doi:10.1016/j.ymthe.2005.02.021

ARTICLE

Identification of Factors that Contribute to Recombinant AAV2 Particle Aggregation and Methods to Prevent Its Occurrence during Vector Purification and Formulation

J. Fraser Wright,* Tannie Le, Joseph Prado, Jennifer Bahr-Davidson, Peter H. Smith, Zhu Zhen, Jurg M. Sommer, Glenn F. Pierce, and Guang Qu

Avigen, Inc., 1301 Harbor Bay Parkway, Alameda, CA 94502, USA

**To whom correspondence and reprint requests should be addressed at the Abramson Research Bldg, 302C, Children's Hospital of Philadelphia, 3615 Civic Center Boulevard, Philadelphia, PA 19104, USA. Fax: (215) 590 3660. E-mail: wrightf@email.chop.edu.*

Available online 9 April 2005

Aggregation of recombinant AAV2 results in reduced yield during purification and may have deleterious effects on vector transduction efficiency, biodistribution and immunogenicity following *in vivo* administration. Studies to elucidate the mechanism of vector aggregation and methods to prevent its occurrence are reported. In excipient screening studies, the sugars sorbitol, sucrose, mannitol, trehalose, or glycerol at concentrations of up to 5% (w/v), or surfactants Tween 80 or Pluronic F68, did not prevent aggregation. Aggregation was prevented by the use of various salts at concentrations corresponding to solution ionic strengths of >200 mM. AAV2 vectors purified by double cesium chloride gradient centrifugation, cation-exchange chromatography, or combined chromatography and gradient centrifugation each demonstrated a similar requirement for ionic strength to prevent aggregation. AAV2 vectors concentrated to 6.7×10^{13} vector genome (vg)/mL in neutral-buffered isotonic saline resulted in 59 ± 6.0% recovery of nonaggregated material compared to 96 ± 4.4% recovery in an isotonic formulation with elevated ionic strength. The latter showed no aggregation following storage or after 10 freeze–thaw cycles at −20°C. AAV2 vectors stored for an extended period in an elevated ionic strength formulation retained a high infectivity titer (13 vg/infectious unit) and transduction efficiency. Nuclease digestion of purified AAV2 vectors reduced aggregation, implicating trace amounts of vector surface nucleic acids in interparticle binding.

Key Words: adeno-associated virus, vector aggregation, ionic strength

INTRODUCTION

Recombinant adeno-associated virus (rAAV) is a promising vector for human gene transfer [1–3]. A member of the *Dependovirus* genus of the parvoviruses, AAV Type 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 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 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 particles is limited, and aggregation of concentrated AAV2 vectors has been reported [6–9]. Aggregation can lead to purification losses and inconsistencies in the testing of purified vector. The *in vivo* administration of AAV2 vectors to certain sites, such as the central nervous system, may require small volumes of concentrated vector, and the maximum achievable dose is limited due to low vector solubility. Vector aggregation is also likely to influence biodistribution following *in vivo* administration and may cause unwanted immune responses to vectors, as has been reported for proteins [10]. Reports of immune responses that limit transgene expression following AAV vector administration in preclinical [11–13] and clinical [14] studies emphasize the need to address factors that may contribute to vector immunogenicity. Hence, an important objective for the development of AAV2 vectors is to optimize vector purification methods and formulations to prevent aggregation when concentrated vector stocks are prepared. To achieve this objective, the biochemical

1525-0016/$30.00

doi:10.1016/j.ymthe.2005.02.021

mechanisms that contribute to aggregation should be elucidated. In this study we have investigated AAV2 vector aggregation by examining the influence of different classes of excipients and by identifying impurities that contribute to aggregation. Methods to prevent the aggregation of AAV2 vectors are described.

RESULTS

Excipient Screening by Dynamic Light Scattering

Initial screening experiments were performed to identify classes of excipients that could reduce aggregation and thereby provide information on the mechanism of AAV vector aggregation. We observed that vector aggregation could be caused by the dilution of our purified AAV2 vector preparations with a low-concentration buffer (10 mM sodium phosphate, pH 7.2). Based on this "dilution-stress" method, we screened for excipients that, when included in the diluent, were able to prevent vector aggregation. For this screening, aggregation was measured by dynamic light scattering (DLS), a method that is highly sensitive, requires only small volumes (20 μL) of sample, and provides a semiquantitative measure of aggregation adequate for comparison studies. Excipients examined included selected inorganic salts, amino acids, uncharged simple carbohydrates, and surfactants. The results are shown in Table 1. Charged excipients (inorganic salts and amino acids) were able to prevent aggregation when present at sufficient concentrations. However, concentrations required to prevent vector aggregation varied, ranging from 180 mOsm for magnesium sulfate to 320 mOsm for sodium chloride. The amino acids Arg, Asp, Glu, Gly, His, and Lys were each found to be unable to prevent aggregation when initially screened at 200 mOsm. Three amino acids (Lys, Asp, and Glu) were examined at higher concentrations and found to prevent aggregation at 300–320 mOsm. Several carbohydrates were tested at concentrations of up to 5% w/v and found to have no effect on vector particle aggregation. For example, 5% w/v glycerol (543 mOsm) did not prevent aggregation under the dilution-stress method. The surfactants Pluronic F68 (to 10% w/v) and Polysorbate 80 (to 1% w/v) similarly had no effect.

TABLE 1: Screening for excipients that prevent AAV2 vector aggregation using a 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.

Vector Aggregation at Reduced Ionic Strength

A more detailed analysis of AAV2 vector aggregation as a function of the concentration of selected excipients was performed. Shown in Fig. 1A is the dependence of aggregation on the osmolarity of these excipients. For charged excipients a concentration-dependent inhibition of aggregation was observed. Salts of multivalent ions were required at lower concentrations to prevent aggregation than was NaCl. For example, magnesium sulfate at ~200 mOsm prevented aggregation, while NaCl was required at ~350 mOsm to achieve a similar effect. Sodium salts of citrate, sulfate, and phosphate were intermediate in their potency. These data suggested that the ionic strength (μ) of the solution, a parameter that depends on charge valency as well as concentration, was the excipient characteristic affecting vector aggregation. In Fig. 1B, the data were plotted to show vector aggregation as a function of the calculated ionic strength of solution for each excipient. This transformation showed that the dependence of vector aggregation on ionic strength was the same regardless of which salt was used, and aggregation was prevented in all cases in which the ionic strength was ~200 mM or greater.

Effect of Purification Method on AAV Vector Aggregation

Recombinant AAV2 purified using different methods (e.g., density-gradient purification versus ion-exchange chromatography) would be expected to have differing impurity profiles. To investigate the effect of purification method, aggregation as a function of ionic strength was measured for vectors purified by three methods. In these studies, NaCl was used to vary ionic strength. As shown in Fig. 1C, AAV2 vectors purified by double cesium chloride gradient ultracentrifugation (Method 1), by cation-exchange column chromatography (Method 2), or by combined column and CsCl gradient ultracentrifugation (Method 3) each aggregated to a similar degree at low ionic strengths. In contrast, AAV2-FIX purified by the column method and then subjected to an additional nuclease digestion step (Method 2 plus nuclease) to further degrade and remove DNA impurities showed a reduced degree of aggregation at low ionic strengths.

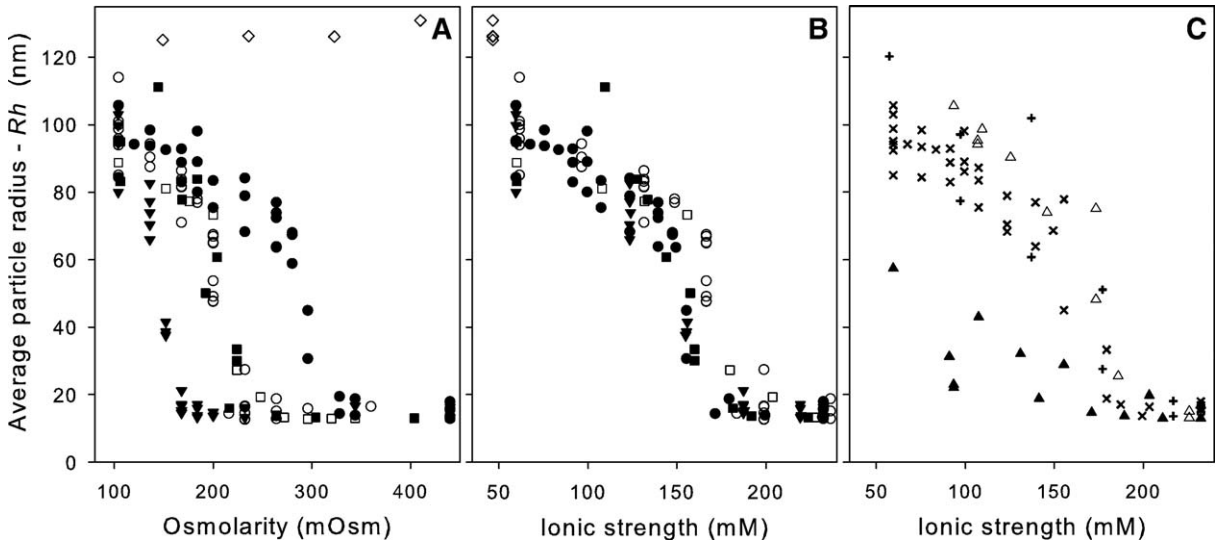

FIG. 1. Dependence of AAV2 vector aggregation on osmolarity and ionic strength of selected excipients and on the method of purification. The average particle radius of AAV2-FIX vectors was measured by DLS following vector dilution in varying concentrations of excipients buffered with 10 mM sodium phosphate, pH 7.5. (A) Aggregation of vectors purified by Method 3 (see Materials and Methods) as a function of the osmolarity of sodium chloride (●), sodium citrate (○), sodium phosphate (■), sodium sulfate (□), magnesium sulfate (▼), and glycerol (◇). (B) Vector aggregation as a function of the ionic strengths of the same solutions. (C) AAV2 vector aggregation as a function of ionic strength of sodium chloride for vectors purified by Method 1 (double CsCl gradient) (+); Method 2 (cation exchange chromatography) (△); Method 2 plus nuclease digestion (▲); or Method 3 (chromatography plus CsCl gradient) (×).

Nuclease digestion performed at an earlier stage of the purification process (clarified HEK cell lysate as in Method 1) did not reduce the aggregation of subsequently purified vector even though the amount of residual nonvector DNA was not reduced significantly following additional nuclease digestion of the purified vector when measured by real-time quantitative (Q)-PCR.

Effects of Ionic Strength and Nuclease on a Preparative Scale

The preceding results describing the dependence of vector aggregation on ionic strength and nuclease treatment were performed on an analytical scale, employing a method to measure aggregation (DLS) that is semiquantitative. The effects of elevated ionic strength and nuclease treatment on AAV2 vector aggregation were next tested on a larger scale using methods to induce and quantify vector aggregation relevant to preparative-scale vector purification. Purified AAV vectors were diafiltered into solutions of varying ionic strengths, the volume was reduced to reach high target vector concentrations, and aggregation was then assessed by our measuring vector recovery following filtration of the product using a 0.2-μm filter. Aliquots from a single pool [1.7×10^{15} vector genome (vg) in 91 mL ~3 M CsCl, 1.9×10^{13} vg/mL] of AAV2-AADC vector purified by Method 1 through the second CsCl gradient centrifugation step were used as the starting material in the experiments described in Table 2. Tangential-flow filtration using hollow fibers was used for diafiltration because this method is scalable and enabled the accurate preparation of small volumes (minimum ~1.4 mL) of concentrated vectors. In Experiment 1, three hollow-fiber units were used to diafilter AAV2-AADC vector into formulations CF, TF1, or TF2 (Control Formulation, Test Formulation 1, Test Formulation 2), respectively, and then the volume was reduced to a value corresponding to 2.5×10^{13} vg/mL. Formulation ionic strengths and vector recoveries following 0.2-μm

TABLE 2: AAV vector recovery on a process scale following diafiltration and concentration in control and elevated-ionic-strength formulations, followed by 0.2-μm filtration

Experiment	Formulation	μ (mM)	Target (vg/mL)	Actual (vg/mL)	Recovery % (RSD)
1	CF	160	2.5×10^{13}	1.93×10^{13}	77 (6.6)
	TF1	310	2.5×10^{13}	2.38×10^{13}	95 (7.4)
	TF2	510	2.5×10^{13}	2.33×10^{13}	93 (7.4)
2	CF	160	6.7×10^{13}	3.98×10^{13}	59 (6.0)
	TF2	510	6.7×10^{13}	6.42×10^{13}	96 (4.4)
3	CF (−Bz)	160	3.6×10^{13}	2.46×10^{13}	68 (11)
	CF (+Bz)	160	3.6×10^{13}	3.29×10^{13}	91 (12)

doi:10.1016/j.ymthe.2005.02.021

filtration are shown in Table 2. Recoveries using both elevated ionic strength formulations TF1 (95 ± 7.4%) and TF2 (93 ± 7.4%) were significantly higher than that using CF (77 ± 6.6%). In Experiment 2, AAV2-AADC was concentrated to a higher target value (6.7×10^{13} vg/mL) in CF or TF2. Vector recovery using TF2 (96 ± 4.4%) was 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 (Experiments 1 and 2) using TF2, indicating that aggregation was prevented. In contrast, significant aggregation was observed at these target concentrations using CF, and the extent of aggregation (i.e., loss following 0.2-μm filtration) was proportional to the target vector concentration. Formulation TF1 was not used in Experiment 2 because of its poor stability following freeze–thaw cycling (Table 3), coupled with the limited supply of vector. In Experiment 3, the effect of prior nuclease digestion of purified vector on aggregation was examined. In the absence of nuclease digestion, recovery of AAV2-AADC was 68 ± 11%, indicating a degree of aggregation consistent with that observed using CF in Experiments 1 and 2. In contrast, purified vector treated with nuclease and then concentrated in CF gave a greater recovery (91 ± 12%). The results obtained on a preparative scale are concordant with the effect of nuclease on vector aggregation observed using the analytical-scale dilution-stress method (Fig. 2), confirming that efficient nuclease digestion of purified AAV2 vectors results in reduced aggregation.

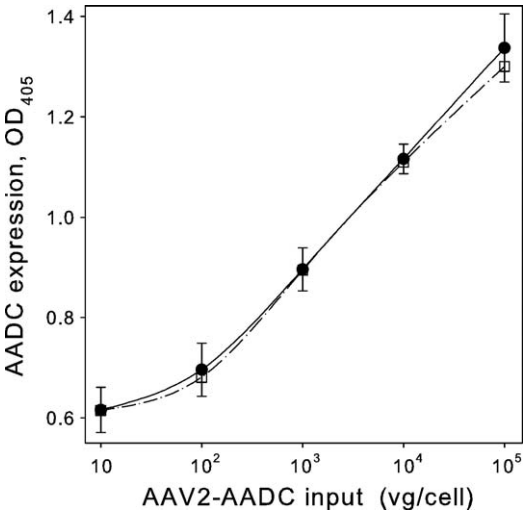

FIG. 2. Transduction by AAV2 vectors prepared and stored in elevated-ionic-strength formulation TF2. D7/4 cells were infected with AAV2-AADC prepared in control (●) or TF2 formulations (□) at 10-fold serial multiplicities. After 72 h, the concentration of AADC in each well was measured by ELISA. Error bars indicate the standard deviation of the average value ($n = 5$) measured at each dilution.

Stability of AAV2 Vectors Following Storage Or Freeze–Thaw Cycling

The concentrated vectors prepared in CF, TF1, and TF2 (Table 2, Experiment 1) were subjected to a short stability study to investigate whether aggregation would occur during refrigerated storage or following multiple freeze–thaw (F/T) cycles. Aggregation was assessed by DLS, and *Rh* values >20 nm were deemed to indicate the occurrence of aggregation. As shown in Table 3, AAV2-AADC vector prepared in CF showed 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 occurred after 5 days at 4°C; however, aggregation occurred after one or more F/T cycles at −20 or −80°C, as indicated by the high DLS signal intensity (too high to measure in neat samples). Visual inspection indicated slight cloudiness in these samples, which is consistent with aggregation. For vector prepared in TF2, no aggregation was observed after storage at 4°C or follow-up to 10 F/T cycles at −20°C. Some aggregation was observed after 5 and 10 F/T cycles at −80°C.

Functional Studies

As described above, the high-ionic-strength isotonic formulation TF2 effectively prevented vector aggregation during concentration and storage and represented a promising candidate for further study. An important question was whether preparation and storage of the vector in high-ionic-strength TF2 would adversely affect its functional activity. To assess this, assays were performed to measure the infectious titer and transduction

TABLE 3: Stability of AAV2 vectors prepared in control and elevated-ionic-strength formulations after 4°C storage or freeze–thaw (F/T) cycling

		Particle radius—*Rh* (nm)						
		4°C	−20°C			−80°C		
Formulation	Pre[a]	5 days	1 F/T	5 F/T	10 F/T	1 F/T	5 F/T	10 F/T
CF[b]	14.5	27.0	22.4	56.1	94.5	20.6	57.5	141
TF1	13.8	16.3	TH[c]	TH	TH	TH	TH	TH
TF2	13.8	14.4	14.2	14.0	14.1	13.8	21.3	50.9

[a] Pre, DLS radius measured immediately following 0.2-μm filtration.
[b] Vector concentrations (vg/mL): CF, 1.93×10^{13}; TF1, 2.38×10^{13}; TF2, 2.33×10^{13}.
[c] TH, signal intensity too high to measure.

efficiency of vectors prepared and stored for an extended period of time in TF2. For infectivity, a highly sensitive assay capable of detecting single infectious events was used [15]. AAV2-AADC prepared in TF2 at a concentration of 6.4×10^{13} and stored at 4°C for 45 days was assayed and determined to have 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 the assay (RSD ~50%). Transduction efficiency was assessed by measuring the expression of AADC protein by ELISA following the transduction of D7/4 cells. As shown in Fig. 2, at vector inputs ranging from 10 to 10^5 vg/cell there was no significant difference between vector prepared in TF2 and the reference control. Together, these data indicate that preparation and storage of AAV2 vectors in high-ionic-strength TF2 does not have a detrimental effect on vector infectivity or transduction efficiency.

Discussion

Critical to the success of clinical gene transfer vectors is the development of purification methods and final product formulations that ensure high safety, consistency, and potency appropriate for preclinical and clinical applications. An important requirement is to maintain product solubility during purification and storage, as is well established for protein therapeutics [16–19]. For AAV2, vector particle aggregation is a problem that has been previously recognized [6–9]. Factors influencing vector aggregation, including the intrinsic characteristics of the virus particle as well as the role of trace impurities, need to be well defined to optimize vector purification methods and product formulation and storage conditions.

In the current study we found that some degree of aggregation of AAV2 vectors purified by our laboratory occurred in neutral-buffered solutions such as phosphate- and Tris-buffered saline when particle concentrations reached approximately $1–2 \times 10^{13}$ particles/mL. In studies that aimed to increase vector solubility in low-ionic-strength solutions to support crystal structure determination, Xie and colleagues reported that 25% (w/v) glycerol enabled concentration of AAV2 to very high concentrations ($4.4–18 \times 10^{14}$ particles/mL) [9]. In our preliminary screen of excipients, we tested glycerol and other sugars at concentrations of up to 5% and did not observe a reduction of vector aggregation induced by low ionic strength. The mechanism by which glycerol improves the solubility of AAV2 may be most effective at higher concentrations. Croyle and colleagues reported a significant loss of the titer of AAV and adenovirus, possibly due to aggregation, following multiple freeze–thaw cycles in sodium phosphate buffer [6]. The results of our freeze–thaw stability study using sodium phosphate are consistent with their findings. We found that while 150 mM sodium phosphate provided sufficient ionic strength to prevent aggregation during preparation and nonfrozen storage of concentrated AAV2-AADC vector, even a single freeze–thaw cycle resulted in aggregation. Particle aggregation is also an unresolved issue for adenovirus. A field-use stability study of a recently established adenovirus reference material was reported [20] in which variable levels of virus aggregation were reported.

We have shown that solution ionic strength is a parameter affecting the solubility of our AAV2 vector preparations, implicating ionic interactions between virus particles in aggregation. The observation that elevated ionic strength increased AAV2 vector solubility regardless of the identity of the charged excipient supports the hypothesis that ionic strength per se, rather than interactions involving a specific ion species, is important. The low solubility of AAV2 particles might be caused by its highly symmetrical structure in conjunction with the stabilizing effect of interactions between oppositely charged moieties such as amino acid side chains on neighboring particles. A pH dependence of AAV2 vector aggregation that is consistent with the participation of charged-vector-surface amino acids has been reported previously [9,21]. However, in the current study we observed that the addition of free amino acids with charged side chains prevented AAV2 vector aggregation only at ionic strengths at which aggregation was prevented using other salts. The absence of a specific effect using soluble amino acids suggests that other mechanisms contributed to interparticle interactions in our studies. Vector aggregation at low ionic strength was found to be reduced by nuclease treatment of already purified vector particles, suggesting that nucleic acid impurities (e.g., host cell and plasmid DNA fragments) associated with the surface of virus particles can form ionic bonds to neighboring particles. We previously reported [22] that AAV2 vector stocks prepared by transient transfection and purified by density gradient ultracentrifugation contained approximately 46 pg/10^9 vg of nonvector DNA (plasmid and mammalian). This nucleic acid was found to be resistant to nuclease digestion, and we concluded that it was packaged. However, the observation reported here that nuclease treatment of our purified AAV2 vectors reduced aggregation implicates residual vector surface nucleic acids. An explanation for this discrepancy is that while most of the impurity DNA in our preparations is packaged, a small amount of DNA that cannot be resolved by our Q-PCR assay is present on the vector surface and contributes to aggregation. For a preparation with 46 pg nonvector DNA per 10^9 vg, we estimate that the amount of surface-associated nucleic acid is less than the standard deviation of the Q-PCR assay (RSD ~10%), therefore <4.6 pg/10^9 vg.

The conditions that we used for Benzonase digestion of crude cell lysates did not achieve the degree of removal

doi:10.1016/j.ymthe.2005.02.021

of vector surface DNA required to prevent aggregation in subsequently purified vector. This may pertain specifically to our protocol, which required an elevated salt concentration to efficiently extract vector from cells, conditions not optimal for Benzonase activity. Nuclease treatment of purified vector was effective in reducing aggregation and may provide a useful purification step for optimizing the stability of concentrated vectors preparations. This use of a nuclease would require additional steps to ensure its subsequent removal, such as diafiltration with an appropriate membrane that retains vector but not the nuclease molecule.

Commonly used buffered-saline solutions have ionic strengths (μ ~150 mM) that may be insufficient to prevent aggregation of concentrated AAV2 with trace amounts of vector surface-associated nucleic acids. In our screening studies, an ionic strength of $\geq$200 mM was required to prevent aggregation at the vector particle concentrations examined, and higher ionic strengths (300–500 mM) may be preferred for optimal solubility and stability. Formulations for preclinical and clinical studies should be approximately isotonic, especially for *in vivo* administration of vector to sites at which solute diffusion may be slow. The exponential relationship of ionic strength with charge valency can be used to achieve this objective. Compared to monovalent salts such as sodium chloride, salts with multiple valencies (e.g., some salts of sulfate, citrate, and phosphate) that are established parenteral excipients (www.accessdata.fda.gov/cder/iig/index.cfm) used at isotonic concentrations can provide higher ionic strengths and thereby enhance the solubility of concentrated AAV2 vectors. For example, the sodium citrate formulation (TF2) characterized in these studies was isotonic (315 mOsm) and provided an ionic strength (510 mM) that enabled AAV2 vector concentration to 6.4×10^{13} vg/mL without evidence of aggregation.

In summary, our studies show that trace amounts of nucleic acid impurities associated with the vector surface can contribute to ionic interactions between vector particles contributing to AAV2 vector aggregation. Efficient removal of residual vector surface nucleic acids during purification and the use of elevated-ionic-strength solutions during AAV2 vector purification and formulation are useful strategies to achieve stable, concentrated solutions of AAV2 vectors.

MATERIALS AND METHODS

AAV purification. AAV2 vectors expressing human coagulation factor IX (FIX) or human amino acid decarboxylase (AADC) were produced by triple transfection of HEK293 cells as previously described [23], with modifications. For large-scale preparations, cells were cultured and transfected in 850-cm^2 roller bottles (Corning). Vectors were purified by one of three methods. In purification Method 1, transfected HEK293 cells from roller bottles were collected by centrifugation (1000*g*, 15 min), resuspended in 500 mM NaCl, 2.5mM $MgCl_2$, 50 mM Tris, pH 8.5, and lysed by three freeze/thaw cycles (alternating an ethanol/dry ice bath and a 37°C water bath). The cell lysate was clarified by centrifugation (8000*g*, 15 min). The pH of the clarified cell lysate was approximately 8.0. The supernatant was then diluted to 200 mM NaCl by the addition of sterile water and digested with Benzonase (purity Grade 1, Merck, Darmstadt, Germany; 200 U/mL, 1 h, 37°C). The lysate was adjusted to 25 mM $CaCl_2$ using a 1 M stock solution and incubated (1 h, 4°C). The mixture was centrifuged (8000*g*, 15 min) and the supernatant containing vector collected. To precipitate virus from the clarified cell lysate, polyethylene glycol (PEG 8000) was added to a final concentration of 8%, and the mixture was incubated (3 h, 4°C) and then centrifuged (8000*g*, 15 min). The pellets containing vector were resuspended with mixing in 0.15 M NaCl, 50 mM HEPES, 25 mM EDTA, pH 8.0, and incubated (16 h, 4°C). The resuspended material was pooled, and dry CsCl was added to a final density of 1.40 gm/mL. Vector was then banded by ultracentrifugation (SW28, 25,000 rpm, 24 h, 20°C) using a Beckman Model LE-80 centrifuge. The centrifugation tubes were fractionated, and densities from 1.38 to 1.42 gm/mL containing vector were pooled. The material was banded a second time by ultracentrifugation (NVT65 Rotor, 60,000 rpm, 16 h, 20°C), and fractions containing AAV2 vectors were pooled. Ultrafiltration/diafiltration (UF/DF) by tangential-flow filtration was used to achieve concentration and buffer exchange as described in the following section. In purification Method 2, cell harvests containing AAV were microfluidized and filtered through 0.65-and 0.2-μm filters (low protein binding, Sartorius, Goettingen, Germany). Virus was purified from the clarified cell lysates by chromatography using Poros 50HS cation-exchange resin (PE Biosystems, Foster City, CA, USA) as previously described [24]. For the nuclease digestion described in Fig. 2, column-purified vectors were incubated (4 h, RT) with 100 U/mL Benzonase and 10 U/mL DNase I (RNase free, Roche Applied Science, Penzberg, Germany). For purification Method 3, vectors obtained following chromatography were further purified by CsCl gradient ultracentrifugation (SW28, 25,000 rpm, 24 h, 20°C) to remove empty capsids.

Real-time quantitative PCR was used to quantify AAV preparations as previously described [25]. Vectors purified by each method were assessed by SDS–PAGE/silver staining analysis, and in all cases VP1, VP2, and VP3 were 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) contained empty capsids at a level ranging from 3 to 10 empty capsids per vector genome. We previously reported characterization of the quantity and size distribution of DNA impurities in AAV2 vectors prepared by these methods [22] (Smith et al., manuscript in preparation). The levels of residual plasmid and mammalian DNA were 11 and 35 $pg/10^9$ vg, respectively, for CsCl gradient-purified vector preparations, and 31 and 100 $pg/10^9$ vg, respectively, for column-purified vector. The higher level of DNA impurities in vectors purified by column chromatography corresponded to fragments of nonvector nucleic acids associated with empty/partially filled capsids that copurified with vector particles in the absence of a density gradient separation step. To measure the level of vector surface nucleic acid impurities, Benzonase digestion was performed by the mixing of 100 μL of purified vector with 400 μL of digestion buffer (10mM Tris, pH 8.0, 10mM $MgCl_2$, 500U Benzonase) and then incubation (60 min, 37°C). Within the limit of precision of the Q-PCR assay used (RSD ~10%), no significant reduction in plasmid and genomic DNA was observed following this nuclease treatment of column-or cesium gradient-purified vectors. In spike controls in which plasmid (250 pg) or genomic (85 ng) DNA was added to the vector, spiked DNA was fully digested. Southern blot analysis of the nonvector plasmid and mammalian DNA indicated a range of sizes up to the packaging limit of the vector (not shown).

Ultrafiltration/diafiltration. Disposable hollow-fiber tangential-flow filtration devices (8-in. Midgee, 100 kDa MW cutoff, Amersham Biosciences, Uppsala, Sweden) were 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 10× the product volume was used, which was added in ~1-mL increments to approximate continuous diafiltration.

Using this method, the calculated amount of residual CsCl after diafiltration was <0.5 mM. The following three formulations were used for UF/DF: Control Formulation (CF; 140 mM NaCl, 10 mM sodium phosphate, 5% sorbitol, pH 7.3, 592 mOsm); Test Formulation 1 (TF1; 150 mM sodium phosphate, pH 7.5, 355 mOsm); and Test Formulation 2 (TF2; 100 mM sodium citrate, 10 mM Tris, pH 8.0, 315 mOsm). For Experiment 1 shown in Table 2, diafiltration was performed at a volume corresponding to a concentration of 1×10^{13} vg/mL and then reduced to a value corresponding to 2.5×10^{13} vg/mL (assumes no vector loss). For Experiment 2, diafiltration was performed at a volume corresponding to a 2×10^{13} vg/mL and then reduced to a value corresponding to 6.7×10^{13} vg/mL. For Experiment 3 (CF ± Bz), AAV2-AADC vector (approximately 1.2×10^{14} vg) was first diafiltered into TF1 and then passed through a 0.2-μm filter. The titer was determined and the volume adjusted to 1×10^{13} vg/mL. To 10 mL of this material, $MgCl_2$ was added to a final concentration of 2 mM, and the material was then divided into two 5-mL aliquots. One aliquot was incubated with Benzonase (200 U/mL, 4 h, RT), and the second was mock-incubated. Each aliquot was then diafiltered into CF at a vector concentration of 2×10^{13} vg/mL and then concentrated to a 3.6×10^{13}-vg/mL target. Following all UF/DF protocols, Pluronic F-68 (BASF, Mount Olive, NJ, USA) was added to the vector product from a 1% stock to a final concentration of 0.001%, and the solution was passed through a 0.2-μm syringe filter (Sartorius).

Measurement of vector aggregation by dynamic light scattering. Purified vectors were assessed for aggregation by dynamic light scattering using Protein Solutions *DynaPro 99* (λ = 825.4 nm). Primary data (particle radius *Rh*, average value measured over 30 cycles, 10 cycles/min) were used for all analyses reported. A dilution-stress method was developed to assess the effect of varying excipients on vector aggregation. In this method, 80 μL of test excipient was added to 20 μL of purified vector with rapid mixing in the cuvette used for DLS measurement, and data collection was initiated within 10 s of mixing. Prior to the addition of excipients, the *Rh* value for AAV2 vector preparations was measured and confirmed to be <15 nm. Samples that were not 100% monomeric were passed through a 0.2-μm syringe disc filter (Sartorius) to remove aggregates. The osmolarity and ionic strength values shown in Fig. 1 were weighted calculations of test excipients (80%) and the starting vector formulations (20%). The osmolarity was calculated according to the equation: osmolarity = Σc_i, where c_i is the molar concentration of each solute species. The ionic strength (μ) was calculated according to the equation: $\mu = 1/2\Sigma c_i z_i^2$, where z_i is the charge on each species. Under conditions that resulted in vector aggregation (e.g., low μ), a progressive increase in *Rh* was observed over time. To validate the use of average *Rh* measured over the 3-min interval following dilution as a measure of aggregation, the average rate of increase of *Rh* ($\Delta Rh/\Delta t$) over the same time interval was also assessed, giving conclusions that were concordant with those reported in Fig. 1.

Functional studies. The infectivity of AAV2-AADC vectors was determined using a sensitive assay as previously described [15]. The test sample was run concurrently with an AAV2-AADC reference previously prepared in CF and stored at −80°C. The transduction efficiency of AAV2 vectors was measured using a whole-cell ELISA. Briefly, D7/4 cells grown in 96-well plates were infected with 10-fold serial dilutions of the test sample and reference vector at inputs ranging from 10 to 10^5 vg/cell (five replicates/dilution). After 48 h, the culture medium was removed, and cells were washed twice with 200 μL PBS (10 mM sodium phosphate, 140 mM NaCl, pH 7.2). Cells were then permeabilized and fixed by the addition of 100 μL PBS containing 0.5% Triton X-100 and 4% paraformaldehyde to each well (15 min). Cells were then washed twice with PBS containing 0.5% Triton X-100. Nonspecific sites were blocked by adding PBS containing 3% bovine serum albumin (BSA) and 0.5% Triton X-100 (60 min). After washing, cells were incubated (60 min) with rabbit anti-AADC IgG antibody (AB136, Chemicon, Temecula, CA, USA) and washed. Cells were then incubated (60 min) with alkaline phosphatase-conjugated goat anti-rabbit IgG and washed. Antibodies were diluted 1:1000 in PBS containing 1% BSA, 0.5% Triton X-100. The substrate para-nitrophenylphosphate (PNPP, Pierce Biotechnology, Rockford, IL, USA) was added (1 mg/mL in diethanolamine buffer, Pierce), and after 30 min the concentration of cleaved substrate was measured spectrophotometrically (λ = 405 nm). Human AADC expression as a function of vector input was fitted using a spline curve (SigmaPlot9.0, Systat Software, Point Richmond, CA, USA). The AAV2-AADC reference vector was measured concurrently with the test sample.

ACKNOWLEDGMENTS

The authors thank Rajiv Nayar (HTD Systems) and Mark D'Andrea (D'Andrea Consulting) for helpful contributions, Kenneth Chahine, Michael Lochrie, Shangzhen Zhou, and Hoc Nguyen (Avigen Inc.) for valuable scientific discussions, and Weidong Xiao (University of Pennsylvania) for review of the manuscript.

RECEIVED FOR PUBLICATION DECEMBER 9, 2004; ACCEPTED FEBRUARY 18, 2005.

REFERENCES

1. Grimm, D., and Kleinschmidt, J. A. (1999). Progress in adeno-associated virus type 2 vector production: promises and prospects for clinical use. *Hum. Gene Ther.* **10:** 2445–2450.
2. High, K. A. (2001). AAV-mediated gene transfer for hemophilia. *Ann. N.Y. Acad. Sci.* **953:** 64–67.
3. Pfeifer, A., and Verma, I. M. (2001). Gene therapy: promises and problems. *Ann. Rev. Genomics Hum. Genet.* **2:** 177–211.
4. Berns, K. I. (1996). Parvoviridae: the viruses and their replication. In *Fields Virology* (B. N. Fields, D. M. Knipe, P. M. Howley, *et al.*, Eds.), pp. 2173–2197. Lippincott–Raven, Philadelphia.
5. Xie, Q., *et al.* (2002). The atomic structure of adeno-associated virus (AAV-2), a vector for human gene therapy. *Proc. Natl. Acad. Sci. USA* **99:** 10405–10410.
6. Croyle, M. A., Cheng, X., and Wilson, J. M. (2001). Development of formulations that enhance physical stability of viral vectors for gene therapy. *Gene Ther.* **8:** 1281–1290.
7. Huang, J., *et al.* (2000). Aggregation of AAV vectors, its impact on liver directed gene transfer and development of vector formulations to prevent and dissolve aggregation and enhance gene transfer efficiency. *Mol. Ther.* **1:** S286.
8. Wright, J. F., Qu, G., Tang, C., and Sommer, J. M. (2003). Recombinant adeno-associated virus: formulation challenges and strategies for a gene therapy vector. *Curr. Opin. Drug Discov. Dev.* **6:** 174–178.
9. Xie, Q., Hare, J., Turnigan, J., and Chapman, M. S. (2004). Large-scale production, purification and crystallization of wild-type adeno-associated virus-2. *J. Virol. Methods* **122:** 17–27.
10. Braun, A., Kwee, L., Labow, M. A., and Alsenz, J. (1997). Protein aggregates seem to play a key role among the parameters influencing the antigenicity of interferon alpha (IFN-alpha) in normal and transgenic mice. *Pharm. Res.* **14:** 1472–1478.
11. Chenuaud, P., *et al.* (2004). Autoimmune anemia in macaques following erythropoietin gene therapy. *Blood* **103:** 3303–3304.
12. Flotte, T. R. (2004). Immune responses to recombinant adeno-associated virus vectors: putting preclinical findings into perspective. *Hum. Gene Ther.* **15:** 716–717.
13. Gao, G., *et al.* (2004). Erythropoietin gene therapy leads to autoimmune anemia in macaques. *Blood* **103:** 3300–3302.
14. High, K. A., *et al.* (2004). Human immune responses to AAV-2 capsid may limit duration of expression in liver-directed gene transfer in humans with hemophilia B. *Blood* **104:** 121a.
15. Zhen, Z., Espinoza, Y., Bleu, T., Sommer, J. M., and Wright, J. F. (2004). Infectious titer assay for adeno-associated virus vectors with sensitivity sufficient to detect single infectious events. *Hum. Gene Ther.* **15:** 709–715.
16. Chen, B., Arakawa, T., Hsu, E., Harhi, L. O., Tressel, T. J., and Chien, S. L. (1994). Strategies to suppress aggregation of recombinant keratinocyte growth factor during liquid formulation development. *J. Pharm. Sci.* **83:** 1657–1661.
17. Shire, S. J., Shahrokh, Z., and Liu, J. (2004). Challenges in the development of high protein concentration formulations. *J. Pharm. Sci.* **93:** 1390–1402.
18. Wang, W. (1999). Instability, stabilization, and formulation of liquid protein pharmaceuticals. *Int. J. Pharm.* **185:** 129–188.
19. Won, C. M., Molnar, T. E., McKean, R. E., and Spenlehauer, G. (1998). Stabilizers against heat-induced aggregation of RPR 114849, an acidic fibroblast growth factor (aFGF). *Int. J. Pharm.* **167:** 25–36.
20. Adadevoh, K., Croyle, M., Malarme, D., Bonfils, E., and Bowe, M. A. (2002). A short-term field use and shipping stability study of a wild type Ad5 adenoviral reference material. *BioProcessing* **Fall:** 62–69.

doi:10.1016/j.ymthe.2005.02.021

21. Qu, G., Connolly, C., Koblansky, A., Sommer, J. M., McClelland, A., and Wright, J. F. (2003). Evidence that ionic interactions are involved in concentration-induced aggregation of recombinant adeno-associated virus. *Mol. Ther.* **7:** S238.
22. Smith, P., Wright, J. F., Qu, G., Patarroyo-White, S., Parker, A., and Sommer, J. M. (2003). Packaging of host cell and plasmid DNA into recombinant adeno-associated virus particles produced by triple transfection. *Mol. Ther.* **7:** S348.
23. Matsushita, T., *et al.* (1998). Adeno-associated virus vectors can be efficiently produced without helper virus. *Gene Ther.* **5:** 938–945.
24. Wright, J. F., and Qu, G. (2003). Large-scale recombinant adeno-associated virus (rAAV) production and purification. *United States Patent* **6,593,123**.
25. Sommer, J. M., *et al.* (2003). Quantification of adeno-associated virus particles and empty capsid by optical density measurement. *Mol. Ther.* **7:** 122–128.