RESEARCH ARTICLE

Novel anion exchange membrane chromatography method for the separation of empty and full adeno-associated virus

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Abstract
A challenge in the production of recombinant Adeno-Associated Virus (AAV) for gene therapies is the presence of capsids that lack the required gene of interest. The impact of these empty vectors in therapies is not fully understood, however the ability to control the ratio of empty to full particles, which contain the genetic payload, is a necessary step in the purification of these viruses. In this study, a novel anion exchange chromatography elution method for enrichment of full AAV particles is demonstrated. A step gradient with small conductivity increases of around 1 mS cm$^{-1}$ provides more efficient separation of empty and full AAV serotype 5 across membrane media as compared to conventional linear gradient method. The use of this approach in optimizing a simpler method for manufacturing processes and scalability to a larger chromatographic volume is explored. With this approach, the authors achieved greater than 4-fold enrichment of full capsids, to give a total of $\approx 50\%$–$60\%$ full capsids, using a 25 mM Bis-Tris Propane pH 9.0 buffer system with NaCl as the eluting salt. Results suggest that this elution method can be implemented into a scalable process and can provide insight into development of elution methods for other AAV serotypes.

KEYWORDS
adeno-associated virus, empty particles, gene therapy, membrane chromatography, step gradient elution

1 | INTRODUCTION

Adeno-associated virus (AAV) continues to hold potential as a delivery vector of therapeutic genes for multiple diseases. The recent FDA gene therapy treatment approvals for Luxturna (for retinal dystrophy) and Zolgensma (for spinal muscular atrophy) have accelerated AAV based research and this is highlighted by plethora of clinical trials involving AAV$^{[1–3]}$.

AAV is an icosahedral-shaped virus consisting of a protein capsid composed of three protein subunits (VP1, VP2, VP3) which house a single-stranded DNA genome inserted enzymatically$^{[4,5]}$. The encapsidation process results in virus particles that contain the desired genome (full) and virus particles without any genome (empty) or with fragmented DNA (partial). Empty AAV particles can greatly outnumber full or semi-filled particles up to 30-fold$^{[5]}$. Since these particles lack the gene of interest (GOI), they are not inherently functional for the gene therapy. As such, empty particles may be considered a process related impurity and can result in an unnecessary immune response.
causing reduced infectivity of full AAV\textsuperscript{[6,7]} However, empty particles may provide a beneficial impact in the form of decoys for neutralizing antibodies.\textsuperscript{[8,9]}

The role empty capsids may play for a therapy is unclear, however, to have a consistent drug product the level of empty and full capsids must be controlled through the downstream manufacturing process. There are a range of methods for separating empty and full AAV particles including analytical ultracentrifugation which relies upon density gradients, typically cesium chloride or iodixanol. This process often yields highly purified AAV particles making it a reliable approach in terms of product quality.\textsuperscript{[10,11]} The drawback to ultracentrifugation is application to a manufacturing environment where operational costs and limited instrument size do not scale with material load volumes. As a result, there has been a push to develop more scalable methodologies for AAV purification.

Chromatography methods including affinity, ion exchange, and size exclusion have been adapted for multiple AAV serotypes in downstream purification. However, the order and conditions of the methods used vary greatly among different groups. Given full AAV contain a negatively charged genome, there are inherent charge differences between empty and full particles which may be exploitable by IEX.\textsuperscript{[12]} Okada et al. reported a process using cation exchange followed by anion exchange using Mustang S and Q membranes respectively enriching a vector stock to less than 1% empty capsids for AAV1.\textsuperscript{[13]} Tomono et al instead used CEX/AEX tandem followed by SEC in their downstream process with a 98% full AAV1 product.\textsuperscript{[14]} Qu et al. showed separation of empty and full AAV2 particles using a linear gradient salt elution using only POROS 50HQ resin resulting in an 86-fold reduction in empty capsids.\textsuperscript{[15]} Using a dual-AEX adsorbers, Kaludov et al. achieved 90% full recovery of multiple serotypes including AAV5.\textsuperscript{[16]} Chromatographic methods have also been performed pre- or post-centrifugation methods to ensure collection of primarily full capsids and removal of contaminants.\textsuperscript{[6,8,15]}

The multitude of methods requiring development and optimization attest to the formidable task of separating empty and full particles by ion exchange chromatography. Many groups note differing buffer reagents necessary for ideal chromatographic purification of each AAV serotypes, suggesting the need to optimize chromatographic methods per serotype.\textsuperscript{[11,16,17]} Wang et al. detailed their optimization process for AAV6.2 using a CIMac empty/full monolith.\textsuperscript{[18]} Biogen recently reported successes with analytical strategies for full AAV quantification and two step isocratic anion exchange approach for AAV2.\textsuperscript{[19,20]} This knowledgebase provides a starting point for the development of separation methods for other serotypes via AEX.

In this paper, we describe our attempts to enrich full AAV5 using Mustang Q membrane anion exchange chromatography and the exploration of a step gradient elution method using 1 mS cm\(^{-1}\) conductivity steps. Mustang Q membrane-based chromatography relies on convective transport which enables processing at lower pressures and higher flow rates, when compared to bead-based resins. The 1 mS cm\(^{-1}\) step elution strategy was attempted as an alternative to commonly used linear gradient elution methods and allowed us to further assess empty and full capsid AAV separation potential.

## EXPERIMENTAL SECTION

### 2.1 AAV5 production

AAV5 was generated by transient transfection of a HEK293A cells with a triple plasmid complex at a molar ratio of 1:1:1 (AAV-GFP : R/C-AAV5 : pHelper) with a PEI:DNA mass ratio of 1:1. Transfected cells were further grown in DMEM [CAT 12–614Q/12], 10% fetal bovine serum [CAT 26140095], 1x Non-essential amino acids [CAT 11140076], 4 mM Glutamax [CAT 35050661] for 4 days. Transfected cells and media were harvested 4 days post transfection by isonic lysis (100 mM Tris, 20 mM MgCl\(_2\), 10% Tween 20) followed by benzonase (final concentration 25 µg ml\(^{-1}\)) treatment for 24 h before being pooled into a homogeneous solution.

This mixture was clarified using a PDK11 depth filter (PALL) at 200 L m\(^{-2}\) into a KA02EKV sterile filter (PALL) at 450 L m\(^{-2}\). Both filters were flushed and chased with 1X phosphate-buffered saline at 100 L m\(^{-2}\). The clarified sample was then concentrated 10-fold by volume via SU-TFF using an Omega PES 100 kDa membrane (PALL) with a crossflow of 5 L m\(^{-2}\) min\(^{-1}\) aiming for a transmembrane pressure of 15 psi.

AAV5-CMV-eGFP empty and full standards were purchased from Vector Biolabs (Malvern, PA).

### 2.2 Affinity and anion exchange chromatography

Chromatography experiments were performed on either AKTA AVANT 25 (Cytiva, formerly GE) (affinity and 0.86 mL membrane) or 150 systems (5 mL membrane). Concentrated bioreactor harvest was applied to a Thermo Fisher 1 mL AAVX affinity resin column aiming for a transmembrane pressure of 15 psi.

Quantitative polymerase chain reaction (qPCR) for AAV genome content was performed using the Takara AAVpro Titration Kit [CAT 6233]. ddPCR for AAV genome content was performed using the BIORAD QX200 AutoDG Droplet Digital PCR System. Non-encapsidated DNA was digested at 37°C for 1 h using RNase Free DNase I kit (Qiagen).
**TABLE 1** Affinity chromatography method

| Step                  | Buffers used/details                                                | Column/Membrane volumes used |
|-----------------------|---------------------------------------------------------------------|------------------------------|
| 1 – Equilibration of column | 50 mM Tris, 500 mM NaCl, pH 8.0                                     | 10                           |
| 2 – Application of sample | Post-TFF AAV5 concentrated feedstock, pH 7.5                       | N/A                          |
| 3 – Wash              | 50 mM Tris, 500 mM NaCl, pH 8.0                                     | 10                           |
| 4 – Elution           | 50 mM Citric Acid, pH 2.0 (Added 5 CV of 1 M Tris, pH 8.5 to elution tube) | 25                           |

**TABLE 2** Chromatographic method using a linear gradient elution

| Step                  | Buffers used/details                                                | Column/Membrane volumes used |
|-----------------------|---------------------------------------------------------------------|------------------------------|
| 1 – Equilibration of membrane | 20 mM Bis-Tris Propane, pH 9.0                                     | 20                           |
| 2 – Application of sample | 0.5 mL post-affinity AAV5 eluate diluted 20-fold into 20 mM Bis-Tris Propane, pH 9.0 | N/A                          |
| 3 – Washing of membrane | 20 mM Bis-Tris Propane, pH 9.0                                     | 20                           |
| 4 – Linear Gradient   | Buffer A – 20 mM Bis-Tris Propane, pH 9.0                          | 40                           |
|                       | Buffer B – 20 mM Bis-Tris Propane, 1 M NaCl, pH 9.0                |                              |
| 5 – CIP               | 20 mM Bis-Tris Propane, pH 9.0                                     | 10                           |
|                       | 1N NaOH                                                             | 10, 15 min hold              |
|                       | 1X PBS, 1 M NaCl                                                    | 10                           |
|                       | 20 mM Bis-Tris Propane, pH 9.0                                     | 20                           |

**TABLE 3** Chromatographic method for 1 mS cm^{-1} step gradient elution

| Step                  | Buffers used/details                                                | Column/Membrane volumes used |
|-----------------------|---------------------------------------------------------------------|------------------------------|
| 1 – Equilibration of membrane | 20 mM Bis-Tris Propane, pH 9.0                                     | 10                           |
| 2 – Application of sample | 0.5 ml post-affinity AAV5 eluate diluted 20-fold into 20 mM Bis-Tris Propane, pH 9.0 | N/A                          |
| 3 – Washing of membrane | 20 mM Bis-Tris Propane, pH 9.0                                     | 10                           |
| 4 – Step Gradients    | Buffer A – 20 mM Bis-Tris Propane, pH 9.0                          | 15 per step                  |
|                       | Buffer B – 20 mM Bis-Tris Propane, 1 M NaCl, pH 9.0                |                              |
|                       | Step 1 = 7.3% Buffer B, 92.7% Buffer A                             |                              |
|                       | Step 2 = 8.3% Buffer B, 91.7% Buffer A                             |                              |
|                       | Step 3 = 9.4% Buffer B, 90.6% Buffer A                             |                              |
|                       | Step 4 = 10.4% Buffer B, 89.6% Buffer A                            |                              |
|                       | Step 5 = 11.4% Buffer B, 88.6% Buffer A                            |                              |
|                       | Step 6 = 12.4% Buffer B, 87.6% Buffer A                            |                              |
|                       | Step 7 = 13.4% Buffer B, 86.6% Buffer A                            |                              |
|                       | Step 8 = 14.4% Buffer B, 85.6% Buffer A                            |                              |
|                       | Step 9 = 15.4% Buffer B, 84.6% Buffer A                            |                              |
| 5 – CIP               | 20 mM Bis-Tris Propane, pH 9.0                                     | 10                           |
|                       | 1N NaOH                                                             | 10, 15 min hold              |
|                       | 1X PBS, 1 M NaCl                                                    | 10                           |
|                       | 20 mM Bis-Tris Propane, pH 9.0                                     | 20                           |

Once digested, the samples were diluted 1:100 in 1X TE solution [CAT 11-05-01-09], supplemented with Pluronic PF-68 [CAT 24040032] to 0.01% and ddPCR was performed under the following conditions:

- 12.5 µL ddPCR Supermix for probes [CAT 186-3027]
- 3.75 µL Nuclease Free Water [CAT 11-05-01-04]
- 1.25 µL FWD primer (900 nM final conc)
- 1.25 µL REV primer (900 nM final conc)
- 1.25 µL Probe (250 nM final conc)
- 10 µL sample

The following primers (Integrated DNA Technologies) were used:

Forward 5’ GAACCGCATCGAGCTGAA 3’
Reverse 5’ TGCTTGTCGGCCATGATATAG 3’
Probe 5’ /56-FAM/ATCGACTTTC/ZEN/AAGGAGGACGGCAAC//31ABkFQ/3’

The PCR conditions used were:
- 1 cycle at 95°C for 10 min
- 42 cycles of 1) 95°C, 30 s; 2) 54.9°C, 60 s; 3) 72°C, 15 s
- 1 cycle at 98°C for 10 min, followed by 8°C hold
### TABLE 4 Chromatographic method used for the two-step elution method

| Step | Buffers used/details | Membrane volumes used |
|------|----------------------|-----------------------|
| 1 – Equilibration of membrane | 20 mM Bis-Tris Propane, pH 9.0 | 20 |
| 2 – Application of sample | 50 mL post-affinity AAV5 eluate diluted 20-fold into 20 mM Bis-Tris Propane, pH 9.0 | N/A |
| 3 – Washing of membrane | 20 mM Bis-Tris Propane, pH 9.0 | 20 |
| 4 – Step Gradients | Buffer A – 20 mM Bis-Tris Propane, pH 9.0 | 20 per step |
| | Buffer B – 20 mM Bis-Tris Propane, 1 M NaCl, pH 9.0 | |
| | Step 1 = 8.0% Buffer B, 92.0% Buffer A | |
| | Step 2 = 12.0% Buffer B, 88.0% Buffer A | |
| 5 – CIP | 20 mM Bis-Tris Propane, pH 9.0 | 10 |
| | 1N NaOH | 10, 15 min hold |
| | 1X PBS, 1 M NaCl | 10 |
| | 20 mM Bis-Tris Propane, pH 9.0 | 20 |

ELISA for AAV capsid content was performed using the PROGEN AAV5 Titration ELISA kit [CAT PRAAV5] per kit instructions.

Purity of sample for the 5 mL Mustang Q XT5 scale-up run was determined using: (A) Quant-iT PicoGreen dsDNA Assay Kit [CAT P7589], (B) HEK 293 HCP ELISA Kit [CAT F650S], (C) SDS-PAGE using 10% Criterion XT Bis-Tris Protein Gel [CAT 3450111]. Samples for SDS–PAGE were prepared by mixing with 4X reducing buffer [CAT J60015-AC]. SDS–PAGE was performed using 1X MOPS buffer [CAT 1610788] for 60 min at 150V. The gel was stained following the SYPRO Ruby Protein Gel Stain [CAT S12000] basic protocol.

### 3 | RESULTS

#### 3.1 | Linear gradient elution method

Our initial work to develop full capsid enrichment for AAV5 began with linear gradient elutions from Mustang Q anion exchange membrane. Qualitative assessment of the purification was performed via 260–280 nm UV absorbance. This provides a guide to full capsid content as proteins absorb primarily at 280 nm and nucleic acids absorb primarily at around 260 nm. Thus, an increase in the ratio 260:280 nm indicates an enrichment of “full” capsids containing DNA. A representative chromatogram from a linear gradient elution experiment is shown in Figure 1. The chromatogram shows a single broad peak of low intensity with no change in 260:280 nm absorbance (Maxima 0.2 mAU : 0.3 mAU). Over ten combinations of chromatographic media, buffers, salts, and gradient conditions were tested including low molarity Tris buffer, sodium phosphate eluting salt, steeper/shorter linear gradient elution step (data not shown), without any apparent enrichment of full capsids.

#### 3.2 | Step gradient elution method

To further improve chromatographic resolution and to be able to identify discrete chromatography peaks an alternative approach was developed using conductivity steps. A similar step approach had proven successful before for the identification of an elution design space for a mAb from a mixed mode sorbent.

To optimize the separation of empty and full capsids a range of different conductivity steps was explored with different combinations of elution duration, 5 CV up to 40 CV, and conductivity increments, 0.5 mS cm⁻¹ versus 1.5 mS cm⁻¹ versus 2 mS cm⁻¹ (data not shown). As judged by UV absorbance the most successful separations were achieved with 1 mS cm⁻¹ steps each of 10 membrane volumes, Figure 2. Here we can see that the 1 mS cm⁻¹ steps generate a series of discrete peaks which facilitate visual analysis of relative amounts of full capsids via the UV ratio 260:280. In Figure 2 we see the first two AAV elution peaks at ≈9 and 10 mS cm⁻¹: These peaks have low 260:280 and appear to be primarily empty capsids. The following two peaks eluting at ≈11–13 mS cm⁻¹ have much higher 260:280 nm ratios indicating full capsid enrichment.

To further assess separation, ELISA and qPCR were performed on each peak to determine total capsids and full capsids respectively (Table 5). ELISA shows that the amount of total capsids reaches a maximum in the second peak ≈10 mS cm⁻¹. qPCR shows that the genome copies, corresponding to full capsids reach a maxima in the fourth peak at 12 mS cm⁻¹. Thus, there is a clear separation of empty and full capsids. An estimate of this can be determined from the ratio of gc/capsids which peaks at 71% in peak 4 at ≈12 mS cm⁻¹. Caution must be taken in trusting this ratio of ELISA and qPCR as there is some error expected in both assays, however, the relative trends appear to be valuable within a purification.

#### 3.3 | Empty and full AAV5 standards

With the relative success of the 1 mS cm⁻¹ step elution approach we wanted to confirm the separation observed. To simplify the analysis of the separation, density gradient purified empty and full AAV5 particles were obtained. An identical step gradient was performed for both the empty and full standards, as shown in Figure 3A and B, respectively. The empty standards eluted primarily in steps 2 and 3 (8 and 9 mS cm⁻¹, respectively) with 260/280 absorbance ratios of 0.7 and 0.6. The full
**FIGURE 1**  Separation of AAV5 using a linear gradient elution with Mustang Q XT Acrodisc (0.86 mL). Only a small absorbance peak (0.2 mAU/0.3 mAU : 260/280 nm) was observed with no change in A280/A260 measurements

**FIGURE 2**  Separation of AAV5 using Mustang Q XT Acrodisc (0.86 mL) with a 1 mS cm\(^{-1}\) conductivity step gradient elution method. A visible shift in A260/A280 appeared during steps starting at conductivity of 9 mS cm\(^{-1}\) up to 13 mS cm\(^{-1}\)

**TABLE 5**  Resulting capsid ELISA and qPCR results from 1 mS cm\(^{-1}\) steps using 0.86 mL device

| Step Conductivity (mS cm\(^{-1}\)) | 7  | 8  | 9  | 10 | 11 | 12 | 13 | 14 | 15 | Empty Peaks | Empty Yield (%) | Full Peaks | Full Yield (%) |
|-----------------------------------|----|----|----|----|----|----|----|----|----|--------------|-----------------|------------|----------------|
| 260:280 signal ratio              | -- | -- | -- | 0.5 | 0.6 | 0.8 | 0.9 | 0.9 | 0.9 | N/A          | N/A             | N/A        | N/A            |
| Total GC (1E10)                   | 0  | 0  | 0  | 6  | 23 | 24 | 13 | 5  | 3  | 14           | 19%             | 60         | 81%            |
| Total Capsids (1E10)              | 8  | 0  | 11 | 263| 59 | 34 | 19 | 18 | 14 | 314          | 74%             | 112        | 26%            |
| GC/Capsids                        | 0% | 0% | 1% | 2% | 39%| 71%| 70%| 29%| 20%| 4%           | N/A             | 54%        | N/A            |

Capsid content is largest at conductivities of 10 and 11 mS cm\(^{-1}\) while the genomic content is highest at conductivities of 11 and 12 mS cm\(^{-1}\).

3.4 Scale-up to 5 ml capsule

The 1 mS cm\(^{-1}\) step elution method worked well at a smaller scale of 1 ml or smaller chromatography devices, however we wanted to know if this method would also be applicable with larger devices. Larger
membrane devices may have increased holdup volume and alternative binding properties from an increased surface area. A Mustang Q XT5 capsule (5 mL) was used to test the elution method using the same reagents and a larger load pool of AAV5. Figure 4A shows the resulting chromatogram while Table 6 shows the ddPCR and ELISA results from our trial. Overall, the separation looks comparable to that achieved with the Mustang Q XT Acrodisc 0.86 mL device, Figure 2. With the 5 mL device we closed our mass balance for total capsids and full capsids with ELISA and ddPCR. While it is encouraging to be able to get 97% yield of total capsids, as measured by ddPCR, a challenge remains in balancing the yield with the purity. The second peak at 12 mS cm\(^{-1}\) contains a significant proportion of full capsids 31%, but also contains 35% of the total capsids. Thus, full capsids are not enriched in peak 2. However, if we consider peak 3 and 4 combined, we recover 60% of the full capsids and 13% of the total capsids which allows us to estimate a greater than four-fold enrichment of full capsids, Figure 4.

### 3.6 Limiting to two-step elution

While \(\approx 1\) mS cm\(^{-1}\) elution steps provide enrichment of full AAV5 capsids and reduction of contaminants using anion exchange membrane chromatography, such a method may be difficult to implement in a manufacturing suite. The ability to maintain such small conductivity changes in larger volumes may prove difficult and failure to do so may result in inconsistent elution. To address this, we took the learnings from the 1 mS cm\(^{-1}\) step gradient and used the approach as a de facto process development technique. This facilitated the implementation of a two-step elution at conductivities of 10 and 14 mS cm\(^{-1}\). Figure 5A shows the resulting chromatogram where the elution peak at 10 mS cm\(^{-1}\) was large with a higher A280 than A260 and the elution peak at 14 mS cm\(^{-1}\) was smaller with near equal A260 and A280. Titration ELISA and ddPCR show the same pattern of empty particles eluting first and full particles eluting later with the 14 mS cm\(^{-1}\) elution have a VG/Capsid ratio of 48% (Table 8).

To additionally verify a separation was occurring based solely on the conductivity of the gradient, the two elutes were reloaded and allowed to elute with the same method, Figure 5B and 5C. Reloading the 10 mS cm\(^{-1}\) peak resulted in a major peak at 10 mS cm\(^{-1}\) and a minor peak at 14 mS cm\(^{-1}\). Reloading the “full peak” which originally eluted at 14 mS cm\(^{-1}\) results in a single peak at 14 mS cm\(^{-1}\) with a very minor increase in UV absorbance at the 11 mS cm\(^{-1}\) elution. This confirms the removal of a large population of empty capsids from this fraction.

### 4 DISCUSSION

Our goal was to identify a potential method for empty and full AAV5 separation using AEX chromatography to implement into an AAV
FIGURE 4  (A) 1 mS cm\(^{-1}\) step conductivity gradient elution chromatogram using AAV5 on a 5 mL Mustang Q device. (B) SDS-PAGE image of samples from our AAV purification platform including post-conductivity step gradient elution show the purity of the sample generated where less banding and smearing is visible post affinity purification.

TABLE 6 Resulting capsid ELISA and qPCR results from 1 mS cm\(^{-1}\) steps using 5 mL device

| Load                  | Peak 1 | Peak 2 | Peak 3 | Peak 4 | Peak 5 | Peak 6 | SUM |
|-----------------------|--------|--------|--------|--------|--------|--------|-----|
| 260:280 Signal Ratio  | N/A    | 0.6    | 0.6    | 1.0    | 0.9    | 0.3    | 0.6 | N/A |
| Total Capsids (1E12)  | 695    | 140    | 245    | 59     | 30     | 55     | 31  | —   |
| Total VG (ddPCR, 1E12)| 35     | 0      | 11     | 17     | 4      | 1      | 1   | —   |
| E/F                   | 5%     | 0%     | 5%     | 29%    | 12%    | 2%     | 2%  | —   |
| ELISA Yield           | —      | 20%    | 35%    | 9%     | 4%     | 8%     | 4%  | 81% |
| ddPCR Yield           | —      | 0%     | 31%    | 49%    | 11%    | 3%     | 2%  | 97% |

VG and capsid content of each eluted fraction.
At peak 3, we measured the highest ddPCR yield of 49% and an estimated empty full ratio of 29%.

platform. The chromatographic strategies found in the literature led us to the application of linear salt gradient elution.\(^{[18]}\) Our initial results applying the approach did not meet our expectations for separation and led us to consider alternative approaches. This may not be entirely surprising as membrane chromatography devices tend not to have a large number of theoretical plates due to the small effective bed height. Therefore, elution peaks from linear gradients would not be focused on a membrane in the same way they might with a chromatographic...
FIGURE 5  Separation of AAV5 using a two-step salt gradient elution method on Mustang Q device (0.86 mL). The chromatogram (A) shows the initial elution peak contains a higher A280/A260 ratio compared to the second elution peak. Reloads of elution 1 (B) and elution 2 (C) from the two-step elution method. Both reloaded fractions eluted primarily at their original elution step with minor peaks present at their respective other step.

column which has a longer bed height and consequently more theoretical plates. Also, a linear gradient approach may lead to broad elution peaks which could explain the low absorbance signals observed in our attempt (<1 mAU) making it difficult to assess full capsid enrichment by UV absorbance. This led us to consider other approaches that might overcome the limitation of low theoretical plates. By adjusting the linear elution into small discrete conductivity steps, we were able to break up the single elution peak into a series of discrete peaks.

While switching to a step gradient provided better enrichment of full AAV5, the method does have some limitations. As we observed when running the purchased standards, there remained an elution overlap for the empty and full AAV capsids suggesting the approach
TABLE 7  Contaminant HCP and dsDNA measurement from AAV5 platform process

| Gel Lane Sample                  | dsDNA Concentration (ng mL⁻¹) | HCP Concentration (ng mL⁻¹) |
|----------------------------------|------------------------------|-----------------------------|
| 1 Crude Harvest                  | 2.7x10³                      | 1.9x10⁴                     |
| 2 Post Clarification/TFF         | 6.9x10³                      | 9.4x10⁴                     |
| 3 Post Affinity                  | 60                           | 109                         |
| 4 Post Mustang Q Empty Capsids   | < 0.2                        | < 10                        |
| 5 Post Mustang Q Full Capsids    | < 0.2                        | < 50                        |
| 6 Post ILDF/TFF                  | < 0.2                        | < 10                        |
| 7 Post Sterile Filtration        | < 0.2                        | < 10                        |

The chromatography was performed using the 1 mS cm⁻¹ step elution method on a 5 mL device. Measuring the HCP and dsDNA (double stranded DNA) content confirms the purity with readings nearing the Limit of Detection for both the HCP ELISA kit and DNA Picogreen assay.

TABLE 8  Capsid ELISA and ddPCR results for the two-step elution method

| Step | 1     | 2     |
|------|-------|-------|
| Conductivity (mS cm⁻¹) | 10    | 14    |
| Total Capsids (1x10¹¹) | 13    | 7     |
| Total VG (1x10¹¹)       | 1     | 3     |
| VG/Capsids              | 10%   | 48%   |

A near 5-fold increase in the empty/full ratio was noted for the second step of the two-step elution method.

will require optimization of buffer conductivity and composition to improve resolution. Additionally, with a step gradient the full enriched fractions may extend over multiple eluates, leading to an overall lower concentration. These challenges can be mitigated by using the 1 mS cm⁻¹ steps as a process development tool to identify buffer conditions and/or ideal conductivity thresholds which may also facilitate a two-step elution.

We observed effective scalability from 0.86 mL to a 5 mL capsule, suggesting this approach can also be used in a processing setting. This may help intensify the AAV purification process and fulfill an unmet need as Mustang Q and other membrane columns can be operated at high flow rates, up to 10 MV per minute. This is desirable for AEX as it is normally positioned as the second AAV chromatography step after affinity purification and typically a large dilution maybe expected after the affinity step to raise pH and lower conductivity before the AEX step.

Additionally, the use of UV absorbance, capsid ELISA, and qPCR/ddPCR were instrumental in confirming and evaluating empty and full AAV content. However, these methodologies tend to suffer from poor accuracy. More definitive methods for ratio determination of empty and full AAV particles are primarily limited to AUC or TEM which are often low throughput and expensive. As AAV continues to show promise in gene therapy, alternative analytical instrumentation will surely emerge to solve empty and full characterization struggles.

Ultimately, the method of applying a conductivity step gradient was found to provide better enrichment of empty and full AAV5 particles compared to a conductivity linear gradient elution using a membrane device. Combined with the advantages of being commercially available, pre-packed PALL Mustang Q membrane devices have high performance at higher flow rates. This step conductivity gradient using membrane chromatography can find major benefit in manufacturing process development as well as a processing strategy. By adjusting this conductivity step gradient, manufacturing pipelines would have a method to identify ideal conductivities for an optimized three or fewer step elution strategy. We speculate this method can be successfullly applied to other AAV serotypes and further optimizations of the method in terms of buffer compositions and step elution volume will lead to a consistent ratio of empty and full AAV particles.

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CONFLICT OF INTERESTS

Adam Hejmowski is an inventor for the 1 mS cm⁻¹ step method and has filed a patent. All authors are Pall employees.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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