Tuning Mobile Phase Properties to Improve Empty Full Particle Separation in Adeno-associated Virus by Anion Exchange Chromatography

Dennis Chen[1], James Warren[1], and Chao Huang[1]

[1]Ultragenyx Pharmaceutical Inc

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Abstract

In the past decade, recombinant adeno-associated virus (rAAV) has gained increased attention as a prominent gene therapy technology to treat monogenetic disease. One of the challenges in rAAV production is the enrichment of full-rAAV particles containing the gene of interested (GOI) payload. Herein, we demonstrated that by adjusting the mobile phase properties of anion-exchange chromatography (AEX), Empty and Full separation of rAAV was improved in monolith based preparative AEX chromatography. When compared to the baseline method using NaCl, the presence of tetraethylammonium acetate (TEA-Ac) in the AEX mobile phase resulted in enhanced resolution (from 0.75 to 1.23) between Empty and Full peaks by salt linear gradient elution, as well as increased the percentage of full-rAAV particles from 20% to 36% and GOI genome recovery (from 59% to 62%). Furthermore, a dual wash + step elution AEX method was developed to harness TEA-Ac contribution on Empty and Full separation in the first wash (wash1) step while removing TEA-Ac in the second wash (wash2) step to ensure product safety. The resulting optimized AEX purification method could be easily adapted in scaled-up manufacturing and could also be applied to purification processes involving other AAV serotypes facing similar Empty and Full rAAV separation challenges.

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Dennis P. Chen, James C. Warren, Chao Huang

Correspondence should be addressed to C.H. (chuang@ultragenyx.com)

Pharmaceutical Development, Ultragenyx Pharmaceutical Inc., 19 Presidential Way, Woburn MA 01801

Chao Huang

Director of Downstream Process Development

chuang@ultragenyx.com

19 Presidential Way

Woburn MA 01801

ABSTRACT

In the past decade, recombinant adeno-associated virus (rAAV) has gained increased attention as a prominent gene therapy technology to treat monogenetic disease. One of the challenges in rAAV production is the enrichment of full-rAAV particles containing the gene of interested (GOI) payload. Herein, we demonstrated that by adjusting the mobile phase properties of anion-exchange chromatography (AEX), Empty and Full separation of rAAV was improved in monolith based preparative AEX chromatography. When compared

to the baseline method using NaCl, the presence of tetraethylammonium acetate (TEA-Ac) in the AEX mobile phase resulted in enhanced resolution (from 0.75 to 1.23) between Empty and Full peaks by salt linear gradient elution, as well as increased the percentage of full-rAAV particles from 20% to 36% and GOI genome recovery (from 59% to 62%). Furthermore, a dual wash + step elution AEX method was developed to harness TEA-Ac contribution on Empty and Full separation in the first wash (wash1) step while removing TEA-Ac in the second wash (wash2) step to ensure product safety. The resulting optimized AEX purification method could be easily adapted in scaled-up manufacturing and could also be applied to purification processes involving other AAV serotypes facing similar Empty and Full rAAV separation challenges.

Keywords: AEX Chromatography; AEX Mobile Phase; Adeno-associated Virus; Empty Full Separation

abbreviation: **rAAV** , Recombinant Adeno-Associated Virus;**AEX** , Anion Exchange Chromatography; **QA** , Quaternary Ammonium

1. INTRODUCTION

Recombinant adeno-associated virus (rAAV) vectors have demonstrated significant promise for *in vivo* gene delivery due to their unique features, including non-pathogenicity to humans, low immunogenicity, and long-term gene expression.[1,2] Although scalable manufacturing of rAAVs has been demonstrated using mammalian and insect cell culture,[3,4] the rAAV production systems inherently form empty-rAAV particles that do not contain the desired therapeutic gene sequences. These empty-rAAV can pose several clinical challenges. A cell-mediated immune response that targets the rAAV capsid can cause the clearance of transduced tissue infected with therapeutic rAAV. This cytotoxic effect has been attributed to the introduction of high ratios of empty- to full-rAAV particles.[5]In addition to immune response challenges, the excess number of empty particles limits the rAAV genome titer in a drug product. This is particularly important for the treatment of diseases which require high doses of the therapeutic rAAV.[6]

The traditional approach of using density gradient ultracentrifugation methods has proven to be effective in removing empty particles.[7–9] However, the ability to scale and validate gradient ultracentrifugation methods to consistently deliver large doses of full-rAAV particles is challenging. In contrast, ion-exchange chromatography, particularly anion-exchange (AEX) chromatography, has been reported as a means of separating empty- and full-rAAV particles.[10] The ability for an AEX chromatography process to enrich full-rAAV particles is dependent on various factors including the properties of AEX stationary phase,[11] AEX mobile phase,[12] and properties of rAAV, such as serotype,[13] genome size,[14] and surface charge alteration.[15] AEX chromatography exploits the surface charge difference between empty- and full-rAAVs, whereby full-AAVs have a slightly lower isoelectric point (pI) than empty-rAAVs, therefore a shallow, linear-gradient elution (LGE) by increasing the salt (*e.g* ., sodium chloride [NaCl]) concentration could provide the resolution required for separation. In cases where sufficient resolution between Empty and Full populations was achieved in LGE, step elution methods have been reported.[10,16]

In addition to the elution methodology, various mobile phase salts have been used to enhance the separation between empty- and full-rAAVs in AEX chromatography. The strength of the eluent salt is adjusted by either increasing the concentration of the elution buffer or through the selection of a modifier with specific physicochemical properties that promote/decrease specific molecular interactions.[17] Reported salts include ammonium acetate (NH_4Ac),[18] magnesium sulfate ($MgSO_4$),[10] sodium acetate (NaAc),[19] and various quaternary ammonium (QA) salts.[11,19–21] Separation of Empty and Full peaks using a single solution gradient consisting of NaCl and $MgCl_2$ has been previously reported, hypothesizing an interaction between Mg^{2+} and rAAV capsids.[16] In line with this notion, Nam*et. al* . reported the cryo-EM structure of AAV8 suggested that there is a potential divalent ion interaction position in the 2-fold symmetry axis region.[22] Furthermore, Gagnon*et al* . were able to separate empty-rAAV from full-rAAV using a novel multimodal metal (including Mg^{2+}) affinity chromatography method,[23] corroborating the interaction between Mg^{2+} and rAAV capsids. In instances where QA salts are compared with other salts, the QA salt tends to outperform their counterparts in the ability to resolve empty- and full- rAAV.[11,19,21]However, the role

of the QA salt in the interaction between the rAAV particles and AEX stationary phase remains unclear. Wang *et al* . explored tetraalkylammonium chlorides with various alkyl-chain lengths and observed improved peak-to-peak resolution with increasing alkyl-chain length in analytical AEX chromatography.[11] While it is encouraging to see QA salts contribution in analytical AEX chromatography, its utilization in preparative AEX methods is not yet realized.[20]

Herein, we present a preparative AEX chromatography method for the separation of empty- and full-rAAV8 particles using the CIMmultus-QA monolith column. The method has a combination of wash1 step with QA salt to remove empty-rAAVs, wash2 step with NaCl salt to remove QA salt, and elution step with NaCl to elute full-rAAVs. Eventually, a scalable and manufacturing-friendly AEX process was developed and demonstrated to provide improved full-rAAV particle enrichment in rAAV downstream purification process.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

All aqueous solutions were prepared using ultrapure water (18.2 MΩ[?]cm) obtained from a Milli-Q water purification system (Millipore Sigma). All chemicals and reagents were used as received, without further purification. Buffers containing 0.001% (w/v) Pluronic F-68 were prepared from a 10% (w/v) Gibco Pluronic F-68 (Thermo Fisher Scientific) stock solution. Sodium chloride (NaCl), [?]99%; and magnesium chloride ($MgCl_2$) hexahydrate, [?]98%, USP grade were purchased from J.T. Baker. Tetramethylammonium chloride (TMAC), [?]98%; tetrabutylammonium chloride (TBAC) hydrate, [?]96%; tetraethylammonium acetate (TEA-Ac), [?]97%; and tetraethylammonium tetrafluoroborate (TEA-BF_4), [?]99% were purchased from Acros Organics. Tetraethylammonium chloride (TEAC) monohydrate, [?]98% and tetraethylammonium bromide (TEAB), [?]98% were purchased from Alfa Aesar. Tetrapropylammonium chloride (TPAC), [?]97% was purchased from TCI America. Bis-tris propane (BTP), [?]98% was purchased from Sigma-Aldrich. Tris(hydroxymethyl) aminomethane (Tris), Emprove Essential, USP grade was purchased from Millipore Sigma. Tris(hydroxymethyl)aminomethane hydrochloride), (Tris-HCl), [?]99%, USP grade; and 6 N hydrochloric acid solution were purchased from J.T. Baker.

2.2 Production and Preparation of rAAV Samples

The rAAV samples were of the AAV8 serotype and produced by Ultragenyx using triple transfection of HEK293 cells following procedures similar to those described in the literature.[32,33] Briefly, three plasmids, an AAV helper plasmid containing the Rep and Cap genes, an adenovirus helper plasmid containing the adenovirus helper genes, and a transgene plasmid containing the sequence to be packaged, were transfected into HEK293 cells. Prior to harvest, a nuclease treatment is performed. After 5 days, the cells were harvested and clarified through a series of depth filters. The clarified harvest was subsequently concentrated and diafiltered via tangential flow filtration (TFF, 300 kDa MWCO membrane) and further purified over affinity chromatography. The AEX load material has an empty-, intermediate-, and full-AAV capsid ratio of 88:6:6.

2.3 Anion Exchange Chromatography

To prepare the AEX load, 1.5 mL of affinity eluate was diluted 8-fold (to 15 mL) with 20 mM Bis-tris-propane (BTP) to adjust pH to 9.0 and to reduce the load conductivity to approximately 4 mS/cm. The AEX experiments were performed on a 1-mL strong-base, CIMmultus-QA monolith (Lot: 20-GE03-005-001A-P1-2A-8-MI-2-MQ17, BIA Separations) with a nominal channel size of 2 μm, using three basic buffering formats:

Linear-gradient Elution (NaCl, with and without $MgCl_2$) – 10 mL of the diluted affinity eluate was loaded on AEX media pre-equilibrated with pH-matched equilibration buffer (NaCl buffer: 20 mM BTP, 25 mM NaCl, 0.001% (w/v) Pluronic F-68, pH 9.0). After washing with 10 column volumes (CV) of equilibration buffer, the column-bound material was eluted with a linear gradient from 25 to 182.5 mM NaCl generated over 90 CVs. AEX chromatography experiments utilizing Mg-containing buffers were performed identically except for the constituents of the equilibration (Buffer A) and end-point buffer (Buffer B) contained 2 mM

$MgCl_2$. AEX chromatography experiments utilizing 10% (v/v) isopropyl alcohol buffers were performed identically except for the constituents of the Buffer A and Buffer B contained 10% (v/v) isopropyl alcohol. The eluate was collected in fractions and fractions corresponding to "Empty" and "Full" peaks were pooled accordingly.

Linear-gradient Elution (QA, with and without $MgCl_2$) – 10 mL of the diluted affinity eluate was loaded on AEX media pre-equilibrated with pH-matched equilibration buffer (QA buffer: 20 mM BTP, 25 mM QA, 0.001% (w/v) Pluronic F-68, pH 9.0). After washing with 10 CVs of equilibration buffer, the column-bound material was eluted with a linear gradient from 25 to 340 mM QA generated over 90 CVs. AEX chromatography experiments utilizing Mg-containing buffers were performed identically except for the constituents of the Buffer A and Buffer B contained 0.2, 1, 5, or 10 mM $MgCl_2$. The eluate was collected in fractions and fractions corresponding to "Empty" and "Full" peaks were pooled accordingly.

Isocratic Wash and Elution – 10 mL of the diluted affinity eluate was loaded on AEX media pre-equilibrated with pH-matched equilibration buffer (TEA-Ac buffer: 20 mM BTP, 25 mM TEA-Ac, 0.001% (w/v) Pluronic F-68, pH 9.0). After washing with 10 CVs of equilibration buffer, the column was washed with 190–200 mM TEA-Ac and the corresponding concentration of $MgCl_2$ (either 0, 1, or 5 mM). The QA buffer was then removed in a second wash step (NaCl buffer: 20 mM BTP, 25 mM NaCl, 2 mM $MgCl_2$, 0.001% (w/v) Pluronic F-68, pH 9.0). Elution was carried out in two manners: 1) a linear gradient was generated over 30 CVs from 25 to 200 mM NaCl; or 2) a step elution was employed using a buffer containing 20 mM BTP, 117 mM NaCl, 2 mM $MgCl_2$, 0.001% (w/v) Pluronic F-68, pH 9.0. In gradient operation, the eluate was collected in fractions and fractions corresponding to "Empty" and "Full" peaks were pooled accordingly. In isocratic mode, the full elution peak was collected, which typically corresponded to a 3 CVs pool.

Following elution, the column was then washed with 10 CVs of a high-salt strip solution (20 mM BTP, 2 M NaCl, pH 9.0), followed by 10 CVs of a sanitation solution (3 M NaCl, 1 M NaOH), and subsequently 10 CVs of a column-storage solution (50 mM Tris, 150 mM NaCl, 20% ethanol, pH 7.5). The column is refrigerated in 2–8 °C after use.

2.4 Analysis of Chromatography Fractions

Quantitative Polymerase Chain Reaction (qPCR) – The quantity of encapsulated vector genome was determined using a qPCR method applicable to rAAV samples that contain the SV40 Poly-A sequence. To quantify only encapsulated vector genome, rAAV samples were treated with salt-activated nuclease (SAN) to remove non-encapsulated polynucleotides. The SAN were then inactivated and the rAAV samples were treated with Proteinase K to digest the capsid protein. This is followed by qPCR using the primer/probe set that is specific to the SV40 Poly-A sequence of the rAAV genomes in the rAAV therapeutic products prepared at Ultragenyx. The qPCR is performed on an Applied Biosystems Flex 7 instrument and quantification is made using the QuantStudio 7 software. For a given experiment (e.g., alkyl series study or anion series study) the qPCR measurements were performed on the same multi-well plate.

Gyrolab xPand – The quantity of rAAV capsid particles was determined using the Gyrolab xPand (Gyros Protein Technologies) nanoliter-scale, high-throughput immunoassay system, in combination with the Gyrolab AAVX Titer kit (Gyros Protein Technologies, P0020695). The AAVX ligand included in Gyrolab AAVX Titer kit is based on the selective affinity ligands developed with CaptureSelect technology (Thermo Fisher Scientific). Typically, a dilution plate containing rAAV samples, calibrants required to generate a standard curve, controls, and wash buffers are prepared ahead of time. The xPand's liquid handling system transfers these solutions into microfluidic channels that contain biotin-labelled CaptureSelect AAVX ligand functionalized on streptavidin-coated particles. As the solutions pass through the channels, rAAV capsid particles are bound to the capture media. Then, fluorescent-conjugated material is passed across the channel to generate a fluorescence signal in the form of response units. The signal obtained from the calibration solutions are then fitted with a four-parameter logistic regression to generate a standard curve to convert response units to particle titer units [vp mL^{-1}]. For a given study (e.g., alkyl series study or anion series study) particle titer measurements were performed on the same Gyrolab disc.

Sedimentation Velocity Analytical Ultracentrifugation (SV-AUC) – The capsid content in rAAV preparations and AEX elution fractions were quantified by SV-AUC using absorption optics at 230 nm. SV-AUC was performed using the ProteomeLab LX-A analytical ultracentrifuge from Beckman Coulter (Indianapolis, IN). Samples were loaded into AUC cells equipped with sapphire windows and 12 mm double-sector charcoal-filled EPON centerpieces. 420 µL of sample was loaded in the sample sector, whereas 430 µL of the buffer matrix was loaded in the reference sector. AUC cells containing the samples were equilibrated at 20 °C for 1.5 h before the rotor was brought up to 12,000 rpm. UV scans were collected at 230 nm every 20 s at a radial step-size of 3 µm. Sedimentation coefficients and relative amounts of each species present in solution were determined by processing the data with SEDFIT (v15.01b) using a c(s) model. The empty, intermediate, and full species were identified using established reference sedimentation coefficients.[34]

3. RESULTS

3.1 Effect of Quaternary Ammonium Salt in Preparative AEX Chromatography

The AEX salt LGE runs were performed on CIMmultus-QA column with either baseline condition (using NaCl) and QA condition (using tetramethylammonium chloride, TMAC). As shown in **Figure 1A** and **1B** , in both conditions, the empty peak elutes earlier than the full peak, which is then followed by a third peak (attributed as empty capsids or capsid debris,[23]). As TMAC requires a higher ionic strength (191 mM) to elute rAAV than that of NaCl (124 mM), TMAC salt is considered a weaker eluent than NaCl. In terms of chromatographic separation, the AEX run with TMAC has higher empty and full peak resolution (1.06) than that of the NaCl run (0.75), which is consistent with a previous report.[21] However, the work from Yang *et al* . was conducted using 0.1-mL CIMac column with analytical loading and high operational pressure to ensure high resolution. In contrast, we employed a 1-mL CIMmultus-QA column with preparative loading and low operational pressure (<50 psi). As 1-mL CIMmultus-QA column is a qualified scale-down model for up to 8000-mL CIMmultus-QA (internal data, not shown), the AEX method established in this work is appropriately scalable.

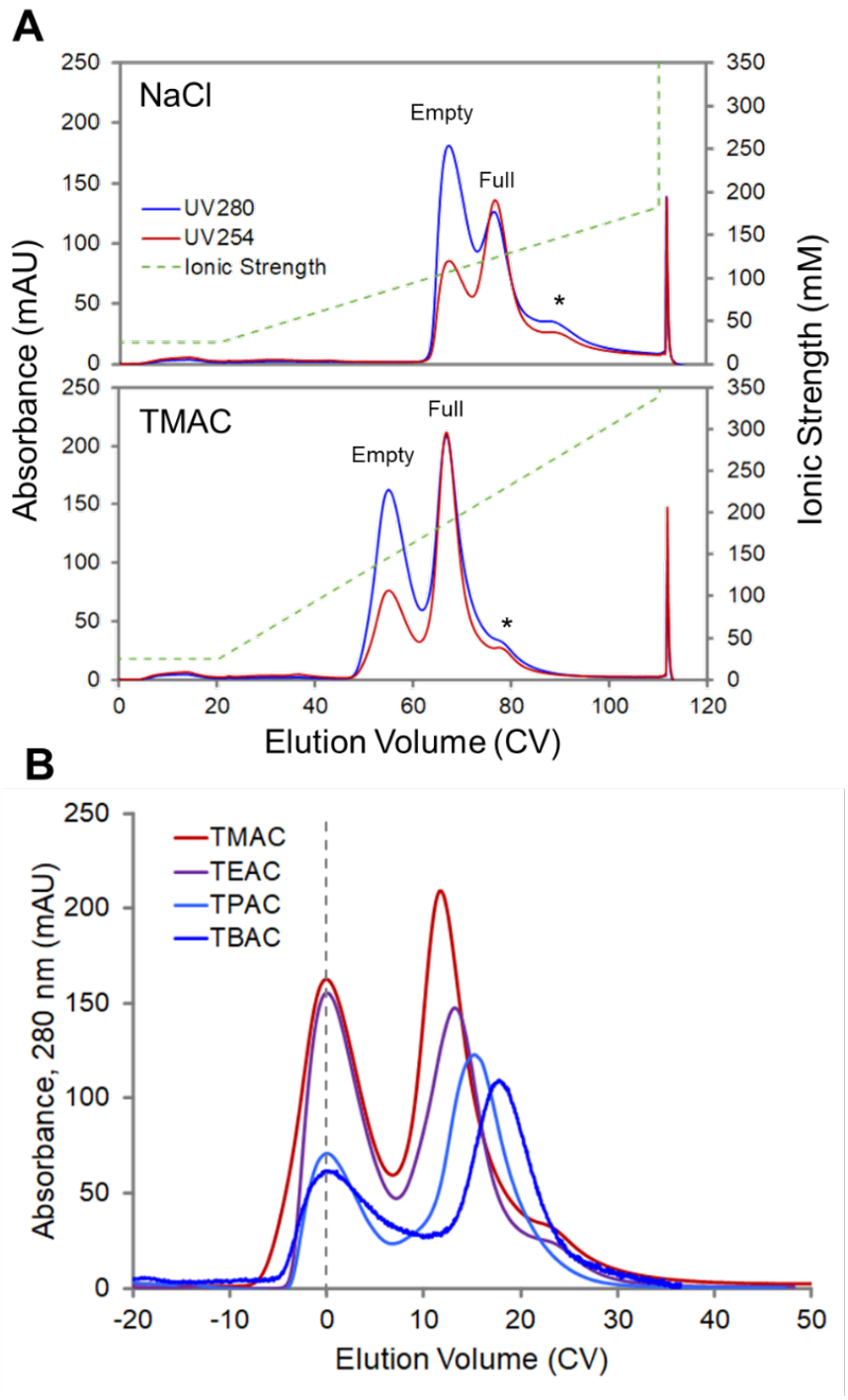

Figure 1 . (A) Elution profiles from AEX processes employing a linear gradient elution containing 20 mM BTP, 0.001% (w/v) Pluronic F-68, and 25–182.5 mM NaCl (top panel) or 25–340 mM TMAC (bottom panel) at pH 9 over 90 CVs. Blue and red traces correspond to A_{280} and A_{254}, respectively. The dashed, green trace corresponds to the solution ionic strength. (B) Elution profiles from AEX processes employing a linear gradient elution containing 20 mM BTP, 0.001% (w/v) Pluronic F-68, and 25–340 mM QA chloride at pH

9 over 90 CVs. The tetra-alkyl chain was varied across the QA chloride runs with the red, purple, light-blue, and blue traces corresponding to tetra-methyl (TMAC), tetra-ethyl (TEAC), tetra-propyl (TPAC), and tetra-butyl (TBAC) QA-chloride salt, respectively (listed from top to bottom). The traces were normalized to the elution volume corresponding to the maximum A_{280} signal of the empty peak, which is indicated by the vertical dashed line. The empty peak is denoted as "Empty", while the full peak is denoted as "Full".

3.2 Effect of Alkyl-chain Length in Quaternary Ammonium Salt

To investigate the effect of alkyl-chain length on Empty and Full separation in preparative AEX chromatography, AEX salt LGE chromatography runs were conducted using a series of tetraalkylammonium chlorides, including tetramethylammonium chloride (TMAC), tetraethylammonium chloride (TEAC), tetrapropylammonium chloride (TPAC), and tetrabutylammonium chloride (TBAC). As evident in **Figure 1B** , the empty peak to full peak separation increases with increasing alkyl-chain length. The differential retention time of various tetraalkylammonium salt also indicated that increasing alkyl-chain length could weaken tetraalkylammonium salt ability to elute rAAV particles. Among all four tetraalkylammonium salts evaluated in this study, TBAC exhibited the highest level of empty full peaks separation, while also suffering from broadest peak width and largest peak collection volume. In addition, due to the high UV_{280nm} absorbance of both TPAC and TBAC salt, they were not considered process feasible since their concentration will interfere with the UV_{280nm} based peak collection criteria of rAAV product. In this case, TEAC was deemed as the optimal salt to perform further experiments in this study.

3.3 Effect ofAnionic Counter-ioninQuaternary Ammonium Salt

With solvation effects assumed to be at play, it is likely that the counter-anion in the QA compound may contribute to the separation of empty- and full-rAAV. To this end, AEX chromatography runs were performed using a series of TEA salts with different anions, including acetate (Ac), bromide (Br), and tetrafluoroborate (BF_4), which were chosen on the basis of their relative affinity to quaternary ammonium ligands, with Ac, Br, and BF4 exhibiting low, moderate, and high affinity, respectively.[24] As seen in **Figure 2** , among all three runs, TEA-BF_4 run has the lowest degree of empty-to-full peak separation, whereas eluting with TEA-Ac resulted in the highest degree of empty-to-full peak separation. It is well known that acetate is a weak eluent in AEX chromatography. Furthermore, given the observation that elution with TEAC resulted in longer retention volumes than NaCl, the overall weak eluent property of TEA-Ac may be leveraged for separation of empty- and full-rAAV species by AEX chromatography.

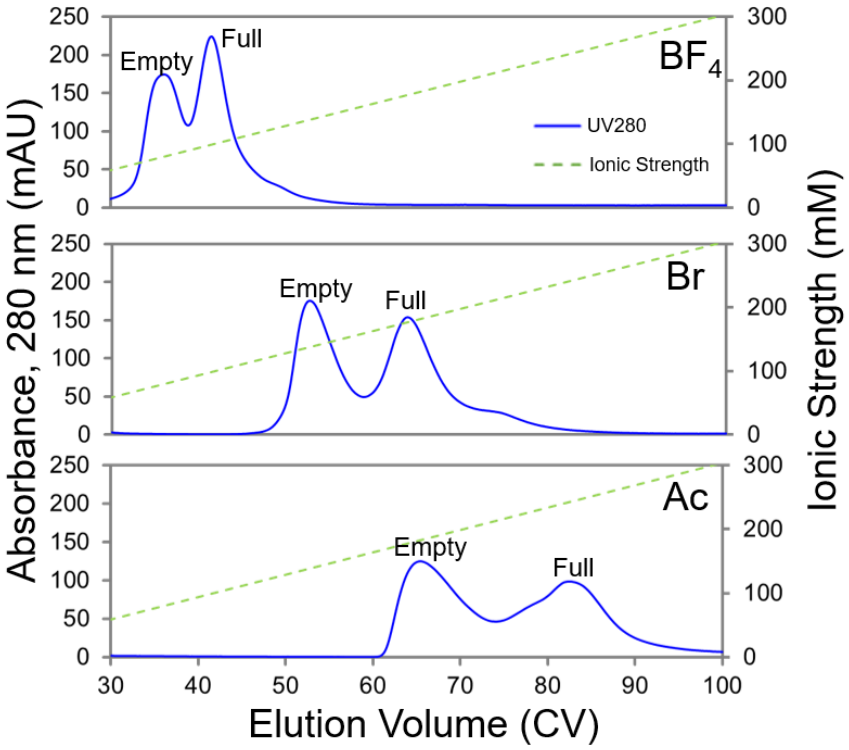

Figure 2 . Elution profiles from AEX processes employing a linear gradient elution containing 20 mM BTP, 0.001% (w/v) Pluronic F-68, 25–340 mM TEA-*X* at pH 9 over 90 CVs, where *X* is the counter-anion associated with tetraethylammonium (TEA). Depicted from top to bottom, *X* is tetrafluoroborate (BF_4), bromide (Br), and acetate (Ac). The blue and dashed green trace correspond to A_{280} and the solution ionic strength, respectively. The empty peak is denoted as "Empty", while the full peak is denoted as "Full".

3.4 Effect of Magnesium Chloride in Combination with Quaternary Ammonium Salt

Previous data indicated when $MgCl_2$ was added to AEX mobile phase, the full peak yield was increased.[16] Therefore, the TEA-Ac salt LGE runs were performed with $MgCl_2$ presence in elution mobile phase with various concentrations, in an aim to identify the optimal AEX condition. As shown in **Figure 3,** the presence of $MgCl_2$ as low as 1 mM was sufficient to observe a noticeable increase of UV_{280nm} area for full peak. In addition, the difference in the ionic strength at which the empty and full peaks are eluted appear to taper off at 5 mM of Mg^{2+}, suggesting that it is likely that the binding sites associated with Mg^{2+} may be saturated at that concentration, and long-range interactions (charge screening effects) begin to dominate afterward and consequently, the retention volumes of empty and full peaks become equally affected (e.g., the AEX run with 10 mM Mg^{2+} in the mobile phase). It should be mentioned that a hump observed between empty and full peaks (indicated by an asterisk in **Figure 4**) gradually disappears when mobile phase $MgCl_2$ concentration increases. It is likely that certain charge variants of empty-rAAVs eventually merged into the major empty peak in the presence of $MgCl_2$, which is evidenced by the increase of viral particle counts in empty peak (from 1.4 x 10^{15} to 2.6 x 10^{15}) with increase of $MgCl_2$ concentration.

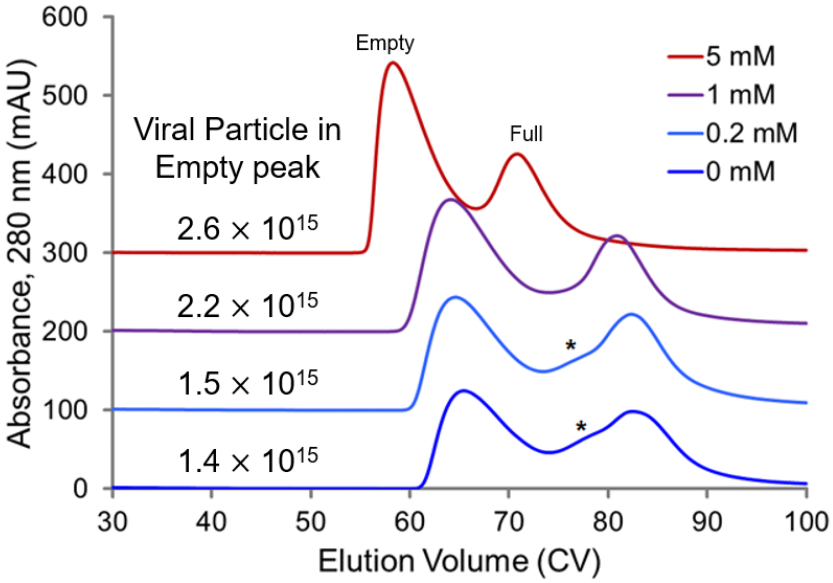

Figure 3 . Elution profiles from AEX processes employing a linear gradient elution (LGE) containing 20 mM BTP, 0.001% (w/v) Pluronic F-68, 25–340 mM TEA-Ac at pH 9 over 90 CVs, while the $MgCl_2$ concentration was held constant at 0, 0.2, 1, and 5 mM for the blue, light-blue, purple, and red traces, respectively (listed from bottom to top; each trace is offset by 100 mAU). Traces correspond to absorbance at 280 nm (A_{280}) and asterisks denote a population not apparently visible in traces containing 1 and 5 mM $MgCl_2$. The total viral particle measured in the empty peak are displayed adjacent to their corresponding UV trace. The empty peak is denoted as "Empty", while the full peak is denoted as "Full".

3.5 Quaternary Ammonium Salt in Wash Phase of AEX Chromatography

As the TEA-Ac salt has dose dependent toxicity,[25] it is imperative to remove it from rAAV product stream. To this end, converting AEX LGE to step elution appears to be a feasible solution, since QA salt could be placed only in the wash phase to facilitate empty-rAAVs reduction while allowing a non-QA-containing buffer to elute the full peak. In addition, to ensure maximum removal of TEA-Ac, a second wash phase (using NaCl) could be implemented to flush residual TEA-Ac off the column. To achieve similar full-rAAV enhancement through AEX chromatography, the TEA-Ac concentration in wash buffer was set referencing its concentration observed at the empty peak apex in LGE run.

In the AEX step elution run, to demonstrate the effectiveness of TEA-Ac in reduction of empty-rAAVs, the elution phase was executed using either single-buffer isocratic elution (intended for final AEX step elution method) or two-buffer LGE (intended to resolve empty and full peaks to assess empty-rAAV reduction). As shown in **Figure 4A** and**4C** , the elution phase with two-buffer linear gradient resolved only the full and third peaks, demonstrating significant amount of empty-rAAVs had been removed by TEA-Ac washes. In comparison, the elution phase with a single-buffer isocratic elution resulted in a sharp elution peak with slight tailing. For better comparison, the magnified images of wash and elution peaks from both runs were shown in**Figures 4B** and **4D** , respectively. As linear gradient operation may encounter manufacturing facility constraints when scale-up,[26] isocratic elution is a preferred operation that enables elution volume reduction and rAAV concentration increase in AEX pool, which is beneficial for subsequent downstream operation, since less concentration factor could be used in the final concentration and buffer exchange unit operation.

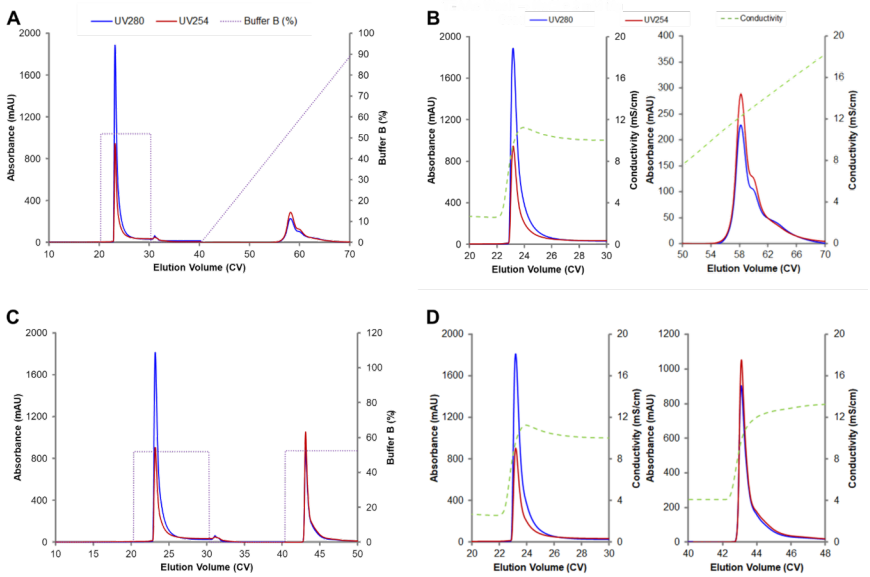

Figure 4 . Chromatograms from AEX processes employing the following: (A–B) an isocratic wash containing 20 mM BTP, 0.001% (w/v) Pluronic F-68, and 206.5 mM TEA-Ac at pH 9 over 10 CVs, and a LGE containing 20 mM BTP, 0.001% (w/v) Pluronic F-68, 2 mM $MgCl_2$, and 25–182.5 mM NaCl at pH 9 over 30 CVs; (C–D) an isocratic wash containing 20 mM BTP, 0.001% (w/v) Pluronic F-68, 206.5 mM TEA-Ac at pH 9 over 10 CVs, a second isocratic wash containing 20 mM BTP, 0.001% (w/v) Pluronic F-68, 2 mM $MgCl_2$, and 25 mM NaCl at pH 9 over 10 CVs, and an isocratic elution containing 20 mM BTP, 0.001% (w/v) Pluronic F-68, 2 mM $MgCl_2$, 116.7 mM NaCl at pH 9 over 10 CVs. (B) and (D) are magnified images of empty (left panel) and full-capsid peaks (right panel) from chromatograms (A) and (C), respectively. Blue and red traces correspond to A_{280} and A_{254}, respectively. The dotted purple traces and dashed green traces correspond to the volumetric fraction of running buffer B pumped through the system and the solution conductivity, respectively.

3.6 Process Performance and Product Quality of AEX Run with Quaternary Ammonium Salt

As there is typically a tradeoff between full-rAAV enhancement and rAAV genome (full capsid) recovery in AEX chromatography, the effectiveness of TEA-Ac was assessed by plotting the percentage of full-rAAV (determined by AUC assay) in the AEX elution pool as a function of the AEX-step genome recovery (calculated based on genome copy determined by qPCR) for a total of 8 runs. As shown in **Figure 5** , two linear regressions were generated corresponding to either TEA-Ac containing runs (R^2=0.75) or non-TEA-Ac containing runs (R^2=0.79), respectively. The slopes of the two regression lines are similar, indicating that the tradeoff rate between full-rAAV percentage to genome recovery is TEA-Ac independent. However, the intercepts of the two regression lines are quite different, projecting TEA-Ac containing runs has ˜50% more genome yield than that of non-TEA-Ac containing runs under same full-rAAV percentage. Taken together, TEA-Ac demonstrated its effectiveness in AEX preparative process operation through not only better chromatographic peak separation, but also enhanced process performance and product quality, holding the promise for future implementation in scale-up AEX operation.

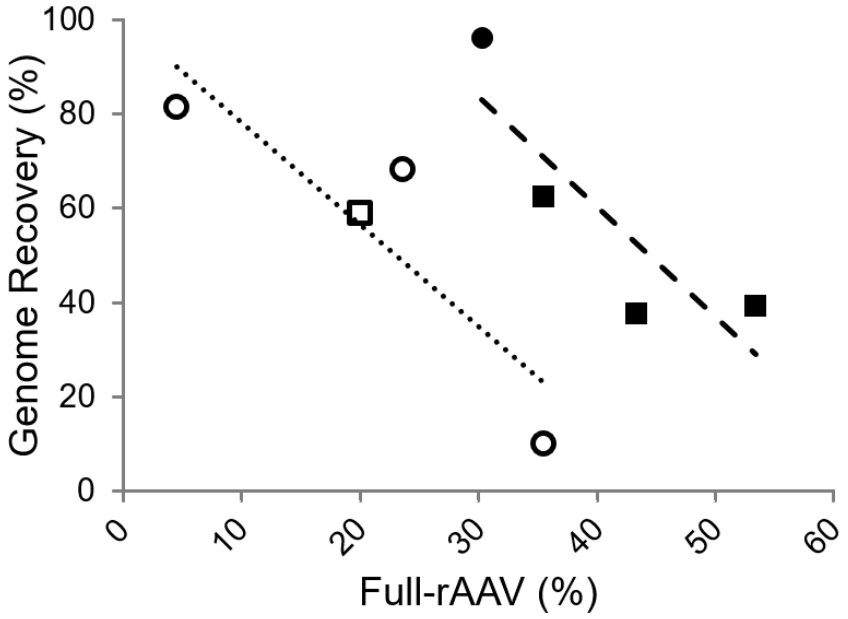

Figure 5 . Regression plot depicting the tradeoff between GOI genome recovery and full-rAAV enrichment. Circles correspond to step elution, whereas the squares correspond to gradient elution. Open markers correspond to AEX chromatography runs using NaCl in process buffers. Closed markers correspond to AEX chromatography runs that employ an isocratic TEA-Ac wash, followed by either a step or gradient elution using NaCl and $MgCl_2$. The genome recovery is calculated using the genome copy determined by qPCR assay, while the full-rAAV percentage is determined by AUC assay.

4. DISCUSSION

Chromatographic separation of empty- and full-AAV particles is a resolution challenge. This combination of TEA and Ac displays the behavior of a weak eluent and is observed to be more effective to discriminate empty- and full-rAAV species in AEX chromatography. In a qualitative sense, if the eluting power of a modifier is too high, the eluent may fail to distinguish the species retained on the stationary phase. In addition, a strong eluent may produce retention times that are too short with the result of poorly resolved elution peaks. Conversely, a weaker eluent is more likely to discriminate between the species retained on the stationary phase and increase the retention time, and in return increase resolution. Similarly, the weak AEX stationary phase, whose ligand is derived from a weak acid and partially ionized in a narrow pH range, may also be able to provide enhanced selectivity between empty- and full-rAAV particles. In line with this notion, Zhou *et al.*reported that a DEAE-based weak AEX stationary phase, POROS 50D, provided markedly improved resolution between empty and full-rAAV particles of AAV-6, when compared with other strong AEX stationary phases.[27] It is foreseeable that a more systematic study around AEX stationary phase may provide further insight on the delicate separation of empty and full-rAAV in AEX chromatography.

Most recently, it was reported that the empty-rAAV species may have preferential binding to a process-related impurity, chromatin, in Sf9 cell line generated rAAV viral vector product.[28]As chromatin consists of histone octamers whose protruded tails carry positive charged lysine residues, it is possible that these positively charged histones could preferentially bind empty-rAAV. In line with this thought, it is also possible that TEA-Ac preferentially interacts with empty-rAAV, either through electrostatic interaction using TEA positive amine group or through enforced charge pairing [24] using both TEA positive amine group and TEA hydrophobic alkyl-chain. Both of these interactions may lead to the decrease of net charge of the empty-rAAV species and thus benefiting empty- and full-rAAV separation in AEX chromatography.

An important consideration of using QA salt in purification process is that its sufficient clearance needs to be demonstrated to assure product safety. As aforementioned, since sodium ion has stronger elution power than that of TEA ion, the second wash with NaCl could replace and flush out residual TEA-Ac salt from AEX column prior to elution phase. In addition, the volume of the second wash buffer could be further optimized to achieve extra clearance of TEA ions. Furthermore, the final concentration and buffer exchange step in downstream process usually could achieve >99.9% buffer exchange efficiency,[29] which offers at least 3 more log reduction value (LRV) for TEA-Ac, providing feasibility for TEA-Ac to be used as a potent process buffer salt to enhance full-rAAV percentage.

Regarding the implementation of AEX step elution method, the lot-to-lot variability (in terms of peak elution retention volume) of monolithic column may add one more layer of complexity when converting LGE to isocratic elution, as the isocratic elution buffer may not consistently elute full-rAAV species when AEX column changed, resulting in either low full-rAAV percentage or low genome recovery. As a mitigation, packed column with AEX resin may provide more consistent peak elution behavior, although the resolution of empty- and full-rAAV separation using AEX resin may suffer certain level of decrease when compared to AEX monolithic column. In addition, it has been communicated that a new line of AEX monolithic column product will be lunched by CIMultus-QA vendor whose manufacturing procedure was specifically improved to reduce lot-to-lot variability (personal communication), which may offer process developer an extra option to achieve enhanced process robustness while still maintaining optimal empty full separation in AEX monolithic column operation.

Furthermore, AEX chromatography is usually employed to clear process related impurities, such as host cell protein (HCP).[30] In AEX LGE run, the HCP species are eluted off column by higher ionic strength post full peak, thus delicate peak cutting criteria need to be implemented to exclude HCP from entering product stream. Similarly, in AEX step elution run, elution salt concentration needs to be carefully optimized to ensure equivalent HCP clearance as achieved by LGE. When implementing TEA-Ac in an AEX step, due to the unique property of TEA ion comparing to other cations in process salts, TEA ion may be able to interact with HCP through its hydrophobic alkyl-chain. Therefore, the HCP reduction capability of AEX step needs to be re-evaluated to assure both product-related impurity and process-related impurity are in control.

Lastly, certain challenges still remain in purification of certain AAV serotype. For instance, Joshi *et al* . reported that AAV9 empty and full peaks could be separated using POROS HQ column with 0.93 peak resolution, compared to 0.91 for rAAV8 serotype, indicating that the rAAV8 and rAAV9 viruses behave similarly in their study in terms of empty full separation.[10] However, Lock and Alvira found that their rAAV9 product possess two empty species with distinct chromatography behavior on CIMmultus QA column using salt LGE, with the major empty peak (early elution species) almost co-eluted with full peak.[31] The discrepancy observed in rAAV9 Empty and Full separation may be contributed by the variation of multiple factors between research labs, including DNA genome sequence, DNA genome size, viral protein amino acid sequence, viral protein post-translation modification,[13] and other factors, which may warrant researchers to consider a case-by-case implementation and optimization of this TEA-Ac and step elution AEX technology.

AUTHOR CONTRIBUTIONS

D.P.C. : Conceptualization, Methodology, Investigation, Writing – Original Draft, Writing – Review Editing. **C.H.** : Supervision, Resources, Writing – Original Draft, Writing – Review Editing.**J.C.W.** : Resources, Writing – Review Editing.

CONFLICTS OF INTEREST

D.P.C. , **C.H.,** and **J.C.W.** are inventors on a patent that includes the work in this manuscript

Data Availability Statement

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

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5. REFERENCES

1. Daya, S., & Berns, K. I. (2008). Gene therapy using adeno-associated virus vectors. *Clinical Microbiology Reviews* , *21* (4), 583–593. https://doi.org/10.1128/CMR.00008-08

2. Asokan, A., Schaffer, D. V, & Jude Samulski, R. (2012). The AAV Vector Toolkit: Poised at the Clinical Crossroads. *Molecular Therapy* , *20* (4), 699–708. https://doi.org/https://doi.org/10.1038/mt.2011.287

3. Samulski, R. J., & Muzyczka, N. (2014). AAV-Mediated Gene Therapy for Research and Therapeutic Purposes. *Annual Review of Virology* ,*1* (1), 427–451. https://doi.org/10.1146/annurev-virology-031413-085355

4. Naso, M. F., Tomkowicz, B., Perry, W. L., & Strohl, W. R. (2017). Adeno-Associated Virus (AAV) as a Vector for Gene Therapy.*BioDrugs* , *31* (4), 317–334. https://doi.org/10.1007/s40259-017-0234-5

5. Gao, K., Li, M., Zhong, L., Su, Q., Li, J., Li, S., He, R., Zhang, Y., Hendricks, G., Wang, J., & Gao, G. (2014). Empty Virions In AAV8 Vector Preparations Reduce Transduction Efficiency And May Cause Total Viral Particle Dose-Limiting Side-Effects. *Molecular Therapy. Methods & Clinical Development* , *1* (9), 20139. https://doi.org/10.1038/mtm.2013.9

6. Kishimoto, T. K., Samulski, R. J., & Samulski, R. J. (2022). Expert Opinion on Biological Therapy Addressing high dose AAV toxicity – ' one and done ' or ' slower and lower '? *Expert Opinion on Biological Therapy* , *00* (00), 1–5. https://doi.org/10.1080/14712598.2022.2060737

7. Hermens, W. T. J. M. C., Brake, O. Ter, Dijkhuizen, P. A., Sonnemans, M. A. F., Grimm, D., Kleinschmidt, J. A., & Verhaagen, J. (1999). Purification of recombinant adeno-associated virus by iodixanol gradient ultracentrifugation allows rapid and reproducible preparation of vector stocks for gene transfer in the nervous system. *Human Gene Therapy* , *10* (11), 1885–1891. https://doi.org/10.1089/10430349950017563

8. Ayuso, E., Mingozzi, F., Montane, J., Leon, X., Anguela, X. M., Haurigot, V., Edmonson, S. A., Africa, L., Zhou, S., High, K. A., Bosch, F., & Wright, J. F. (2010). High AAV vector purity results in serotype- and tissue-independent enhancement of transduction efficiency.*Gene Therapy* , *17* (4), 503–510. https://doi.org/10.1038/gt.2009.157

9. Crosson, S. M., Dib, P., Smith, J. K., & Zolotukhin, S. (2018). Helper-free Production of Laboratory Grade AAV and Purification by Iodixanol Density Gradient Centrifugation. *Molecular Therapy - Methods and Clinical Development* , *10* (September), 1–7. https://doi.org/10.1016/j.omtm.2018.05.001

10. Joshi, P. R. H., Bernier, A., Moço, P. D., Schrag, J., Chahal, P. S., & Kamen, A. (2021). Development of a scalable and robust AEX method for enriched rAAV preparations in genome- containing VCs of serotypes 5 , 6 , 8 , and 9. *Molecular Therapy: Methods & Clinical Development* , *21* (June), 341–356. https://doi.org/10.1016/j.omtm.2021.03.016

11. Wang, C., Mulagapati, S. H. R., Chen, Z., Du, J., Zhao, X., Xi, G., Chen, L., Linke, T., Gao, C., Schmelzer, A. E., & Liu, D. (2019). Developing an Anion Exchange Chromatography Assay for Determining

Empty and Full Capsid Contents in AAV6.2. *Molecular Therapy - Methods and Clinical Development* , *15* (December), 257–263. https://doi.org/10.1016/j.omtm.2019.09.006

12. Wright, J. F., Le, T., Prado, J., Bahr-Davidson, J., Smith, P. H., Zhen, Z., Sommer, J. M., Pierce, G. F., & Qu, G. (2005). Identification of factors that contribute to recombinant AAV2 particle aggregation and methods to prevent its occurrence during vector purification and formulation. *Molecular Therapy* , *12* (1), 171–178. https://doi.org/10.1016/j.ymthe.2005.02.021

13. Mary, B., Maurya, S., Arumugam, S., Kumar, V., & Jayandharan, G. R. (2019). Post-translational modifications in capsid proteins of recombinant adeno-associated virus (AAV) 1-rh10 serotypes. *FEBS Journal* , *286* (24), 4964–4981. https://doi.org/10.1111/febs.15013

14. Grieger, J. C., & Samulski, R. J. (2005). Packaging Capacity of Adeno-Associated Virus Serotypes: Impact of Larger Genomes on Infectivity and Postentry Steps. *Journal of Virology* ,*79* (15), 9933–9944. https://doi.org/10.1128/jvi.79.15.9933-9944.2005

15. Giles, A. R., Sims, J. J., Turner, K. B., Govindasamy, L., Alvira, M. R., Lock, M., & Wilson, J. M. (2018). Deamidation of Amino Acids on the Surface of Adeno-Associated Virus Capsids Leads to Charge Heterogeneity and Altered Vector Function. *Molecular Therapy* ,*26* (12), 2848–2862. https://doi.org/10.1016/j.ymthe.2018.09.013

16. Dickerson, R., Argento, C., Pieracci, J., & Bakhshayeshi, M. (2021). Separating Empty and Full Recombinant Adeno-Associated Virus Particles Using Isocratic Anion Exchange Chromatography.*Biotechnology Journal* , *16* (1). https://doi.org/https://doi.org/10.1002/biot.202000015

17. Johnson, A. R., & Vitha, M. F. (2011). Chromatographic selectivity triangles. *Journal of Chromatography. A* , *1218* (4), 556–586. https://doi.org/10.1016/j.chroma.2010.09.046

18. Qu, G., Bahr-Davidson, J., Prado, J., Tai, A., Cataniag, F., McDonnell, J., Zhou, J., Hauck, B., Luna, J., Sommer, J. M., Smith, P., Zhou, S., Colosi, P., High, K. A., Pierce, G. F., & Wright, J. F. (2007). Separation of adeno-associated virus type 2 empty particles from genome containing vectors by anion-exchange column chromatography.*Journal of Virological Methods* , *140* (1–2), 183–192. https://doi.org/10.1016/j.jviromet.2006.11.019

19. Khatwani, S. L., Pavlova, A., & Pirot, Z. (2021). Anion-exchange HPLC assay for separation and quantification of empty and full capsids in multiple adeno-associated virus serotypes. *Molecular Therapy: Methods & Clinical Development* , *21* (June), 548–558. https://doi.org/10.1016/j.omtm.2021.04.003

20. Urabe, M., Xin, K. Q., Obara, Y., Nakakura, T., Mizukami, H., Kume, A., Okuda, K., & Ozawa, K. (2006). Removal of empty capsids from type 1 adeno-associated virus vector stocks by anion-exchange chromatography potentiates transgene expression. *Molecular Therapy* ,*13* (4), 823–828. https://doi.org/10.1016/j.ymthe.2005.11.024

21. Yang, H., Koza, S., & Chen, W. (2020). Anion-Exchange Chromatography for Determining Empty and Full Capsid Contents in Adeno-Associated Virus. *Waters Application Note* , 1–7.

22. Nam, H.-J., Lane, M. D., Padron, E., Gurda, B., McKenna, R., Kohlbrenner, E., Aslanidi, G., Byrne, B., Muzyczka, N., Zolotukhin, S., & Agbandje-McKenna, M. (2007). Structure of Adeno-Associated Virus Serotype 8, a Gene Therapy Vector. *Journal of Virology* ,*81* (22), 12260–12271. https://doi.org/10.1128/jvi.01304-07

23. Gagnon, P., Leskovec, M., Prebil, S. D., Žigon, R., Štokelj, M., Raspor, A., Peljhan, S., & Štrancar, A. (2021). Removal of empty capsids from adeno-associated virus preparations by multimodal metal affinity chromatography. *Journal of Chromatography A* ,*1649* , 462210. https://doi.org/10.1016/j.chroma.2021.462210

24. Fritz, J. S. (2005). Factors affecting selectivity in ion chromatography. *Journal of Chromatography A* , *1085* , 8–17. https://doi.org/10.1016/j.chroma.2004.12.087

25. GRUHZIT, O. M., FISKEN, R. A., & COOPER, B. J. (1948). TETRAETHYLAMMONIUM CHLORIDE. ACUTE AND CHRONIC TOXICITY IN EXPERIMENTAL ANIMALS. *Journal of Pharmacology and Experimental Therapeutics* ,*92* (2), 103 LP – 107. http://jpet.aspetjournals.org/content/92/2/103.abstract

26. Singh, N., & Heldt, C. L. (2022). Challenges in downstream purification of gene therapy viral vectors. *Current Opinion in Chemical Engineering* , *35* , 100780. https://doi.org/10.1016/j.coche.2021.100780

27. Zhou, J., Hauck, B., Wright, J. F., & High, K. A. (2007). Weak Anion Exchange Column Chromatography Enhances the Resolution of Separation of AAV Empty Capsid and Full Vectors. *Molecular Therapy* , *15* , S36. https://doi.org/10.1016/s1525-0016(16)44297-8

28. Gagnon, P., Goricar, B., Mencin, N., Zvanut, T., Peljhan, S., Leskovec, M., & Strancar, A. (2021). Multiple-monitor HPLC assays for rapid process development, in-process monitoring, and validation of AAV production and purification. *Pharmaceutics* , *13* (1), 1–14. https://doi.org/10.3390/pharmaceutics13010113

29. Schwartz, L. (2003). Diafiltration: A Fast, Efficient Method for Desalting or Buffer Exchange of Biological Samples. *Pall Scientific & Technical Report* , 6. http://www4.pall.com/pdf/02.0629_Buffer_Exchange_STR.pdf

30. Levy, N. E., Valente, K. N., Lee, K. H., & Lenhoff, A. M. (2016). Host cell protein impurities in chromatographic polishing steps for monoclonal antibody purification. *Biotechnology and Bioengineering* , *113* (6), 1260–1272. https://doi.org/10.1002/bit.25882

31. Lock, M., & Alvira, M. R. (2019). SCALABLE PURIFICATION METHOD FOR AAV9 (Patent No. US 2019 / 0002842 A1). In *United States Patent Application Publication* (US 2019 / 0002842 A1).

32. Ayuso, E., Mingozzi, F., & Bosch, F. (2010). Production, purification and characterization of adeno-associated vectors.*Current Gene Therapy* , *10* (6), 423–436. https://doi.org/10.2174/156652310793797685

33. Grieger, J. C., Soltys, S. M., & Samulski, R. J. (2016). Production of recombinant adeno-associated virus vectors using suspension HEK293 cells and continuous harvest of vector from the culture media for GMP FIX and FLT1 clinical vector. *Molecular Therapy* , *24* (2), 287–297. https://doi.org/10.1038/mt.2015.187

34. Fu, X., Chen, W. C., Argento, C., Clarner, P., Bhatt, V., Dickerson, R., Bou-Assaf, G., Bakhshayeshi, M., Lu, X., Bergelson, S., & Pieracci, J. (2019). Analytical Strategies for Quantification of Adeno-Associated Virus Empty Capsids to Support Process Development. *Human Gene Therapy Methods* , *30* (4), 144–152. https://doi.org/10.1089/hgtb.2019.088