Purification of Adeno-Associated Virus Using Ion Exchange and Mixed-Mode Resins

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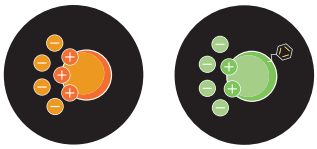

Abstract

Recombinant adeno-associated viruses (AAV) are among the most promising vectors for long-term gene transduction. Process-scale purification of AAVs can be quite challenging, and there remains an unmet need for cost-effective purification strategies. Affinity chromatography is currently the most adopted approach for the capture step of AAV purification in the gene therapy industry due to the high selectivity of affinity resins and their ease of integration into a platform process. Following the affinity capture step, a polishing step is utilized for the enrichment of full particles. This is typically performed by anion exchange chromatography. Affinity resins can be costly due to their high manufacturing cost and also lead to an expensive downstream process. The work described here demonstrates an economical and efficient nonaffinity capture and polishing step for AAV8. A workflow using an anion exchange (AEX) resin, Nuvia HP-Q Resin, and a hydrophobic anion exchange mixed-mode (MM) resin, Nuvia aPrime 4A Resin, was optimized to maximize viral recovery and full AAV8 particle enrichment. Nuvia HP-Q Resin captured 100% of the virus particles and provided exceptional purity by removing host cell protein (HCP) and DNA. Nuvia aPrime 4A Media separated the empty capsids from the full capsids with excellent yield and further removal of HCP and DNA.

Introduction

Adeno-associated virus has recently become a preferred viral vector for gene therapy. In gene therapy, functional gene copies can be delivered by viral vectors using one of two routes: ex vivo or in vivo. AAV vectors are the delivery choice for in vivo gene transfer therapy due to a milder course of innate immune reactions, infrequent chromosome integration, and availability in many serotypes for specific delivery routes and target tissues (Asher et al. 2020). Currently, 263 gene therapy clinical trials worldwide use AAV vectors to address a wide range of areas such as cancer and ocular, infectious, monogenic, cardiovascular, and neurological diseases (https://a873679.fmphost.com/fmi/webd/GTCT, accessed October 24, 2023). Table 1 shows a few representative AAV drug products in clinical or commercial production, with the AAV serotype and dose based on the target organ.

As indicated by the number of gene therapy products in clinical phases, there is a growing market demand for AAV-based therapeutics. If successful, these will ultimately require cost-effective large-scale production processes. Upstream production of viruses begins by generating cells that express the components necessary to create functional viral particles. There are many ways of accomplishing this, but the most common method for viruses such as lentivirus and AAV involves transiently transfecting human embryonic kidney 293T (HEK 293T) or HEK 293 cells with multiple expression plasmids. AAV production commonly makes use of three separate plasmids: a cis-plasmid that encodes the AAV inverted terminal repeats (ITRs) along with the gene therapy of interest, a trans-plasmid that encodes the AAV *Rep* and *Cap* genes, and a helper plasmid (commonly encoding adenovirus helper genes), which AAV is dependent on for viral production. The transiently transfected cells are then allowed to produce virus particles for several days before virus harvesting by chemical or mechanical lysis of the producer cells and treatment with a nuclease to remove any free DNA (de Rooij et al. 2019). The cell lysate is then moved to downstream processes for virus particle purification.

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Table 1. Representative list of AAV-based drug products in clinical trials and commercial use.

Target Organ	Drug Product	Disease	Company	AAV Serotype	Dose	Production Cell	Phase
Eye	Luxturna	Retinal dystrophy	Spark Therapeutics, Inc.	AAV2	1.5 x 10^{11} vg/eye	Triple transfection in HEK 293	Commercial
CNS	PTC-AADC	L-amino acid decarboxylase deficiency	PTC Therapeutics, Inc.	AAV2	1.8 x 10^{11} vg/brain	Triple transfection	Phase 3
Liver	ATM-061	Hemophilia B	uniQure N.V.	AAV5	2 x 10^{13} vg/kg	Insect cell (Sf9) with baculovirus expression technology	Phase 3
Liver	BENGENE-2	Hemophilia B	Pfizer Inc.	AAV Spark100	5 x 10^{11} vg/kg	Triple transfection in HEK 293	Phase 3
Liver	BMN 270	Hemophilia A	BioMarin Pharmaceutical Inc.	AAV5	4–6 x 10^{13} vg/kg	Insect cell (Sf9) with baculovirus expression technology	Phase 3
Neuro-muscular	Zolgensma	Spinal muscular atrophy	Novartis Gene Therapies, Inc.	AAV9	1 x 10^{14} vg/kg	Triple transfection in HEK 293	Commercial
Muscle	SRP-9001	Duchenne muscular dystrophy	Sarepta Therapeutics, Inc.	AAV8	2 x 10^{14} vg/kg	Transfection in HEK 293	Phase 3
Muscle	SGT-001	Duchenne muscular dystrophy	Solid Biosciences Inc.	AAV9	Start at 5 x 10^{13} vg/kg	Transient transfection	Phase 1 and 2
Muscle	PF-06939926	Duchenne muscular dystrophy	Pfizer Inc.	AAV9	3 x 10^{14} vg/kg	Suspension in HEK 293	Phase 3

AAV, adeno-associated virus; CNS, central nervous system; vg, vector genome.

The material produced during the upstream process contains several process- and product-related impurities and purification steps are required to obtain a safe, pure drug product. Historically, centrifugation-based protocols (CsCl- or iodixanol-based) have been shown to effectively separate AAV particles and remove impurities such as empty capsids. These procedures are versatile and quickly implemented in research settings but can become cost-inefficient for larger batch sizes and are challenging to validate through quality control release. The purification step may, therefore, be replaced by controllable, scalable chromatography processes (Dobrowsky et al. 2021).

Affinity chromatography is currently the most common approach for the capture step of AAV purification in the gene therapy industry due to high selectivity and ease of integration into a platform process. Following the capture step, a polishing step, typically AEX chromatography, is utilized for the enrichment of full particles. The high costs of affinity resins can result in an expensive downstream process. Indeed, downstream processing accounts for roughly 60 percent of the cost of producing a biological drug, and there is a need for cost control, process efficiency, and speed to market. In recent years, multimodal, or mixed-mode, chromatography — in which ligands in the resin interact with the protein product in multiple ways — has gained attention for its potential to provide optimized downstream purification workflows (Deorkar 2022). Here, an economical and efficient downstream process is presented using a nonaffinity capture step (Nuvia HP-Q Resin) and an MM polishing step (Nuvia aPrime 4A Resin) for AAV8. The workflow was optimized to maximize viral recovery and full AAV8 particle enrichment.

Materials and Methods

AAV8 carrying DNA that encodes green fluorescent protein (GFP) was produced by suspension cultures of human embryonic kidney 293T (HEK 293T) cells. Triple transfection of the appropriate expression plasmids was performed when the cell concentration reached 2 x 10^6 cells/ml. At the time of harvest (72 hr after transfection), cells were lysed, treated with nuclease, filtered, and the clarified stock was stored at –80°C. Before each purification experiment, clarified stock was thawed and treated with 50 U/ml of nuclease for 1 hr at 37°C under agitation. After tenfold concentration by diafiltration with four volumes of equilibration buffer (10 mM Tris, 10 mM $MgCl_2$, and 0.1% Tween 20, pH 8.8), the retentate was diluted fivefold with equilibration buffer for loading onto a Foresight™ Nuvia HP-Q Column (Bio-Rad Laboratories, Inc., catalog #12007020 or 12007021). Elution buffer (10 mM Tris, 10 mM $MgCl_2$, 0.1% Tween 20, and 2 M NaCl, pH 8.8) was used at a flow rate of 1 ml/min (residence time = 1 min). Initially, a gradient elution step was assessed, followed by the development of a step elution protocol. Foresight Nuvia HP-Q Column elution steps were carried out using 200, 500, and 2,000 mM NaCl.

The elution peak at 200 mM NaCl was diluted 1:5 with equilibration buffer and loaded onto a Foresight Nuvia aPrime 4A Column (Bio-Rad, #12007392 or 12007393). Foresight Nuvia aPrime 4A Column elution steps were performed using 100, 200, 500, and 2,000 mM NaCl.

Total AAV particles were quantified with an AAV8 Titration ELISA (PROGEN Inc., #PRAAV8), and genome-containing particles were quantified by real-time PCR following iBET internal protocols. Total protein was assessed with a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc., #23225), and total DNA was quantified with a Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, #P11496). Host cell protein was determined with a HEK 293 HCP ELISA Kit (Cygnus Technologies, #F650R). AAV8 full-to-empty ratio was determined for some samples using a Refeyn Samux mass photometer (Refeyn Ltd.).

Results and Discussion

Evaluation of Nuvia HP-Q Resin as a Capture Step

Preliminary runs were performed on 1 ml Foresight Nuvia HP-Q Columns using 10 mM Tris, pH 8.8, with NaCl for elution. A linear NaCl concentration gradient indicated that elution steps at 200 and 500 mM NaCl were suitable to assure high AAV8 recoveries, followed by resin stripping with 2 M NaCl. The effect of adding 10 mM $MgCl_2$ and 0.1% Tween 20 was evaluated. Additives had no significant impact on AAV8 recovery or impurity clearance but promoted AAV8 stability and

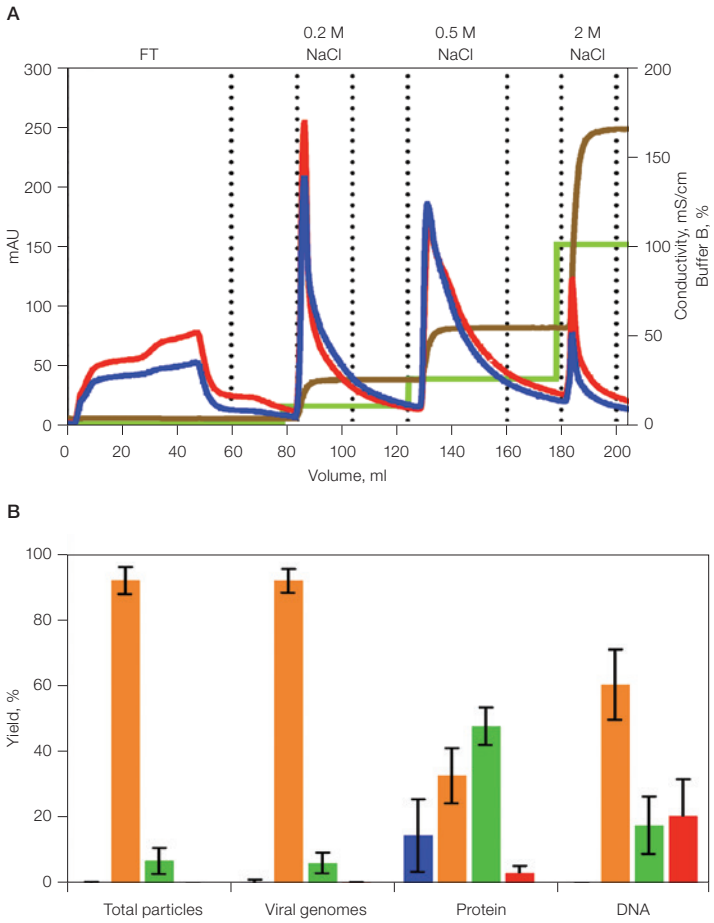

Fig. 1. Capture of AAV8 using Foresight Nuvia HP-Q chromatography. A, chromatogram of AAV capture and elution with buffer b, 2 M NaCl (—), in steps of 0.2, 0.5, and 2 M NaCl. OD 260 (—); OD 280 (—); conductivity (—). **B**, yield of AAV8 total particles and genome-containing particles from Nuvia HP-Q capture step and residual percentages of protein and DNA. 0.2 M NaCl (■); 0.5 M NaCl (■); 2 M NaCl (■); FT (■). AU, absorbance units; FT, flowthrough.

reduced virus loss due to nonspecific binding to tubing. The elution profile for Nuvia HP-Q Resin is shown in Figure 1A.

Following elution with 200 mM NaCl, the total recovery of AAV8 was >90%; however, there was no separation of full and empty particles (Figure 1B). Regarding impurities, approximately 35% of eluted proteins and 60% of eluted DNA were present in the 200 mM NaCl fraction.

After optimization of the purification conditions at a 1 ml scale, purification runs were performed using 5 ml Foresight Nuvia HP-Q Columns. The results obtained were comparable to the 1 ml column runs, indicating the scalability of the capture step. Additionally, at the 5 ml scale, increased residence time from 1 to 3 min was assessed with no significant impact on AAV8 recovery or impurity clearance (data not shown).

Dynamic binding capacity (DBC) determination was attempted at 1 min residence time. Still no viruses were found in the column's flowthrough upon loading 3 x 10^{13} total particles/ml resin, indicating a DBC higher than this amount.

Evaluation of Nuvia aPrime 4A Resin as a Capture Step

Like the Nuvia HP-Q AEX capture step, the MM resin, Nuvia aPrime 4A, with Tris, pH 8.8, and NaCl for elution of AAV8, was also evaluated as a potential capture step. Following initial runs with a linear NaCl gradient, elution steps at 0.1, 0.2, and 0.5 mM NaCl were determined to separate full and empty particles and column stripping was performed with 2 M NaCl. The effect of adding 10 mM $MgCl_2$ and 0.1% Tween 20 on Nuvia aPrime 4A Resin performance was also evaluated. As observed for Nuvia HP-Q Resin, no significant impact was observed on AAV8 recovery or impurity clearance.

When Nuvia aPrime 4A Resin was used for the capture step, results showed that viruses elute in the three elution steps with recoveries of 30, 40, and 25% in the 100, 200, and 500 mM NaCl, respectively (Figure 2A). However, approximately 60% of genome-containing particles elute in the 200 mM NaCl step, leading to full-particle enrichment in this fraction. Observing contaminating proteins, 3, 15, and 65% of the loaded amount eluted in the 100, 200, and 500 mM NaCl fractions respectively. Only very minor amounts of contaminating DNA were found in the 100 and 200 mM NaCl steps. Approximately 60% of loaded DNA eluted in the 500 mM NaCl fraction and the remaining in the 2 M NaCl strip fraction (Figure 2B).

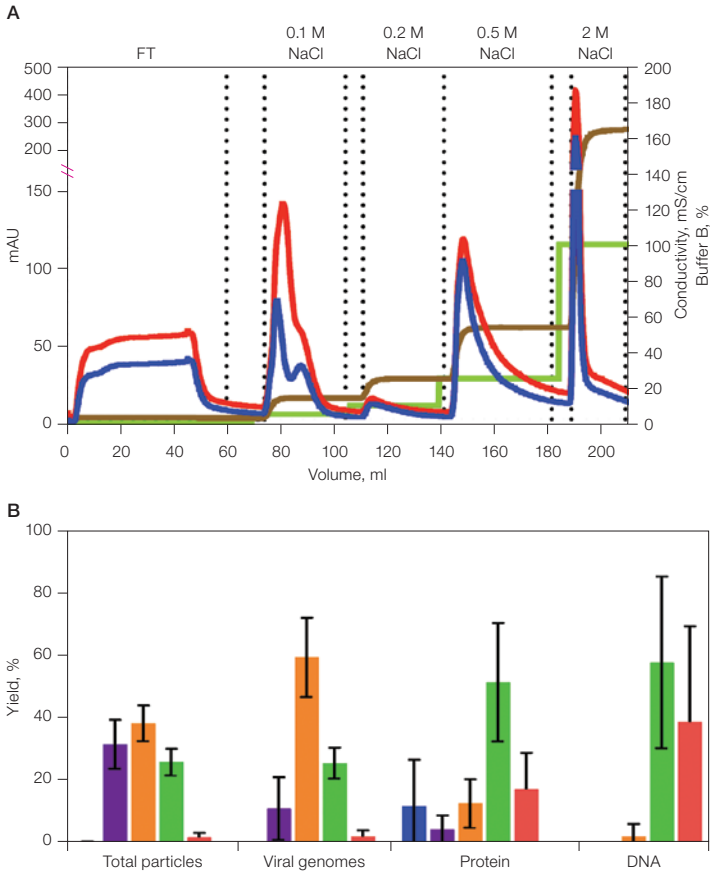

Fig. 2. Capture of AAV8 using Foresight Nuvia aPrime 4A chromatography. A, chromatogram of AAV8 capture and elution with buffer B, 2 M NaCl (—), in steps of 0.1, 0.2, 0.5, and 2 M NaCl. OD 260 (—); OD 280 (—); conductivity (—). **B**, yield of AAV8 total particles and genome-containing particles from Nuvia aPrime 4A capture step and residual percentages of protein and DNA. 0.1 M NaCl (■); 0.2 M NaCl (■); 0.5 M NaCl (■); 2 M NaCl (■); FT (■). AU, absorbance units; FT, flowthrough.

The established conditions were used to scale up to 5 ml Foresight Nuvia aPrime 4A Columns. As observed in 1 ml column experiments, full-particle enrichment was obtained in the 200 mM NaCl fraction. The effect of increasing residence time from 1 to 3 min was evaluated at a 5 ml scale, and the results showed no relevant impact on AAV8 recovery or impurity clearance (data not shown).

Dynamic binding capacity (DBC) at 1 min residence time was determined for Nuvia aPrime 4A Resin, and a value of 2.4 x 10^{13} total particles/ml resin was obtained.

Evaluation of Nuvia aPrime 4A Resin as a Polishing Step

The use of Nuvia aPrime 4A Resin as a polishing resin after Nuvia HP-Q chromatography was evaluated by loading the 200 mM NaCl fraction of the capture step (after 1:5 dilution to decrease conductivity to ~5 mS/cm) onto a Foresight Nuvia aPrime 4A Column. Enrichment of full particles was observed in the 200 mM NaCl eluate of the Nuvia aPrime 4A Column (Figure 3), similar to when this resin was used as a capture step. The recovery yield of full particles in this fraction was higher in the 5 ml columns than in the 1 ml columns (~67 vs. 32%). To recover AAV8 particles eluting in the 500 mM NaCl step (22% of total particles and 18% of genome-containing particles), a linear NaCl gradient between 200 and 500 mM over 60 column volumes was run. The results indicate that the majority of AAV8 particles eluted in the 100 and 200 mM NaCl steps (58 and 14% of total particles and 54 and 32% of genome-containing particles, respectively) and, confirming results from previous runs, full-particle enrichment occured in the 200 mM NaCl step. More than 70% of proteins in the eluate from the capture column eluted in the 500 mM and 2 M NaCl steps. The residual amount of DNA in the enriched full-particle fraction was below the 10 ng/ml limit of detection, indicating Nuvia aPrime 4A Resin to be very efficient for DNA clearance.

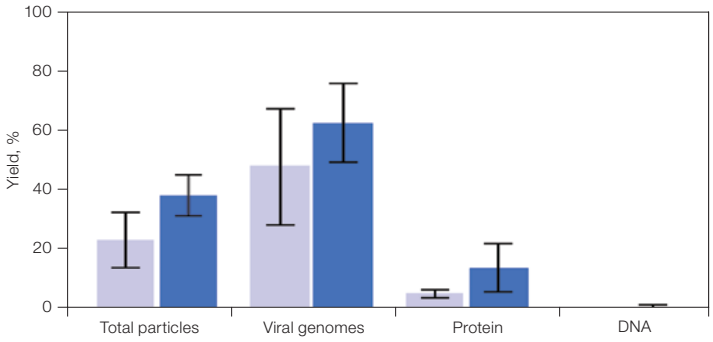

Fig. 3. Comparison of AAV8 recovery yields and residual impurities from a two-step purification with Nuvia HP-Q and Nuvia aPrime 4A chromatography (■), and single-step purification with Nuvia aPrime 4A chromatography (■).

Evaluation of Nuvia HP-Q and Nuvia aPrime 4A Resins for Separation of Full and Empty Capsids

While no full-capsid enrichment was seen with Nuvia HP-Q Resin as a capture step, Nuvia aPrime 4A Resin separated empty from full capsids during the elution steps, specifically in the 0.2 M NaCl step elution. As seen in Figure 4, Nuvia aPrime 4A Resin showed a similar separation of full and empty AAV virus particles when used as either a capture step or a polishing step. Full-particle enrichment was 1.7-fold when Nuvia aPrime 4A Resin was used as a polishing step and 1.6-fold when used as a capture step.

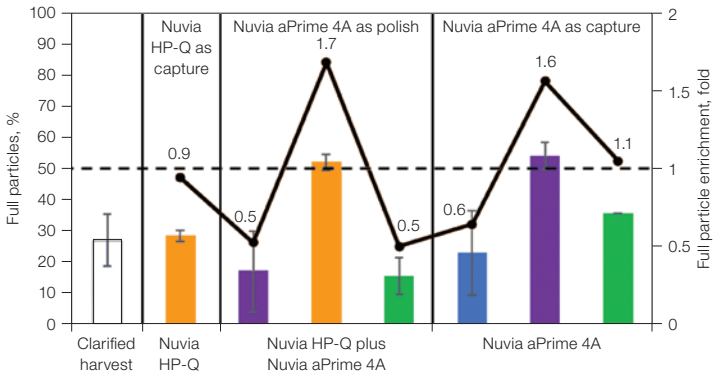

Fig. 4. Recovery and enrichment of full AAV8 particles from various purification steps. Clarified harvest purification was purified over Nuvia HP-Q, Nuvia HP-Q plus Nuvia aPrime 4A, or Nuvia aPrime 4A Resins as indicated, with step elution of 0.1 M NaCl (■); 0.2 M NaCl (■); 0.5 M NaCl (■). Full particles 50% yield (**---**); fold enrichment (—).

Conclusions

In the work described, we show that anion exchange chromatography following multimodal chromatography was able to purify AAV8 effectively. AAV8 elution in the 200 mM NaCl step using Nuvia HP-Q Resin as a capture step led to high recovery yields with no apparent separation between empty versus full particles. DNA and proteins were eluted along with the virus. Using Nuvia aPrime 4A chromatography as a polishing step following Nuvia HP-Q chromatography led to enrichment in the 200 mM NaCl fraction. High recovery yields and excellent impurity clearance were observed. Both resins have high DBCs. This workflow can be a more flexible, economical alternative to traditional affinity and ultracentrifugation gradient purification processes.

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