion was set up at Sarepta to confirm the deamidation levels: 20 mM Tris HCl, pH 7.5 was used as the buffer; 10% Acetonitrile was added to the digestion solution as it is known to reduce deamidation artifacts; and 10 mM Methionine was also added to the digestion solution to reduce the oxidation artifacts

[0198] Therefore, the method of this disclosure is more accurate in measuring deamidation, oxidation, or other post-translation modification with the Tris-HCl buffer.

[0199] Example 6: Characterization of host cell proteins using LC-QTOF-MS

[0200] The purity of a rAAV based gene therapy drug product was analyzed by characterizing the host cell proteins remaining in the AAV composition through LC-QTOF-MS.

[0201] 6.1 Preparation of Sample

[0202] An AAVrh74 sample was spiked with a known amount of human thioredoxin 1 (HTI) protein standard, Invitrogen, (Catalog No. LF-P0001) and bovine carbonic anhydrase II (BCAII) protein standard, Sigma, (Catalog No. C7749). Human Thioredoxin 1 (HTI) and bovine carbonic anhydrase II (BCAII) were selected as the spiking protein standards for quantitate human and bovine HCPs found, respectively. For immune-depletion process, pipette 100 µL of 0.05 mg/mL Anti-Adeno-associated virus (AAV), VP1/VP2/VP3 antibody, and 100 µL of the sample solution, along with 20 µL of 0.05 mg/mL BCAII and 10 µL of 0.1 mg/mL HTI into 270 µl of IP-MS cell lysis buffer from Pierce MS-compatible magnetic IP kit. AAV capsid proteins were then immunoprecipitated from the sample with anti-adeno-associated virus (AAV), VP1/VP2/VP3 from American Research Products, Inc. (Catalog # 03-6105) and the Pierce MS-compatible magnetic IP kit (Catalog # 90409). The sample was then passed through the Pierce detergent removal spin columns (Catalog # 87777).

[0203] The samples were then buffer exchanged into Promega rapid digestion buffer (Catalog # VA1060). The samples were reduced, alkylated, and digested with rapid digestion trypsin at 70° C. for 60-180 minutes.

[0204] 6.2 LC Operating Conditions

[0205] Digested residual host cell protein separation was performed on an Agilent 1290 HPLC system using a Waters Acquity peptide BEH C18, 1.7 µm, 2.1×150 mm column. Mobile phases used are:

[0206] First mobile phase (A): 0.1% formic acid in water; and

[0207] Second mobile phase (B): 0.1% formic acid in 90% acetonitrile and 10% water.

[0208] The column temperature was maintained at about 45° C., and separation was achieved by using mobile phase B increased from 2% to 50%, and from 50% to 100% at a flow rate of 0.3 mL/minute, followed by flushing with 100% mobile phase B for 3 minutes and re-equilibrating with the starting mobile phase composition (2% mobile phase B) for another 5 minutes.

[0209] The LC operation conditions are listed in Table 8.

TABLE 8

LC Operation Conditions

Parameter	Setting		
Mobile Phase A	0.1% Formic Acid in water		
Mobile Phase B	0.1% Formic Acid in 90% Acetonitrile		
Flow Rate	0.3 mL/minute		
Column	Waters ACQUITY peptide BEH C8 Column, 2.1 × 150 mm, 1.7 µm;		
Column Temperature	45° C.		
Autosampler Temperature	4° C.		
Injector Volume	100 µL		
Needle Wash	50% Acetonitrile/50% water		
Acquisition Time	130 minutes		
Post time	5 minutes		
Gradient Program	Time (minutes)	% A	% B
	0.0	98	2
	115.0	50	50
	120.0	0	100
	123.0	0	100
	125.0	98	2

[0210] 6.3 Mass Spectrometer (MS) Operating Conditions

[0211] Mass spectroscopy was performed using Agilent 6545XT AdvanceBio Quadrupole Time of Flight Mass Spectrometer (Q-ToF), using API-ES ionization in a survey scan in the range of m/z values 50-3000 m/z. The capillary voltage, nozzle voltage, fragmentor voltage, and skimmer voltage were set at 4 kV, 500 V, 135 V, and 65 V, respectively. The drying gas temperature and drying gas flow were set at 325° C. and 12 L/min, respectively.

[0212] The mass spectrometer operating conditions are listed in Table 9.

TABLE 9

Mass Spectrometer (MS) Operating Conditions

Parameter	Setting
Gas Temperature	325° C.
Dry Gas	12 L/min
Nebulizer	35 psig
Sheath Gas Temperature	275° C.
Sheath Gas	12 L/min
Vcap	4000 V
Nozzle voltage	500 V
Fragmentor	135 V
Skimmer	65 V
Oct 1 RF vpp	750 V
MS acquisition	300 to 3000 m/z
MS/MS acquisition	50 to 3000 m/z
MS storage threshold	400 absolute/0.01% relative threshold
MS/MS storage threshold	5 absolute/0.02% relative threshold

TABLE 9-continued

Mass Spectrometer (MS) Operating Conditions

Parameter	Setting		
Auto MS/MS acquisition rate			
MS	3 spectra/s		
MS/MS	2 spectra/s		
Isolation width	Narrow		
Collision Energy	Charge	Slope	Offset
	2	3.1	1
	3	3.6	−4.8
	>3	3.6	−4.8
Precursor selection I	6 max precursor per cycle		
Absolute threshold	2000 counts		
Relative threshold	0.001%		
Active exclusion	Enabled, excluded after 1 spectra, release after 0.15 min		
Iterative MS/MS	Use PC for MS/MS		
Mass error tolerance	±20 ppm		
RT exclusion tolerance	±0.3 min		
Isotope model	Peptides		
Precursor charge state selection	2, 3, >3		
Abundance dependent accumulation	Scan speed varied based on precursor abundance		
Target	45000 counts/spectrum		
	Use MS/MS accumulation time limit		
Purity Stringency	100%		
Purity Cutoff	30%		
Reference Mass	322 and 2422		
Detection Window	500 ppm		
Minimum height	400 counts		
Time segments	0 to 2 min to waste		
	2 to 120 min to MS		
	120 to 130 min to waste		

[0213] 6.4 Analysis and Results

[0214] The data generated in Example 6.3 were processed by Byos software from Protein Metrics to search against certain Uniprot protein database. The identity and relative quantity of each residual protein were calculated against the amount of the amount of spiked protein standards. HCP analysis indicated that only few residual host cell proteins (two bovine proteins, but no human proteins) were identified by MS for three lots of AAV viral particles (Table 10). The concentrations for the proteins are in the order of ng/mL, or ppm level based on the spiked protein standards.

TABLE 10

three lots of Host Cell Protein analyzed by LC/MS

Protein	Uniprot ID	Protein Identified	Species	Size (kDa)	Peptide coverage (%)	B634-0220-002	NCH G02B1117	NCH G22A0219
1	O46375	Transthyretin	Bovine	16	61.9	2.0 ng/mL	0.0 ng/mL	14.0 ng/mL
2	Q3SZR3	Alpha-1-acid glycoprotein	Bovine	23	39.1	133.0 ng/mL	27.0 ng/mL	69.0 ng/mL

[0215] The following references are incorporated herein in their entirety:

[0216] 1. Buller R M, Rose J A. Characterization of adenovirus-associated virus-induced polypeptides in K B cells. J Virol 25: 1978, Pages 331-338.

[0217] 2. Johnson FB, Ozer H L, Hoggan M D. Structural proteins of adenovirus-associated viruses. J Virol 8:1971, Pages 776-770.

[0218] 3. D. W. Bauer. et.al. Exploring the Balance between DNA Pressure and Capsid Stability in Herpesviruses and Phages. J Virol 2015, 9288-98.

[0219] 4. Vamseedhar Rayaprolu.et.al. Comparative Analysis of Adeno-Associated Virus Capsid Stability and Dynamics. J Virol 2013, 13150-60.

[0220] 5. Xiaoying Jin et.al. Direct Liquid Chromatography/Mass Spectrometry Analysis for Complete Characterization of Recombinant Adeno-Associated Virus Capsid Proteins. Human Gene Therapy Methods, Volume 38 Number 5 2017, 255-267.

SEQUENCE LISTING

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<212> TYPE: PRT
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<223> OTHER INFORMATION: AAV capsid peptide sequence

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Phe Gly Tyr Ser Thr Pro Trp Gly Tyr Phe Asp Phe Asn Arg
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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: AAV capsid peptide sequence

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<220> FEATURE:
<223> OTHER INFORMATION: AAV capsid peptide sequence

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<220> FEATURE:
<223> OTHER INFORMATION: AAV capsid peptide sequence

<400> SEQUENCE: 4

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Gly Ala Thr Lys
20

<210> SEQ ID NO 5

-continued

<211> LENGTH: 13
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: AAV capsid peptide sequence

<400> SEQUENCE: 5

Thr Trp Ala Leu Pro Thr Tyr Asn Asn His Leu Tyr Lys
1 5 10

<210> SEQ ID NO 6
<211> LENGTH: 11
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: AAV capsid peptide sequence

<400> SEQUENCE: 6

Tyr Asn His Ala Asp Ala Glu Phe Gln Glu Arg
1 5 10

1. A method to characterize VP1, VP2 and VP3 capsid proteins in an adeno-associated virus (AAV) particle, the method comprising:

subjecting the AAV particle to a liquid chromatography at about 70° C. to about 90° C.;

wherein the ratio of VP1, VP2 and VP3 capsid proteins are determined by mass spectrometry and/or ultraviolet (UV)-visible spectroscopy.

2. The method of claim **1**, wherein individual masses of VP1, VP2 and VP3 capsid proteins are determined by the mass spectrometry.

3. The method of claim **1** wherein the ratio of the VP1, VP2 and VP3 capsid proteins is determined by comparing ultraviolet chromatogram (UV) of the VP1, VP2 and VP3 capsid proteins.

4. The method of claim **1**, wherein the liquid chromatography is a reverse phase liquid chromatography.

5. The method of claim **4**, wherein the reverse phase liquid chromatography is performed using a C18 column, a C8 column, or a C4 column.

6. (canceled)

7. The method of claim **5**, wherein the column comprises particles of about 1.2-3.5 μm.

8. (canceled)

9. The method of claim **5**, wherein the column is from about 50 mm to about 300 mm long and has an internal diameter of from about 1 mm to about 4.6 mm.

10. (canceled)

11. The method of claim **1** wherein the AAV particle is of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, AAV 13, AAVrh10, AAVrh74 serotype, or any naturally occurring, recombinant, or synthetic AAV particle.

12-17. (canceled)

18. The method of claim **1**, wherein the liquid chromatography comprises a second mobile phase that comprises trifluoroacetic acid in a mixture of acetonitrile and water.

19-20. (canceled)

21. The method of claim **18**, wherein the second mobile phase comprises about 80-95% of acetonitrile by volume.

22. (canceled)

23. The method of claim **18**, wherein the percentage of the second mobile phase, as compared to a combination of the first mobile phase and the second mobile phase, in the liquid chromatography is increased over time.

24-31. (canceled)

32. The method of claim **1**, wherein the characterization further comprises determining a post translational modification of at least one of VP1, VP2 and VP3 capsid proteins.

33. The method of claim **32**, wherein the post translational modification comprises one or more of loss of amino acid, glycosylation, sialylation, acetylation, phosphorylation, deamidation, oxidation, formylation, hydroxylation, methylation, and sulfation.

34. (canceled)

35. The method of claim **1**, wherein the method is performed with a gradient program listed in Table 2.

36. The method of claim **32**, wherein the characterization uses a buffer comprising Tris-HCl.

37. The method of claim **36**, wherein the buffer comprises acetonitrile.

38. The method of claim **36**, wherein the buffer comprises methionine.

39. The method of claim **36**, wherein the buffer comprises Tris-HCl at 5mM to 50 mM, 5%-20% acetonitrile, and methionine at 1 mM to 50 mM.

40-41. (canceled)

42. The method of claim **32**, wherein the post-translation modification comprises deamidation at one or more of N263, N514, N57, N502, N254, and N94 of AAV8 or its equivalent residue at AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV9, AAV10, AAV11, AAV12, AAV 13, AAVrh10, AAVrh74, or a recombinant or synthetic AAV particle.

43-44. (canceled)

45. An AAV composition comprising an AAV capsid that comprises deamidation at one or more of N57, N255, N256, and N263 of AAV. Rh74 or the equivalent residues of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, AAV 13, AAVrh10, or a recombinant or synthetic AAV particle.

46. An AAV composition comprising an AAV capsid that comprises oxidation at one or more of M437, M473, M526, M544, M560, and M637 of AAV. Rh74 or the equivalent residues of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, CAAV11, AAV12, AAV 13, AAVrh10, or a recombinant or synthetic AAV particle.

47-97. (canceled)

* * * * *

Exhibit V

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
8178.WO00		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US2020/067395	30 December 2020 (30-12-2020)	3 January 2020 (03-01-2020)
Applicant		
SAREPTA THERAPEUTICS, INC.		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 9 sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**
   a. With regard to the **language,** the international search was carried out on the basis of:
      ☒ the international application in the language in which it was filed
      ☐ a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))
   b. ☐ This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6*bis*(a)).
   c. ☒ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. ☐ **Certain claims were found unsearchable** (See Box No. II)

3. ☒ **Unity of invention is lacking** (see Box No III)

4. With regard to the **title,**
   ☒ the text is approved as submitted by the applicant
   ☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**
   ☒ the text is approved as submitted by the applicant
   ☐ the text has been established, according to Rule 38.2, by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority

6. With regard to the **drawings,**
   a. the figure of the **drawings** to be published with the abstract is Figure No. _____
      ☐ as suggested by the applicant
      ☐ as selected by this Authority, because the applicant failed to suggest a figure
      ☐ as selected by this Authority, because this figure better characterizes the invention
   b. ☒ none of the figures is to be published with the abstract

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2020/067395

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

   b. [ ] furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. [ ] furnished subsequent to the international filing date for the purposes of international search only:

      [ ] in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      [ ] on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2020/067395

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-46

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2020/067395

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/68 C07K14/14 C12N15/86 G01N30/72
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2018/035059 A1 (GENZYME CORP [US]) 22 February 2018 (2018-02-22) cited in the application the whole document paragraphs [[0003][0010][14], [0017] - [0019], [0021] - [0030], [0040] - [0043], [0059], [0061] - [0069] paragraphs [0106], [0113] - [0117], [0120], [0121]; claims 1-180 paragraphs [0098], [0083], [0205] paragraph [0178] -----	1-46
Y	WO 2019/168961 A1 (UNIV PENNSYLVANIA [US]) 6 September 2019 (2019-09-06) the whole document -----	1-46

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 April 2021	09/06/2021

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Boiangiu, Clara

2

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2020/067395

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	XIAOYING JIN ET AL: "Direct Liquid Chromatography/Mass Spectrometry Analysis for Complete Characterization of Recombinant Adeno-Associated Virus Capsid Proteins", HUMAN GENE THERAPY METHODS, 16 June 2017 (2017-06-16), XP055416361, ISSN: 1946-6536, DOI: 10.1089/hgtb.2016.178 the whole document ----	1-46
Y	Yang Yu: "High-Temperature Liquid Chromatography Solubility of drug active ingredients under subcritical water conditions View project", , 1 April 2008 (2008-04-01), XP055793361, Retrieved from the Internet: URL:https://www.chromatographyonline.com/view/high-temperature-liquid-chromatography [retrieved on 2021-04-07] the whole document ----	1-46
Y	STEVEN J. BARK ET AL: "High-Temperature Protein Mass Mapping Using a Thermophilic Protease", JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, vol. 123, no. 8, 1 February 2001 (2001-02-01), pages 1774-1775, XP055417355, US ISSN: 0002-7863, DOI: 10.1021/ja002909n the whole document ----	1-46
Y	VAN VLIET K ET AL: "Adeno-associated virus capsid serotype identification: Analytical methods development and application", JOURNAL OF VIROLOGICAL METHODS, ELSEVIER BV, NL, vol. 159, no. 2, 1 August 2009 (2009-08-01), pages 167-177, XP026159973, ISSN: 0166-0934, DOI: 10.1016/J.JVIROMET.2009.03.020 [retrieved on 2009-03-26] the whole document ----	1-46
Y	WO 01/83692 A2 (UNIV PENNSYLVANIA [US]; HILDINGER MARKUS [US] ET AL.) 8 November 2001 (2001-11-08) cited in the application the whole document ---- -/--	1-46

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2020/067395

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2013/158879 A1 (PHILADELPHIA CHILDREN HOSPITAL [US]) 24 October 2013 (2013-10-24) the whole document	1-46
Y	BOSMA BAS ET AL: "Optimization of viral protein ratios for production of rAAV serotype 5 in the baculovirus system", GENE THERAPY, NATURE PUBLISHING GROUP, LONDON, GB, vol. 25, no. 6, 1 August 2018 (2018-08-01), pages 415-424, XP036858971, ISSN: 0969-7128, DOI: 10.1038/S41434-018-0034-7 [retrieved on 2018-08-01] the whole document	1-46
Y	APRIL R. GILES ET AL: "Deamidation of Amino Acids on the Surface of Adeno-Associated Virus Capsids Leads to Charge Heterogeneity and Altered Vector Function", MOLECULAR THERAPY, vol. 26, no. 12, 1 December 2018 (2018-12-01), pages 2848-2862, XP055635211, ISSN: 1525-0016, DOI: 10.1016/j.ymthe.2018.09.013 the whole document	1-46
Y	WO 2019/169004 A1 (UNIV PENNSYLVANIA [US]) 6 September 2019 (2019-09-06) the whole document	1-46

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Form PCT/ISA/210 (continuation of second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2020/067395

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No
PCT/US2020/067395

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
		SI 2839014 T1	31-05-2021
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International Application No. PCT/ US2020/ 067395

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-46

   Method to characterize VP1, VP2 and VP3 capsid proteins in an adeno-associated virus (AAV) particle.

   ---

2. claims: 47-95

   Method of characterizing host cell proteins in an AAV composition

   ---

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:

see form PCT/ISA/220

PCT

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing
(day/month/year) see form PCT/ISA/210 (second sheet)

Applicant's or agent's file reference
see form PCT/ISA/220

FOR FURTHER ACTION
See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US2020/067395	30.12.2020	03.01.2020

International Patent Classification (IPC) or both national classification and IPC
INV. G01N33/68 C07K14/14 C12N15/86 G01N30/72

Applicant
SAREPTA THERAPEUTICS, INC.

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☒ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☒ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☐ Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will usually be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notifed the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA:	Date of completion of this opinion	Authorized Officer
European Patent Office P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk - Pays Bas Tel. +31 70 340 - 2040 Fax: +31 70 340 - 3016	see form PCT/ISA/210	Boiangiu, Clara Telephone No. +31 70 340-0

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No. PCT/US2020/067395

Box No. I Basis of the opinion

1. With regard to the **language**, this opinion has been established on the basis of:

   ☒ the international application in the language in which it was filed.

   ☐ a translation of the international application into , which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1 (b)).

2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43*bis*.1(a))

3. ☒ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:

      ☒ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

4. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No. PCT/US2020/067395

Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been examined in respect of

☐ the entire international application

☒ claims Nos. 47-95

because:

☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international search *(specify)*:

☐ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. are so unclear that no meaningful opinion could be formed *(specify)*:

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed *(specify)*:

☒ no international search report has been established for the whole application or for said claims Nos. 47-95

☐ a meaningful opinion could not be formed without the sequence listing; the applicant did not, within the prescribed time limit:

- ☐ furnish a sequence listing in the form of an Annex C/ST.25 text file, and such listing was not available to the International Searching Authority in the form and manner acceptable to it; or the sequence listing furnished did not comply with the standard provided for in Annex C of the Administrative Instructions.
- ☐ furnish a sequence listing on paper or in the form of an image file complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in the form and manner acceptable to it; or the sequence listing furnished did not comply with the standard provided for in Annex C of the Administrative Instructions.
- ☐ pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rule 13*ter*.1(a) or (b).

☐ See Supplemental Box for further details

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No. PCT/US2020/067395

Box No. IV Lack of unity of invention

1. ☒ In response to the invitation (Form PCT/ISA/206) to pay additional fees, the applicant has, within the applicable time limit:

   ☐ paid additional fees

   ☐ paid additional fees under protest and, where applicable, the protest fee

   ☐ paid additional fees under protest but the applicable protest fee was not paid

   ☒ not paid additional fees

2. ☐ This Authority found that the requirement of unity of invention is not complied with and chose not to invite the applicant to pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rule 13.1, 13.2 and 13.3 is

   ☐ complied with

   ☒ not complied with for the following reasons:

   see separate sheet

4. Consequently, this report has been established in respect of the following parts of the international application:

   ☐ all parts.

   ☒ the parts relating to claims Nos. 1-46

Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-46
	No:	Claims	
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-46
Industrial applicability (IA)	Yes:	Claims	1-46
	No:	Claims	

2. Citations and explanations

see separate sheet

Re Item IV

Lack of unity of invention

Reference is made to the following document:

D1 WO 2018/035059

This Authority considers that the application does not meet the requirements of unity of invention and that there are 2 inventions covered by the claims indicated as follows:

claims: 1-46: Method to characterize VP1, VP2 and VP3 capsid proteins in an adeno-associated virus (AAV) particle.

claims: 47-95: Method of characterizing host cell proteins in an AAV composition

The reasons for which the inventions are not so linked as to form a single general inventive concept, as required by Rule 13.1 PCT, are as follows:

1 The problem to be solved in the present application and shared by all embodiments of all independent claims is the provision of methods for characterising proteins in adeno-associated virus (AAV) composition, comprising viral capsid proteins.

2 The common concept which can be identified as linking the various claimed inventions and which forms a solution to the above mentioned problem is based on the idea that proteins from a AAV composition can be determined using liquid chromatography.

3 This common concept is not novel over D1 disclosing already methods for characterising proteins of an AAV composition, such as one or more capsid proteins, by using liquid chromatography (see references in the search report, such as for example paragraphs: [0017]-[0019]). The method of D1 characterises the capsid proteins by determining for example the masses of said one or more capsid proteins of the viral particle. The masses of VPI, VP2 and VP3 of the AAV particle are determined by " injecting the denatured AAV particle to liquid chromatography/mass spectrometry (LC/MS)". The capsid protein of D1 are further characterised by comparing calculated masses of the one or more capsid proteins to the theoretical masses of the one or more capsid proteins of one or more virus serotypes.

4 In view of the above, the common concept does not comprise any same or corresponding special technical features and is consequently not a single general inventive concept.

5 Starting from the common concept, the following group of inventions are identified:

5.1 Invention 1 (claims 1-46): Method to characterize VP1, VP2 and VP3 capsid proteins in an adeno-associated virus (AAV) particle.

Starting from the common concept, this group of possible inventions comprises the additional features of "subjecting the AAV particle to a liquid chromatography at about 70°C to about 90 °C;wherein the ratio of VP1, VP2 and VP3 capsid proteins are determined by mass spectrometry and/or ultraviolet (UV)-visible spectroscopy". These features are related to the solution to the problem of characterising VP1, VP2 and VP3 capsid proteins.

5.2 Invention 2 (claims 47-95): Method of characterizing host cell proteins in an AAV composition

Starting from the common concept, this group of possible inventions comprises the additional features of "immunoprecipitating viral capsid proteins from the composition; digesting residual host cell proteins; analyzing the digested proteins with liquid chromatography quadrupole time of flight mass spectrometry (LC-QTOF-MS) to identify host cell proteins.". These features are related to the solution to the problem of characterizing host cell proteins.

6 Consequently, the features of inventions 1 and 2 which make a contribution over the common matter are different.

7 As the problems they solve are different, these features are not corresponding either.

8 There is thus no relationship among these inventions which would involve the same or corresponding special technical features, such that the requirement of unity of invention is not met (Rule 13.1 PCT in conjunction with Rule 13.2 PCT).

9 The search for subject 2 represent a major extra search burden. In consequence the applicant was invited to pay one additional search fees but he refused to do so. An opinion will be given on the searched subject-matter (the subject-matter of claims 1-46).

Re Item V

Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following documents:

D1 WO 2018/035059 A1 (GENZYME CORP [US]) 22 February 2018 (2018-02-22)cited in the application

D2 WO 2019/168961 A1 (UNIV PENNSYLVANIA [US]) 6 September 2019 (2019-09-06)

D3 XIAOYING JIN ET AL: HUMAN GENE THERAPY METHODS, 16 June 2017 (2017-06-16), XP055416361, ISSN: 1946-6536, DOI: 10.1089/hgtb.2016.178

D4 Yang Yu: , 1 April 2008 (2008-04-01), XP055793361, [retrieved on 2021-04-07]

D5 STEVEN J. BARK ET AL: JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, vol. 123, no. 8, 1 February 2001 (2001-02-01), pages 1774-1775, XP055417355, US ISSN: 0002-7863, DOI: 10.1021/ja002909n

D6 VAN VLIET K ET AL: JOURNAL OF VIROLOGICAL METHODS, ELSEVIER BV, NL, vol. 159, no. 2, 1 August 2009 (2009-08-01), pages 167-177, XP026159973, ISSN: 0166-0934, DOI: 10.1016/J.JVIROMET.2009.03.020 [retrieved on 2009-03-26]

D7 WO 01/83692 A2 (UNIV PENNSYLVANIA [US]; HILDINGER MARKUS [US] ET AL.) 8 November 2001 (2001-11-08)cited in the application

D8 WO 2013/158879 A1 (PHILADELPHIA CHILDREN HOSPITAL [US]) 24 October 2013 (2013-10-24)

D9 BOSMA BAS ET AL: GENE THERAPY, NATURE PUBLISHING GROUP, LONDON, GB, vol. 25, no. 6, 1 August 2018 (2018-08-01), pages 415-424, XP036858971, ISSN: 0969-7128, DOI: 10.1038/S41434-018-0034-7 [retrieved on 2018-08-01]

D10 APRIL R. GILES ET AL: MOLECULAR THERAPY, vol. 26, no. 12, 1 December 2018 (2018-12-01), pages 2848-2862, XP055635211, ISSN: 1525-0016, DOI: 10.1016/j.ymthe.2018.09.013

D11 WO 2019/169004 A1 (UNIV PENNSYLVANIA [US]) 6 September 2019 (2019-09-06)

10 **Novelty**

10.1 Documents D1 to D3, each document taken alone, disclose (see references in the search report and citations here below):

A method to characterize VP1, VP2 and VP3 capsid proteins (such as, e.g., in D1: "determining the masses of one or more capsid proteins of the viral particle", or "determine the serotype of a viral particle") in an adeno-associated virus (AAV) particle, the method comprising:

subjecting the AAV particle to a liquid chromatography wherein the ratio (in D1: the combination) of VP1, VP2 and VP3 capsid proteins are determined by mass spectrometry (D1 recites "wherein the specific combination of masses of VPI, VP2 and VP3 are indicative of the AAV serotype").

Thus, for example D1 recites:

"[0003] The present invention relates to **methods for serotyping and/or determining the heterogeneity of a viral particle** (e.g., an adeno-associated virus (AAV) particle) using mass determination, e.g., by employing liquid chromatography/mass spectrometry (LC/MS) or liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS). In some aspects, the present invention relates to methods to improve the stability of AAV particles. (...)

[0010] (...) the invention provides a method to **determine the serotype of a viral particle** comprising a) **denaturing** the viral particle, b) subjecting the denatured viral particle to **liquid chromatography/mass spectrometry** (LC/MS), and c) **determining the masses of one or more capsid proteins of the viral particle**; wherein the **specific combination of masses of the one or more capsid proteins are indicative of the virus serotype**. In some

embodiments, the **calculated masses of the one or more capsid proteins are compared to the theoretical masses** of the one or more capsid proteins of one or more virus serotypes. (...)

[0012] In some embodiments of the above aspects, the liquid chromatography is **reverse phase liquid chromatography**, (...)

[0013] (...) d) subjecting the **fragments** of the one or more capsid proteins to **liquid chromatography/mass spectrometry-mass spectrometry** (LC/MS/MS), and e) determining the masses of fragments of the one or more capsid proteins of the viral particle; wherein the **specific combination of masses of fragments of the one or more capsid proteins are indicative of the viral serotype**. In some embodiments, the calculated masses of the fragments of the one or more capsid proteins are compared to the theoretical masses of fragments of the one or more capsid proteins of one or more viral serotypes. (...)

[0017] In some aspects, the invention provides a method to **determine the serotype of an adeno-associated virus** (AAV) particle comprising a) **denaturing** the AAV particle, b) subjecting the denatured AAV particle to **liquid chromatography/mass spectrometry (LC/MS)**, and c) **determining the masses of VP1, VP2 and VP3** of the AAV particle; wherein the **specific combination of masses of VP1, VP2 and VP3 are indicative of the AAV serotype**. In some embodiments, the calculated masses of VP1, VP2 and VP3 are compared to the theoretical masses of VP1, VP2 and VP3 of one or more AAV serotypes. (...)

[0023] In some aspects, the invention provides a method to determine the serotype of an adeno-associated virus (AAV) particle comprising a) denaturing the AAV particle, b) subjecting the denatured AAV particle to reduction and/or alkylation, c) subjecting the denatured AAV particle to digestion to generate fragments of VPI, VP2 and/or VP3 of the AAV particle, d) subjecting the fragments of VPI , VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), and e) determining the masses of fragments of VPI, VP2 and VP3 of the AAV particle; wherein the specific combination of masses of fragments of VPI , VP2 and VP3 are indicative of the AAV serotype. In some embodiments, the calculated masses of the fragments of VPI, VP2 and/or VP3 are compared to the theoretical masses of fragments of VPI, VP2 and/or VP3 of one or more AAV serotypes. (...)

[0115] A **variety of mass analyzers suitable for LC/MS** and/or LC/MS/MS are known in the art, including without limitation time-of-flight (TOF) analyzers, **quadrupole mass filters, quadrupole TOF (QTOF)**, "

10.2 The subject-matter of claim 1 therefore differs from this known D1 (or D2, or D3) in that in the present application the liquid chromatography is realised at about 70°C to about 90°C.

10.3 The subject-matter of claim 1 is new according to Article 33(2) PCT.

11 **Inventive step**

11.1 No technical effect derives form the above mentioned difference.

11.2 The problem to be solved could be seen as providing an alternative liquid chromatography process.

11.3 The solution is that of present claim 1.

11.4 D1 already recites in paragraph [0183], that "AAVs can be denatured through a number of methods using detergent, heat, high salt, or buffer with low or high pHs." Moreover, high temperature liquid chromatography is a well know technique (see for example documents D4 and D5). For example, D4 relates to High-Temperature Liquid Chromatography wherein "the most frequently used temperature in HTLC normally ranges from: 50°C to 150°C" (see D4 the entire document).

11.5 The feature "high temperature" is merely one of several straightforward possibilities from which the skilled person would select, in accordance with circumstances, without the exercise of inventive skill, in order to solve the problem posed. Because document D4 describes that the additional technical feature solves the problem posed combining it with the method of document D1 is obvious for a person skilled in the art, therefore the subject-matter of claim 1 does not involve an inventive step in the sense of Article 33(3) PCT.

11.6 Dependent claims 2-46 do not contain any features which, in combination with the features of any claim to which they refer, meet the requirements of the PCT in respect of novelty and/or inventive step (Article 33(2) and (3) PCT) for the following reasons: The features of dependent claims 2-46, 48-95 are either known from D1-D11 or fall within the knowledge and the ability of a person skilled in the art.

11.7 It seems that all features of claims 1-12 and 15-46 are already known from D1. The same type of columns (C8) and particle sizes are disclosed in D1 (see for example paragraph [0178]; or the the same reverse phase liquid chromatography (see claim 6 of D1); or AAV particles(see claim 23 to 27 of D1); or the same type of modifications (see claims 142 to 157 of D1).

11.8 The features of claims 13 and 14 of present set of claims are apparently known from D4 or D5 (see references in the search report and above).

12 **Industrial applicability**

The subject-matter of claims 1-46 is considered as industrially applicable, because it can be used or made in the medical or pharmaceutical industry or research (Article 33(4) PCT).

Exhibit W

Direct Liquid Chromatography/Mass Spectrometry Analysis for Complete Characterization of Recombinant Adeno-Associated Virus Capsid Proteins

Xiaoying Jin,[1,*] Lin Liu,[1] Shelley Nass,[2] Catherine O'Riordan,[2] Eric Pastor,[1] and X. Kate Zhang[3]

[1]Biopharmaceutics Development; [2]Gene Therapy Research; [3]Translational Science; Sanofi, Framingham, Massachusetts.

The requirement for robust analytical methods to characterize adeno-associated virus (AAV) vectors is immediate, as the field advances more AAV gene therapies into the clinic and onto commercialization. AAV capsid proteins (VPs) are critical for viral infectivity and vector potency. Thus, complete characterization of the constituent viral capsid proteins of AAV vectors, including their sequences and post-translational modifications (PTMs), is highly recommended to ensure AAV product quality and consistency. Typically, SDS-PAGE analysis followed by in-gel enzymatic digestion and liquid chromatography/tandem mass spectrometry (LC/MS/MS) is used for the characterization of viral capsid proteins. However, due to the limited recovery of digested peptides from the gel, determination of N-terminal sequences of VPs has not been reported to date. In this study, a direct liquid chromatography/mass spectrometry (LC/MS) intact protein analysis was developed to characterize viral capsid proteins in a variety of AAV serotypes. Both N- and C-terminal sequences of six AAV serotypes have been identified based on accurate mass measurement. This method can be used to confirm the identity of AAV serotype and monitor potential capsid protein heterogeneity. Complete sequence confirmation of AAV2 VPs was achieved through LC/MS/MS analysis of peptides generated using multiple enzymatic digestions. LC/MS/MS analysis confirmed the sequences for both N- and C-termini of capsid VPs and revealed acetylation on the N-termini of VP1 and VP3, consistent with LC/MS intact protein analysis.

Keywords: AAV, LC/MS, identity test, accurate mass measurement, peptide mapping, gene therapy

INTRODUCTION

Recombinant adeno-associated viruses (rAAVs) have become popular gene-therapy vectors due to their nonpathogenic nature, ability to infect both dividing and nondividing cells, and ability to provide sustained, long-term gene expression. Currently, AAV-based gene therapies are being evaluated in clinical trials for numerous disease indications, including muscular dystrophy, hemophilia, Parkinson's disease, Leber congenital amaurosis, and macular degeneration.[1]

AAV is a small virus with a single-stranded DNA genome encapsidated in an icosahedral protein capsid shell. To date, 13 AAV serotypes and ~150 gene sequences have been isolated from human and nonhuman primate tissues. These serotypes show 51–99% identity in capsid amino acid sequence.[1–4] Each AAV serotype has a specific tissue tropism, which is complemented by a corresponding sequence-specific receptor(s) and co-receptor(s).[5–9]

Each AAV capsid consists of 60 copies of three viral capsid proteins VP1 (87 kDa), VP2 (73 kDa), and VP3 (62 kDa) at an approximate 1:1:10 ratio.[10–12] The three viral capsid proteins are expressed from the same open reading frame by using alternative mRNA splicing of the transcript and translational start codon. Consequently, the three capsid proteins share overlapping sequences.[13–15] VP1 of AAV2 has a unique N-terminal region of 137 amino acid residues, while all the VPs share a common C-terminal sequence of 533 amino acid residues within VP3. The entire sequence of VP3

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is contained within VP2, and all of the VP2 sequence is contained within VP1. Different AAV production methods may result in different relative expression levels of VP1, VP2, and VP3. Urabe *et al*. reported that the first-generation baculovirus AAV production had a reduced level of VP1 relative to VP2 and VP3,[16] an attribute that resulted in decreased vector potency.

The AAV capsid, in addition to protecting the viral genome, plays an important role in mediating receptor binding, escape of the virus from the endosome, and transport of the viral DNA to the nucleus. For example, heparin sulfate proteoglycan was identified as a major receptor for AAV2.[17–20] A group of basic amino acids (R484, R487, R585, R588, and K532) were identified as heparin-binding motifs.[21] The N-terminal region of VP1 in AAV2 contains a phospholipase 2 domain (PLA2; amino acid 52–97), which is critical for the release of the virus from the endosome.[22–24] Moreover, the N-terminal regions of VP1 and VP2 also contain three clusters of basic amino acid that function as nuclear localization signals. Site-directed mutagenesis of these basic amino acid clusters resulted in reduced infectivity, underscoring the importance of this region in viral transduction.[25]

Zhong *et al*. also reported that surface tyrosine residues on the AAV2 capsid can be phosphorylated *in vitro* by the epidermal growth factor receptor-protein tyrosine kinase (EGFR-PTK), and they postulated that the phosphorylation had a negative effect on viral intracellular trafficking and transgene expression, presumably through the ubiquitin–proteasome pathway.[26] Additionally, site-directed mutagenesis on capsid surface serine, threonine, and lysine residues has resulted in increased transduction efficiencies of various AAV serotypes.[27–29] In summary, viral capsid proteins play an important role in cellular targeting and trafficking as part of the viral infection cycle, and thus any changes in the viral capsid protein sequence or post-translational modifications (PTMs) might impact viral targeting and infectivity.

The structures of multiple AAV serotypes have been resolved through X-ray crystallography. However, unique sequences for VP1 and VP2 have not been observed in the structures. In addition, the first 14 N-terminal amino acid residues of VP3 were not traceable in the crystal structure, possibly due to the intrinsic disorder in the region.[30–32] Edman sequencing analysis of VP bands after blotting from SDS-PAGE determined that the VP2 N-terminus in AAV2 was A139 but failed to identify N-terminal sequences of VP1 and VP3, suggesting N-termini of VP1 and VP3 may be blocked.[15] When VP3 of AAV2 was synthesized *in vitro*, under conditions that prevented N-terminal acetylation, the VP3 N-terminus was sequenced to be A204.[15]

Mass spectrometry has become an increasingly powerful technique to study protein structures and PTMs in AAV VPs. For example, in 2006, Murray *et al*. used liquid chromatography/tandem mass spectrometry (LC-MS/MS), after in-gel enzymatic digestion of VP1, VP2, and VP3 bands following SDS-PAGE separation, to confirm the absence of glycosylation on the AAV2 capsid proteins.[33] Although VP1, VP2, and VP3 bands were partially sequenced by LC-MS/MS, the N-termini of VP1, VP2, and VP3 have not been confirmed due to the limited recovery of peptides after protein in-gel digestion.[4,34,35] In 2014, Snijder *et al*. reported that the entire AAV1 viral particles could be detected with a modified Orbitrap mass spectrometer. The study showed that there was no defined VP1/VP2/VP3 stoichiometry for AAV1, and the relative abundance of VP1/VP2/VP3 incorporated in the capsid might depend mainly on their relative expression levels.[36] In another report, using charge detection mass spectrometry, empty capsids lacking a vector genome or capsids harboring a partial genome were separated and quantified from capsids containing a full vector genome.[37] Notably, in the two reports above, only the masses of the whole AAV particles were reported, without accurate mass measurement of constituent VP1, VP2, and VP3 proteins.

Since AAV serotypes differ in their tropism or the types of cells they infect, a particular AAV serotype is selected for preferentially transducing a specific cell type or organ. AAV serotype is directly related to the efficacy and safety of clinical AAV gene therapies. The identity test of AAV serotype is therefore recommended for AAV vector product release, especially those manufactured in multi-product/multi-serotype facilities. Currently, the common AAV serotype identity tests are SDS-PAGE, enzyme-linked immunosorbent assay (ELISA), or Western blot. However, the gel banding patterns on SDS-PAGE are not specific enough to differentiate every AAV serotype. Serotype-specific antibodies were developed against some AAV serotypes, including AAV2/AAV3, AAV1/AAV6, AAV4, AAV5, AAV8, and AAV9.[38–40] Nonetheless, some closely related AAV serotypes such as AAV1/AAV6 cannot be differentiated due to high sequence homology and overlapping epitopes.[31,41,42] With an increasing number of AAV serotypes and engineered capsids being developed for clinical applications, antibodies that do not cross-react

may be difficult to obtain, as reported.[4] Therefore, there is an urgent need to develop robust analytical methods for characterization of viral capsid proteins to support AAV serotype identification.

In this study, AAV capsid proteins were directly analyzed by reverse-phase liquid chromatography/mass spectrometry (LC/MS) and characterized in detail by LC/MS/MS after in-solution enzymatic digestion. For AAV2, complete sequence coverage of VP1, VP2, and VP3 was achieved, and acetylation at N-terminal alanine in VP1 and VP3 was identified. The LC/MS analysis of capsid proteins of six AAV serotypes demonstrated the accurate mass measurement of capsid proteins as a fast and straightforward AAV serotype identity test.

MATERIALS AND METHODS

Materials and reagents

Dithiothreitol (DTT), 4-vinylpyridine, ultrapure formic acid, acetic acid, guanidine-HCl, Tris-HCl, and Tris base were from Sigma–Aldrich (St. Louis, MO). Amicon ultra-4 filters (10 kDa MWCO) were purchased from Millipore (Billerica, MA). Sequencing grade porcine trypsin was purchased from Promega (Milwaukee, WI). Endoproteinase Lys-C and Asp-N were from Roche Diagnostics (Indianapolis, IN). Slide-A-Lyzer cassettes with 10 kDa MWCO were purchased from Pierce (Rockford, IL). Sample reducing agent (10×), NuPAGE 4–12% Bis Tris gel, MOPS buffer, and Mark 12 standard were purchased from Life Technologies (Woburn, MA). SYPRO Ruby protein gel stain was from Sigma–Aldrich.

Vector production and purification

AAV vectors were produced using the transient triple transfection method, as previously described.[43] Briefly, HEK293 cells were transfected using polyethyleneimine (PEI), and a 1:1:1 ratio of three plasmids (ITR vector, AAV rep/cap, and Ad helper plasmid). The vector plasmid contains the vector genome CBA-EGFP and ITR sequences from AAV2. EGFP expression is driven by the cytomegalovirus (CMV) enhancer, chicken beta actin hybrid promoter (CBA), as described.[44,45] The AAV rep/cap helpers contained rep sequences from AAV2 and serotype-specific capsid sequences with the nomenclature rep2/cap2, rep2/cap5, rep2/cap7, and so on. The pAd helper used was pHelper (Stratagene/Agilent Technologies, Santa Clara, CA). AAV vectors were purified by affinity column chromatography (AVB Sepharose High Performance medium; GE Healthcare, Aurora, OH), as previously described.[46,47]

SDS-PAGE analysis

Briefly, AAV2 CBA-EGFP vectors (5E10 vector genomes) were reduced in a 10× sample reducing agent by boiling for 5 min. The sample, along with Mark 12 protein standard, was run in a NuPAGE 4–12% Bis Tris gel in 1×MOPS buffer for 40 min at a constant voltage of 200 V. The gel was then stained using SYPRO Ruby Protein Gel Stain following the manufacturer's instructions and imaged using a FUGIFILM LAS-4000 imager.

LC/MS intact protein analysis

One milliliter of AAV virions at a concentration of 1E12 vg/mL was first concentrated with an Amicon ultra-4 filter (4 mL, 10 kDa MWCO) and then washed with 1 mL of 25 mM of Tris, pH 8.0, three times using a centrifuge with a swinging bucket rotor at a spin speed of 4,000 *g*. The concentrated AAV virions (~0.04 mg/mL protein) were denatured with 10% acetic acid, vortexed, and further diluted with an equal volume of HPLC-grade water. The final acetic acid concentration was 5%; the pH of the AAV virion solution was increased from 2.5 to 2.6 after dilution. Fifty microliters of AAV solution (~2 μg of proteins) was injected to Acquity UPLC coupled with a Xevo G2 QTOF MS instrument (Waters, Milford, MA). The separations were performed on a UPLC BEH C4 (1 mm×100 mm) at a flow rate of 50 μL/min or a BEH C8 column (2.1 mm×100 mm) at a flow rate of 250 μL/min. Mobile phase A and B were 0.1% formic acid in water and acetonitrile, respectively. The final gradient for C8 column was as follows: from 10% B to 20% B over 6 min, from 20% B to 30% B over 10 min, then from 30% to 38% B over 40 min. The capillary voltage and sampling cone voltage of the mass spectrometer were set at 3.5 kV and 45 V, respectively. The mass spectra were acquired in the positive sensitivity mode over m/z 500–4,000. MaxEnt1 in MassLynx software v4.1 was used for protein deconvolution.

Enzymatic digestions of AAV2 VPs

Concentrated AAV2 virions in 25 mM of Tris buffer (~5 μg proteins total) were denatured with 6 M of Guanidine-HCl, 0.1 M of Tris at pH 8.5. The proteins were reduced with 30 mM of DTT at 55°C for 1 h in darkness and then alkylated with 0.07% 4-vinylpyridine at room temperature for 2 h. The alkylation was quenched by the addition of excess DTT. The samples were dialyzed overnight with Slide-A-Lyzer cassettes (10 kDa MWCO, 0.5 mL; Thermo Fisher Scientific, Waltham, MA) against 25 mM of Tris at pH 8.5. After dialysis, the samples were split into three aliquots. Each aliquot was digested with trypsin at 1:25 or with Lys-C at 1:50

or with Asp-N at 1:100 enzyme: protein ratio (wt/wt) at 37°C for 18 h, respectively.

Peptide mapping

NanoLC/MS/MS peptide mapping. LC/MS/MS analysis for AAV2 was performed on a NanoAcquity HPLC system (Waters) in conjunction with an Orbitrap Velos mass spectrometer (Thermo Fisher Scientific). The separation was performed on a home-packed nanoLC column (75 μm × 10 cm) with a PicoFrit tip (New Objective, Woburn, MA) packed with Magic C18 material (5 μm, 100 Å; Bruker, Billerica, MA) at a flow rate of 300 nL/min. The peptides were eluted using a linear gradient from 2% to 60% B (0.1% formic acid in acetonitrile) over 121 min.

The source parameters for Velos were as follows: source voltage: 2.5 kV; capillary temperature 275°C; S-lens RF level: 55%. Data were acquired using a top 10 data-dependent method with accurate MS at 60,000 resolution and MS/MS in the ion trap.

The peptide identification was performed using Mascot for database searching against AAV2 viral capsid protein sequences with MS tolerance of 10 ppm and MS/MS tolerance of 0.8 Da.[48] N-terminal acetylation, methionine oxidation, and asparagine deamidation were included as variable modifications for the database search.

UPLC/MS^E peptide mapping. The protein digests were also analyzed by UPLC/MS^E in an Acquity UPLC-Xevo G2XS QTOF MS system (Waters). The separation was achieved using a BEH300 C18 column (2.1 mm × 150 mm) with a linear gradient from 2% to 55% B (0.1% formic acid in acetonitrile) over 48 min at a flow rate of 250 μL/min. For MS, the capillary voltage and sampling cone voltage were set 2.8 kV and 30 V, respectively. The mass spectra were acquired in the positive sensitivity MS^E mode in the m/z range of 200–2000.

RESULTS

Intact protein analysis

Intact protein analysis of AAV2 capsid proteins was initially performed using a UPLC BEH C4 column at a fast gradient of 1.7% B/min, as shown in Fig. 1A. Only VP3 was observed, while VP1 and

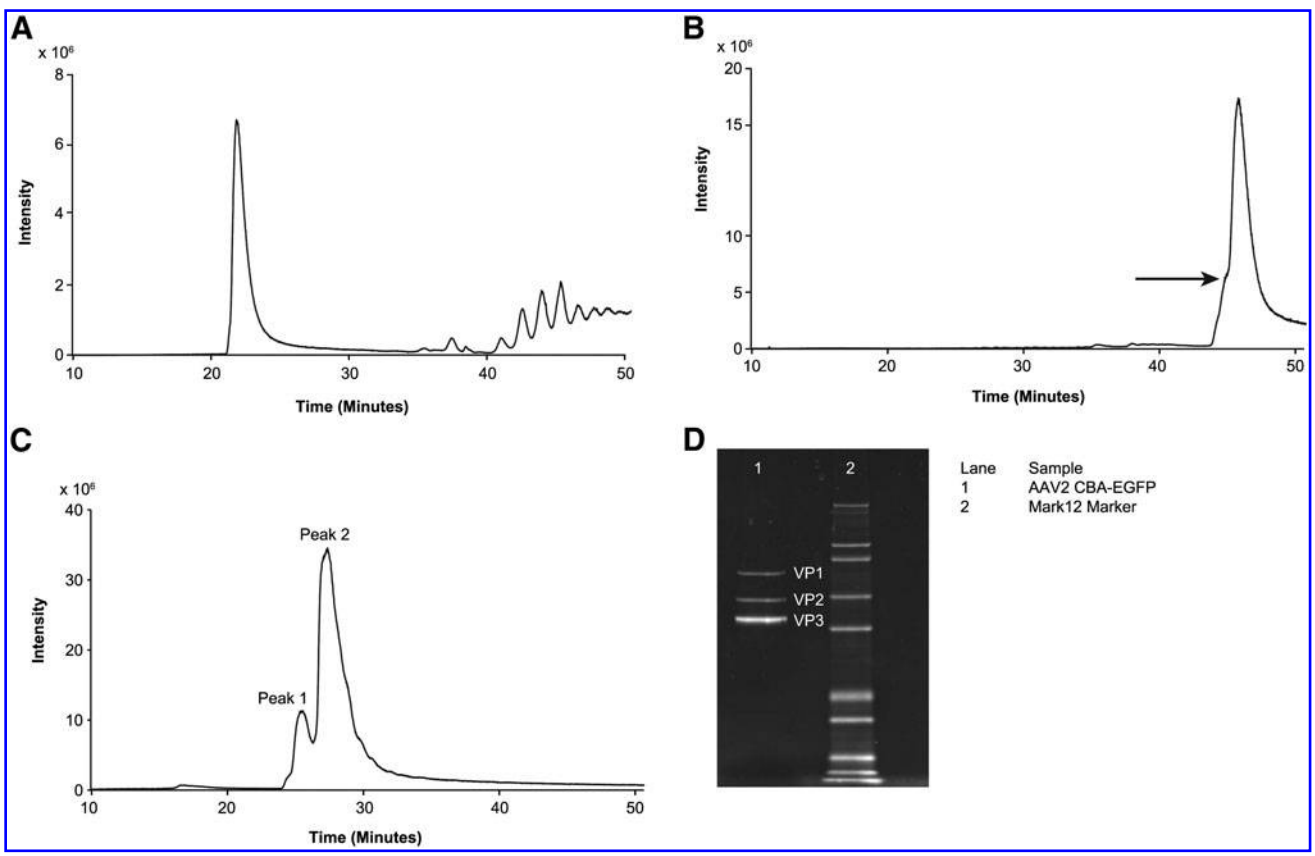

Figure 1. Liquid chromatography/mass spectrometry (LC/MS) total ion chromatograms of AAV2 capsid proteins (VPs) using: **(A)** a BEH C4 column (1.0 mm × 100 mm), **(B)** a BEH C4 column (1.0 mm × 100 mm), **(C)** a BEH C8 column (2.1 mm × 100 mm) with a 0.5% B/min gradient, and **(D)** SDS-PAGE of AAV2 CBA-EGFP.

VP2 were masked by the predominant and co-eluting VP3. The stoichiometry of VP1/VP2/VP3 is estimated as 1:1:10 by SDS-PAGE analysis, as illustrated in Fig. 1D. Increased sample loading ($\sim$1.7 μg of protein) with a change in gradient to 0.5% B/min resulted in a shoulder peak, shown with an arrow in Fig. 1B. The shoulder peak was further resolved from the main peak using a BEH C8 column, as shown in Fig. 1C. The VP1 and VP2 were detected in the shoulder peak at measurable signal intensities, as shown in Fig. 2B. Three major peaks were detected, with masses of 81,852 Da, 66,486 Da, and 59,971 Da, respectively. The measured mass of 66,486 Da matches the amino acid (aa) sequence 139–735 of VP2, starting at A139 right after the predicted N-terminus T138 from DNA sequence. The other two masses of 81,852 Da and 59,971 Da correspond to, respectively, the aa sequences 2–735 of VP1 and 204–735 of VP3, with a 42 Da mass shift, indicating one acetylation on each protein. The acetylation was suspected to be present at VP1 and VP3 N-termini because Edman sequencing of VP1 and VP3 bands directly from the gel was not successful prevoiusly.[15]

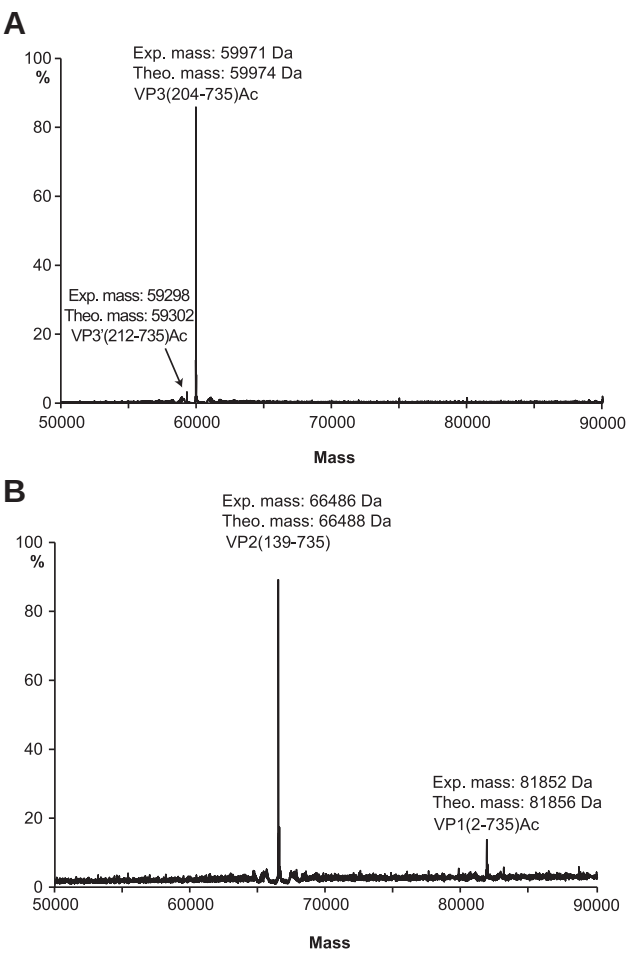

Figure 2. Deconvoluted mass spectra of AAV2 VPs separated in Fig. 1C. **(A)** Peak 2. **(B)** Peak 1. Ac, acetylation.

A minor peak with a mass of 59,302 Da was detected, as shown in Fig. 2A. The mass corresponds to a VP3 variant of aa 212–735, with +42 Da mass shift. The DNA sequence of AAV2 VP3 has two potential *ATG* initiation codons: *ACGATGGCTA CAGGCAGTGGCGCACCAATGGCAGAC*, resulting in two possible protein N-termini *M*ATGS GAP*M*AD. Since the first initiation codon has an optimal Kozak sequence (both A in –3 position and G in +4 position shown in italic relative to A as number 1 nucleotide in *ATG* codon),[49] the first initiation codon is favored and becomes the major N-terminus for VP3. The N-terminal methionine residues are not present in either VP1 or VP3, as measured by intact protein analysis. It is common that the first methionine residue is cleaved off after synthesis, and then the second amino acid residue becomes acetylated.[50]

Intact protein analysis also confirmed that there is no glycosylation in the viral capsid proteins, even though several N-linked glycosylation consensus sequences are present.[33] The AAV capsid proteins have five highly conserved cysteine residues. No evidence of disulfide linkages has been reported using either structural analysis of AAV capsids or by cysteine mutational analysis.[30,32,51–53] Although the experimental mass of AAV2 VP3 (59,974 Da) from intact protein analysis matches with the theoretical mass of VP3 without disulfide bonds, disulfide mapping by LC/MS/MS of native enzymatic digestion is needed to confirm cysteine status further in the AAV capsid proteins.

LC/MS/MS peptide mapping

The AAV2 capsid proteins were digested using a combination of trypsin, Lys-C, and Asp-N enzymes in order to achieve complete sequence coverage. The protein digests were analyzed with nanoLC/MS/MS and UPLC/MS^E methods. The two methods are complementary to ensure the fragmentation occurred at the desired peptide bond to locate the PTM at the specific amino acid residue. The MS/MS spectrum of a triply-charged ion m/z 721.68 is shown in Fig. 3A. The protein database search using the well-distributed b and y ion series identified the peptide as the N-terminal VP1 tryptic peptide AADGYLPDWLEDTLSEGIR plus 42 Da (Ac: Acetylation) mass shift. The fact that the y_{18} ion was not modified but b_3 ion carried 42 Da mass shift indicated that the N-terminal alanine of VP1 was acetylated and was responsible for the addition of 42 Da. The MS/MS of the doubly charged ion m/z

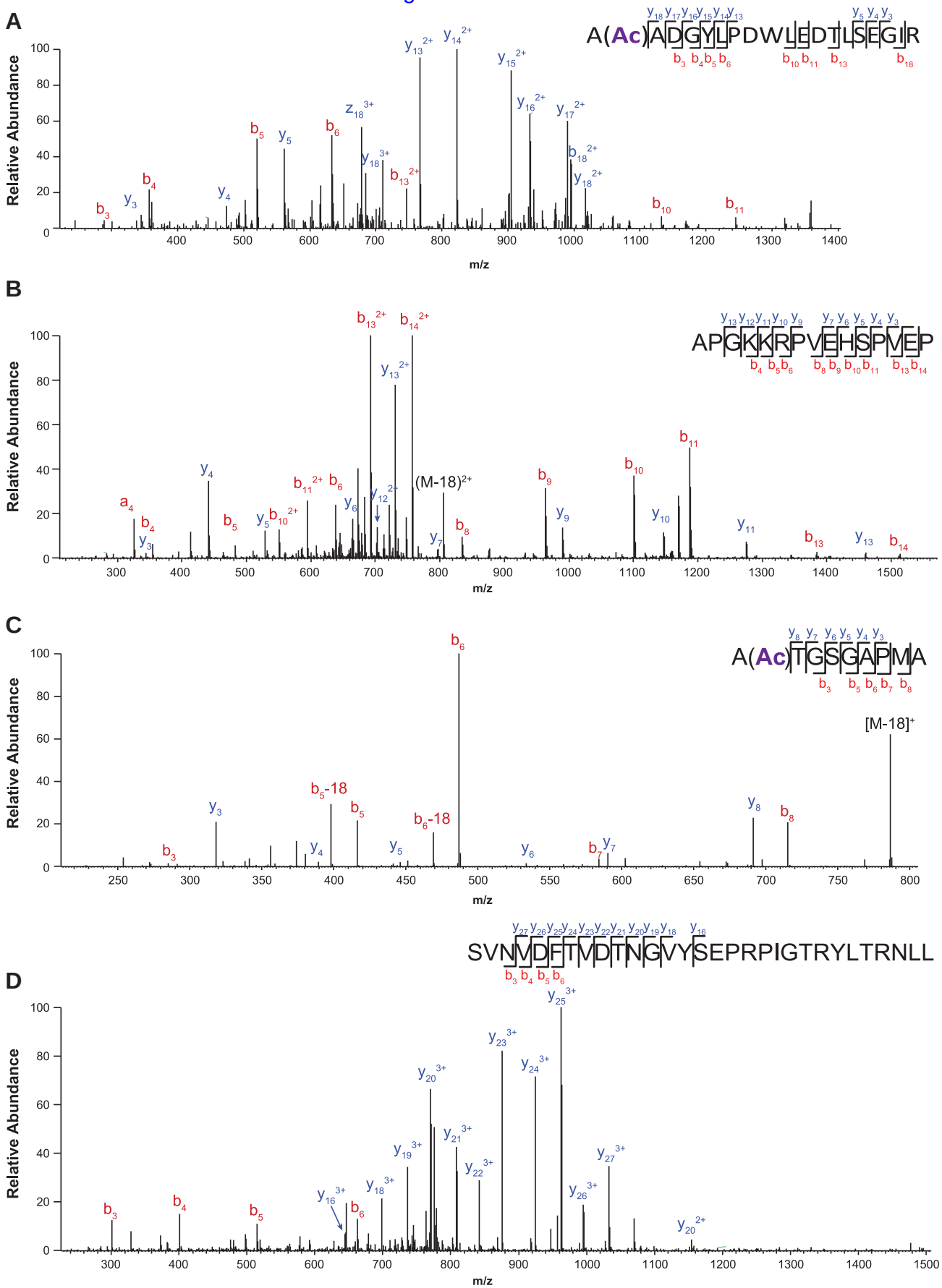

Figure 3. Tandem mass spectrometry (MS/MS) spectra of N- and C-terminal peptides of AAV2 VPs. **(A)** VP1 N-terminal tryptic peptide A(**Ac**)ADGYLPDWLEDTLSEGIR, **(B)** VP2 N-terminal Asp-N peptide APGKKRPVEHSPVEP, **(C)** VP3 N-terminal Asp-N peptide A(**Ac**)TGSGAPM, and **(D)** C-terminal Lys-C peptide SVNVDFTVDTNGVYSEPRPIGTRYLTRNL.

814.45, corresponding to a MW of 1626.88 Da with mass accuracy 0.6 ppm, as shown in Fig. 3B, gives unambiguous confirmation of the N-terminal Asp-N peptide APGKKRPVEHSPVEP of VP2. Figure 3C is MS/MS of the singly charged m/z 804.36, which was identified as N-terminal Asp-N peptide A(Ac)TGSGAPM with mass accuracy 0.6 ppm. Similar to N-terminal peptide of VP1, the location of acetylation at the N-terminal alanine residue of VP3 was identified with the presence of y_8 ion unmodified and b_3 ion modified with a +42 Da mass shift. The C-terminal Lys-C peptide

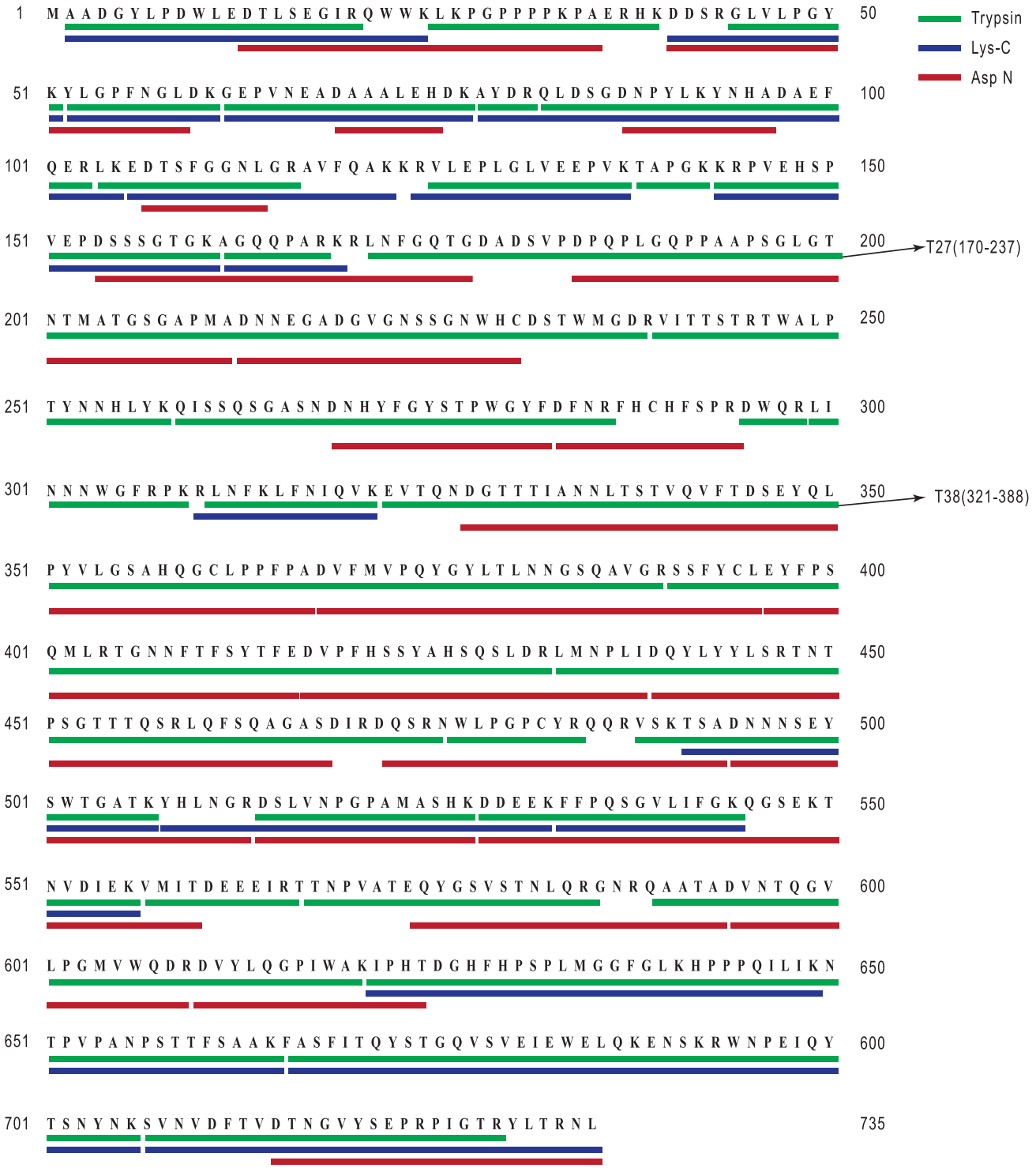

Figure 4. Sequence coverage of AAV2 VP1 by LC/MS/MS using different enzymatic digests. Green line, tryptic peptides; blue line, Lys-C peptides; red line, Asp-N peptides.

S^{707}VNVDFTVDTNGVYSEPRPIGTRYLTRNL of VPs was confirmed by the MS/MS spectrum of the quadruply-charged ion m/z 764.89 shown in Fig. 3D, in which the complete y_{16} to y_{27} ion series verified the N^{709}VDFTVDTNGV sequence. As a result, the peptide map by LC/MS/MS analysis has confirmed the N- and C-termini of VP1, VP2, and VP3 with acetylation on the N-termini of VP1 and VP3.

A Mascot search against AAV2 VP database using nanoLC/MS/MS data of tryptic peptides yielded 78% sequence coverage, as indicated by green lines in Fig. 4. The two large tryptic peptides—T27 (aa 170–237) and T38 (aa 321–388)—were not detected by nanoLC/MS/MS but were successfully detected in the UPLC/MS^E analysis with the same digests. Most of T27 and T38 amino acid sequences were also covered by nanoLC/MS/MS of Asp-N digests, as shown in red lines in Fig. 4. The N- and C-terminal peptides of VP1 were confirmed by Lys-C digests, as shown in blue lines in Fig. 4. Therefore, 100% sequence coverage of AAV2 VP1 was achieved through multiple enzymatic digestions and two LC/MS/MS methods.

Comparison of AAV VP N-termini of 13 AAV serotypes

Five other AAVs—AAV1, AAV5, AAV7, AAV9, and AAVRh10—were analyzed by intact protein analysis in the current study alongside AAV2. The experimental and predicted masses of VPs of the six AAV serotypes are summarized in Table 1. The mass differences between the predicted and corresponding detected values are no more than 5 Da, which are well within the assay variation. N-termini, as well as their PTM, were highly conserved among the AAV serotypes analyzed. The N-termini of all VPs of six serotypes, except VP3 of AAV7, start at one residue after the predicted N-termini based on DNA sequences. All VP1 and VP3 of six AAV serotypes contain one acetylation.

The N-terminal sequences of VPs from 12 AAV serotypes (AAV1–AAV12) and AAVRh10 were aligned in Fig. 5. Twelve amino acid residues AADGYLPDWLED at the N-terminal region of VP1 are highly conserved in 11/13 AAV serotypes. In AAV4, the two residues AA were replaced with one residue T, and the rest N-terminal sequence remains the same. VP1 of AAV5 has a unique N-terminus starting with SFVDHPPDWLED, confirmed by the intact protein analysis shown in Table 1. VP2 of all 12 AAV serotypes has an identical N-terminal sequence consensus starting at APGKK, with AAV5 starting at APTGK. The N-termini of VP3 are the most diverse comparing to VP1 and VP2. Seven AAV serotypes—AAV1, AAV2, AAV3, AAV6, AAV8, AAV10, and AAVRh10—have two potential *ATG* initiate codons, translating to the N-termini starting at $\underline{M}AX_{(S,T,A)}GX_{(G,S)}GAP\underline{M}AD$ sequence consensus. The DNA and amino acid sequences of VP3 N-terminus of AAV2 are illustrated as an example in Fig. 6A. The intact protein analysis showed the dominant peak series in the mass spectra corresponded to the protein with amino acid sequence initiated at the first ATG in AAV2 (Fig. 6B) and in AAV1 and AAVRh10 (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/hgtb). Interestingly, the two potential initiation codons for

Table 1. *Comparison of the experimental and predicted masses of capsid proteins (VPs) for AAV1, AAV2, AAV5, AAV7, AAV9, and AAVRh10*

Serotype	*Isoform*	*Predicted amino acid sequence*	*Actual amino acid sequence*	*Theoretical mass (Da)*	*Experimental mass (Da)*
AAV1	VP1	1–736	2(Ac)–736	81,286	81,287
	VP2	138–736	139–736	66,093	66,095
	VP3	203–736	204(Ac)–736	59,517	59,518
AAV2	VP1	1–735	2(Ac)–735	81,856	81,852
	VP2	138–735	139–735	66,488	66,486
	VP3	203–735	204(Ac)–735	59,974	59,971
AAV5	VP1	1–724	2(Ac)–724	80,336	80,336
	VP2	137–724	138–724	65,283	65,284
	VP3	193–724	194(Ac)–724	59,463	59,463
AAV7	VP1	1–737	2(Ac)–737	81,564	81,567
	VP2	138–737	139–737	66,372	66,374
	VP3	204–737	213(Ac)–737	59,101	59,103
AAV9	VP1	1–736	2(Ac)–736	81,291	81,288
	VP2	138–736	139–736	66,210	66,209
	VP3	203–736	204(Ac)–736	59,733	59,733
AAVRh10	VP1	1–738	2(Ac)–738	81,455	81,455
	VP2	138–738	139–738	66,253	66,252
	VP3	204–738	205(Ac)–738	59,634	59,634

Ac, acetylation.

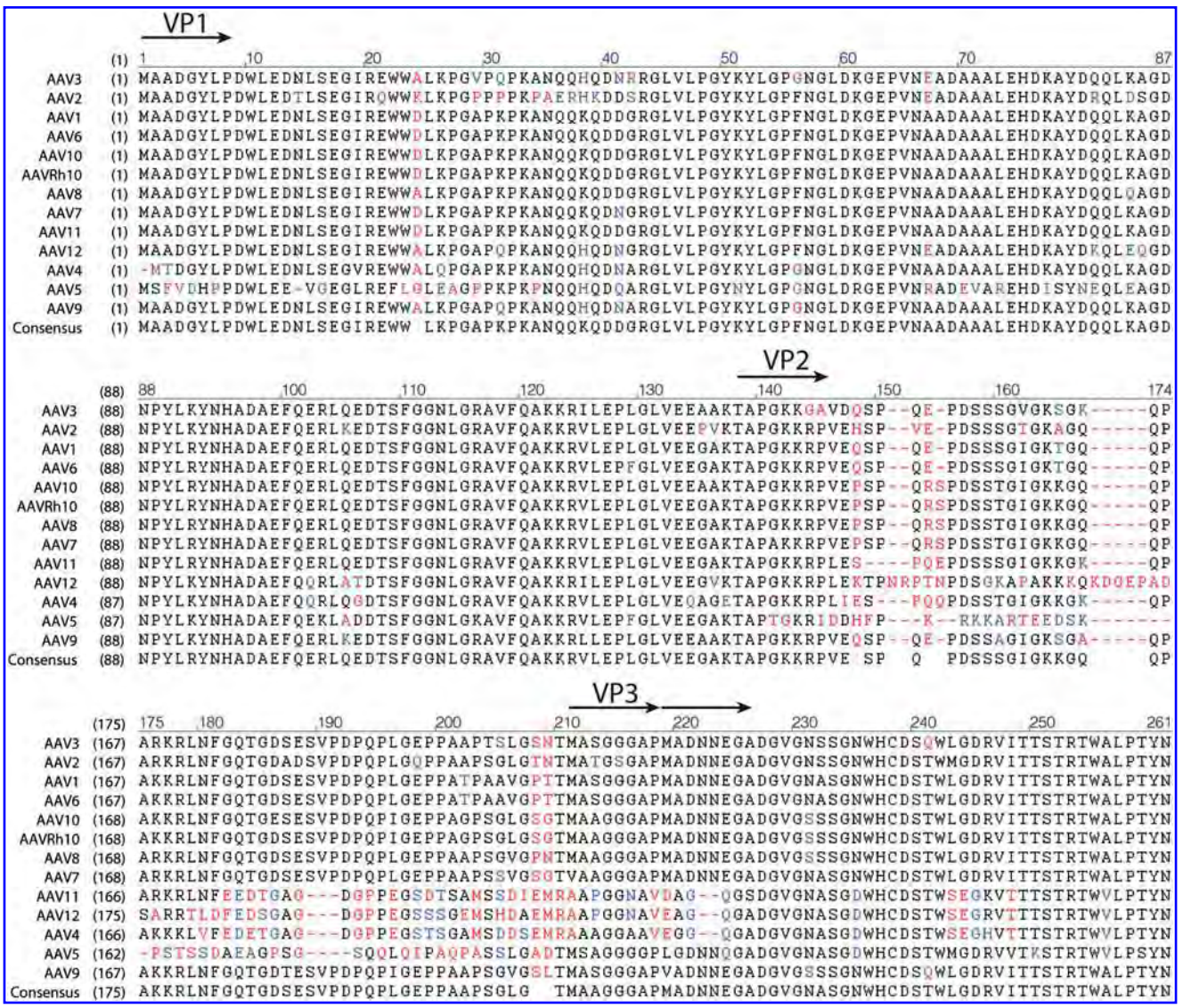

Figure 5. Sequence alignment of VP N-termini of 13 AAV serotypes. The amino acid residues in black are either identical, conservative, or block of similar according to sequence alignment; residues in red are non-similar, and residues in blue are weakly similar.

VP3 of AAV7 are GTG and ATG (Fig. 6A). In this case, the second initiation codon ATG is more favored; the VP3 starting at amino acid residue 213 is a predominant peak, while VP3 starting at amino acid residue 205 is a minor peak shown in Fig. 6B. In contrast the VP3 of AAV5 and AAV9 have only one peak in the intact protein mass spectra (data not shown). The VP3 sequence of AAV4, AAV11, and AAV12 were predicted to share the sequence consensus $\underline{M}$RAAX$_{(P,A)}$GG.

DISCUSSION

The cGMP production of clinical grade AAV vectors for gene therapy requires AAV vectors to be well characterized. The identity test of AAV serotype is one of the key characterization assays to ensure product safety and efficacy. The identity tests can be based on immunochemical, physicochemical, or biological properties of the product and should be highly specific. As mentioned before, the field is lacking a generic method to support capsid serotype identity testing. This study evaluated direct LC/MS intact protein analysis as a serotype identity test because it can provide accurate mass measurement of proteins and confirm protein sequence in a very short time <4 h, including sample preparation, LC/MS, and data analysis. The theoretical masses of VP1, VP2, and VP3 of each serotype are unique based on intact protein analysis of six serotypes, as shown in Table 1. The mass difference between VP1 of AAV1 and AAV9 is

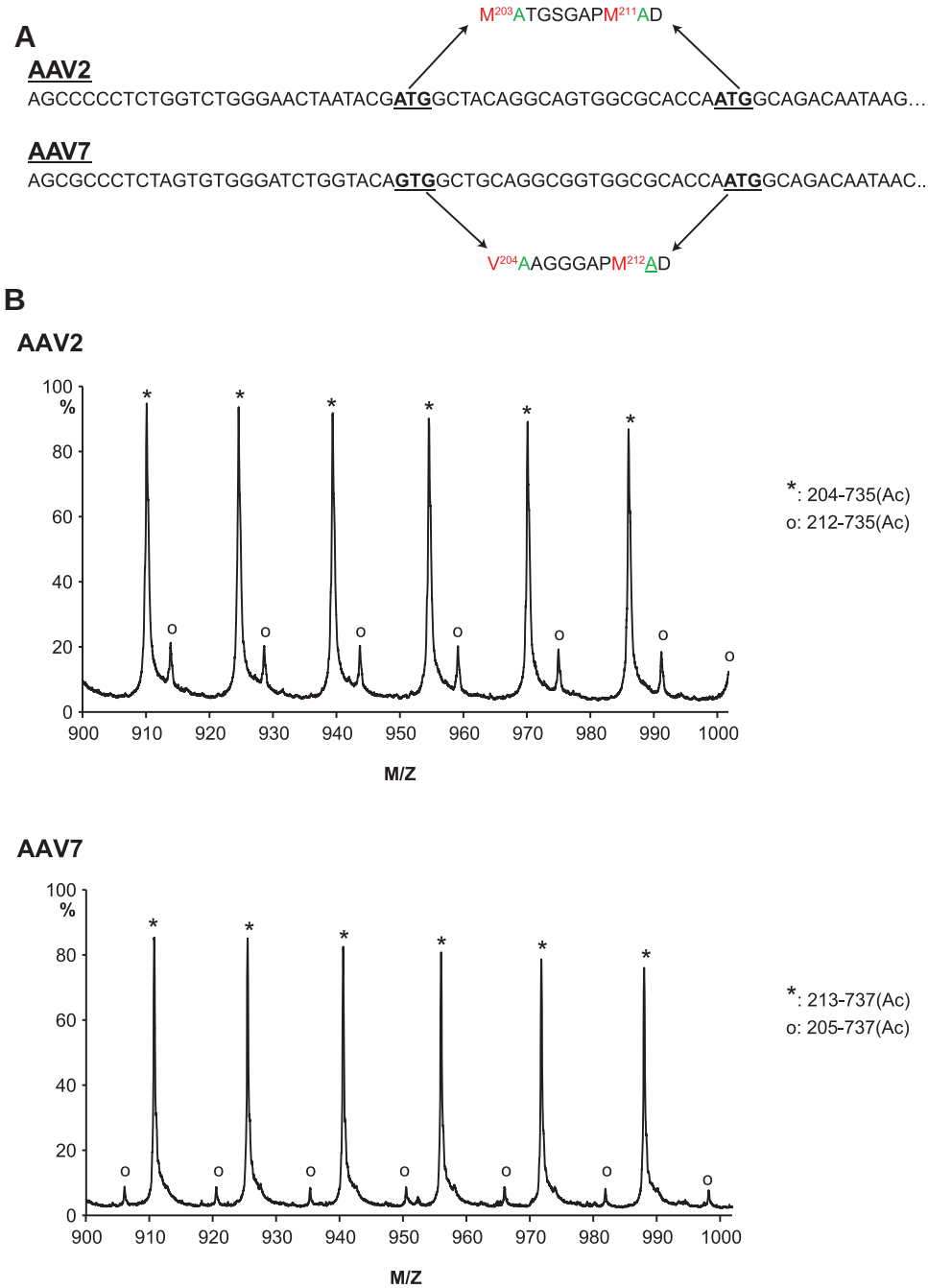

Figure 6. **(A)** DNA sequence alignment of AAV2 and AAV7 in VP3 N-terminal region. **(B)** Averaged mass spectra of VP3 with six charge states of AAV2 and AAV7 show the two peak series corresponding to VP3 with two different N-termini.

smallest (at 5 Da) among all pairs of predicted masses. The measured masses of VP1 for AAV1 and AAV9 are 81,287 and 81,288 Da, respectively, and cannot be differentiated with the current method. However, the measured masses of VP2 and VP3 for AAV1 are 66,095 and 59,518 Da, respectively, which are distinctly different from VP2 and VP3 for AAV9 at 66,209 and 59,733 Da, respectively. Therefore, the combination of VP1, VP2, and VP3 masses can be used to differentiate AAV serotypes unambiguously.

The theoretical masses of 13 AAV serotypes based on sequence alignment and intact protein analysis of six AAV serotypes were calculated in Supplementary Table S1. Table 2 shows the mass differences of VPs among 13 AAV serotypes. Pairs with a mass difference <10 Da are shown in bold. Upon theoretical mass analysis of 13 AAV serotypes, it is

Table 2. *Mass differences of VPs among 13 AAV serotypes*

	AAV1											
AAV2	570	AAV2										
AAV3	285	285	AAV3							VP1		
AAV4	736	1306	1021	AAV4								
AAV5	950	1520	1235	215	AAV5							
AAV6	36	534	249	772	987	AAV6						
AAV7	277	292	**8**	1013	1228	241	AAV7					
AAV8	381	189	96	1117	1332	345	104	AAV8				
AAV9	**5**	565	280	741	955	31	272	376	AAV9			
AAV10	191	379	94	927	1142	155	86	190	186	AAV10		
AAV11	299	869	584	436	651	335	577	681	304	490	AAV11	
AAV12	820	250	535	1555	1770	784	542	439	815	629	1119	AAV12
AAVRh10	169	401	116	905	1119	133	109	212	164	22	468	651
	AAV1											
AAV2	395	AAV2										
AAV3	226	169	AAV3							VP2		
AAV4	467	862	693	AAV4								
AAV5	810	1205	1036	343	AAV5							
AAV6	**2**	392	224	470	812	AAV6						
AAV7	278	116	52	746	1088	276	AAV7					
AAV8	425	31	199	893	1235	423	147	AAV8				
AAV9	117	278	109	584	927	115	161	308	AAV9			
AAV10	177	217	49	645	987	175	101	248	60	AAV10		
AAV11	299	694	525	168	511	301	578	725	416	476	AAV11	
AAV12	812	417	586	1279	1622	810	533	386	695	635	1111	AAV12
AAVRh10	160	235	66	627	970	157	119	266	43	18	459	652
	AAV1											
AAV2	457	AAV2										
AAV3	332	125	AAV3							VP3		
AAV4	12	445	320	AAV4								
AAV5	54	511	386	66	AAV5							
AAV6	**2**	455	330	**10**	56	AAV6						
AAV7	416	873	748	428	362	418	AAV7					
AAV8	288	169	44	276	342	286	704	AAV8				
AAV9	216	241	116	204	270	214	632	72	AAV9			
AAV10	121	336	211	109	175	119	537	167	95	AAV10		
AAV11	179	278	153	167	233	177	595	109	37	58	AAV11	
AAV12	329	128	**3**	317	383	327	745	41	113	208	150	AAV12
AAVRh10	117	340	215	105	171	115	533	171	99	**4**	62	212

Pairs with a mass difference <10 Da are shown in bold.

notable that most of the time, single VP mass can sufficiently differentiate AAV serotypes. For VP3, only 4/78 pairs have mass differences within 10 Da, while 1/78 pairs for VP2 and 2/78 pairs for VP1 have mass differences within 10 Da between two serotypes. No single pair of two AAV serotypes has delta masses <10 Da for all three VPs. AAV1 and AAV6 have high sequence homology, with only 6/735 amino acid residues being different in VP1. Even though there is only a 2 Da mass differences for both VP2 and VP3 between AAV1 and AAV6, the mass difference of VP1 between the two serotypes is 36 Da, large enough to be easily distinguished by accurate mass measurement. Therefore, the combination of mass measurement of intact VP1, VP2, and VP3 proteins is highly specific as an identity test.

The method has its limitations. When there is a contamination of a capsid from a different, known or unknown, serotype or a known serotype with unknown PTMs, intact protein analysis alone may not be sufficient to detect or identify the contaminant. Even though the stoichiometry of VPs can be variable, the commonly estimated ratio of VP1:VP2:VP3 is 1:1:10 by SDS-PAGE analysis. The contaminant may not be detected if (1) the minor contaminant only has distinct mass differences on VP1 and VP2 with the major capsid, and (2) the VP1 and VP2 of the minor contaminant coelute with the VP3 of the major capsid on the chromatogram. In the case of an unknown contaminant, its identity can only be verified by combining with peptide mapping using LC/MS/MS.

Although N-terminal acetylation of proteins is a widely known phenomenon, the biological significance of N-terminal acetylation of viral capsid proteins is not well understood. The predicted N-termini of VP1 and VP3, based on the DNA

sequences, are methionine residues followed mainly by alanine residues. It has been reported that removal of N-terminal methionine by Met-aminopeptidases frequently leads to acetylation on following alanine, valine, serine, threonine, and cysteine residues. The acetylation of the N-terminus acts as a potential degradation signal or ubiquitination site.[50] Ubiquitination of viral capsid proteins was suggested as a signal for processing of the capsid at the time of virion disassembly.[54] The link between N-terminal acetylation of VP1 and VP3 and viral capsid degradation and uncoating before nuclear entry warrants further investigation.

In conclusion, LC/MS intact protein analysis and LC/MS/MS peptide mapping can be used to profile viral capsid proteins and monitor their modifications and expression. The direct assessment of the viral capsid protein ensures product consistency during AAV vector cGMP manufacturing. Additionally, the analytical methods described herein can be used to confirm site-directed mutagenesis of the capsid proteins and are therefore important tools to support the design of more potent capsids with increased tissue specificity.

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No competing financial interests exist.

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Exhibit X

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May 1, 2025

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Re: Elevidys® Patent Infringement

Dear Mr. Berdon and Mr. Livingstone,

I write on behalf of Genzyme Corporation ("Genzyme") as a follow up to John Conway's July 26, 2024 letter, my October 31, 2024 letter, and my March 12, 2025 letter in relation to the manufacture and sale of Elevidys® (delandistrogene moxeparvovec-rokl) by Sarepta Therapeutics, Inc. and Sarepta Therapeutics Three, LLC's (collectively, "Sarepta").

As previously noted, having examined the publicly available information regarding Elevidys®, we have concerns that Sarepta is infringing Genzyme's intellectual property by the manufacture and sale of Elevidys®.

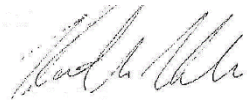
In addition to the patents identified in my March 12, 2025 letter, Sarepta also appears to infringe U.S. Patent Application No. 19/013,863, which published today and will issue as U.S. Patent No. 12,298,313 on May 13, 2025, through its manufacture, use, sale, and/or offer to sell Elevidys®.

Dechert LLP

Genzyme remains willing to license Genzyme's patents covering Elevidys® on fair and reasonable terms, but cannot tolerate continued infringement of the Genzyme patent estate. Please advise whether Sarepta is interested in discussing a license.

We look forward to your prompt reply.

Regards,



Katherine A. Helm, J.D., Ph.D.