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Purification of recombinant adeno-associated virus type 8 vectors by ion exchange chromatography generates clinical grade vector stock☆

Andrew M. Davidoff[a,b], Catherine Y.C. Ng[a], Susan Sleep[b], John Gray[b], Selina Azam[c], Yuan Zhao[c], Jenny H. McIntosh[d], Morteza Karimipoor[d], Amit C. Nathwani[d,e,*]

[a] *Department of Surgery, St. Jude Children's Research Hospital, Memphis, TN, USA*
[b] *Division of Experimental Hematology, St. Jude Children's Research Hospital, Memphis, TN, USA*
[c] *National Institute for Biological Standards and Control, Potters Bar, UK*
[d] *Department of Haematology, University College London, London, UK*
[e] *National Blood Service, UK*

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Abstract

Recombinant vectors based on the recently isolated AAV serotype 8 (rAAV-8) shows great promise for gene therapy, particularly for disorders affecting the liver. Transition of this vector system to the clinic, however, is limited by the lack of an efficient scaleable purification method. In this report, we describe a simple method for purification of rAAV-8 vector particles based on ion exchange chromatography that generates vector stocks with greater than 90% purity. The average yield of purified rAAV-8 from five different vector preparation was 41%. Electron microscopy of these purified stocks revealed typical icosohedral virions with less than 10% empty particles. Liver targeted delivery of ion-exchange purified rAAV-8 vector encoding the human factor IX (hFIX) gene, resulted in plasma hFIX levels approaching 30% of normal in immunocompetent mice, which is 20-fold higher than observed with an equivalent number of rAAV-5 ion exchange purified vector particles. The method takes less then 5 h to process and purify rAAV-8 vector from producer cells and represents a significant advance on the CsCl density centrifugation technique in current use for purification of rAAV-8 vector systems and will likely facilitate the transition of the rAAV-8 vector system to the clinic.

Keywords: rAAV-8 purification; Ion exchange chromatography

1. Introduction

Recombinant adeno-associated viral (rAAV) vectors are a promising system for gene therapy of disorders that affect the liver such as haemophilia B. There are currently eight immunologically distinct serotypes of AAV, however, vectors based on serotype 2 (AAV-2) have been most extensively evaluated in preclinical studies as this was the first serotype to be fully characterized (Samulski et al., 1983). Based on highly encouraging efficacy data in animal models, AAV-2 based vectors are currently being evaluated in a Phase I/II study of liver targeted gene transfer in patients with severe haemophilia (Nathwani et al., 2003; High et al., 2003). Vectors based on the alternative serotypes of AAV are gaining in popularity because their unique tropism may result in more efficient transduction of the target tissue than currently possible with rAAV-2 based vectors (Grimm et al., 2003; Rutledge et al., 1998; Zabner et al., 2000; Davidson et al., 2000). Indeed, the most recent isolate, AAV-8, mediates

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* Corresponding author. Tel.: +44 207 679 6225; fax: +44 207 679 6221.
E-mail address: a.nathwani@ucl.ac.uk (A.C. Nathwani).

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between 10- to 100-fold higher transduction of murine liver then observed with equivalent numbers of AAV-2 particles (Gao et al., 2002; Sarkar et al., 2004). Consequently, fewer vector particles will be required to achieve transgene expression at therapeutic levels which will ease pressure on vector production whilst adding a measure of safety as biodistribution of rAAV to non-target sites, including the gonads, is directly proportional to the dose of rAAV administered (Nathwani et al., 2001). An equally important advantage afforded by AAV-8 vector system is an ability to circumvent pre-existing immunity to AAV-2 that is prevalent in over 70% of humans as a consequence of infection with wild type virus. In animal model, neutralizing anti-AAV-2 antibodies block transduction with rAAV-2 based vectors, but not with vectors based on rAAV-8 (Gao et al., 2002). Hence, vectors based on alternative serotypes may prove to be a valuable resource for gene therapy.

A major limitation to the clinical use of rAAV-8 vectors is the lack of efficient methods for generating clinical grade vector particles. Key to the transition of the rAAV-2 vector system to the clinic was the establishment of heparin affinity chromatography purification method based on the finding that heparan sulphate proteoglycans served as a natural receptor for AAV-2 (Summerford and Samulski, 1998; Clark et al., 1999).

As the cellular receptor for AAV-8 has not been described, it is not amenable to purification using affinity chromatography. Currently, rAAV-8 vectors are purified by density gradient centrifugation using CsCl, the traditional approach for isolating rAAV vectors. Density centrifugation is time consuming, difficult to scale-up and yields vector stocks that are heavily contaminated with cellular proteins, thus, rendering them unsuitable for clinical use. In addition, CsCl has been shown to denature rAAV vector particles leading to a substantial reduction of the therapeutic potency (Auricchio et al., 2001a). As an alternative to affinity purification methods, many investigators have shown that ion exchange chromatography can be used to purify rAAV-2 and 5 based vectors without compromising potency (Kaludov et al., 2002; Zolotukhin et al., 2002; Smith et al., 2003; Brument et al., 2002). An ion exchange method for purification of rAAV-5 vector using membrane immobilised resin that generates vector stock with purity and potency that is comparable to that achieved with mucin affinity chromatography has recently been described (Sleep et al., 2003). However, to date, a scaleable chromatographic method for purification of rAAV-8 vectors has not been described.

In this report, a simple but versatile method for purification of rAAV-8 vectors based on ion exchange chromatography is described. The purity of the resulting vector stocks is in excess of 80% as assessed by SDS PAGE electrophoresis. Additionally, the ion-exchange purified rAAV-8 particles are highly potent in murine models after liver targeted delivery of vector. This novel method is amenable to scale-up under good manufacturing practice (GMP) conditions and will be valuable in the development of AAV-8 based gene transfer vectors for clinical applications.

2. Methods and materials

2.1. Production of pseudotyped vectors

Pseudotyped AAV vector stocks in which an AAV-2 genome was cross-packaged with AAV-8 capsid proteins were prepared according to the method described previously (Gao et al., 2002). In brief, subconfluent 293T cells were co-transfected using calcium phosphate with pAV-2 vector plasmids together with adenoviral helper plasmid [80-XX6 (Nathwani et al., 2000)] and a chimeric packaging plasmid [pAAV8-2 (Gao et al., 2002)] kindly provided by Dr James Wilson (Philadelphia, PA). Vector plasmid pAV-2 CAGG-FIX encoding human FIX and pAV-2 CMV-GIL, a biscistronic vectors encoding GFP have been described before (Nathwani et al., 2000, 2001). pAV-2 HCR hAAT-FIX is similar to pAV-2 CAGG-FIX except that the CAGG enhancer–promoter has been replaced with the human apolipoprotein E/C-I gene locus control region (HCR) and the human (1 antitrypsin promoter (hAAT). rAAV producer cells were harvested between 50 and 60 h post transfection, washed with PBS and re-suspended in TD buffer (140 mM NaCl, 5 mM KCl, 0.7 mM K_2HPO_4, 25 mM Tris HCl (pH 7.4)) at a concentration of 2×10^7 cells/ml. rAAV-2/8 vector was liberated from the producer cells by five cycles of freeze thaw and incubated with 0.5% deoxycholate (Sigma, Poole, UK) in the presence of 50 U/ml benzonase (Sigma, Poole, UK), for 30 min at 37 °C. The cellular debris was removed by centrifugation (2000 g) and the supernatant was then filtered through a 0.2 μm PVDF filter (Millipore, UK Ltd, Watford, UK) prior to dilution with 20 mM Bis Tris Propane buffer to reduce salinity to between 20 and 50 mM. Pseudotyped rAAV-2/5 vectors were prepared in a similar fashion using previously described pAAV5-2 (Chiorini et al., 1999) and pRep-Ad (Nathwani et al., 2000) packaging plasmids. Pre-purification processing of the rAAV-2/5 vectors consisted of incubation with 0.1% octylglucopyranoside (Sigma, Poole, UK) for 30 min at 37.0 °C instead of deoxycholate and benzonase. The rAAV-2/5 clarified lysate was diluted with 20 mM Bis Tris propane buffer to reduce salinity to between 20 and 50 mM and adjusted to pH 6.0, prior to chromatography.

2.2. Ion-exchange chromatography

Purification of rAAV-2/5 was as per the method described originally using Mustang® S and Q ion exchange chromatography “coin” devices (Pall Corp., Portsmouth, UK) (Sleep et al., 2003). The Mustang coins consist of layers of membrane coated with modified hydrophilic polyethersulphone containing either sulphonic acid (Mustang-S) or quaternary amines (Mustang-Q) ion exchange groups. Efficient

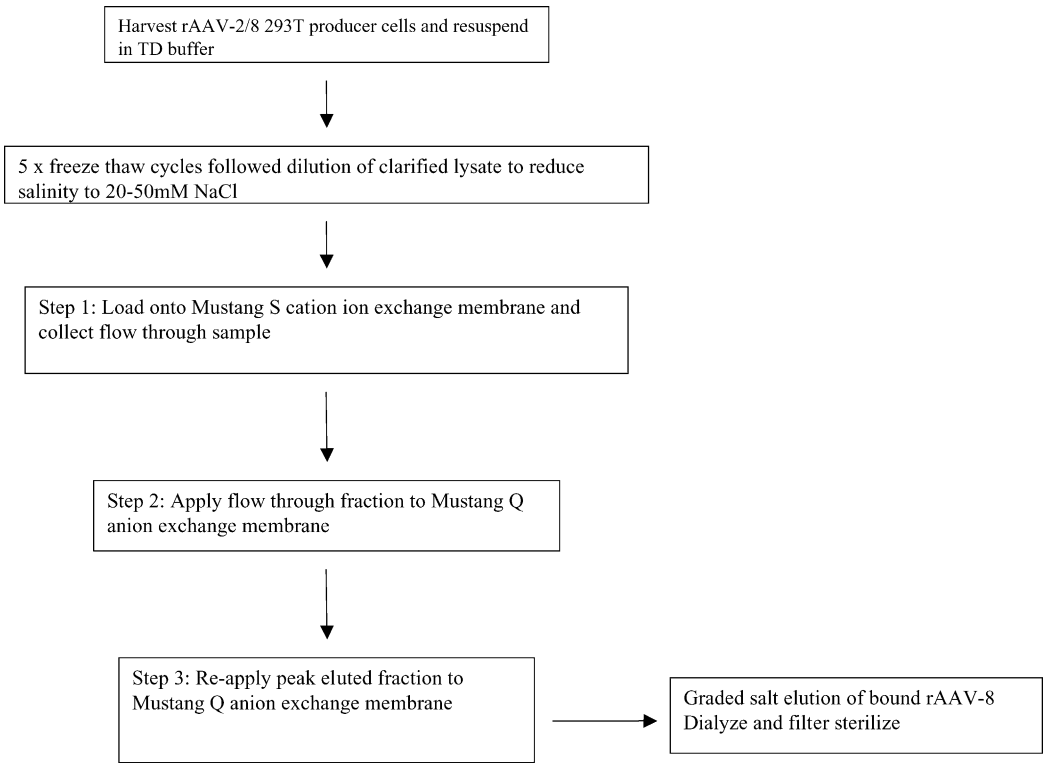


Fig. 1. Scheme for purification of rAAV-2/8: 293T cells were transfected with vector, packaging and helper plasmids to generate chimeric rAAV-2/8 vector. The viral particles were released by five cycles of freeze-thaw and the clarified lysate processed by ion exchange chromatography as outlined in the figure.

purification of rAAV-5 vector particles with these devices required a two-step purification method where the crude clarified cellular lysate, adjusted to pH 6.0, was initially passed through a Mustang-S cation exchange coin which had the critical role of removing some of the cellular proteins but not rAAV vector. The flow through sample was then applied directly onto a Mustang-Q anion exchange coin, which efficiently captured the rAAV vector particles. Failure to pass the crude clarified lysate through cation ion exchange matrix substantially reduced the eventual yield and purity of rAAV-5 vector indicating that this step was not dispensable. We, therefore, adopted a similar dual ion exchange method for purification of rAAV-2/8 vector particles (Fig. 1). The initial experiments were designed to determine the optimal pH for efficient binding of rAAV-8 particles to the Mustang membrane. Briefly, the membranes were placed in their housing, clamped and equilibrated as per manufacturer's instructions with 10 ml of 1 M NaOH followed by 10 ml of 1 M NaCl and then 20 ml of 20 mM Bis Tris propane at appropriate pH (between 6.0 and 9.0) using a syringe. The diluted, filtered and clarified rAAV-8 lysate was adjusted to the appropriate pH (between 6.0 and 9.0) and applied first to the Mustang® S membrane (see Fig. 1) using a syringe at approximately one drop per second and then washed with 1.5 ml of base buffer. The Mustang-S flow through sample was then loaded onto equilibrated Mustang-Q membranes. After washing with 1.5 mls of base buffer, the anion membrane bound proteins were eluted with increasing concentration of NaCl (100–1000 mM) and the vector yield determined by standard slot blot technique. Additionally some of the rAAV-2/8 preparations were passed twice through Mustang-Q followed by concentration using appropriately sized devices containing 10 kd Ultracel-YM membrane (Millipore, UK Ltd, Watford, UK). Once suitable parameters for purification of rAAV-8 had been defined, several rAAV-8 preparations were purified using the Mustang system to establish reproducibility of yields and purity.

2.3. Physical and biological characterization of rAAV vector

rAAV-5 and 8 vector stocks were titrated by standard slot blot analysis to determine the number of genome containing vector particles as described before (Nathwani et al., 2000). Contamination of rAAV vector stocks with cellular proteins was assessed by SDS PAGE and silver staining. In brief, approximately, 0.5–1 $\times$ 10^{10} vector particles in 5 $\times$ Laemmli buffer were denaturated at 98 °C for 5 min prior to electrophoresis on 10% SDS-PAGE gel. Total proteins were visualised using the silver stain plus kit according to the manufacturer's instruction (BIO-RAD Laboratories Ltd, Hemel Hempstead, UK). Percentage purity was determined by standard densitometry methodology. For electron microscopy, aliquots of purified rAAV 5 and 8 were dropped onto 200 mesh electron microscopy grids, double coated with Formvar and thin carbon films. The grids were then washed

Table 1
Effect of pH on recovery of rAAV-2/8 in peak fraction using ion exchange chromatography

pH	rAAV-2/8 particles loaded on membrane ($\times 10^{11}$)	Mustang-S (cation)			Mustang-Q (anion)		
		rAAV-8 particles recovered in peak fraction ($\times 10^{11}$)	Particle recovery (%)	rAAV-8 particles in flow through sample ($\times 10^{11}$)	rAAV-8 particles recovered in peak fraction ($\times 10^{11}$)	Particle recovery (%)	rAAV-8 particles in flow through sample ($\times 10^{11}$)
6.0	20	5	25	15	0.2	1	11
7.5	20	0.24	1.2	19	2.9	15	9
8.0	20	0.14	0.7	17	4.8	24	7.7
8.5	20	0.14	0.7	17	5.8	29	1.3
9.0	20	0.08	0.4	17	8.7	43	0.2

with 5 mM Tris-HCl, pH 7.5 followed by distilled water, stained with 1% uranyl acetate and then air dried, The rAAV particles were visualized over a number of grids using JEOL 100 CX-II TEM (Hitachi, Tokoyo, Japan) to determine the proportion of full and empty vector particles. In-vivo transduction efficiency was assessed by injecting 1×10^{11} rAAV vector genomes (vg) into the tail vein of 7–10 week old male C57Bl/6 mice. Blood samples were collected at regular intervals from the retro-orbital plexus and assayed for human factor IX (hFIX) by an ELISA methods described previously (Nathwani et al., 2001). The probability of statistical difference between experimental groups was determined by one-way ANOVA and paired student *t* test using GraphPad Prizm version 3.0 software (GraphPad, San Diago, CA).

3. Results and Discussion

Preliminary experiments indicated that rAAV-8 vectors did not bind to either heparin or mucin matrixes that have been used effectively to purify rAAV-2 and 5 vectors, respectively (Summerford and Samulski, 1998; Auricchio et al., 2001b) (data not shown). To establish if ion-exchange chromatography could be used to purify rAAV-8 vector particles we used our previously described two-step ion exchange chromatography method for purification of rAAV-5 (Sleep et al., 2003). This method utilizes the "Mustang® coin" ion exchange chromatography units which differ from conventional chromatography columns in that the ion exchange resin is conjugated on membranes as opposed to beads. The Mustang coins are available commercially and are ideally suited for small scale evaluation without the need for FPLC or HPLC systems. Pseudotyped AAV-2/8 vector in which the AAV-2 genome is packaged in an AAV-8 capsid were generated as described previously using adenovirus free, 293T based transient transfection method (Gao et al., 2002). The yield of rAAV-2/8 chimeric vector in the crude clarified lysate was in the range of $1–3 \times 10^4$ particles/cells, consistent with the published reports (Gao et al., 2002). These crude vector stocks were adjusted to four different pH conditions (pH 6.0–9.0) prior to processing by ion exchange chromatography. In our initial experiments crude lysate from ten 15 cm plates containing approximately 2×10^{12} vector genomes (vg) were loaded onto a single coin. Previous experience with rAAV-5 indicated that this protein load was within the capacity of the coin system. After applying the crude lysate to Mustang-S cation exchange units (Fig. 1), the proportion of rAAV-2/8 vector that flowed through unbound was determined using slot-blot analysis. Consistent with our previous experience with rAAV-5, most of the rAAV-2/8 vector particles passed though the cation resin without binding except at pH 6.0 when approximately 25% of input vector was present in the eluted fraction (Table 1). In contrast, binding of rAAV-2/8 vector to anion resin was more efficient and appeared to improve with increasing pH from 1% at pH 6.0 to 43% at pH 9.0 (Table 1). Over 90% of the bound vector particles

Table 2
Recovery of rAAV-2/8 using the two-step ion exchange chromatography

Vector preparation	Total number of particles in crude lysate (10^{11})	Total number of particles in Mustang-S F/T sample (10^{11})	Total number of particles in purified preparation (10^{11})	Particle recovery (%)
rAAV-2/8 CAGG-FIX	30	30	12	40
rAAV-2/8 CMV GIL	200	178	50	25
rAAV-2/8 HCR hAAT FIX	53	48	28	53
rAAV-2/8 HCR hAAT FIX	200	188	62	31
rAAV-2/8 HCR hAAT-FIX	40	37	23	58

were eluted at 150 mM NaCl from the anion exchange matrix (Fig. 2A).

The overall purity of the eluted rAAV-2/8 vector was estimated (by densitometry) at approximately 40% as in addition to the three rAAV capsid proteins, a number of contaminating bands that were less than 100 kd were observed on silver stained SDS-PAGE gels (lane Q1, Fig. 2B). Re-applying the rAAV-2/8 vector preparations to Mustang-Q membrane followed by concentration of the virus by centrifugation using a 10 kd Ultracel-YM membrane, improved vector purity to over 80% (Fig. 2B, lanes Q2a and b). Electron microscopy of the double anion exchange purified rAAV-2/8 preparation (Fig. 2C) over multiple fields, consistently showed full virions with empty capsids accounting for between 5 and 10% of particles. Several different rAAV-2/8 vector preparations were purified using the method described above (Table 2). The average recovery was 41% which compares favourably with chromatography purification of other rAAV vectors (Kaludov et al., 2002; Zolotukhin et al., 2002; Smith et al., 2003; Brument et al., 2002). The best recovery of rAAV-2/8 with the coin system was observed with crude vector preparations containing less then 5×10^{12} vg, based on the limited data set shown in Table 2. This limitation can be overcome by applying our method to larger commercially available membrane based ion exchange units using either FPLC or HPLC devices.

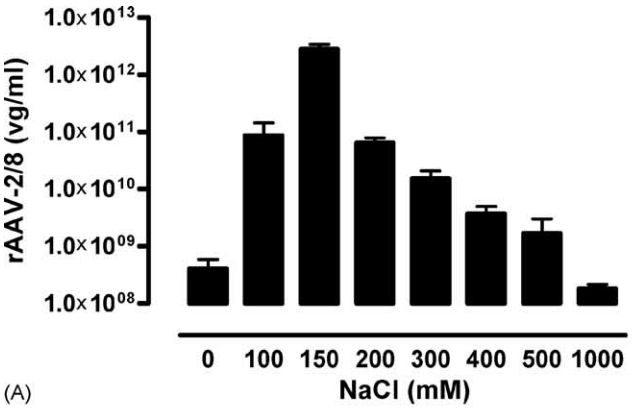
For comparison, several preparations of rAAV-2/5 were purified by the two-step ion exchange method using Mustang coin devices described previously (Sleep et al., 2003). The mean recovery of rAAV-5 vector particles using the two-step ion exchange method (Table 3) was substantially higher at 67% with maximum elution of vector particles from anion resin occurring at 200 mM NaCl (Fig. 3A). The purity of the ion exchange chromatography prepared rAAV-2/5 particles was consistently >80% as assessed by densitometry of sliver stained SDS PAGE gels which revealed the three capsid proteins at the appropriate ratio as the dominant bands (Fig. 3B). Further analysis of the purified rAAV-5 preparations by electron microscopy over multiple fields showed exclusively full, ~25 nm sized particles with the typical icosohedral structure that were evenly distributed and not clumped (Fig. 3C).

Hence there are distinct physiochemical differences between rAAV-5 and 8 vector particles which influences the efficiency with which these vectors bind to anion exchange matrix and are likely a reflection of the differences in the amino-acid composition of the respective capsid. rAAV-8 vector particles bind to anion exchange resin efficiently at alkaline pH whereas, rAAV-5 requires acidic condition for optimal binding. This suggests that, the rAAV-2/8 capsid is more positively charged then rAAV-5 which should allow more efficient interaction of the former with negatively charged cellular membranes. Therefore, the biological activity of the ion exchange chromatography-purified vector stocks was compared in-vivo following systemic administration of 1×10^{11} chromatography purified rAAV-2/8 CAGG FIX or rAAV-2/5 CAGG FIX vector genomes in C57Bl/6 mice. Plasma levels of hFIX at 6 weeks after vector administration were on average 20-fold higher in cohort of mice (n = 4) that were transduced with rAAV-2/8 CAGG FIX (1420 ± 84 ng/ml) compared with a group (n = 4) that received an equivalent number of rAAV-2/5 FIX vector particles (72 ± 15 ng/ml) (Fig. 4). These results were reproducible with different vector stocks and are consistent with previous reports which indicated that rAAV-8 vectors were more efficient at transducing the liver (Gao et al., 2002).

In summary, a simple, scaleable method for purification of rAAV-8 vector particles based on ion exchange chromatography has been developed. This method generates vector stocks that are greater than 80% pure, without compromising potency. It is at present the only scaleable method for generating clinical grade rAAV-2/8 vector and although further optimisation of vector yield is required, it represents a significant advance on the CsCl density centrifugation method in current use. Transition of our method to GMP purification protocols

Table 3
Recovery of rAAV-5 following ion exchange chromatography methods

Vector preparation	Purification method	Total number of particles in crude lysate (10^{11})	Total number of particles in purified preparation (10^{11})	Particle recovery (%)
rAAV-2/5 CAGG-FIX	Ion exchange	145	97	67
rAAV-2/5 CAGG-FIX	Ion exchange	79	57	72
rAAV-2/5 HCR hAAT FIX	Ion exchange	15	9	61



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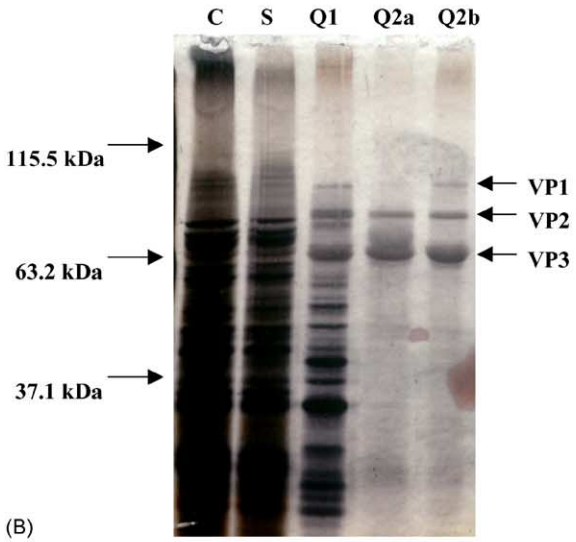

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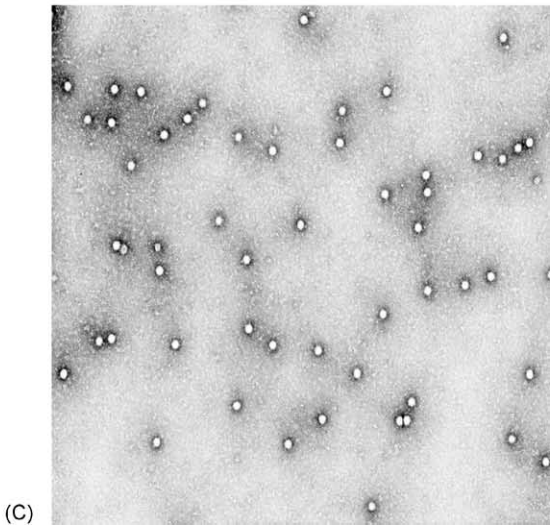
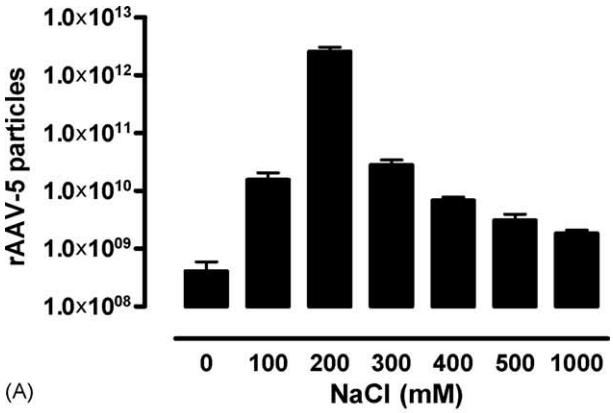
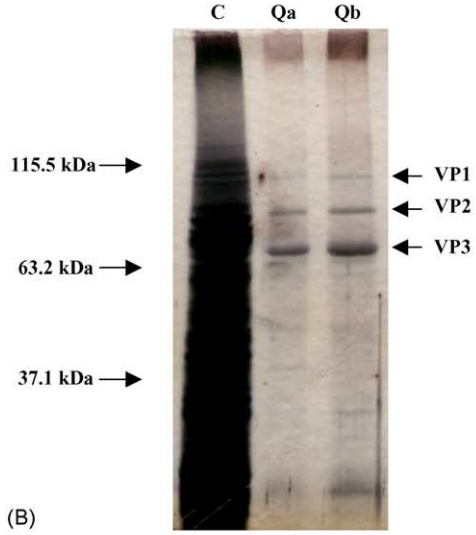


(C)

Fig. 2. Characterization of rAAV-8 purified by Mustang ion exchange chromatography: (A) elution profile of rAAV-8 using Mustang-Q coin units with graded increase in NaCl concentration; (B) silver-stained SDS PAGE analysis of rAAV-2/8 samples (C: crude cell lysate; S: post cation flow through sample; Q1: $\sim 1 \times 10^{10}$ vg from peak elute from Mustang-Q coin; Q2a and b are two individual eluated samples (7×10^9 and 1.3×10^{10} vg, respectively) that have been put through the Mustang-Q coin twice) and (C) electron microscopic assessment of ion exchange purified rAAV-2/8 vector.



(A)



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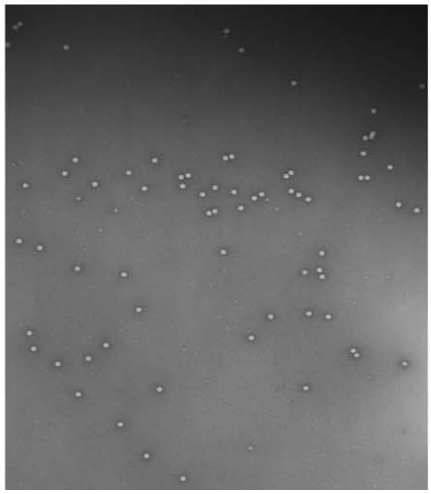

(C)

Fig. 3. Characterization of rAAV-5 purified by Ion exchange chromatography: (A) elution profile of rAAV-5 using Mustang-Q coin units with graded increase in NaCl concentration; (B) SDS PAGE gradient gel visualised by silver staining (C: crude cell lysate; Qa and Qb are two individual samples that have been purified by the two-step ion exchange purification method) and (C) electron microscopic assessment of ion exchange purified rAAV-2/5 vector.

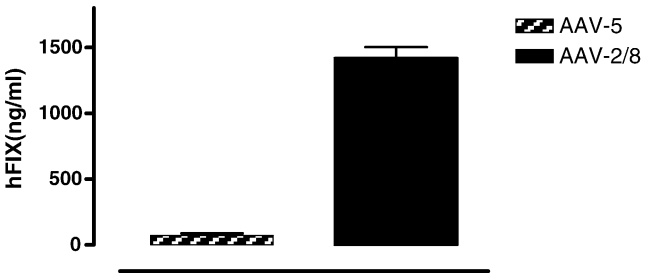

Fig. 4. Comparison of the biological activity of purified rAAV-5 and 8 vector particles. Stable hFIX levels were determined in C57Bl/6 competent mice 10 weeks after tail vein administration of 1×10^{11} rAAV-2/5 CAGG-FIX and rAAV-2/8 CAGG-FIX vector particles purified by mucin (AAV-5) or ion exchange (AAV-8) methodsm, respectively. Transgene expression values represent means together with the standard error for each cohort.

will be assisted by the availability of larger validated disposable membrane based ion exchange units. This method will likely expedite preclinical testing of the rAAV-2/8 vector system in larger animal models that are a prerequisite to well designed clinical studies.

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