Recombinant adeno-associated virus: Formulation challenges and strategies for a gene therapy vector

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Recombinant adeno-associated virus (AAV)-based vectors capable of expressing therapeutic gene products in vivo have shown significant promise for human gene therapy. One challenge facing the field is the development of vector formulations to achieve optimal vector safety, stability and efficacy. Formulation challenges for AAV vectors can be divided into those relating to maintaining vector activity during purification and storage, and those relating to efficient target tissue transduction in vivo. AAV vectors are potentially susceptible to loss of activity through aggregation, proteolysis and oxidation, as well as through non-specific binding to product contact materials used for vector purification and storage. These deleterious changes need to be thoroughly characterized, and the conditions and excipients to prevent them need to be identified. For in vivo administration, major vector formulation challenges include optimization of efficiency and specificity of target tissue transduction, and the ability to overcome host immune responses.

Keywords AAV vectors, excipient, formulation, gene therapy, stability

Abbreviations

AAV	Adeno-associated virus
ARM	Adenovirus reference material
cp	Capsid particles
FGFR1	Fibroblast growth factor receptor-1
HSPG	Heparin sulfate proteoglycan
HSV	Herpes simplex virus
PEG	Polyethylene glycol
rAAV	Recombinant adeno-associated virus
vg	Vector genome

Introduction

Recombinant adeno-associated virus (rAAV) is a promising vector for human gene transfer. A member of the family *Parvoviridae*, the wild-type virus (AAV-2) is composed of a single strand (positive or negative) DNA molecule of 4680 nucleotides encoding proteins involved in replication (*rep*) and encapsidation (*cap*) [1]. Flanking the *rep*/*cap* sequences in the virus genome are two 145-nucleotide inverted terminal repeat (ITR) sequences, which are the essential cis-elements for replication, packaging and rescue [2]. The genome is packaged by three capsid proteins (VP1, VP2 and VP3), which consist of amino-terminal variants of the cap open reading frame [1]. The virus particles have an icosahedral structure of ~ 26 nm in diameter [1] and, relative to other viruses, are resistant to inactivation by changes of temperature, pH and other physicochemical factors. Adeno-associated virus (AAV) replication requires co-infection of cells with a helper virus, such as adenovirus or herpes simplex virus (HSV) [1]. Adenovirus gene products contributing to AAV gene expression products include those encoded by regions E1a, E1b, E2a and E4 [3]. Similarly, the subset of HSV-1 genes required for AAV replication and packaging has been established [4,5]. In 1982, isolation of a molecular clone for AAV-2 by Samulski and co-workers resulted in the development of the first AAV-based vector [6]. AAV vectors are generated by deleting the viral *rep* and *cap* genes of the wild-type virus, and replacing them with the gene of interest, as well as associated transcriptional control elements, making AAV vectors free from all viral genes.

rAAV vectors have received considerable attention in the field of gene therapy, because of their ability to mediate long-term gene transfer in the absence of significant toxicity. AAV can infect both dividing and non-dividing cells and establish a latent state with high frequency [7], characteristics which contribute to its utility for vector development. AAV vectors mediate efficient and long-term gene transduction in a variety of tissues, including lung [8], liver [9], muscle [10], central nervous system [11] and eye [12,13]. AAV vectors appear less immunogenic than other viral vectors, such as vectors based on adenoviruses [14], a factor which may contribute to enhanced duration of therapeutic gene expression *in vivo* [15,16••,17••,18]. To date, most studies have been performed using AAV-2. However, a number of additional serotypes have been described [19-23] that provide the potential for alternative tissue specificity and reduced recognition by pre-existing antibodies. These features make AAV-based vectors particularly promising for human gene transfer. Evidence for vector activity and gene expression in human gene therapy for hemophilia B has recently been reported [24,25••].

Formulation development for virus-based gene transfer vectors is a relatively recent area of investigation, and few studies have been published that describe systematic efforts to address the optimization of AAV vector formulation and stability. While lessons can be learned from the formulation experiences for the development of vaccine products and purified recombinant proteins, formulation development for viral vectors provides a number of unique challenges. Compared to rAAV, more extensive formulation development has been performed with recombinant adenovirus vectors and, because AAV and adenovirus are both non-enveloped viruses developed as gene transfer vectors, studies on the latter can provide guidance for AAV vector formulation development. Therefore, some reference will be made in this review to studies performed using adenovirus vectors, as a basis for comparison with AAV vectors.

Establishing vector formulations that minimize biochemical changes and ensure full vector activity during purification and extended storage periods is a major challenge in the formulation development of AAV vectors. Formulations and

storage conditions must be amenable to commercial development of a biological product. Optimizing the efficiency and specificity of vector-mediated *in vivo* gene transfer constitutes the second major challenge. Current progress and outstanding issues relating to these challenges will be discussed in this review.

Vector formulation development for optimal stability during purification and storage

Storage temperature

AAV vectors are typically formulated and stored in neutral-buffered saline solutions. In some cases, a cryoprotectant (eg, glycerol or sorbitol) is added to preserve vector activity following storage at -20 or -80°C, and a surfactant (eg, Polysorbate 80, Pluronic F68) is included to minimize non-specific binding of the vector to plastic or glass surfaces. Croyle *et al* reported a systematic investigation of buffer systems, cryoprotectants and storage temperature for AAV and adenovirus vectors expressing the marker gene β-galactosidase (*LacZ*) [26••]. These authors reported that the use of buffer systems that do not effectively buffer against pH changes during freezing correlated with a significant loss of vector activity following a freeze-thaw cycle. In particular, they reported that the use of the common sodium phosphate buffer system resulted in a shift from pH 7 to 4 upon freezing at -80°C, and that a freeze-thaw cycle under these conditions resulted in a 3-fold (0.5 log) loss of AAV vector infectious titer. The pH shift was attributed to precipitation of disodium hydrogen phosphate decahydrate upon freezing. A modest but still significant pH shift was observed following freezing to -20°C. The authors reported that the use of potassium phosphate rather than sodium phosphate improved pH buffering and reduced loss of infectious titer during freeze-thaw cycling. They also indicated that inclusion of either 1 M sucrose, 1 M trehalose, 0.5% β-cyclodextrin (BCD) or 5% tertiary amine β-cyclodextrin (TMBCD) could maintain physiological pH upon freezing for potassium or sodium phosphate buffer systems. Xu *et al* reported that AAV vectors expressing luciferase, formulated without a cryoprotectant and frozen at -80°C for 3 months, showed similar activity to that measured using freshly prepared vector following *in vivo* administration to rat brain [27].

While the development of buffer systems that can minimize the loss of vector infectious titer during freeze-thaw cycling is important, formulations that support non-frozen storage of vectors will ideally be established and used for commercial development of AAV gene transfer. For such non-frozen formulations, a reasonable target for AAV vector stability for commercial applications is minimal loss (≤ 10%) of infectious activity during a period of ≥ two years. Croyle *et al* reported initial screening studies examining the stability of AAV vectors in non-frozen conditions [26••]. They discovered that a formulation of phosphate buffered saline containing 0.4% sucrose, 0.4% mannitol, 0.001% Span 20 (sorbitan monolaurate) and 1.0 mg/ml protamine resulted in ≤ 10% loss of initial infectious titer during 150 days of storage at both 4 and 25°C. These authors also reported initial studies on lyophilization of AAV vectors, demonstrating that the actual lyophilization process resulted in an immediate ~ 2-fold loss of titer; however, after this initial loss, vector infectivity was stable for 90 days at 25°C (maximum time point measured). Our research team at Avigen Inc (USA) observed that AAV vector formulated in neutral phosphate buffered saline containing 5% sorbitol and 0.1% Polysorbate 80 showed no significant loss of transduction activity when stored at 2 to 8°C for up to one year [Zhen Z, Kao H, Sommer J, unpublished data]. Additional studies are required to characterize AAV vector stability in non-frozen conditions, including assessment of functional activity over extended periods of time, and characterization of biochemical changes, such as proteolysis and oxidation. Based on these data, formulation excipients that minimize deleterious changes can be further developed and optimized.

Vector aggregation

Aggregation of AAV particles, which constitutes a significant issue for vector formulation and stability, has been described. The mechanism of vector aggregation is not well understood, and purification conditions that may affect aggregation include buffer ionic strength and pH, shear and vector concentration. Huang *et al* reported that rAAV undergoes concentration-dependent aggregation [28]. We observed a similar phenomenon, with aggregation of vector frequently occurring in a time- and concentration-dependent manner in vector preparations at concentrations ≥ 10^{14} capsid particles (cp)/ml [29]. For AAV vector preparations that do not contain empty capsids, this corresponds to a protein concentration of ~ 0.62 mg/ml. Our and other research teams have observed that freeze-thaw cycling exacerbates vector aggregation, and can lead to aggregation at vector concentrations significantly lower than 10^{14} cp/ml. For example, using dynamic light scattering, we observed that highly purified vector preparations at concentrations of 5×10^{13} cp/ml that are stable in a non-aggregated, monomeric state when stored at 2 to 8°C, can be induced to undergo some aggregation following a single freeze-thaw cycle to -20°C [Kao H, Sommer J, Wright F, unpublished data].

Reduced yield is one of the deleterious consequences of aggregation during the vector purification process. We observed that loss of rAAV following a 0.2-µm filtration step correlates with the extent of vector aggregation. The tendency of AAV vectors to aggregate at high concentrations is a factor to consider during the development of vector purification methods. Based on ease of scalability, column chromatographic methods have been developed for the purification of AAV vectors [30-34]. In the absence of a density-based method, such as cesium chloride or iodixanol gradient ultracentrifugation, column-based methods do not separate full (genome-containing) vector particles from the lighter empty capsids that are typically generated in excess (usually ranging from 5- to 50-fold) during vector production in cell culture. These empty capsids, whose size and surface characteristics are similar to that of genome-containing vector particles [35], contribute to particle aggregation, and their presence may result in aggregation at lower vector genome (vg) concentrations than would be observed in their absence. Assuming that full vector particles and empty capsids aggregate by a similar mechanism (an assumption that requires testing), a preparation of AAV vectors containing a 10-fold excess of empty capsids should have a similar risk of aggregation at

concentrations of ≥ 10^{13} vg/ml (corresponding to ≥ 10^{14} cp/ml). Depending on the ratio of empty capsids in a vector preparation, and the concentration requirements for the final purified vector, concentration limitations associated with aggregation in vector preparations containing empty capsids may be a significant challenge for vector purification process development.

Another potentially deleterious consequence of vector aggregation is inconsistency in the functional activity of AAV vectors following their administration *in vivo*. For example, in order to deliver an AAV vector to hepatocytes following systemic administration, vector particles must pass through the endothelial lining of hepatic sinusoids to reach target hepatocytes. The endothelial cell lining contains intercellular gaps of 0.1 to 1 µm [36] that could allow the passage of non-aggregated AAV particles (diameter of 0.026 µm), but may significantly inhibit the passage of aggregated vectors. In mice, following vascular delivery (portal or tail vein injection) of AAV, vector aggregation resulted in vector uptake by macrophages and clearance [37]. These investigators further reported that PEGylation of AAV could prevent reverse aggregation of AAV vectors. For other routes of administration, the variability in particle size associated with aggregation may also adversely affect *in vivo* gene transfer.

The nature of the interparticle interactions that result in aggregation has not been well characterized. In preliminary experiments, we observed that aggregation appeared to be a multistep process, with an easily reversible 'initial' aggregation followed by transition to a more stable aggregation state. Initial aggregation could be reversed by adjusting buffer pH, suggesting that interparticle salt bridges between charged amino acid side chains might be involved. Moreover, non-ionic surfactants do not prevent or reverse aggregation induced at high vector concentrations or elevated temperatures [29], suggesting that hydrophobic interactions alone do not mediate the strong interparticle interactions involved in this phenomenon. Additional studies are required to elucidate the full mechanism of vector aggregation, and to identify formulation excipients and conditions to prevent its occurrence.

Aggregation is a significant and not fully resolved issue for adenovirus vectors. A recently established adenovirus reference material (ARM) was characterized in a field-use and shipping stability study [38]. In this study, the aggregation state of the adenovirus, formulated in 20 mM Tris, 25 mM NaCl and 2.5% glycerol at pH 8.0, was assessed by dynamic light scattering, photon correlation spectroscopy and visual appearance. The investigators observed variable levels of vector aggregation following freeze-thaw cycling and storage at 2 to 8°C, or 22 to 25°C. Based on these observations, they recommended that, to ensure consistent results, vials of the ARM be thawed only once and used within 4 h of thawing.

Non-specific vector adsorption

AAV vectors are typically prepared at final purified concentrations in the range of 10^{11} to 10^{13} vg/ml. For vectors purified using methods that remove empty capsids, this corresponds to a protein concentration range of ~ 0.62 to 62 µg/ml. For vectors co-purified with empty capsids, the protein concentration will be higher, and proportionate to the empty capsid content. In either case, these protein concentrations are relatively low, and may result in loss of vector due to absorption to surfaces used for storage or transfer, unless appropriate excipients are employed to prevent such non-specific losses. For example, sampling of small volumes (≤ 100 µl into 1.5-ml polypropylene sample tubes) of purified AAV vector (10^{12} to 10^{13} cp/ml), formulated in neutral phosphate-buffered saline in the absence of an added surfactant, could result in significant losses (up to and exceeding 50%), due to non-specific binding [39]. Such loses in the samples used to determine key parameters, such as vector concentration and activity, would result in poor accuracy and high variability in the measured parameters. Similar concerns apply to transfer of purified vectors (eg, during administration to animals), in which the vector solution is exposed to transfer containers, tubing, etc. We found that addition of the surfactants Polysorbate 80 or Pluronic F68 at concentrations as low as 0.01 and 0.001%, respectively, could effectively prevent losses due to non-specific binding during vector sampling and transfer [39]. Croyle *et al* [26••] reported the use of Span 20 at a concentration of 0.001% in an optimized AAV vector formulation, although the issue of non-specific vector loss was not addressed in this study.

Vector formulation for optimal efficacy *in vivo*

Tissue targeting

Targeting of therapeutic genes to specific tissues requires both effective delivery of gene transfer vectors to the tissue of interest, and the ability of the vector to specifically bind and transduce cells in the target tissue. For AAV-2, cell surface heparin sulfate proteoglycan (HSPG), which is broadly distributed on a variety of cell types, and human fibroblast growth factor receptor (FGFR)-1 have been reported as cellular receptors for virus binding [40,41]. However, not all potential target cells express these receptors, and the broad cellular expression of HSPG may preclude selective cell transduction. Hence, novel approaches to target rAAV, based on vector surface modifications, have been developed. These methods include the use of alternative AAV serotypes which, in animal models, give different tissue specificities and transduction efficiencies than AAV-2 [19-23,42••]. For example, vector genomes cross-packaged in AAV-8 capsids were reported to transduce liver cells in mice and non-human primates 100- to 1000-fold more efficiently than AAV-2 vectors, and vector genomes cross-packaged in AAV-7 capsids transduced mouse muscle 20- to 30-fold more efficiently [42••]. Another approach to modify tropism is through the attachment of targeting ligands to the surface of the vector. Vieira *et al* reported that, *in vitro*, PEGylation of AAV vectors resulted in an efficient target cell transduction independent of the AAV-2 receptor molecules HSPG and FGFR [43]. The development of $F(ab'\gamma)_2$ bispecific heterodimers that bind to both AAV-2 capsid protein and to αIIbβ3 integrin, a cell surface molecule expressed on human megakaryocytes, was reported by Bartlett *et al* [44]. This enabled the efficient transduction of megakaryocyte cell lines DAMI and MO7e, which are non-permissive for normal AAV-2 infection, by preformed AAV-2/$F(ab'\gamma)_2$ complexes. Shi *et al* reported an insertional mutagenesis strategy for AAV-2 capsids to

modify viral tropism, and reported one mutant vector displaying a 15-amino acid human luteinizing hormone receptor-binding peptide that could transduce ovarian cancer cells in an HSPG-independent manner [45]. Ponnazhagan *et al* reported that AAV-2 vectors could be biotinylated and then bound to a streptavidin-conjugated cognate peptide ligand, in order to direct binding and transduction of epidermal growth factor receptor-positive SKOV3.ip1 cells [46]. Further development of these approaches, aided by progress in the understanding of the structural biology of AAV (including the recently published crystal structure of AAV-2 at 3 Å-unit resolution [47]) will facilitate progress in AAV vector targeting.

Overcoming host immunity

Pre-existing immunity is one of the major challenges facing the use of rAAV and virus-based vectors for human gene transfer. AAV is a ubiquitous virus, and most potential gene transfer recipients have previously been exposed to it. Furthermore, re-administration of vector may be desirable in some applications, which will be complicated by the boosting of antibody titers resulting from an initial exposure. Hence, most potential recipients are expected to express some level of circulating antibody that should bind to AAV vectors following their administration. Antibodies bound to vectors may reduce target tissue transduction by blocking the binding of vectors to target cell receptors, and mediate binding to antigen-presenting cells via immunoglobulin receptors expressed on these cells. A general strategy to mitigate the effects of pre-existing antibodies is to modify the surface of AAV vector particles, so that they are not recognized by pre-existing antibodies. Modification of vector particles by covalent attachment of polyethylene glycol (PEG) to free lysine groups on the capsid proteins was described for adenovirus and AAV vectors [37]. In this study, the *in vivo* tranduction efficiency of AAV vector administered intravenously or intramuscularly was not affected by PEGylation. In contrast to control vector (non-PEGylated), the modified vector resulted in significant levels of gene transfer in immunocompetent mice previously exposed to the native virus. Compared with non-PEGylated vectors, PEGylated adenovirus vectors ('stealth' adenoviruses) showed no decrease in transduction efficiency in lung [48••] and liver [49] routes of administration. In these studies, activation of helper T-cells, cytotoxic T-cells and neutralizing antibodies to adenovirus were reduced, compared with studies in which non-PEGylated vectors were used. Other approaches to address pre-existing immunity include the use of alternative AAV serotypes that are not recognized by prevalent pre-existing antibodies, and mutagenesis of the AAV capsid to eliminate antibody epitopes [47,50].

Conclusions

While significant progress has been made in the development of formulation strategies for rAAV, several challenges remain to be addressed. For vector purification and storage, a more complete characterization of the mechanisms of vector aggregation, and of time- and temperature-dependent biochemical changes in purified vector preparations, is required to enable development of optimal formulation excipients and storage conditions. To achieve optimal *in vivo* efficacy, further development of current strategies to improve the specificity and efficiency of target tissue transduction, and to address pre-existing immunity to vector, is necessary. Progress in these key areas will contribute to the successful commercial development of this promising vector for human gene transfer.

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