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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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TITLE OF THE INVENTION (500 characters max):

Compositions and Methods to Prevent AAV Vector Aggregation

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ENCLOSED APPLICATION PARTS *(check all that apply)*

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Application Size Fee: If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

METHOD OF PAYMENT OF FILING FEES AND APPLICATION SIZE FEE FOR THIS PROVISIONAL APPLICATION FOR PATENT

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COMPOSITIONS AND METHODS TO PREVENT AAV VECTOR AGGREGATION

FIELD OF THE INVENTION

The present invention relates to compositions and methods of preparing and storing AAV virions that prevent aggregation.

BACKGROUND

Recombinant adeno-associated virus (rAAV) is a promising vector for human gene transfer [1-3]. AAV is a member of the Dependovirus genus of the parvoviruses. AAV serotype 2 (AAV2) is composed of a single-strand DNA molecule of 4680 nucleotides encoding replication (*rep*) and encapsidation (*cap*) genes flanked by inverted terminal repeat (ITR) sequences [4]. The genome is packaged by three capsid proteins (VP1, VP2 and VP3), which are amino-terminal variants of the *cap* gene product. The resulting icosahedral virus particle has a diameter of ~26 nm. A high resolution crystal structure of AAV2 has been reported [5].

The solubility of purified AAV2 virus particles is limited, and aggregation of AAV2 particles has been described as a problem [6-9]. In commonly used buffered-saline solutions, significant aggregation occurs at concentrations of 10^{13} particles/mL, and aggregation increases at higher concentrations. Huang and co-workers reported that AAV vectors undergo concentration-dependent aggregation [7]. Xie and coworkers [9] similarly reported that at concentrations exceeding 0.1mg/mL, AAV2 vectors require elevated concentrations of salt to prevent aggregation. Aggregation of AAV2 vectors occurs at particle concentrations exceeding 10^{13} particles/mL in commonly used neutral-buffered solutions such as phosphate- and Tris-buffered saline. This corresponds to a protein concentration of ~0.06 mg/mL, and emphasizes

the low solubility of AAV2 under these conditions. The effective vector concentration limit may be even lower for vectors purified using column chromatography techniques because excess empty capsids are co-purified and contribute to particle concentration.

Particle aggregation is a significant and not fully resolved issue for adenovirus vectors as well. Stability of a recently established adenovirus reference material (ARM) was recently reported [21]. Aggregation of the reference material, formulated in 20mM Tris, 25 mM NaCl, and 2.5% glycerol at pH 8.0, was assessed by dynamic light scattering, photon correlation spectroscopy and visual appearance. A variable level of vector aggregation following either freeze-thaw cycling or non-frozen storage was observed, resulting in restrictive protocols for the use of the ARM.

Aggregation can lead to losses during purification and inconsistencies in testing of purified vector preparations. The *in vivo* administration of AAV2 vectors to certain sites, such as the central nervous system, may require small volumes of highly concentrated vector, and the maximum achievable dose may be limited by low vector solubility.

Vector aggregation is also likely to influence biodistribution following *in vivo* administration, and cause adverse immune responses to vectors following their administration. As has been reported for proteins [10], aggregation of vector may increase immunogenicity by targeting the vector to antigen presenting cells, and inducing enhanced immune responses to the capsid proteins and transgene product. The reports of immune responses to AAV vectors in pre-clinical [11-13] and clinical [14] studies illustrate the need to address all factors that may contribute to vector immunogenicity.

Testing protocols to characterize purified vectors are also likely to be affected by vector aggregation. Determination of the infectivity titer of vector was reported to be highly sensitive

to vector aggregation [15]. An important concern is that vector aggregates may have deleterious consequences following their *in vivo* administration because their transduction efficiency, biodistribution and immunogenicity may differ from monomeric particles. For example, intravascular delivery of AAV vectors to hepatocytes requires that the vectors pass through the fenestrated endothelial cell lining of hepatic sinusoids. These fenestrations have a radius ranging from 50 to 150 nm [20] that is predicted to allow the passage of monomeric AAV vectors (diameter ~26 nm), but prevent the passage of larger vector aggregates. In biodistribution studies in mice, aggregated AAV2 vectors labeled with the fluorescent molecule Cy3 were sequestered in liver macrophages following vascular delivery [7].

Formulation development for virus-based gene transfer vectors is a relatively recent area of investigation, and only a few studies have been reported describing systematic efforts to optimize AAV vector formulation and stability [6,8,9]. Defining formulations compatible with pre-clinical and clinical applications that minimize changes in vector preparations is an important requirement to achieve consistently high vector safety and functional characteristics. As is well established for protein therapeutics [16-19], an important aspect of vector stability is solubility during preparation and storage, and vector aggregation is a problem that needs to be fully addressed. Vector aggregation leads to losses during vector purification, and while aggregates can be removed by filtration, the loss in yield results in higher costs and capacity limitations when producing vector for pre-clinical and clinical studies. Even after filtration to remove aggregates, new aggregates can form in concentrated preparations of AAV2 vector in buffered-saline solutions.

The need exists for improved formulations and methods for purification and storage of AAV vectors, such as rAAV2, that prevent aggregation of virus particles.

SUMMARY OF THE INVENTION

These and other needs in the art are met by the present invention, which provides high ionic strength solutions for use in preparing and storing AAV vectors that maintain high infectivity titer and transduction efficiency, even after freeze-thaw cycles.

The effect of ionic strength (μ) on virus particle interactions is determined to elucidate the mechanism of vector aggregation. The ionic strength of neutral-buffered isotonic saline (μ = 150mM) is insufficient to prevent aggregation of AAV2 vectors purified by gradient ultracentrifugation or by cation exchange chromatography at concentrations exceeding ~10^{13} particles/mL. Inclusion of sugars (sorbitol, sucrose, mannitol, trehalose, glycerol) at concentrations up to 5% (w/v) or of surfactants Tween80 (1%) or Pluronic® F68 (10%) does not prevent aggregation of vector particles.

In contrast, vector particles remain soluble when elevated ionic strength solutions (μ > 200mM) are used during purification and for final vector formulation. Elevated ionic strength solutions using isotonic excipient concentrations for *in vivo* administration are prepared with salts of multivalent ions, including sodium citrate, sodium phosphate, and magnesium sulfate. An isotonic formulation containing 10mM Tris, 100mM sodium citrate, 0.001% Pluronic® F68, pH 8.0 (μ ~500mM) enables concentration of AAV2-AADC vectors to 6.4x10^{13} vg/mL with no aggregation observed during preparation and following ten freeze-thaw cycles at -20 °C. *See* Table 3, below, and accompanying discussion. AAV2-AADC vectors prepared and stored for an extended period in elevated ionic strength formulation retain high infectivity titer (13 IU/vg) and transduction efficiency.

Nuclease treatment of purified AAV2 vectors reduces the degree of vector aggregation, implicating vector surface nucleic acid impurities in inter-particle interactions. Hence,

purification methods to efficiently remove vector surface residual nucleic acids, coupled with the use of elevated ionic strength isotonic formulations, are useful methods to prevent AAV2 vector aggregation.

In one embodiment the invention comprises AAV storage solutions with ionic strength (μ) greater than 200 mM. In other embodiments the ionic strength is 300, 400 or 500 mM or higher.

In some embodiments of the present invention, the surfactant Pluronic® F68 is added to a solution for preparing or storing AAV vectors, for example to 0.001%. In one embodiment, the solution comprises 10 mM Tris, 100mM sodium citrate and 0.001% Pluronic® F68, pH 8.0.

In one embodiment, AAV vectors can be stored in solutions of the present invention at concentrations up to $6.4x10^{13}$ vg/mL with no aggregation even after ten freeze-thaw cycles at -20°C.

In another aspect, the invention relates to methods of preparing and storing preparations of AAV vectors using high ionic strength solutions. In some embodiments, 0.001% Pluronic® F68 is included in the high ionic strength solutions.

In some embodiments of the present invention, purified AAV vectors are treated with one or more nucleases to reduce aggregation. In one embodiment such nuclease treatment is combined with storage in a high ionic-strength solution of the invention.

In yet another aspect, the invention relates to kits comprising the high ionic strength formulations of the invention. In one embodiment the kits comprise pre-mixed solution. In another embodiment the kit comprises two or more separate components of a high ionic strength solution of the present invention to be mixed by a user. In some embodiments the kit comprises one or more of the following: sodium citrate, sodium phosphate, magnesium sulfate, Tris and

Pluronic® F68. In other embodiments the kit further comprises a recipe for mixing a solution of the present invention.

DESCRIPTION OF THE DRAWINGS

FIGS. 1A and **1B** present data showing aggregation of AAV2-FIX particles as a function of osmolarity (FIG. 1A) or ionic strength (FIG. 1B) for various buffer compositions. AAV2-FIX vectors are prepared by Method 2 of Example 1. Average particle radius is measured by dynamic light scattering (DLS) following vector dilution in varying concentrations of excipients buffered with 10 mM sodium phosphate at pH 7.5. Excipients include sodium chloride (●), sodium citrate (○), sodium phosphate (■), sodium sulfate (□), magnesium sulfate (▲), and glycerol (Δ).

FIG. 2 presents data on AAV2-FIX aggregation as a function of the method of purification. The average particle radius is measured by DLS following vector dilution in varying concentrations of sodium chloride buffered with10mM sodium phosphate at pH 7.5. Vectors are purified by Method 1 (double CsCl gradient) (○); Method 2 (cation exchange chromatography) (□); Method 2 plus nuclease digestion (■); or Method 3 (chromatography plus one CsCl gradient) (Δ). Purification Methods 1-3 are described in Example 1.

FIG. 3 presents data on transgene expression from D7/4 cells transduced with rAAV2-AADC virions prepared and stored in high ionic strength formulation (□) or in a control formulation (●). The concentration of AADC was measured by ELISA (in triplicate for each data point) 72 hours post-transduction. Error bars represent standard deviations.

DETAILED DESCRIPTION OF THE INVENTION

AAV2 vector aggregation is frequently observed in concentrated preparations of vectors and can affect purification recovery, and *in vivo* potency and safety. Hence, an important objective for the development AAV2 vectors is to identify methods and formulations that prevent aggregation of vectors when concentrated stocks are prepared. Unless otherwise indicated, the term "vector" as used herein refers to a recombinant AAV virion, or viral particle.

The present invention is based in part on the observation that solution ionic strength is an important parameter in AAV vector aggregation, implicating the involvement of ionic interactions between virus particles in the aggregation process. The observation that elevated ionic strength increases AAV2 vector solubility regardless of the identity of the charged excipient supports the hypothesis that ionic strength of solution *per se*, rather than interactions involving a specific ionic species, is the relevant physico-chemical parameter. A threshold ionic strength of at least 200mM is required to prevent aggregation at vector particle concentrations examined herein.

Of practical concern, commonly used buffered saline solutions have insufficient ionic strength to prevent AAV2 vector aggregation at concentrations exceeding 10^{13} particles/mL. It is known that high salt concentrations increase AAV2 vector solubility (e.g. highly concentrated AAV2 vectors recovered from gradients generally remain soluble in concentrated CsCl). However, optimal formulations for pre-clinical and clinical studies should be close to isotonic (280-400 mOsm), especially for *in vivo* administration of vector to sites where dilution of hypertonic solutions may be slow. In embodiments of the present invention the exponential relationship of ionic strength with charge valency is used to develop isotonic formulations with high ionic strengths. Salt species with multiple charge valencies (e.g. salts of sulfate, citrate, and

phosphate) that are commonly used as excipients in human parenteral formulations [22] can provide the level of ionic strength needed to prevent AAV2 vector aggregation when used at isotonic concentrations. While isotonic (150mM) sodium chloride has an ionic strength of 150mM, a value insufficient to maintain AAV2 solubility at high vector concentrations, isotonic sodium citrate, with an ionic strength of ~500mM, can support AAV2 vector concentrations of at least 6.4 x 10^{13} vg/mL without aggregation.

Without intending to be limited by theory, the low solubility of AAV2 particles may be caused by their highly symmetrical nature in conjunction with the stabilizing effect of complementary charged regions between neighbouring particles in aggregates. The surface charge density based on the crystal structure of AAV2 [5] reveals a pattern of positive and negative charges on the virus surface. Previous reports showed that AAV2 vector aggregation is pH dependent, and hypothesized that amino acids with charged side groups are involved in inter-particle binding [23]. These reports hypothesized that if charged amino acid side chains are involved in vector aggregation, high concentrations of free amino acids could block vector particle interactions. However, we have found that amino acids with charged side chains are not effective in preventing AAV2 vector aggregation beyond their contribution to ionic strength.

Vector aggregation at low ionic strength was also found to be reduced but not prevented by efficient nuclease treatment of purified vector particles. Digestion at an earlier stage of the purification process (clarified HEK cell lysate) did not reduce aggregation following vector purification. It is likely that digestion of already purified virions is more efficient because of a higher enzyme to nucleic acid substrate ratio. One mechanism to explain these results is that residual nucleic acid impurities (e.g. host cell and plasmid DNA) bound to the vector surface can bridge to binding sites on neighbouring virus particles and thus cause aggregation. Purified

AAV2 vectors (empty capsid free) have been reported to contain approximately 1% non-vector DNA [24]. While >50% of this non-vector DNA was reported to be nuclease resistant and was packaged within capsid particles, some impurity DNA was nuclease resistant and appeared to be associated with the surface of purified vector particles. The observation that efficient nuclease treatment can reduce vector aggregation suggests that nucleic acids associated with the vector surface at an average level not greater than ~25 nucleotides per vector particle can contribute to AAV vector aggregation.

In summary, the use of high ionic strength solutions during AAV2 vector purification and final formulation, and efficient removal of residual vector surface DNA are two effective strategies to achieve highly concentrated solutions of AAV2 vectors for use in pre-clinical and clinical studies. High ionic strength solutions and nuclease treatment can be used in combination or separately. Although data were obtained using AAV2 vectors, the composition and methods of the present invention may also be useful with other AAV serotypes / variants, or other viral vectors such as adenoviruses, lentiviruses and retroviruses.

AAV Aggregation as a Function of Excipient Concentration

Initial screening experiments are performed to elucidate the mechanism of AAV vector aggregation and to identify classes of excipients that can reduce / prevent aggregation. Vector aggregation can be caused by dilution (5-fold) of vector in neutral-buffered saline with low concentration buffer (20mM sodium phosphate, pH 7.2). Excipients are screened using this "dilution-stress" method to identify excipients that are able to prevent vector aggregation when included in the diluent. For screening, aggregation is measured by dynamic light scattering

(DLS). Classes of excipients examined included selected inorganic salts, amino acids, uncharged carbohydrates, and surfactants. Results are presented in Table 1.

TABLE 1

SCREENING FOR EXCIPIENTS THAT PREVENT AAV2 VECTOR AGGREGATION USING DILUTION-STRESS METHOD

Excipient	Osm required to prevent aggregation (max tested)
Magnesium sulfate	180 mOsm
Sodium citrate	220 mOsm
Sodium chloride	320 mOsm
Sodium phosphate	220 mOsm
Sodium sulfate	220 mOsm
Arginine	NIA (200 mOsm)
Aspartic acid	320 mOsm
Glutamic acid	320 mOsm
Glycine	NIA (200 mOsm)
Histidine	NIA (200 mOsm)
Lysine	300 mOsm
Glycerol	NIA (5% w/v, 543 mOsm)
Iodixanol	NIA (5% w/v, 32 mOsm)
Mannitol	NIA (5% w/v, 275 mOsm)
Sorbitol	NIA (5% w/v, 275 mOsm)
Sucrose	NIA (5% w/v, 146 mOsm)
Trehalose	NIA (5% w/v, 146 mOsm)
Pluronic® F68	NIA (10% w/v, 12 mOsm)
Polysorbate 80	NIA (1% w/v)

NIA: No inhibition of aggregation

As illustrated in Table 1, charged excipients (inorganic salts and amino acids) prevent aggregation when present at sufficient concentrations. However, salt concentrations required to prevent vector aggregation vary, ranging from 180 mOsm for magnesium sulfate, to 320 mOsm for sodium chloride. The amino acids arginine, aspartic acid, glutamic acid, glycine, histidine, and lysine do not prevent aggregation at 200 mOsm, but lysine, aspartic acid, and glutamic acid prevent aggregation at 300-320 mOsm. Arginine, glycine and histidine were not tested at concentrations other than 200 mOsm. Selected carbohydrates have no effect on vector particle aggregation when present at concentrations up to 5% w/v. For example, 5% w/v glycerol (543 mOsm) does not prevent aggregation. The surfactants Polysorbate80 (1% w/v) and Pluronic® F68 (10% w/v) similarly have no effect on aggregation using the "dilution-stress" method.

AAV Aggregation as a Function of Osmolarity and Ionic Strength

FIGS. 1A and 1B show the results of a more detailed analysis of vector aggregation as a function of the concentration of various salts. FIG. 1A shows vector aggregation as a function of the osmolarity of selected excipients. For charged species a concentration-dependent inhibition of AAV2 vector aggregation is observed. Salts with multivalent ions achieve a similar degree of inhibition of aggregation at lower concentrations than monovalent sodium chloride. For example, magnesium sulfate prevents aggregation at ≥200 mOsm whereas sodium chloride requires ≥350 mOsm to achieve a similar effect. Sodium citrate, sodium sulfate, and sodium phosphate are intermediate in their potency to prevent vector aggregation.

Although the results in FIG. 1A and Table 1 show no effect of glycerol and certain sugars at concentrations up to 5% on AAV2 vector aggregation induced by low ionic strength, the data

cannot rule out improvement of AAV2 solubility at glycerol concentrations above 5%. For example, Xie and co-workers reported that 25% (w/v) glycerol enabled concentration of AAV2 to very high concentrations (4.4 to 18 x 10^{14} particles/ml) in low ionic strength solutions [9].

FIG. 1B shows the data of FIG. 1A plotted as a function of the calculated ionic strength, rather than osmolarity, for each excipient. FIG. 1B demonstrates that vector aggregation is prevented when ionic strength is ~200 mM or greater regardless of which salt is used. These data suggested that the ionic strength (μ) of a solution, a parameter that depends on both solute concentration and charge valency, is the primary factor affecting aggregation.

Other ionic strengths may also be used in embodiments of the present invention to prevent aggregation, such as 250 mM, 300 mM, 350 mM, 400 mM, 450 mM, 500 mM, 600 mM, 700 mM or higher ionic strengths. Multivalent ions are preferred to achieve these ionic strengths in methods and formulations of the present invention, such as divalent, trivalent, tetravalent, pentavalent ions and ions of even higher valency. The pH buffer in solutions and formulations of the present invention may be phosphate, Tris, or HEPES (or other Good's buffers), but any other suitable pH buffer may be used. In preferred embodiments, the multivalent ions and buffer are selected to be compatible with the target tissue for the vector being prepared.

AAV Aggregation as a Function of the Method of AAV Purification

Recombinant AAV2 purified using different methods (e.g. density gradient purification versus ion-exchange chromatography) would be expected to have different impurity profiles. FIG. 2 shows vector aggregation as a function of ionic strength for several preparations of AAV differing in the purification method. Purification methods are described in Example 1. Sodium chloride is used to vary the ionic strength. AAV2-FIX vectors purified by double cesium

chloride gradient ultracentrifugation (Method 1), by cation exchange column chromatography (Method 2), or by combined column and cesium chloride gradient ultracentrifugation (Method 3) each demonstrate similar aggregation responses as ionic strength is decreased. In contrast, AAV2-FIX purified by the column method and then subjected to a nuclease digestion step (Method 2 + nuclease) shows reduced aggregation at low ionic strength.

AAV Aggregation at Preparative Scale

The data in Table 1 and FIGS. 1A, 1B and 2 involve vector aggregation *at an analytical scale*, employing a method to measure aggregation (DLS) that is difficult to quantify reliably. Table 2, in contrast, shows the effects of elevated ionic strength and nuclease treatment on AAV2 vector aggregation at a larger scale, using methods to induce and quantify vector aggregation that are relevant to *preparative scale* vector purification. Experimental details are provided in Example 2. Purified AAV vectors are diafiltered into solutions of various ionic strengths, the volume is reduced to achieve high vector concentrations, and aggregation is then assessed by measuring vector recovery after filtration through a 0.22μm filter. Aliquots from a single pool of AAV2-AADC vector purified by Method 1 through the second CsCl gradient centrifugation step ($1.8x10^{15}$ vg in 91mL, $1.8x10^{13}$ vg/mL, in ~3M CsCl) are used as starting material in the diafiltration experiments. Tangential flow filtration using hollow fibers is used for diafiltration because it is scalable and yet it still enables preparation of volumes (min. 1.4mL), and thus AAV concentrations, at which aggregation would be expected in neutral buffered saline.

In Experiment 1, three hollow fiber units are used to diafilter AAV2-AADC vector in formulations CF, TF1, or TF2, and the volume is reduced to a target of $2.5x10^{13}$ vg/mL. *See*

Example 2. The samples are then filtered through a 0.22μm filter. Results are shown in Table 2. Vector recovery ("Yield %") for both elevated ionic strength formulations TF1 (95 ± 7.4%) and TF2 (93 ± 7.4%) are significantly higher than the recovery using the control formulation CF (77 ± 6.6%).

TABLE 2
AAV VECTOR RECOVERY AT PROCESS SCALE

Experiment	Formulation	μ (mM)	Target (vg/mL)	Actual (vg/mL)	Yield % (RSD)
1	CF	160	2.5E13	1.93E13	77 (6.6)
1	TF1	310	2.5E13	2.38E13	95 (7.4)
1	TF2	510	2.5E13	2.33E13	93 (7.4)
2	CF	160	6.7E13	3.98E13	59 (6.0)
2	TF2	510	6.7E13	6.42E13	96 (4.4)
3	CF (-Bz)	160	3.6E13	2.46E13	68 (11)
3	CF (+Bz)	160	3.6E13	3.29E13	91 (12)

In Experiment 2, AAV2-AADC is concentrated to a higher target value ($6.7x10^{13}$ vg/mL) in CF or TF2. Vector recovery using TF2 (96 ± 4.4%) is again significantly higher than recovery using CF (59 ± 6.0%). Within the variability of the assays used, vector was recovered fully at both target concentrations using TF2, indicating that aggregation was prevented. In contrast, significant aggregation was observed at both target concentrations using CF, and the extent of aggregation (i.e. loss following 0.22μm filtration) was higher at the higher target vector concentration. In an additional experiment (not shown), 50 μL samples of AAV2 vector are taken following concentration but prior to the 0.22μm filtration step of Experiment 2, and examined by light microscopy. Vector concentrated in CF contains obvious amounts of visible material (not shown), while no such material is seen in vector concentrated in TF2.

Experiment 3 examines the effect of prior nuclease digestion of purified vector on aggregation. In the absence of nuclease digestion recovery of AAV2-AADC in CF is 68 ± 11%, similar to the recoveries in Experiments 1 and 2. In contrast, purified vector treated with nuclease and then concentrated in CF gives higher recovery (91 ± 12%). These prep scale results reflect the same effect of nuclease digestion shown in FIG. 2 using the "dilution-stress" (analytical scale) method.

AAV Stability and Activity Following Storage or Freeze-Thaw Cycling

Croyle and coworkers reported a significant loss of titer of AAV and adenovirus following multiple freeze-thaw cycling in sodium phosphate buffer, and demonstrated that the better pH buffering provided by potassium phosphate during freeze-thaw cycling prevented titer loss [6]. Results of our freeze-thaw stability study using sodium phosphate support these findings. We find that while 150mM sodium phosphate provides sufficient ionic strength to prevent aggregation during preparation and non-frozen storage of concentrated AAV2-AADC vector, even a single freeze-thaw cycle at -20 or -80 °C results in aggregation.

AAV stability after storage or freeze-thaw (F/T) cycling is assessed in buffers of the present invention as follows. The concentrated vectors prepared in CF, TF1, and TF2 (Table 2, Experiment 1) are subjected to a short stability study to investigate whether aggregation will occur during refrigerated storage, or following multiple freeze-thaw (F/T) cycles. Aggregation is assessed by DLS using undiluted samples, and *Rh* values >20nm are deemed to indicate the occurrence of some level of aggregation.

TABLE 3

STABILITY OF AAV2 VECTORS

	Particle radius – Rh (nm)							
Formulation	Pre	4 °C	- 20 °C			- 80 °C		
		5d	1 F/T	5 F/T	10 F/T	1 F/T	5 F/T	10 F/T
CF	14.5	27.0	22.4	56.1	94.5	20.6	57.5	141
TF1	13.8	16.3	TH	TH	TH	TH	TH	TH
TF2	13.8	14.4	14.2	14.0	14.1	13.8	21.3	50.9

Pre: DLS radius measured immediately following 0.2μm filtration.

Vector concentrations (vg/mL): CF: 1.93E13, TF1: 2.38E13, TF2: 2.33E13.

TH: signal intensity is too high to measure because of extensive aggregation.

As shown in Table 3, AAV2-AADC vector prepared in CF shows some aggregation after 5 days of storage at 4 °C, as well as following one or more F/T cycles at -20 or -80°C. For vector prepared in TF1, no aggregation occurs after 5 days at 4°C, but aggregation occurs following a single F/T cycle at -20 or -80 °C as indicated by a DLS signal intensity that is too high to measure. Visual inspection of these samples reveals slight cloudiness, which is consistent with aggregation. For vector prepared in TF2, no aggregation is observed at 4 °C, or following up to 10 F/T cycles at -20 °C. Some aggregation is observed following 5 and 10 F/T cycles at -80 °C.

AAV activity after storage or F/T cycling in TF2 is assessed as follows. As described above, the high ionic strength, isotonic formulation TF2 effectively prevents vector aggregation during concentration and storage, and therefore represents a promising candidate for further study. An important question is whether preparation and storage of the vector in high ionic strength TF2 would adversely affect its functional activity. To assess this, assays are performed

to measure the infectious titer and the transduction efficiency of vectors prepared and stored for an extended period of time in TF2.

For infectivity, a highly sensitive infectivity assay capable of detecting single infectious events is used [15]. AAV2-AADC is prepared in TF2 at a concentration of $6.4x10^{13}$ vg/mL. After being stored for 45 days at 4 °C the preparation has a vector genome to infectious unit ratio (vg/IU) of 13, compared to a value of 16 vg/IU for the reference vector. This difference is not significant given the reported variability of this assay (RSD ~50%).

Transduction efficiency is assessed by measuring the expression of AADC protein by ELISA following transduction of D7/4 cells. FIG. 3 shows no significant difference between vector prepared in TF2 and the reference control for vector input ranging from 10 to 10^5 vg/cell. Together, these data indicate that preparation and storage of AAV2 vectors in high ionic strength TF2 does not have a deleterious effect on vector infectivity or transduction efficiency.

EXAMPLE 1

AAV PURIFICATION METHODS

AAV2 vectors expressing human coagulation factor IX (FIX) or human amino acid decarboxylase (AADC) are produced by triple transfection of HEK293 cells as previously described [25], with modifications. For the large scale preparations, cells are cultured and transfected in 850 mm^2 roller bottles (Corning). Vectors are purified by one of three methods.

In purification Method 1, modified from Matsushita, transfected HEK293 cells in roller bottles are collected by centrifugation (1000 *g*, 15min), resuspended in 10mM sodium phosphate, 500mM sodium chloride, pH 7.2, and lysed by three freeze / thaw cycles (alternating an ethanol / dry ice bath and a 37°C water bath). The cell lysate is clarified by centrifugation (8,000 *g*,

15 min). The supernatant is then diluted to 200mM NaCl by addition of 10mM sodium phosphate, pH 7.2, and digested with Benzonase (Merck, Purity Grade 1; 200 U/mL, 1h, 37 °C). The lysate is adjusted to 25mM $CaCl_2$ using a 1M stock solution, and incubated at 4°C for one hour.

The mixture is centrifuged (8,000 *g*, 15 min), and the supernatant containing vector is collected. To precipitate virus from the clarified cell lysate, polyethylene glycol (PEG8000) is added to a final concentration of 8%, the mixture incubated at 4°C for three hours, and then centrifuged (8,000 *g*, 15 min). The pellets containing vector are re-suspended with mixing in 0.15M NaCl, 50mM Hepes, 25mM EDTA, pH 8.0 and incubated at 4°C for 16 hours. The resuspended material is pooled, and solid cesium chloride is added to a final density of 1.40 gm/ml. Vector is then banded by ultracentrifugation (SW28, 27,000rpm, 24h, 20°C) using a Beckman model LE-80 centrifuge. The centrifugation tubes are fractionated, and densities from1.38 to 1.42 gm/mL containing vector are pooled. This material is banded a second time by ultracentrifugation (NVT65 rotor, 65,000 rpm, 16h, 20°C), and fractions containing purified AAV2 vectors are pooled. To concentrate vector and to perform buffer exchange, vectors in concentrated cesium chloride solution are subjected to ultrafiltration / diafiltration (UF/DF) by tangential flow filtration as described below (Example 2).

In purification Method 2, cell harvests containing AAV are microfluidized and filtered sequentially through 0.65 and 0.22 μm filters (Sartorius). Virus is purified from the clarified cell lysates by cation exchange chromatography using Poros HS50 resin as previously described [26]. For the nuclease digestion described in FIG. 2, column-purified vectors are incubated (4h, RT) with 100 U/mL Benzonase and 10 U/mL DNAse I (RNAse free, Roche Diagnostics, Indianapolis, Indiana).

For purification Method 3, AAV2 vectors purified by cation exchange chromatography are subjected to an additional cesium chloride gradient ultracentrifugation step (SW28, 27,000rpm, 20h) to remove empty capsids prior to UF/DF.

Real time quantitative PCR (Q-PCR) is used to quantify AAV preparations as previously described [27]. Vectors purified by each of the three methods are analyzed by SDS-PAGE / silver staining analysis, and in all cases VP1, VP2 and VP3 are present in the expected ratios, with the capsid proteins representing >95% of total proteins as determined by scanning densitometry. However, unlike gradient-purified AAV2 vectors purified using Methods 1 and 3, vectors purified by Method 2 (column chromatography) contain empty capsids, ranging from 3-10 empty capsids per vector genome.

EXAMPLE 2

ULTRAFILTRATION AND DIAFILTRATION TO DETECT AAV AGGREGATION

Disposable hollow fiber tangential flow filtration devices (Amersham BioSciences 8" Midgee, 100 kDa nominal pore size) are used to concentrate and diafilter AAV2 vectors purified by the methods described above, and for the UF/DF experiments described in Table 2. For all UF/DF procedures a volume of diafiltration buffer corresponding to 10x the product volume is used, and it is added in ~1mL increments to approximate continuous diafiltration. Using this method, the calculated residual CsCl after diafiltration is <0.5mM.

The following three formulations were used for UF/DF: Control Formulation (CF: 140mM sodium chloride, 10mM sodium phosphate, 5% sorbitol, pH 7.3); Test Formulation 1 (TF1: 150mM sodium phosphate, pH7.5); and Test Formulation 2 (TF2: 100 mM sodium citrate, 10mM Tris, pH8.0). For Experiment 1 shown in Table 2, diafiltration is performed at a volume

corresponding to a vector concentration of $1x10^{13}$ vg/mL, and following diafiltration the volume is reduced to a value corresponding to $2.5x10^{13}$ vg/mL (assuming no vector loss).

For Experiment 2, diafiltration is performed at a volume corresponding to a $2x10^{13}$ vg/mL, and the volume is then reduced to a value corresponding to $6.7x10^{13}$ vg/mL.

For Experiment 3 (CF ± Bz), AAV2-AADC (approximately $1.2x10^{14}$ vg) is first diafiltered into TF1 (a formulation compatible with nuclease activity) and then passed through a 0.22 μm filter. The titer of this material is determined, and the volume is adjusted to correspond to a concentration of $1x10^{13}$ vg/mL. To 10 mL of this material, $MgCl_2$ is added to a concentration of 2 mM, and then divided into two equal aliquots. One aliquot is incubated with Benzonase (200 U/mL, 4h, RT), and the second is mock-incubated. Each aliquot is then diafiltered at a volume corresponding to a vector concentration $2x10^{13}$ vg/mL, and then concentrated to a $3.6x10^{13}$ vg/mL target. Following all UF/DF protocols, Pluronic® F-68 (BASF Corp., Mount Olive, NJ) from a 1% stock is added to the vector product to a final concentration of 0.001%, and the solution is passed through a 0.22μm syringe filter (Sartorius). All UF/DF procedures are performed in a laminar flow cabinet.

EXAMPLE 3

MEASUREMENT OF VECTOR AGGREGATION BY DYNAMIC LIGHT SCATTERING

Purified vectors are analyzed for aggregation by dynamic light scattering (DLS) using a Protein Solutions *DynaPro 99* (λ=825.4 nm). Primary data (particle radius – *Rh*, average value measured over 30 cycles, 10 cycles/min) are used for all analyses reported. A "dilution-stress" method is used to assess the effect of varying excipients on vector aggregation. In this method,

80 μL of test diluent is added to 20 μL of vector solution with mixing in the actual cuvette used for DLS measurement, and data collection is initiated within 10 seconds of mixing. Prior to addition of test diluents, the *Rh* value for AAV2 vector preparations is measured and confirmed to be <15 nm to ensure that the starting material is monomeric. Samples that are not 100% monomeric are passed through a 0.22μm syringe disc filter (Sartorius, low protein binding) to remove aggregates.

The osmolarity and ionic strength values given in FIGS. 1 and 2 are calculated using all excipients present in the mixture (i.e. weighted: test diluent (80%) and starting vector formulation (20%)). Osmolarity is calculated according to the equation: Osmolarity = $\sum c_i$, where c_i is the molar concentration of each solute species. The ionic strength (μ) is calculated according to the equation: $\mu = \frac{1}{2} \sum c_i z_i^2$, where z_i is the charge on each species. In conditions that resulted in vector aggregation (e.g. low μ) a progressive increase in *Rh* is observed over the course of data collection. To validate the use of the average *Rh* measured over the 3 minute interval following dilution as a reliable measure of aggregation, the average rate of increase of *Rh* (ΔRh / Δt) over the same time interval is also determined (not shown). Analysis of ΔRh / Δt gives results concordant with those obtained using the average *Rh* value reported in FIGS. 1 and 2.

EXAMPLE 4

AAV VIRION INFECTIVITY

Infectivity of AAV2-AADC vectors is determined using a highly sensitive assay as previously described [15]. Briefly, samples are serially diluted (10-fold dilutions, 10 replicates / dilution) and added to D7/4 cells (modified HeLa cells expressing AAV *rep* and *cap*) grown in

96 well tissue culture plates (Falcon, cat. #353227) in DMEM medium containing 10% FBS. Adenovirus (Ad-5, 100 vp/cell) is added to each well to provide helper functions. After 48h, replication of AAV vector in each well is quantified by Q-PCR using transgene-specific primers and probes, and the frequency of infection at limiting dilution is analyzed by the Karber method to calculate the infectivity titer. The test sample is run concurrently with an AAV2-AADC reference previously prepared in CF and stored at -80 °C.

The transduction efficiency of AAV2 vectors is quantified by a whole cell ELISA. D7/4 cells grown in 96 well plates are infected with 10-fold serial dilutions of the test sample and reference vector, corresponding to 10 to 10^5 vg / cell input (5 replicates / dilution). After 48h, the culture medium is removed, and cells are washed twice with 200 μL PBS (10 mM sodium phosphate, 140mM sodium chloride, pH 7.2). Cells are then permeabilized and fixed by addition of 100μL of PBS containing 0.5% Triton X-100 and 4% paraformaldehyde to each well (15 min). The fixing solution is removed, and the cells are washed twice with PBS containing 0.5% Triton X-100. Non-specific sites are blocked by adding PBS containing 3% bovine serum albumin (BSA) and 0.5% Triton X-100 (60min).

After washing, cells are incubated for one hour with rabbit anti-AADC IgG antibody (Chemicon, AB136), and washed. Cells are then incubated for one hour with alkaline phosphatase-conjugated goat anti-rabbit IgG, and washed. Antibodies are diluted 1:1000 in PBS containing 1% BSA, 0.5% Triton X-100. Substrate (PNPP, Pierce, cat. #34047) is then added (1 mg/mL in 1X diethanolamine substrate buffer, Pierce, cat. #34064), and after incubation for 30min the concentration of cleaved substrate is measured spectrophotometrically (λ=405nm). Human AADC expression as a function of vector input is fitted using a spline curve (SigmaPlot). The AAV2-AADC reference vector is measured concurrently with the test sample.

While preferred illustrative embodiments of the present invention are described, it will be apparent to one skilled in the art that various changes and modifications may be made therein without departing from the invention, and it is intended in the appended claims to cover all such changes and modifications that fall within the true spirit and scope of the invention.

All publications, patents and patent applications referred to herein are hereby incorporated by reference in their entireties.

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We claim:

1. A method of preventing aggregation of virions in a preparation of virions, comprising treating said preparation of virions with a nuclease.

2. The method of claim 1, wherein the virions are AAV virions.

3. The method of claim 2, wherein the virions are AAV2 virions.

4. The method of claim 1, further comprising adding one or more excipients to the preparation of virions to achieve an ionic strength of greater than 200 mM.

5. The method of claim 4, wherein one or more of the excipients is a compound comprising one or more multivalent ions.

6. A solution for the storage of purified virus particles, comprising:

a pH buffer; and

one or more multivalent ions; wherein the ionic strength of the solution is greater than 200 mM.

7. The solution of claim 6, wherein the purified virus particles are AAV virus particles.

8. The solution of claim 6, wherein one of the one or more multivalent ions is citrate.

9. The solution of claim 6, further comprising Pluronic® F68.

10. The solution of claim 9, wherein the Pluronic® F68 is present at 0.001%.

11. The solution of claim 6, comprising:

10 mM Tris, pH 8.0; and

100 mM sodium citrate.

ABSTRACT

Compositions and methods are provided for preparation of concentrated stock solutions of AAV virions without aggregation. Formulations for AAV preparation and storage are high ionic strength solutions (e.g. μ ~500mM) that are nonetheless isotonic with the intended target tissue. This combination of high ionic strength and modest osmolarity is achieved using salts of high valency, such as sodium citrate. AAV stock solutions up to $6.4x10^{13}$ vg/mL are possible using the formulations of the invention, with no aggregation being observed even after ten freeze-thaw cycles. The surfactant Pluronic® F68 may be added at 0.001% to prevent losses of virions to surfaces during handling. Virion preparations can also be treated with nucleases to eliminate small nucleic acid strands on virions surfaces that exacerbate aggregation.

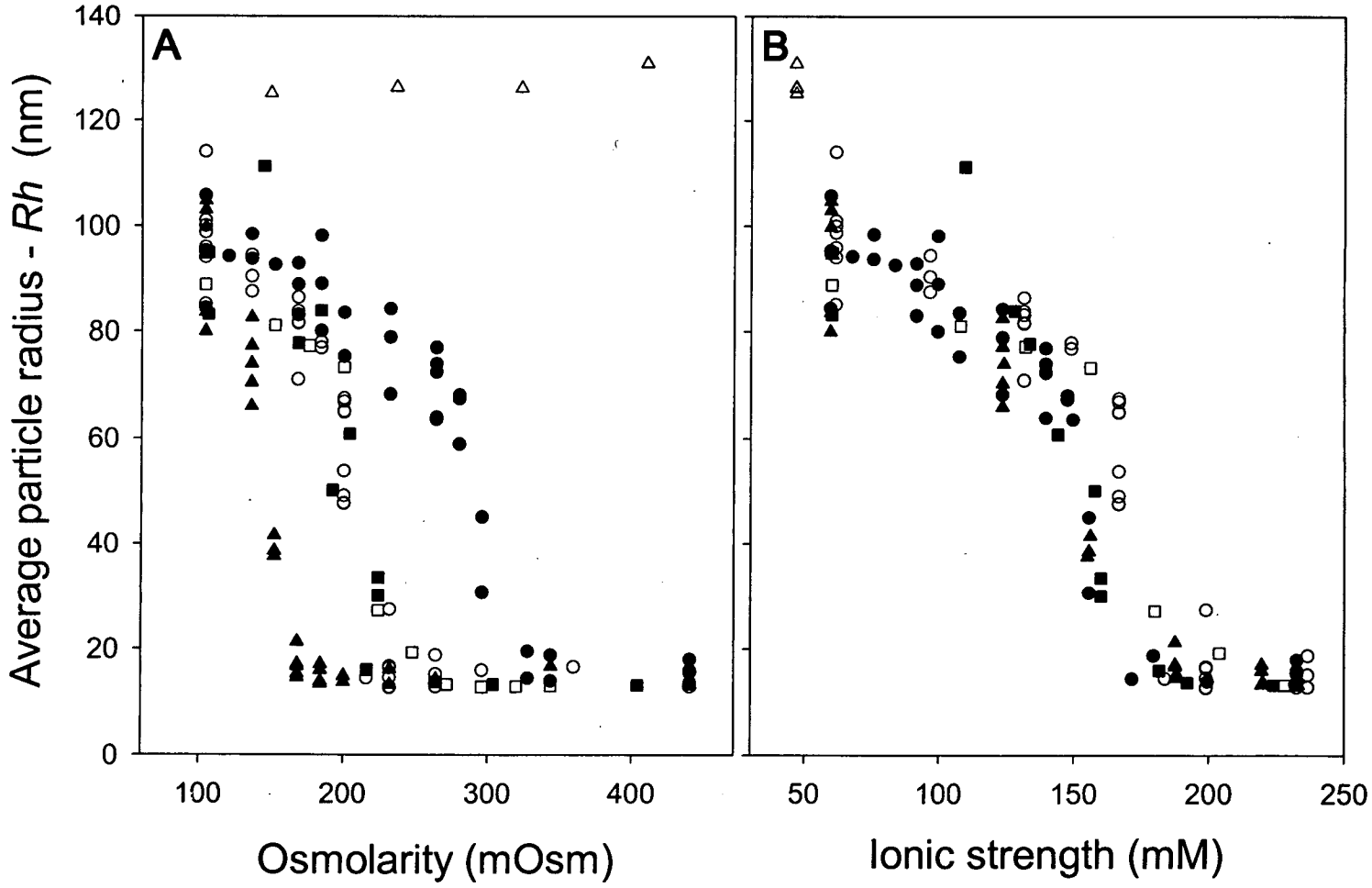

FIGURE 1

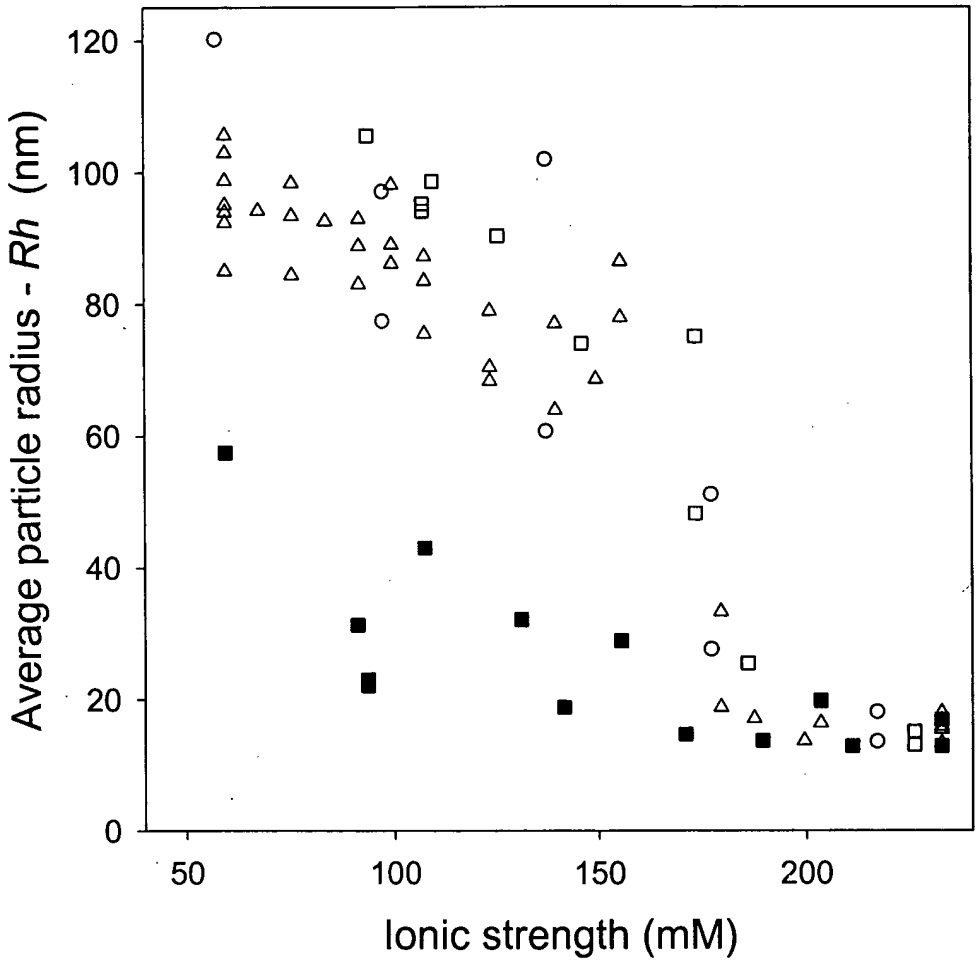
Average particle radius - Rh (nm)
120
100
80
60
40
20
0
50
100
150
200
Ionic strength (mM)

FIGURE 2

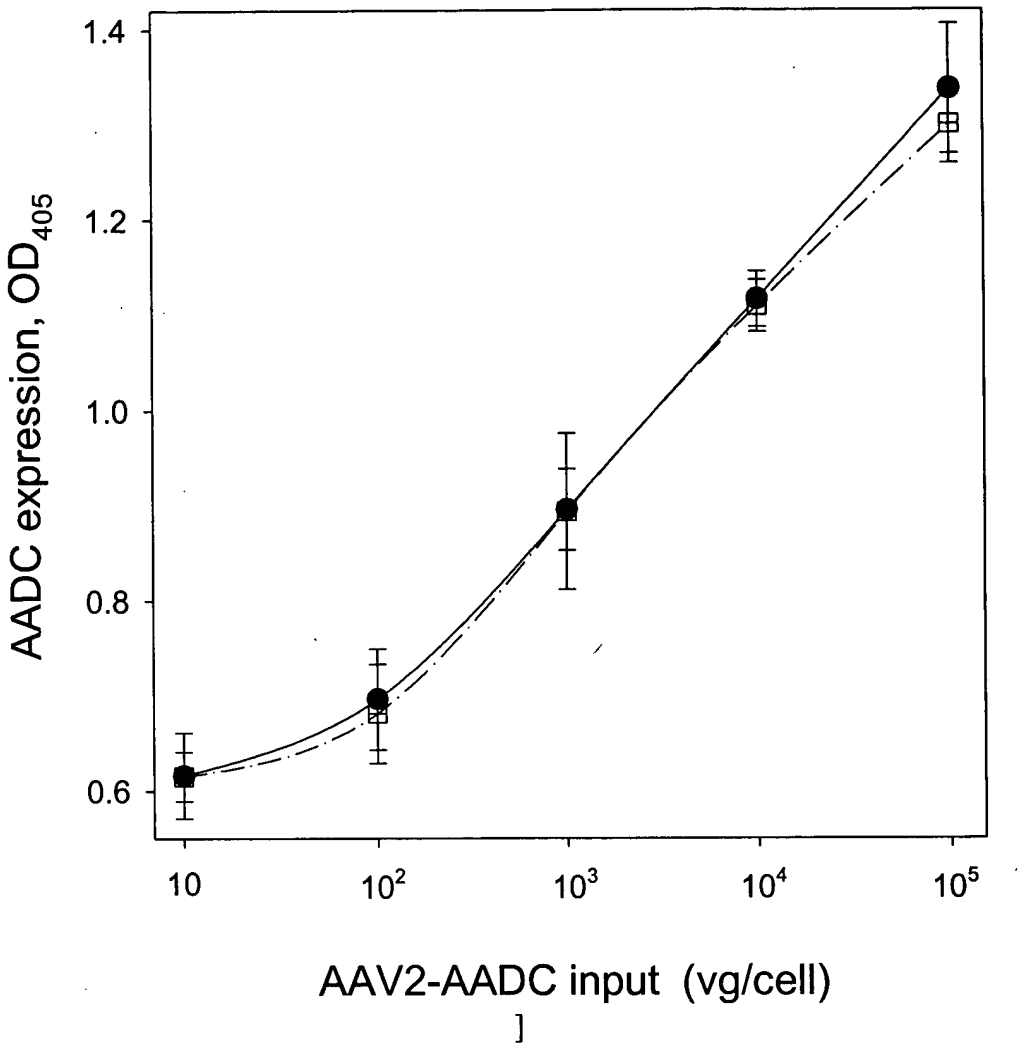

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FIGURE 3

PATENT APPLICATION SERIAL NO. _____

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