

Article

Mechanistic Insights into AAV Capsid–Stationary Phase Interactions Governing Native Stability and Chromatographic Separation Using AAV8 as a Model System

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Abstract

Background/Objectives: Adeno-associated viruses (AAVs) are widely used gene therapy vectors; yet their physicochemical stability and chromatographic behavior are highly sensitive to the solution conditions they are in. Effective separation of full (F), empty (E), and partially filled (P) capsids—most commonly achieved by anion exchange (AEX) chromatography—is essential for standard analytical characterization, process development, and product safety. However, conventional AEX methods rely on low-conductivity alkaline mobile phases with low salt, which promote capsid binding and therefore higher resolution, at the expense of structural stability. Conversely, formulations such as near-neutral buffers might preserve capsid integrity but often impair AEX retention and separation resolution. **Methods:** Here, we extend a mechanistic investigation using AAV8 capsids as a model system, focusing on detailed capsid interactions with strong AEX, and present novel AAV8 separation strategies on a weak AEX stationary phase. **Results:** By systematically varying buffer pH and ionic strength, we identify operational regimes that balance capsid stability with chromatographic separation efficiency. In parallel, we introduce an integrated two-dimensional (2D) in-line buffer exchange configuration that decouples AEX performance from sample formulation, enabling robust separation of stability-optimized, high-salt matrices without off-line desalting. **Conclusions:** By elucidating the roles of capsid charge modulation, ligand physicochemical properties, and local microenvironmental buffering, this study establishes practical design principles for stability-preserving chromatography. It lays a foundation for more reliable analytical and future preparative AAV workflows.

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1. Introduction

Adeno-associated virus (AAV) vectors are central to modern gene therapy development; however, their physicochemical stability is highly dependent on the solution environment and handling conditions. Deviations in formulation pH, ionic strength, or excipient composition can trigger capsid destabilization, aggregation, or loss of biological ac-

tivity. Consequently, AAV materials are commonly stored and processed in formulation-like buffers designed to preserve structural integrity and long-term stability [1].

Anion exchange (AEX) chromatography is a cornerstone technique for AAV characterization and purification, enabling separation of full capsids from empty, partially filled, and other product-related variants [2–14]. Standard AEX workflows typically employ low-salt mobile phases with low ionic strength and a carefully adjusted alkaline pH to maximize electrostatic interactions between the negatively charged capsids and the positively charged stationary phase [14–16]. However, these conditions are increasingly recognized as unfavorable for AAV stability, whereas moderate salt concentrations and near-neutral pH are better suited to preserving capsid integrity [14,17,18]. Achieving effective AEX E–F capsid separation under native, stability-preserving conditions, therefore, remains a significant analytical challenge.

AAV capsid stability is governed by a complex interplay of formulation parameters, including pH, ionic strength, cryoprotectants, and non-ionic surfactants, all of which contribute to minimizing aggregation and preserving infectivity [17]. In particular, maintaining a minimum salt concentration of approximately 150–200 mM has been shown to be critical for preventing capsid aggregation during storage and handling [18]. Conversely, exposure to low-conductivity environments—especially at alkaline pH—has been reported to compromise capsid stability more severely than exposure to moderately conductive solutions at comparable pH [14]. These effects reflect the underlying balance of intermolecular forces governing capsid behavior: electrostatic interactions dominate at low ionic strength, whereas increasing salt concentration progressively shields surface charges and enhances hydrophobic interactions [19]. This physicochemical interplay directly impacts chromatographic separations, where conditions optimized for binding and resolution may simultaneously increase the risk of capsid destabilization.

Strong AEX media currently dominate AAV capsid separation workflows [11], yet their fully ionized functional groups can promote strong electrostatic interactions that may further compromise capsid stability unless stabilizing counterions, such as magnesium, are introduced [11,14]. In contrast, strictly weak AEX phases (like diethylaminoethyl—DEAE) have not yet been demonstrated to achieve near-baseline AAV E–F resolution [11,16], and enhanced separation under near-neutral pH conditions remains largely unexplored. This gap motivated a detailed investigation into how charge regulation, ligand ionization, other ligand physicochemical properties, and local buffering affect the retention and elution behavior of the AAV capsid on AEX columns.

To begin, a thorough evaluation of AEX ligands is required. It is widely assumed that electrostatic interactions dominate in many ion-exchangers (IEXs), with hydrophobic interactions often considered negligible. However, hydrophobic contributions can depend not only on the properties of the resin matrix but also on factors such as the density of charged functional groups, and the length and characteristics of linkers. Importantly, these hydrophobic contributions also depend on the size and hydrophobicity of the functional groups; particularly in the case of AEXs, these contributions remain insufficiently explored [20]. Unlike strong IEXs, whose functional groups remain fully ionized across the operational pH range, weak IEX ligands exhibit pH-dependent ionization behavior [21]. This property enables dynamic protonation–deprotonation equilibria that can modulate both ligand charge density and the local microenvironment within the stationary phase, providing an additional degree of control and tunability over analyte–ligand interactions and potentially facilitating finer separation of closely related viral species [21,22]. In this context, DEAE resins have been reported to provide milder binding conditions and more favorable elution profiles, reducing irreversible absorption and minimizing particle loss during purification [22]. Together, these attributes position weak AEX materials as a

promising but underexplored platform for enhancing AAV chromatographic performance while preserving AAV capsid integrity.

In parallel with stationary phase considerations, formulation-like sample matrices pose a major challenge for AEX-based workflows. Buffers designed to preserve AAV stability—characterized by near-neutral pH and moderate to high salt concentrations—often inhibit effective binding to AEX columns due to the multiple physicochemical mechanisms [23,24]. As a result, such samples typically require buffer exchange (BEX) or desalting before analysis, particularly when AAV concentrations are low, salt levels are elevated, or pH is incompatible with AEX chromatographic binding [15,25–27]. These conditions are common in samples from the early stages of downstream processing (DSP) [28]. Conventional off-line BEX approaches are labor-intensive and introduce variability, sample loss, and reduced reproducibility, especially for dilute or fragile virus preparations [26,27,29]. Consequently, these approaches are expected to be poorly suited for robust and reliable analytical workflows.

We used an in-line two-dimensional (2D) buffer exchange (BEX) configuration to facilitate reproducible BEX, thereby optimally preparing the sample for subsequent robust AEX binding, and allowing a controlled, rigorous investigation of the interactions underlying AAV capsid separation. In the context of BEX, the 2D configuration enables robust AEX analysis independent of initial sample formulation. This approach eliminates the need for off-line pretreatment, tolerates higher salt concentrations and different formulation compositions, and preserves native capsid stability while maintaining chromatographic performance.

We systematically investigated the effects of pH, ionic strength, and formulation-relevant conditions, specifically on AAV8 capsid separation, a process that serves as a well-defined and established AAV model system [9,12,14,30–32]. Following the approach of [33], we employed a pre-purified AAV8 as a single-molecule model system. Such simplified systems allow experimental variables to be controlled more precisely, enabling a more direct and systematic investigation of molecular interactions, including the effects of ionic strength and pH on ion-exchange behavior. Moreover, such an approach facilitates the identification of subtle interaction features that are often obscured in more complex samples. The mechanistic insights obtained from such single-molecule experiments are not limited to a specific molecule-stationary phase system but have proven broadly applicable and relevant for understanding and modeling chromatographic processes [33–35].

The study compared different DSP buffers across low- and high-salt environments and acidic, near-neutral, and alkaline pH regimes. Together, these studies provide both mechanistic insight and practical guidance for balancing capsid stability with chromatographic separation efficiency, laying the groundwork for future analytical and preparative AAV workflows operating under stability-preserving conditions.

2. Materials and Methods

2.1. Chemicals and Reagents

All buffers were freshly prepared using European Pharmacopoeia-grade purified water and analytical-grade reagents. Buffer solutions were filtered through 0.2 µm polyethersulfone (PES) membrane filters (Thermo Fisher Scientific, Waltham, MA, USA) prior to use. Magnesium acetate tetrahydrate was purchased from Thermo Fischer Scientific. The following reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA): 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS or Trizma® base), D-sorbitol, propionic acid, 2-(N-morpholino)ethanesulfonic acid (MES) hydrate, potassium phosphate dibasic trihydrate, sodium citrate tribasic dihydrate, cesium chloride, Bis-Tris propane (BTP), and magnesium chloride hexahydrate. Absolute ethanol and anhydrous sodium acetate were

sourced from Supelco (Bellefonte, PA, USA), while sodium chloride, potassium chloride, and sodium hydroxide were obtained from Honeywell (Charlotte, NC, USA). Ammonium acetate and Poloxamer 188 were purchased from Merck Millipore (Darmstadt, Germany). Finally, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulphonic acid (HEPES) was purchased from VWR (Radnor, PA, USA).

2.2. AAV Samples

The AAV8 model sample serves as a well-controlled and informative testbed for demonstrating the validity and robustness of the approach under study. Consequently, the findings reported in this interaction study are expected to generalize across a broad range of AAV serotypes. This interpretation is further supported by the results of the previous studies [9,36], which reported clear separation across multiple AAV serotypes using a conceptually similar AEX approach to those employed here. Therefore, the present work provides a solid and extensible foundation for applying the methodologies described in the current study to a wide range of AAV samples.

Model AAV8 sample was produced and purified by Sartorius BIA Separations (Ajdovščina, Slovenia) according to the protocol described previously [36]. Briefly, recombinant AAV8 vectors were produced by triple transfection of HEK293 cells, followed by cell lysis, clarification, and DNase treatment. The clarified lysate was processed by tangential flow filtration (TFF) prior to downstream processing (DSP). AAV capture was performed on a CIMmultus SO3 1 mL monolithic column (2 µm channel size; Sartorius BIA Separations) on an ÄKTA Avant 150 chromatography system (Cytiva, Marlborough, MA, USA), yielding SO3-purified AAV8 material (model AAV8 sample). Full AAV8 capsids encapsidated an approximately 3.5 kb GFP reporter genome. All samples used in this study were SO3-purified AAV8 capture fractions formulated in buffer containing 20 mM Tris, 150 mM NaCl, 2 mM MgCl₂, and 0.01% (*w/v*) Poloxamer 188 at pH 7.5. Unless otherwise stated, experiments were performed using AAV8 batch 1 (4.00×10^{12} vg/mL). The stability experiment (Section 3.2.3.) was conducted using an independently produced AAV8 preparation (batch 2, 3.19×10^{13} vg/mL), generated using the same production and purification workflow. Minor differences in capsid composition observed between batches are consistent with expected batch-to-batch variability inherent to recombinant AAV production [37]. Importantly, inclusion of an independent AAV8 preparation enabled evaluation of the reproducibility of the observed trends and confirmed that the reported findings are not specific to a single viral lot, thereby supporting the robustness and generalizability of the method.

2.3. Chromatographic Methods

2.3.1. One-Dimensional (1D) Chromatographic Experiments

Monolithic columns (all 0.1 mL) for testing E–F separation: CIMac SO3 (1.3 µm), CIMac DEAE (2 µm), and CIMac QA HR (2 µm) were supplied by Sartorius BIA Separations. All 1D chromatographic experiments were performed using the PATfix analytical system (Sartorius BIA Separations), equipped with a multi-wavelength UV-Vis detector (MWD), fluorescence (FL) detector (FLD), multi-angle light scattering detector (MALS or LS), and in-line conductivity and pH monitoring, similar to previous studies [9,31,36]. Detector settings were applied as described previously [36]. For clarity, fluorescence served as primary detection signal in most figures. Before analysis, samples were diluted with the appropriate mobile phase A (MPA; compositions listed in Table S3). Unless otherwise specified, chromatographic experiments followed a standardized LC operating procedure. Blank (MPA) and sample solutions were prepared simultaneously and stored in the autosampler at 8 °C. Each sequence began with three blank injections to ensure baseline stability, followed by sample injections. Samples remained in the autosampler during the

initial blank runs. The E–F separation methods were performed following previously established protocols [9]. In brief, AAV samples (approx. 5×10^{10} vg) were loaded onto the ion-exchange (IEX) column and, after a 1 min (10 column volumes—CVs) residence time, eluted using a linear gradient from 100% MPA to 100% MPB over 16 min (160 CVs). This was followed by a 1 min (10 CVs) hold at 100% MPB, a high-salt wash step (100% MPC for 2 min; 20 CVs), and subsequent column equilibration (2 min; 20 CVs 100% MPB), and conditioning back to loading conditions (100% MPA for 8 min; 80 CVs). Blank injections (injections of 100% MPA) were performed before sample analysis to ensure stable detector baselines. All 1D IEX E–F separations were conducted at a constant flow rate of 1.0 mL/min.

For the indicative AAV8 stability assessment (Section 3.2.3.), a bulk-diluted AAV8 sample was incubated in AEX loading buffer and repeatedly analyzed by 1D AEX (see Section 2.3.2.) after defined residence times (0, 1, 2, 4, 6, 8, 12, 24, 48 h and additional subsequently prepared 0 h sample to confirm the overall separation trend) while stored in an autosampler at 8 °C. Because immediate injection was not technically feasible, the earliest sampling point (0 h) corresponded to a buffer exposure time of less than 1 min rather than true time 0.

The resolution between E and F AAV particles was calculated using PATfix software version 3.1, which applies a standard chromatographic resolution equation based on the difference in retention times and the corresponding peak widths at half heights of the E and F particle peaks [38].

A 1D SEC method was performed following the procedure from a previous study [31] to demonstrate the principle of the 2D approach. In general, the SEC separates species based on size, with larger particles (such as aggregates and complexes) eluting first, followed by AAV particles, and finally proteins and smaller components [31]. For this purpose, a BIA SEC Methacrylate-2000 Å column (diameter: 8.0 mm, length: 300 mm, bed column volume (CV): 15.1 mL, particle size: 10 µm, pore radius: 200 nm) from Sartorius BIA Separations was used. An isocratic elution using MPA (Table S3E) was applied from 0 to 30 min (~1.59 CVs) at a flow rate of 0.8 mL/min.

2.3.2. Two-Dimensional (2D) Chromatographic Experiments

Two-dimensional chromatographic analyses were performed using the PATfix AAV 2D system (Sartorius BIA Separations), a custom in-line 2D chromatographic platform integrating a size-exclusion chromatography (SEC) first dimension with an anion exchange chromatography (AEX) second dimension. The system was equipped with the same detector array described in Section 2.3.1 and detailed previously [36].

The first-dimension column was a SEC Methacrylate-2000 Å column, while the second-dimension column was a CIMac QA HR 0.1 mL (2 µm) monolith. Samples were prepared by standard dilution with the appropriate buffer (compositions listed in Table S1) and incubated for 2 h at 8 °C in the autosampler prior to analysis. The AEX method was adapted from previous studies, where it demonstrated excellent recovery and robust performance [9,36].

Samples were injected onto the SEC column and eluted isocratically from 0.00 to 10.90 min at a constant flow rate of 0.8 mL/min using the SEC pump. During this phase, the SEC eluate was merged via a switching valve with a 0.2 mL/min flow from the AEX pump and directed onto the CIMac QA HR column.

At 10.90 min, the switching valve was actuated to separate the two flow paths. The SEC column then underwent continuous washing of the excessive salt and conditioning in preparation for the subsequent injection, while the CIMac QA HR column proceeded to E–F separation. At 11.50 min, the AEX flow rate (100% MPA) was increased from 0.2 to 3.0 mL/min and held for 0.5 min, followed by a linear gradient from 100% AEX MPA to

100% AEX MPB over 7 min (210 CVs) at 3.0 mL/min. CIMac QA HR column regeneration, equilibration, and conditioning steps followed.

Throughout the analysis, the SEC (first dimension) flow rate was maintained at 0.8 mL/min. The total runtime for the combined 2D analysis was 30 min.

For the experiments in Section 3.2.3, a dimensionally reduced AEX method was employed, preserving the same gradient characteristics as those used in the 2D in-line BEX methodology.

2.4. Physicochemical Characterization of Monolithic Materials

2.4.1. Zeta Potential Analysis of Monolithic Materials

Zeta potential analysis was performed to provide quantitative insight into the surface charge properties that strongly influence analyte interactions with the stationary phase. Measurements were performed using a SurPASS 3 electrokinetic analyzer (Anton Paar, Graz, Austria) equipped with a cylindrical cell. Approximately 1 g of monolithic material was washed with 10 mL of 96% ethanol for 15 min, after which the solvent was discarded. The monoliths were then dried at 60 °C for 6 h until completely solvent-free and subsequently crushed coarsely using a mortar and pestle.

A mass of 0.25 g of the crushed monolith was fixed in the cylindrical cell using appropriate support disks and filters. The permeability index was adjusted to approximately 100 by using the micrometer screw. A 1 mM KCl solution was used as the background electrolyte, while 0.05 M NaOH and 0.05 M HCl were used for pH adjustment.

Zeta potential measurements were conducted across the relevant pH range for each column material in 0.3 pH unit increments. At each pH value, three rising cycles were performed, followed by three zeta potential measurements. A pressure gradient of 1500–1000 mbar was applied to generate a streaming potential. Instrument control and data evaluation were performed using SurPASS 3 software version 2.10.270.5111 Release (Anton Paar, Graz, Austria).

2.4.2. Attenuated Total Reflectance—Fourier Transform Infrared Spectroscopy (ATR-FTIR) Analysis of Monolithic Materials

ATR-FTIR analysis was performed to characterize the chemical composition and surface functionality of the stationary phase. ATR-FTIR spectra were recorded using a PerkinElmer Spectrum GX FTIR spectrometer (Waltham, MA, USA). Spectra were acquired at room temperature in the wavenumber range of 400–4000 cm^{-1} with a spectral resolution of 4 cm^{-1} , averaging 32 scans per measurement. Background and sample spectra were collected under identical conditions. Before analysis, the spectra were smoothed and baseline corrected. Each sample was measured in triplicate.

3. Results and Discussion

3.1. Separation of AAV8 Capsids at Near-Physiological pH

3.1.1. pH-Dependent AAV8–Ligand Interactions on Strong and Weak AEX

The interplay between mobile-phase pH, capsid surface charge, and stationary phase physicochemical characteristics critically governs AAV8 retention and empty–full (E–F) separation across both strong and weak anion exchange (AEX) modalities. Previous studies using strong AEX monoliths have consistently shown that E–F resolution improves with increasing operating pH, reaching a maximum within a narrow alkaline window, beyond which resolution deteriorates—particularly above pH 9 [9,11]. This behavior reflects the increasing net negative charge of the capsid at elevated pH, combined with the permanently charged nature of strong AEX ligands. However, alkaline conditions are also

well known to compromise AAV capsid stability [11,14], making high-pH operation undesirable for robust analytics and impractical for preparative chromatography.

The objective of this work was to elucidate the mechanistic role of pH in AAV–stationary phase interactions and to identify conditions that preserve capsid stability while maintaining chromatographic performance. Strong AEX materials such as CIMac QA HR exhibit optimal binding capacity and separation efficiency at elevated pH, typically above pH 9 [2–4,6,8,9,11,31,32,39]. This underlies our working hypothesis that enhanced E–F separation requires operation at higher pH. However, near-neutral pH could provide a more favorable balance between AAV8 E–F resolution and capsid integrity.

As an initial step, we extended and contextualized previously reported observations of enhanced E–F separation on CIMac QA HR at alkaline pH [9]. In that study, raising the loading buffer pH from 7.50 to 9.25 enhanced AAV8 retention and improved E–F resolution, whereas lowering the pH below 8.00 or raising it above 9.00 diminished separation. These findings confirm the existence of an optimal pH window rather than monotonic improvement with alkalinity and underscore the strong dependence of CIMac QA HR performance on capsid charge modulation.

Although strong AEX materials are generally favored for AAV separations, we also considered weak AEX systems to explore alternative interaction mechanisms. To date, near-baseline AAV E–F separation has not been reported with strictly weak AEX ligands such as DEAE. Partial separation has been achieved using multimodal stationary phases that combine AEX with hydrogen-bonding functionality, such as CIMac PrimaS [14,40]. To isolate the fundamental mechanisms governing AAV retention, we aimed to evaluate AEX interactions in their simplest form, deliberately excluding columns officially designated as multimodal and mixed-mode. Nevertheless, minor secondary interactions may still occur in single-mode AEX materials, as previously suggested [20]. In our initial experiment, informed by prior work reported in a patent application [41], DEAE appeared poorly suited for E–F separation at pH 9.0, where weak AAV8 retention and early elution were observed. This behavior is consistent with the known ionization properties of DEAE, which begins to lose charge above pH ~9.5 [16,40].

Despite these unfavorable expectations, we systematically evaluated DEAE across a pH range from 8.5 to 6.5, excluding strongly alkaline conditions where ligand ionization is diminished (Figure 1). At pH 8.5, the AAV8 sample eluted predominantly in the flow-through. As pH decreased, retention increased, and E–F resolution progressively declined, from $R = 2.18$ at pH 8.0 (achieving near-baseline separation) to $R = 0.48$ at pH 6.5. At pH 6.0, most of the sample eluted only during the high-salt wash, indicating substantially stronger binding. Although the decrease in resolution with decreasing pH mirrors trends observed for CIMac QA HR [9], the increase in binding capacity on DEAE at lower pH was unexpected and contradicts earlier reports suggesting weak retention on weak AEX materials [11,14].

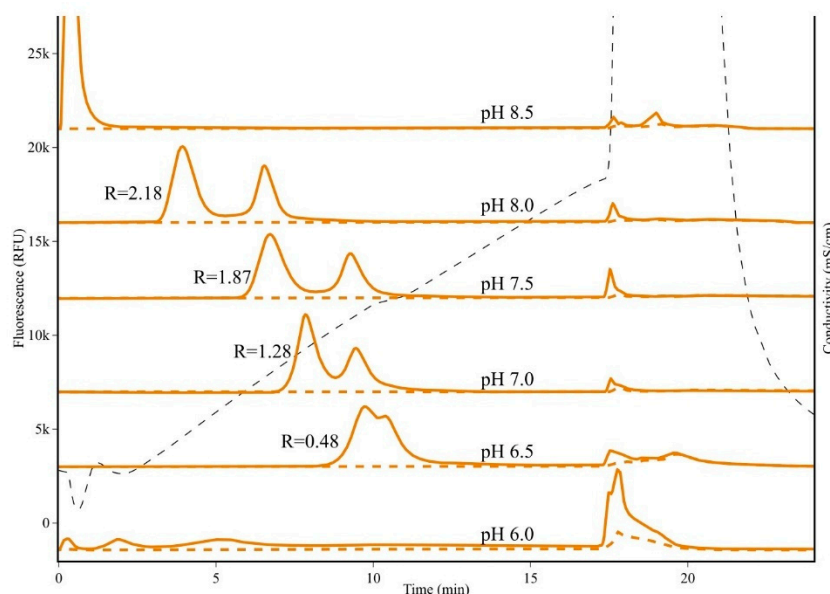


Figure 1. Effect of operating pH (6.5–8.5) on AAV empty–full (E–F) capsid resolution (R) on a CIMac DEAE monolithic column. The chromatogram at pH 8.5 was intentionally cut off for clarity, as most of the sample did not bind and eluted at the beginning of the gradient, resulting in a large early peak. Orange dotted lines indicate the blank run (mobile phase A injection), and the black dotted line represents the conductivity trace.

3.1.2. Role of Strong and Weak AEX Ligand Chemistry and Mixed Interaction Modes

To rationalize previously unexpected behavior, it is necessary to consider the distinct electrochemical and physicochemical properties of the QA and the DEAE ligands (Figures 2, 3 and 4).

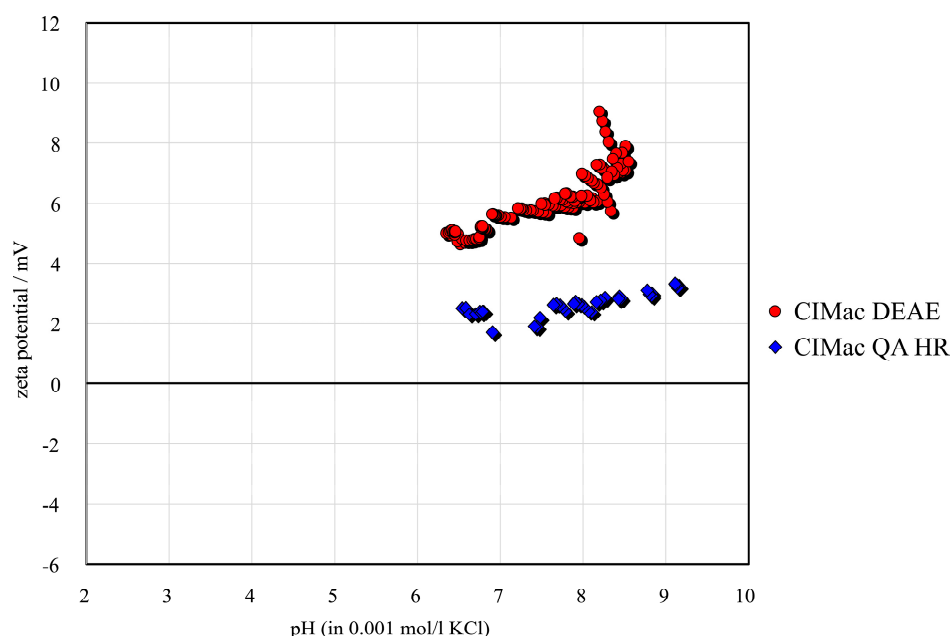


Figure 2. pH dependence of the zeta potential of the diethylaminoethyl (DEAE)–CIMac DEAE, and quaternary amine (QA) monolithic stationary phase–CIMac QA HR. The zero zeta potential is shown as a solid black line.

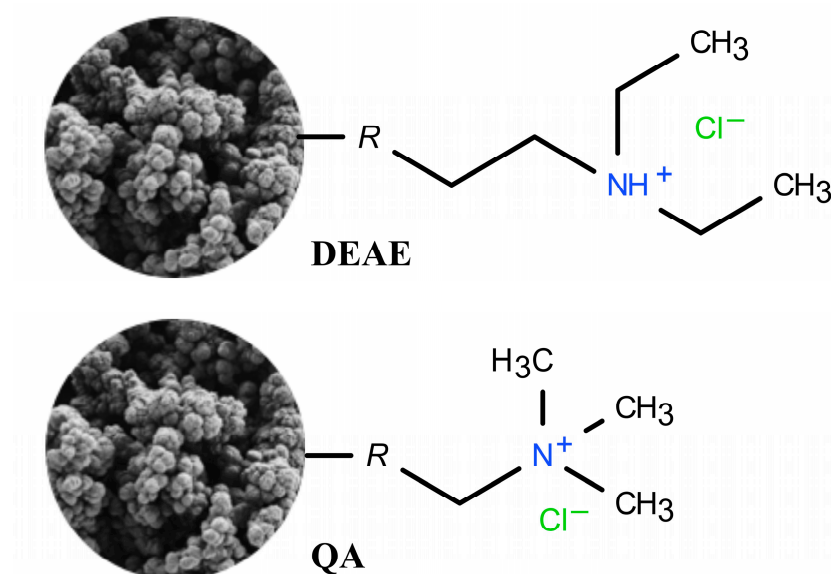


Figure 3. General chemical structures of diethylaminoethyl (DEAE) and quaternary amine (QA) ligands [42], highlighting differences in substituents relevant to ionization behavior and interaction mechanisms. This figure highlights especially the general structural differences in the ion-exchange functional group (charged moiety); other substituents may vary between different manufacturers. R represents a generic organic group or side chain.

Zeta potential measurements (Figure 2) revealed that both QA and DEAE AEX monoliths exhibited a positive surface charge across the investigated pH range (≈ 6.5 – 8.5), with no isoelectric point detected. The absence of an isoelectric point within this range is consistent with the presence of strongly protonated AEX groups, which sustain a positive surface charge under near neutral to mildly alkaline conditions. The DEAE monolith showed systematically higher zeta potential values and a stronger pH-dependent behavior compared to the QA monolith. Conversely, the QA monolith displayed lower but more stable positive zeta potential values, suggesting a more uniform surface charge distribution. These observations align with previous reports indicating that the QA monolith functions as a strong AEX, featuring permanently charged quaternary ammonium groups and a relatively constant positive zeta potential across the operational pH range [40]. In contrast, DEAE ligand functions as a weak AEX whose tertiary amine exhibits pH-dependent ionization, consistent with a previous study [21]. Although zeta potential alone did not fully explain the retention behavior, it highlighted the fundamentally different interaction landscapes between the two ligands.

At the molecular level (Figure 3), DEAE contains two ethyl substituents, resulting in a less hydrophilic surface than the three methyl groups of QA. Moreover, the tertiary amine of DEAE retains a lone electron pair and can act as a weak hydrogen-bond acceptor, whereas the quaternary ammonium of QA lacks a lone pair and therefore cannot engage in classical hydrogen bonding. Instead, its positively charged $N-C-H$ groups interact with anions and organic molecules primarily via ion-dipole interactions and may form weak, directional $N-C-H$ hydrogen bonds [43].

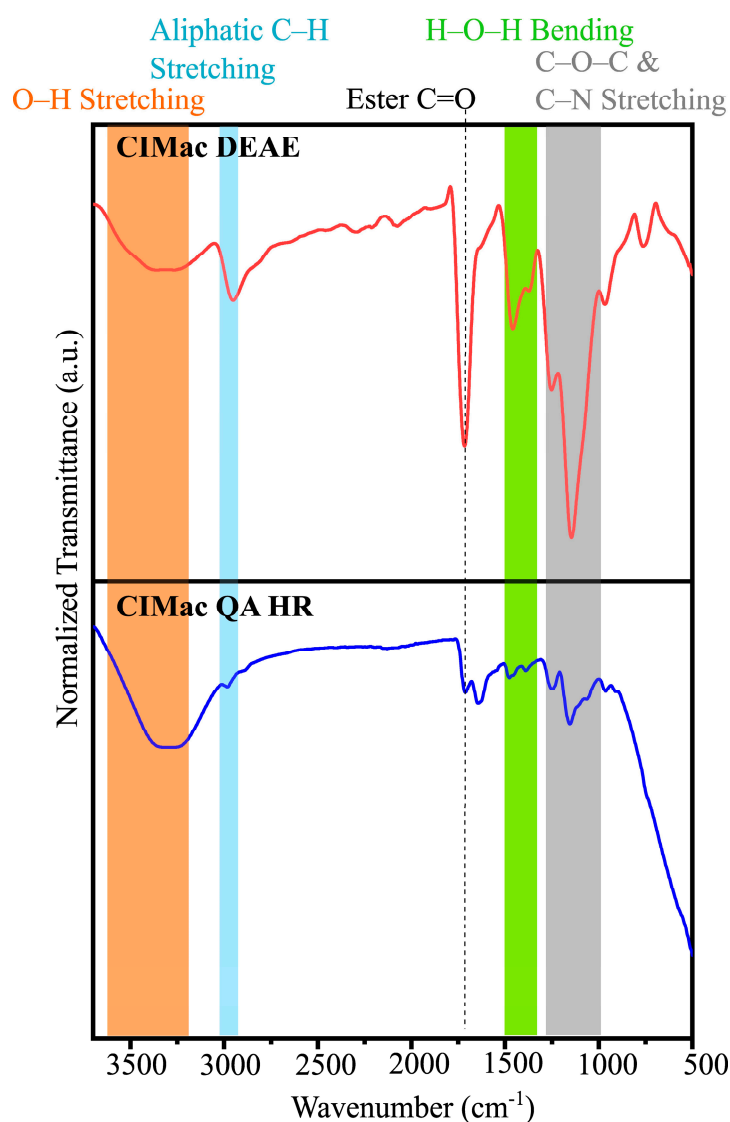


Figure 4. ATR-FTIR spectra of CIMac QA HR (blue) and CIMac DEAE (red) monoliths. The characteristic ATR-FTIR wavenumber regions are highlighted and indicated in the legend at the top of the figure. The dotted vertical line indicates the ester C=O stretching band.

The electrokinetic results (Figure 2) are fully supported by ATR-FTIR spectroscopy (Figure 4). Both monoliths displayed spectral features characteristic of the common inorganic monolithic backbone (bands in the 1200–900 cm^{-1} region), confirming that functionalization does not alter the underlying framework. The CIMac QA HR spectrum was dominated by features related to surface hydration and framework vibrations, consistent with its lower but more stable electrokinetic response (Figure 2). Specifically, the QA monolith exhibited pronounced O–H stretching (3600–3200 cm^{-1}) and H–O–H bending ($\sim 1650 \text{ cm}^{-1}$) bands, indicative of a highly hydrated surface. In contrast, CIMac DEAE exhibited distinct spectroscopic signatures associated with tertiary amine groups, most notably the C–N stretching band around $\sim 1140 \text{ cm}^{-1}$, providing direct evidence of surface chemical functionalization. Additionally, differences observed in the 1700–1600 cm^{-1} region suggested altered/reduced surface hydration behavior for DEAE, which correlates well with its higher zeta potential and enhanced pH sensitivity (Figure 2). Overall, these ATR-FTIR spectral changes indicate altered surface chemistry and reduced surface hydration for DEAE compared to the QA monolith, while the underlying monolithic structure remains unchanged.

Beyond electrostatics, hydrophobic interactions contribute to protein retention on weak AEX columns. A recent systematic study demonstrated that multiple weak AEX materials achieved higher resolution in hydrophobic interaction chromatography (HIC) mode than in classical AEX mode [44], indicating that hydrophobic contributions cannot be fully eliminated from the weak AEX systems. Proteins together with viral capsids present a heterogeneous/complex surface comprising polar and nonpolar, as well as positively and negatively charged residues. By modulating environmental parameters such as pH and salt concentration, different interaction modes (electrostatic, hydrogen bonding, or hydrophobic) can be selectively emphasized. Although multiple interactions occur simultaneously, one interaction type typically dominates under a given set of conditions [44].

At lower pH values, as the AAV capsid approaches its isoelectric point, net electrostatic interactions weaken, while non-electrostatic interactions, such as hydrogen bonding and hydrophobic interactions, become more pronounced relative to those under alkaline conditions—an effect also suggested previously [44]. These mixed interaction modes likely contribute to the unexpectedly stronger retention observed on DEAE at near-neutral and mildly acidic pH.

3.1.3. Comparison of Weak and Strong AEX Performance

Returning to electrostatic contributions, previous work suggested that strong AEX ligands may reduce AAV capsid stability due to stronger binding interactions [11]. However, it is important to clarify that the term “strong” in strong AEX refers to ligand ionization behavior rather than interaction strength with analytes [45]. Strong AEX materials remain fully ionized across a wide pH range and therefore provide consistent charge density, which does not necessarily imply stronger binding [40].

Notably, the chromatograms reported in [9], together with Figure 5 of the present study, show that AAV8 elutes from CIMac QA HR within the first third of the relatively gentle salt gradient, corresponding to comparatively low conductivity. This behavior indicates weak overall interactions between AAV8 and the QA ligand, suggesting that the interpretation proposed in [11] linking strong AEX binding to reduced AAV stability should be treated with caution and evaluated in the broader context of ligand chemistry and operating conditions.

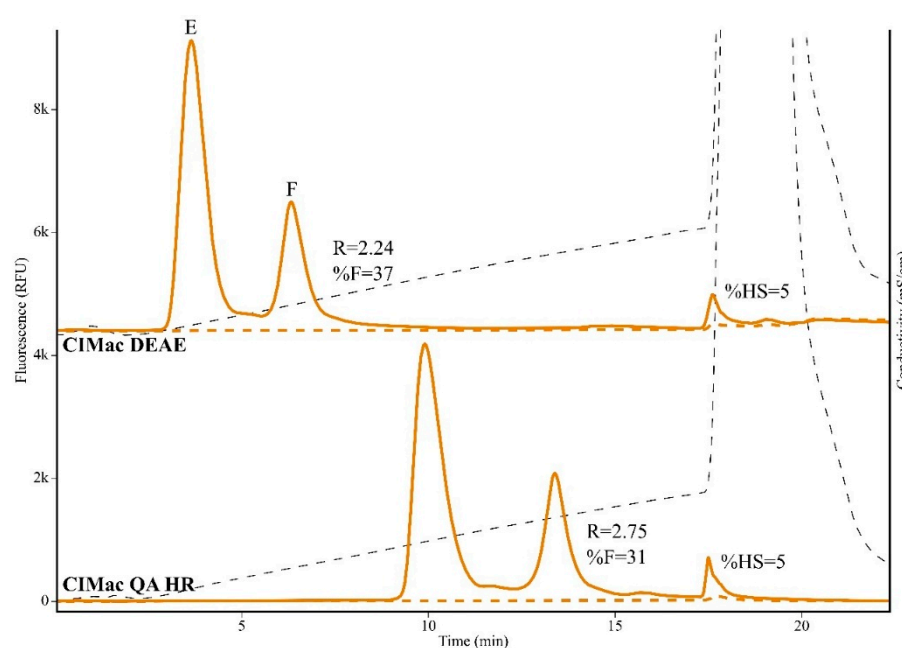


Figure 5. Comparison of currently optimized empty–full (E–F) capsid separation on weak AEX (DEAE) and strong AEX (QA HR) monolithic columns under their respective optimal pH conditions [9]. The percentage of full AAV particles (%F) was calculated as the ratio of the integrated fluorescence signal area of the full (F) fraction to the total AAV fluorescence area, comprising the empty (E), full (F), and high-salt wash (HS) fractions, the latter containing strongly retained AAV species. The black dotted lines represent the conductivity traces; the conductivity of the high salt wash at the end of the method was intentionally truncated. Since the gradients were zoomed to highlight capsid elution, the resulting large conductivity peaks were cut off to preserve figure readability. Orange dotted lines indicate the blank run (mobile phase A injection).

To directly compare column performances, we evaluated DEAE under its current optimal conditions (pH 8.0; Figures 1 and 5) and compared it with QA HR operated at its reported optimal pH of 8.5 [9] (Figure 5). Under these conditions, both columns achieved near-baseline E–F separation, with the CIMac QA HR providing higher E–F resolution. However, the optimized QA HR conditions are not directly transferable to DEAE due to substantial differences in ligand chemistry and interaction modes, as also previously noted [21].

Despite this, the enhanced E–F separation on DEAE—approximately 20% lower than that achieved on QA HR—was obtained by directly applying the optimal QA HR separation conditions and adjusting the operating pH. Importantly, DEAE enabled effective AAV8 binding and separation under near-neutral pH conditions, which is advantageous for stability-preserving workflows. Additional minor adjustments to the salt concentration, the acid type used for pH titration, and the gradient shape resulted in only marginal improvements (Figure S1). Moreover, attempts to further enhance E–F separation on DEAE using pH-gradient elution alone (Figure S2), and on the strong cation-exchanger (CEX) SO3 using a salt gradient (Figure S4), were ineffective (see Sections 2.1 and 2.2 in the Supplementary Material).

Overall, these results confirm that an alkaline pH remains necessary for enhanced E–F separation on ion exchangers, although DEAE enables satisfactory AAV8 E–F operation closer to formulation-relevant pH conditions. However, separation would not be achievable for samples whose pH does not match the pH required for binding to DEAE or AEX. Importantly, E–F separation under near-physiological salt conditions is expected to be unattainable on AEX due to reduced binding capacity, which most likely results in elution of the AAV in the flow-through [28]. Altogether, elution on both QA HR and DEAE is highly sensitive to both pH and buffer salt concentration and therefore occurs at relatively low salt concentrations. Consequently, although DEAE would be expected to provide improved E–F separation compared to QA HR, it would still be highly unlikely to enable separation of E and F in samples with formulation-like salt concentrations, i.e., at ionic strengths exceeding those compatible with column binding and elution. At such elevated ionic strength, increased concentrations of mobile-phase counter ions competitively shield electrostatic interactions between the analytes (AAV) and the charged stationary phase, reducing binding affinity and narrowing the functional adsorption population, thereby diminishing resolution of closely related species such as E and F under standard gradient conditions [33].

3.2. Separation of AAV8 Capsids at Near-Physiological Ionic Strength

3.2.1. Impact of Ionic Strength and Buffer History on AEX Separation

To further understand how formulation-like environments influence AAV capsid behavior and chromatographic separation, we investigated the effect of ionic strength on E–F separation. Since CIMac QA HR provided the most effective separation (Figure 5), sub-

sequent experiments were conducted on this column, while acknowledging the potential of CIMac DEAE for preparative applications at near-neutral pH.

As discussed above, AAV binding to AEX is generally not feasible, or at least not robust, under near physiological conditions, or at salt concentrations or pH values exceeding those required for AAV elution from the column. Therefore, such samples require prior buffer exchange (BEX), a step that imposes several limitations that compromise their suitability for routine and reliable analytical workflows.

To overcome these limitations, we implemented a specialized in-line BEX strategy based on size-exclusion chromatography (SEC), building on the off-line concept reported previously [27]. In the AAV field, approaches combining in-line SEC with subsequent AAV characterization are extremely rare. To our knowledge, the only reported example integrated a non-chromatographic second dimension via mass spectrometry [46]. However, this method may present several limitations, including the requirement for specialized technical expertise, reduced sensitivity, and lower resolution compared with conventional, well-established LC techniques. Furthermore, multi-detector chromatographic configurations measuring physically different principles in parallel enhance the analytical informativeness and enable deeper insight into AAV processes [9,31,36]. In our work, we integrated SEC into the existing two-dimensional (2D) AAV chromatographic system, originally developed for upstream process monitoring [36]. By replacing the first-dimension CIM[®] Trap SO3 (CEX) monolithic column with a non-monolithic non-IEC SEC column, we established a new application of the system, enabling in-line BEX analysis. Moreover, we substantially reduced the analytical loop volume (200 μ L instead of 10,000 μ L) to minimize sample dispersion and carryover. In-line dilution was deliberately omitted because the SEC mobile phase matched the AEX loading buffer, thereby ensuring direct compatibility between dimensions. The SEC-based in-line BEX enabled rapid equilibration of both pH and conductivity, while excess salt eluted separately from the AAV-containing fraction. This fraction (Figure 6A) was then seamlessly transferred to the second dimension AEX E–F capsid separation analysis, performed on the CIMac QA HR column (Figure 6B).

Using this configuration, model AAV8 samples diluted in a range of commonly used in-house downstream process (DSP) buffers (Table S1) were incubated for 2h, equilibrated during the first dimension (SEC), and automatically transferred to the CIMac QA HR column for E–F capsid separation (Figure 7).

Resulting chromatographic performance metrics are summarized in Table 1.

Table 1. Influence of sample dilution buffer composition on empty–full (E–F) resolution (R_s) and conductivity (cond) at elution of empty (σ E) and full (σ F) AAV8 capsids. Annotations: HS + CIP—high-salt wash + cleaning-in-place elution fraction.

Sample	Resolution E–F	Elution cond E [mS/cm]	Elution cond F [mS/cm]	%E	%F	%HS + CIP
Formulation Buffer	1.96	3.52	4.33	70.17	27.67	2.16
SEC Buffer	1.95	3.51	4.32	70.14	27.64	2.22
QA HR Buffer (pH 8.5)	2.21	3.47	4.34	70.75	27.36	1.89
QA HR Buffer (pH 9.5)	2.21	3.45	4.29	70.5	27.38	2.12
SO3 Buffer	1.79	3.61	4.39	71.18	27.16	1.66
DGUC Buffer	1.19	3.53	4.08	68.19	29.24	2.57
% RSD	20.04	1.59	2.53	1.48	2.73	14.68
% RSD (-DGUC)	9.03	1.76	0.84	0.61	0.78	11.55

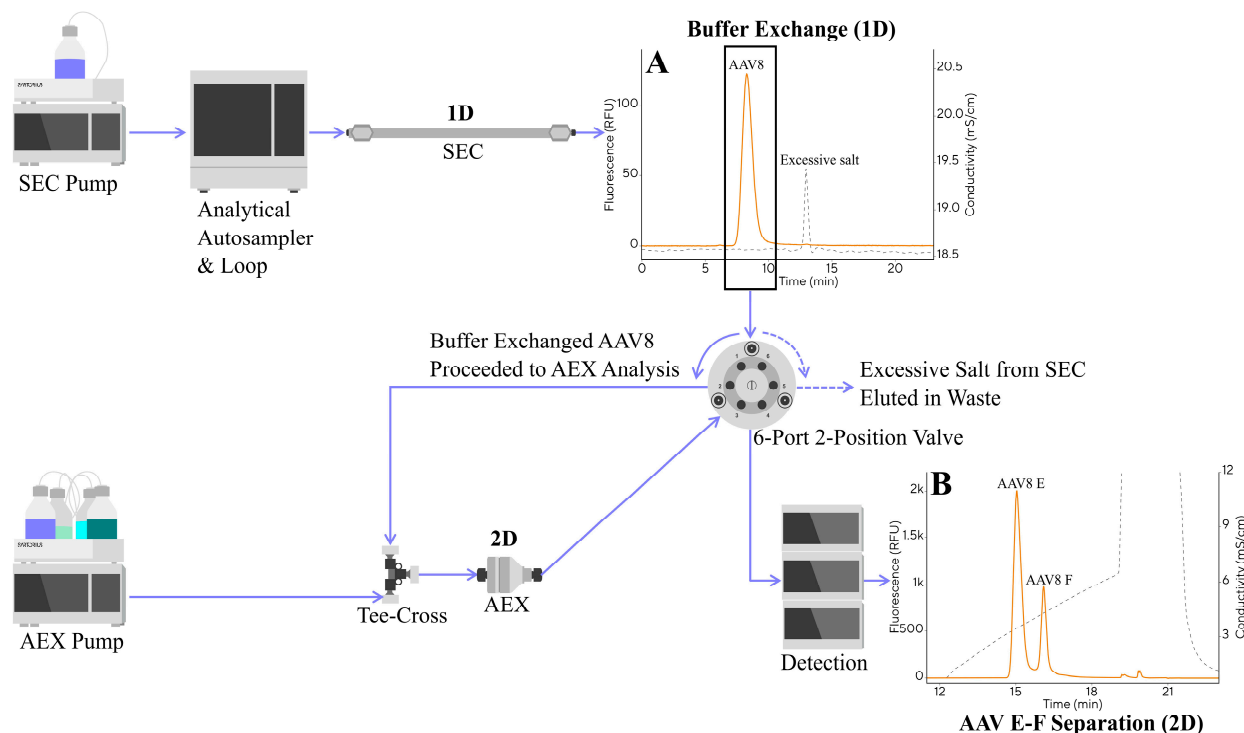


Figure 6. Schematic of the two-dimensional (2D) in-line buffer exchange (BEX) system: (A) SEC first dimension for buffer exchange; (B) AEX second dimension for AAV capsid characterization. The dotted purple line indicates a secondary flow path used to divert excess salt to waste. The black dotted lines in chromatograms represent the conductivity trace. The detailed configuration principle was adapted from a previous study [36].

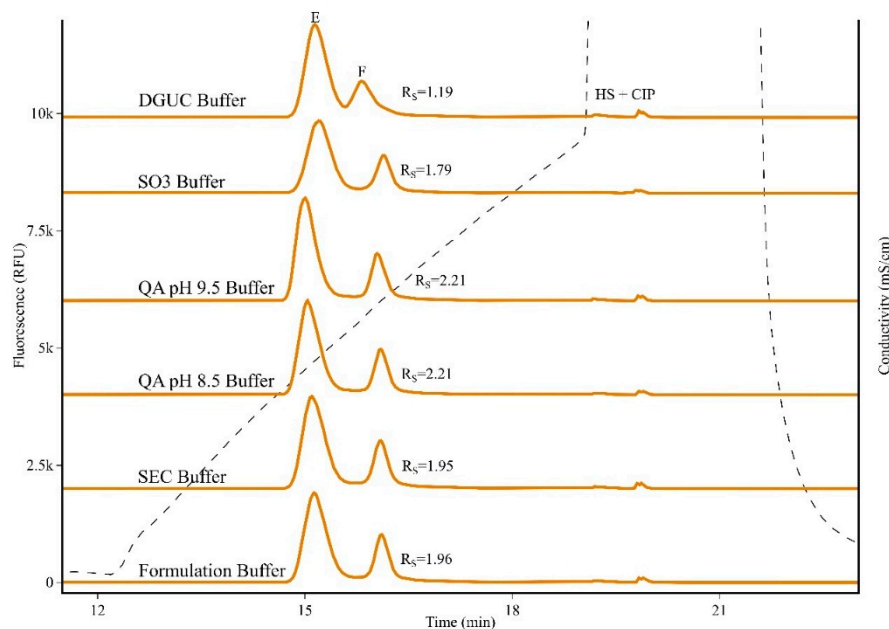


Figure 7. Effect of sample dilution buffer composition on chromatographic behavior and separation performance of AAV8 capsids. Annotations: orange—tryptophan fluorescence; black dashed—conductivity; R_s —resolution between empty and full AAV8 capsids. Annotations: HS + CIP—high-salt wash + cleaning-in-place elution fraction. The black dotted line represents the conductivity trace. Blank runs (mobile phase A injections) were subtracted from these chromatograms to improve readability of the figure.

Across all tested DSP buffer conditions, the elution conductivity of both E and F was highly reproducible (RSD < 3%) and below 2% when excluding the DGUC condition, whereas resolution exhibited substantially greater variability (RSD ~20%, or ~9% excluding DGUC), indicating that buffer composition exerts a stronger influence on separation efficiency than on retention behavior.

The highest resolution ($R = 2.21$) was achieved under alkaline, low-conductivity conditions using CIMac QA HR buffers at pH 8.5 and 9.5. Near-neutral formulation-like buffers containing 150–200 mM NaCl resulted in only a modest (~8%) decrease in resolution, demonstrating that effective separation can still be achieved under conditions relevant to product formulation. In contrast, the SO3 buffer caused a more pronounced decrease in resolution (~16%), while exposure to very high ionic strength in the DGUC buffer (3 M CsCl) led to a substantial reduction of approximately 44%, consistent with the destabilizing and interaction-screening effects of excessive salt concentrations on protein structure [44,47]. This behavior can be attributed to salting-out effects, which alter protein solvation and disrupt protein–stationary phase interactions, promoting the transfer of the protein into the mobile phase. Collectively, these phenomena can decrease retention and broaden elution profiles [23,24]. In addition, high salt concentrations compete with the analyte for binding sites, shielding electrostatic interactions, reducing separation selectivity, and, at sufficiently high ionic strengths, approaching or exceeding the column's binding capacity [23].

Importantly, the relatively small difference in resolution between alkaline and near-neutral buffers indicates that pH alone does not dominate separation performance within this range. Instead, the most significant differences were associated with ionic strength. This conclusion is further supported by a comparison of the SEC and DGUC buffers, which have similar pH values but markedly different salt concentrations, yet exhibit markedly different resolutions. Likewise, among low-conductivity buffers (SO3 and QA HR buffers), samples incubated in the SO3 buffer exhibited only a moderate (~16%) reduction in resolution relative to both QA buffers. The 2D configuration, therefore, enabled robust, repeatable analysis of a model AAV8 sample, achieving comparable E–F separation across buffers varying widely, especially in pH and salt concentration, highlighting its versatility for formulation-relevant conditions.

Overall, these results demonstrate that ionic strength is the dominant factor governing E–F resolution under near-physiological conditions, whereas moderate variations in pH within the tested range have a comparatively smaller impact. This finding underscores the importance of controlling salt concentration when designing AEX-based analytical methods that aim to balance chromatographic performance with capsid stability.

Finally, the SEC-based in-line BEX approach substantially broadens the range of formulation-relevant matrices compatible with AEX analysis while maintaining robustness and throughput and reducing reliance on complex off-line BEX processes. Extreme conditions, such as DGUC buffers, remain problematic; however, most formulation-relevant environments yielded separation performance comparable to conventional AEX loading conditions. While minor parameter adjustments—for example, a reduced SEC flow rate—could further improve matrix-independent alignment, the current conditions already provide robust and practically relevant performance.

3.2.2. Mechanistic Interpretation and Stability Considerations

Buffer-dependent differences observed in AAV8 empty–full (E–F) separation are primarily driven by ionic strength-mediated modulation of electrostatic and hydrophobic interactions. Moderate salt concentrations partially shield capsid surface charges and stabilize AAV particles [17], whereas very high salt concentrations reduce hydration of hydrophobic regions on proteins (like AAVs), promoting hydrophobic interactions, aggreg-

gation, precipitation, and diminishing chromatographic resolution. In other words, proteins are displaced from the solvent shell by water molecules, which remain strongly attracted to ions [44,47]. Conversely, low-salt conditions reduce ionic shielding, making the capsid surface more exposed to solvent and thus increasing the prominence of electrostatic interactions with the AEX stationary phase.

Importantly, separation differences persisted despite rapid in-line buffer exchange (~15 min), indicating that incomplete re-equilibration of the capsid microenvironment influences subsequent AEX behavior. Samples incubated in different buffers retained distinct separation profiles after SEC-based in-line BEX, consistent with slow equilibration of ionic microenvironments surrounding the capsid. Such residual ionic environments likely modulate capsid–ligand interactions, analogous to ion-mediated surface effects reported for lipid nanoparticle (LNP) systems [48]. According to this study, at elevated salt concentrations, an “ionic layer” may be formed around the LNP (or AAV capsid in our case), effectively coating its surface and altering interactions with the stationary phase.

However, this process is suggested to be a slow interplay between hydrophobic and electrostatic interactions. In our case, when a model AAV8 sample is diluted with a low-ionic strength buffers (e.g., QA buffers), the final salt concentration decreases. The ions surrounding the capsid may become sparser, reducing ionic shielding of charged residues on the capsid surface. As a result, the electrostatic interactions may become more dominant. In contrast, diluting the same AAV8 sample with a buffer containing additional salt (e.g., DGUC buffer) increases the final salt concentration around the capsid. Under these conditions, an ionic layer can form around the capsid, enhancing hydrophobic contributions that may compromise resolution on AEX. Equilibration during subsequent in-line BEX was therefore insufficient to fully homogenize ionic environments and interaction states.

Encouragingly, no major capsid degradation or debris formation (observed in the form of a distinct peak eluting between 19 and 21 min in Figure 7, see %HS + CIP in Table 1 or the lack of additional peaks during demonstrational SEC analysis in Figure 6A) was observed under any tested DSP buffer condition, in contrast to previous reports of degradation under alkaline, low-conductivity conditions [14].

Turning to the stability consideration of AAV8 under alkaline pH conditions—an area that remains insufficiently investigated—previous studies have reported serotype-dependent sensitivity in alkaline conditions, with AAV8 generally exhibiting greater resilience in capsid integrity and infectivity compared with AAV2 [49]. Conversely, acidic conditions have been shown to reduce AAV8 transduction efficiency, with the most pronounced decrease occurring between pH 7.4 and 6.4, and a dramatic loss of capsid stability observed at pH 3.0 [50,51]. These observations are consistent with general formulation principles, whereby aggregation and solubility loss are minimized by avoiding pH conditions near the isoelectric point, estimated to be approximately 5.9 for AAV capsids [51,52]. Together, these findings emphasize the importance of maintaining pH within a physiologically relevant range to preserve AAV8 structural integrity and functionality.

Ionic strength represents an additional critical determinant of AAV8 stability. Moderate salt concentrations stabilize AAV8 capsids by screening surface charges and reducing electrostatically driven aggregation. Specifically, increasing NaCl concentrations up to approximately 0.3 M has been shown to mitigate aggregation relative to low-salt conditions, whereas at low ionic strength (e.g., 0.05 M NaCl), electrostatic interactions between capsids may promote aggregation [53]. Although the effects of very high ionic strength on AAV8 remain largely uncharacterized, established protein chemistry principles suggest that excessive salt concentrations may reduce solubility and promote precipitation (‘salting out’) of AAV8, as observed for other macromolecular assemblies.

In the present study, pH and ionic strength screening indicated that AAV8 maintains high apparent structural stability across the tested conditions, as evidenced by the absence of additional later-eluting species in 2D chromatographic analyses. Nonetheless, subtle effects on biological activity, such as transduction efficiency—particularly relevant at preparative scales—cannot be excluded and were beyond the scope of this work.

3.2.3. Indicative Stability Assessment of AAV8 Under Alkaline pH and Low-Conductivity Conditions

To further evaluate AAV8 capsid stability under alkaline and low-conductivity conditions—commonly employed to enhance E–F resolution in AEX—the AAV8 samples were incubated in AEX loading buffer at pH 8.5 and analyzed at defined time points using the 1D AEX method. In this study, we focused on assessing AAV8 stability using a liquid chromatography approach similar to a previous study [11], while other complementary techniques—such as dynamic light scattering (DLS), mass photometry, ultraviolet/optical density (UV/OD), variable temperature charge detection mass spectrometry (VT-CD-MS) and cell-based assays—can also be applied to evaluate particle integrity, thermal stability, or functional potency [54,55].

Our targeted analytical approach enabled systematic monitoring of potential capsid degradation or disassembly through peak morphology, retention behavior, and E–F resolution, consistent with a previously reported AEX-based AAV stability study approach [11]. Despite prior reports suggesting that alkaline, low-conductivity buffers may compromise AAV8 stability [14], chromatographic profiles remained largely consistent throughout the investigated time course, with clearly distinguishable and chromatographically well-resolved empty (E), full (F), and strongly retained (HS + CIP) fractions, with no evidence of aggregation, fragmentation, peak broadening, or fronting (Figure 8).

Detailed evaluation showed that across the evaluated time points, AEX chromatographic performance remained robust (confirmed also by 0 h duplicate injections), maintaining a resolution of >2.2 from 1 to 48 h (%RSD = 4.65; Table 2A). E–F resolution (R_s) exhibited a non-monotonic dependence on residence time, with the most pronounced increase occurring within the first hour (Figure 8, Table 2A), indicating that short-term exposure plays a critical role in modulating chromatographic behavior. The t_0 and t_0'' samples, injected immediately (practically within less than one minute) after dilution, highlight how immediate processing captures this effect. This behavior is consistent with the mechanism proposed earlier in this study for in-line BEX, whereby the slow equilibration of the AAV capsid microenvironment, such as surface charges or interaction interplay, requires some time to fully stabilize, particularly given the complexity of the AAV samples. Notably, the elution conductivity of F AAV8 capsids remained largely constant (%RSD = 0.26), whereas empty capsids exhibited a progressive retention shift (%RSD = 1.60), particularly during the initial incubation period of 1 h (Table 2A). Quantitative analysis also showed that the relative proportion of F capsids remained stable over time (%RSD = 1.68), while the apparent proportion of E capsid fraction increased, accompanied by a concomitant decrease in the HS + CIP fraction. Importantly, absolute fluorescence losses were observed in the HS + CIP fraction and, to a lesser extent, the F capsids (Table 2B), whereas the E capsids remained comparatively stable, exhibiting a %RSD as low as 1.66. These findings indicate that E AAV8 capsids are structurally more stable under alkaline, low-conductivity conditions, while genome-containing populations—particularly the heterogeneously charged HS + CIP fraction—exhibit increased metastability. Previous studies have shown that later-eluting fractions comprise approximately equal proportions of empty and full capsids but display pronounced surface charge heterogeneity and may include metastable or structurally strained species or aggregates [9,36]. Prolonged exposure to AEX loading buffer conditions may therefore promote partial capsid disassembly,

possible particle sedimentation, protein dissociation, or fluorescence quenching due to structural relaxation [56,57]. Another possible explanation for the observed signal loss is that later-eluting, highly charged capsids may experience increased nonspecific adsorption to sample-contact surfaces. Nevertheless, the original method, as described in previous studies [9,36,58] and adapted here, remains well-established for its strong recovery and robustness.

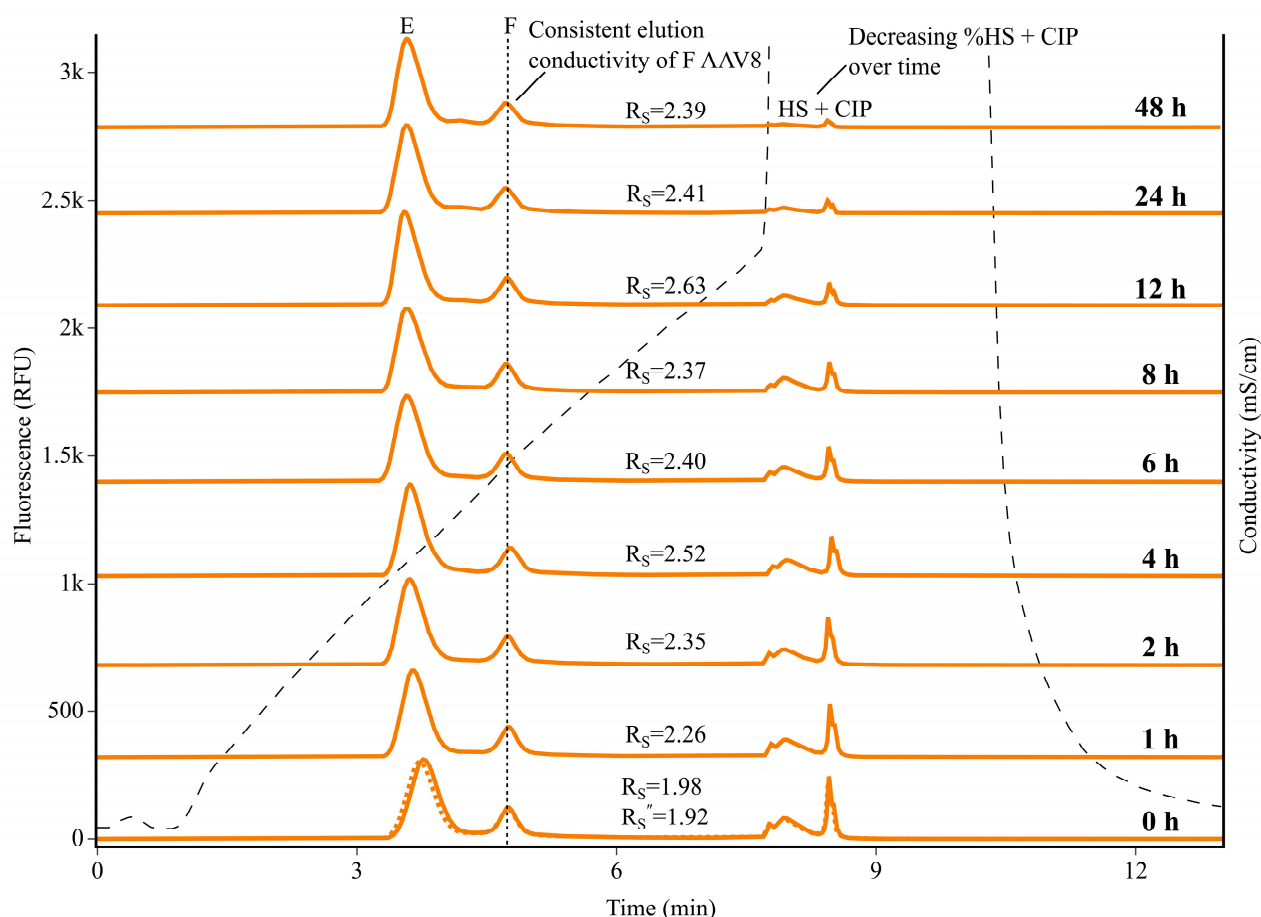


Figure 8. Evaluation of AAV8 capsid stability during incubation under alkaline and low-conductivity AEX loading conditions. Representative AEX chromatograms of AAV8 samples incubated in QA loading buffer for up to 24 h, showing empty (E), full (F), and more strongly retained species eluting during high-salt and CIP steps (HS + CIP). Annotation: The initial time point (0 h) was injected at the start of the stability experiment (solid line) and again at the end of the experiment using a freshly prepared aliquot of the AAV8 sample (dotted line, and its resolution noted as R_s''). This served as an internal control to assess the reproducibility and consistency of the observed stability trend. The dotted black lines represent the conductivity trace. Blank runs (mobile phase A injections) were subtracted from these chromatograms to improve readability of the figure.

Our stability observations are therefore consistent with prior reports showing higher stability of empty AAV particles compared to genome-containing AAV capsids, likely arising from internal pressure or assembly-related strain [54,59]. Consequently, the observed increase in the relative proportion of empty capsids reflects preferential instability of HS + CIP, and, to a lesser extent, full populations, rather than actual enrichment of empty particles. Considering the absolute fluorescence areas of empty capsids, these remain stable, indicating that the capsid shell without genomic load is structurally robust, whereas genome-containing populations—particularly the structurally metastable HS + CIP fractions—are susceptible to alkaline and low-conductivity buffers.

Table 2. Time-dependent chromatographic parameters for AAV8 capsids incubated under alkaline and low-conductivity AEX loading conditions. **(A)** Summary of E–F resolution, elution conductivity (cond) of empty and full capsids, capsid composition (%E, %F), and proportion of later-eluting species (%HS + CIP) as a function of incubation time. **(B)** Summary of tryptophan fluorescence areas for each separated peak and the proportion of gain/reduction over time relative to the initial state (t0). Annotations: t0''—0 h time point for replicate sample injection.

(A)								
Sample	Resolution E–F	Elution cond E [mS/cm]	Elution cond F [mS/cm]	%E	%F	%HS + CIP		
t0	1.98	3.72	4.59	54.52	22.97	22.51		
t1	2.26	3.64	4.57	56.94	22.13	20.93		
t2	2.35	3.64	4.60	58.02	22.09	19.89		
t4	2.52	3.64	4.61	59.40	22.05	18.55		
t6	2.40	3.61	4.60	60.94	22.17	16.89		
t8	2.37	3.58	4.60	62.34	21.93	15.73		
t12	2.63	3.60	4.60	65.37	22.15	12.49		
t24	2.41	3.59	4.59	70.09	22.21	7.70		
t48	2.39	3.58	4.59	72.94	22.82	4.23		
t0''	1.92	3.75	4.61	54.51	22.78	22.71		
%RSD	9.50	1.60	0.26	10.22	1.68	38.79		
%RSD (-t0 and -t0'')	4.65	0.74	0.26	9.13	1.20	41.05		
(B)								
Sample	Area FLD E [mVs]	Area FLD F [mVs]	Area FLD HS + CIP [mVs]	Area FLD Total [mVs]	FLD Gain E [%]	FLD Reduction F [%]	FLD Reduction HS + CIP [%]	FLD Reduction Total [%]
t0	7.399,28	3.085,31	3.030,11	13.514,69	/	/	/	/
t1	7.725,33	3.002,63	2.840,36	13.568,32	+4	–3	–6	0
t2	7.685,64	2.926,02	2.635,18	13.246,83	+4	–5	–13	–2
t4	7.728,38	2.868,39	2.413,45	13.010,21	+4	–7	–20	–4
t6	7.565,32	2.752,52	2.097,55	12.415,39	+2	–11	–31	–8
t8	7.556,28	2.658,08	1.906,59	12.120,96	+2	–14	–37	–10
t12	7.478,06	2.533,41	1.428,48	11.439,95	+1	–18	–53	–15
t24	7.461,15	2.364,79	819,65	10.645,59	+1	–23	–73	–21
t48	7.447,65	2.330,46	432,22	10.210,33	+1	–24	–86	–24
%RSD	1.66	10.01	46.16	10.15	/	/	/	/

Extended residence times enhanced E–F resolution while introducing a progressive stability stress. Importantly, AAV8 capsids remained intact during AEX analysis, as evidenced by reproducible peaks, particularly confirmed with duplicate 0h injections. Additionally, AEX revealed the inherent heterogeneity and selective fragility of AAV8 capsid subpopulations. Considering the comparatively lower stability of the HS + CIP fraction, a practically relevant residence time window balancing stability and separation performance appears to lie between 2 and 4 h (Table 2B). Beyond this window, instability can occur, which is consistent with prior reports [14]. For complex biological samples such as AAV, variability of up to $\pm 20\%$ is generally considered acceptable, reflecting the inherently higher analytical variability of biologic analytes [60]. Overall, these results indicate that short-term exposure to alkaline, low-conductivity AEX conditions does not significantly compromise AAV8 capsid integrity or the reliability of analytical separations, whereas long-term exposure necessitates formulation-optimized conditions. For preparative-scale applications, where capsid integrity is critical, tailored compromises between

formulation stability and separation efficiency will be required, particularly given the serotype-dependent nature of AAV stability [61,62].

Ultimately, an appropriate balance must be achieved between AAV sample stability and chromatographic separation performance to ensure reliable separation and accurate analytical outcomes. While maximal capsid stability is not always a strict requirement for analytical-scale methods [55,63], it becomes critical for preparative-scale purification, where tighter quality control and preservation of biological functionality are essential [11,63]. Given the well-documented serotype-dependent nature of AAV stability, future analytical and preparative workflows will likely require condition-specific compromises between formulation stability and separation efficiency. Although the present study did not directly assess long-term stability or biological activity under all investigated conditions, the results establish a mechanistic and experimental framework for guiding preparative-scale optimization and support the rational design of robust AAV8 formulations.

Within this context, operation at near-neutral pH on weak anion exchange media, such as DEAE, emerges as a promising starting point for further development of stability-preserving preparative AEX methods.

4. Conclusions

Effective chromatographic separation is essential for achieving accurate analytical results and the required purity of AAV products, yet it must be carefully balanced against the need to preserve capsid stability. Using an AAV8 model system, this study demonstrates that anion exchange (AEX) separation of AAV capsids is governed by a complex interplay among capsid charge state, ligand ionization, physicochemical surface properties, and the surrounding ionic microenvironment.

Elevated pH enhances empty–full (E–F) resolution on AEX materials by increasing the net negative charge of the capsid and strengthening electrostatic interactions; however, such conditions—particularly at low conductivity—may introduce stability constraints. Importantly, the stability experiments added in this study show that AAV8 remains structurally preserved under alkaline, low-conductivity loading conditions for analytically relevant time windows, with no evidence of aggregation, fragmentation, or loss of chromatographic integrity. These findings indicate that improved separation performance does not necessarily come at the expense of capsid stability under controlled and defined exposure.

Weak AEX media further mitigate stability concerns by introducing pH-dependent ligand ionization and secondary interaction modes, enabling meaningful capsid retention and partial E–F separation under milder, near-physiological pH conditions. Buffer ionic strength emerged as a dominant determinant of separation efficiency: moderate salt concentrations stabilize capsids, low ionic strength enhances resolution, and excessive salt suppresses electrostatic discrimination while promoting unfavorable secondary interactions. Additionally, we demonstrate that the ionic environment surrounding the capsid equilibrates slowly, such that prior buffer conditions influence subsequent chromatographic behavior even after rapid in-line buffer exchange.

By integrating mechanistic insights with direct stability evaluation, this work defines practical design principles for balancing separation efficiency with capsid integrity. The implementation of a dedicated 2D in-line buffer exchange system enabled consistent AEX retention of AAV8 and comparable separation across diverse buffer compositions, establishing a robust and reproducible platform for final AAV characterization. Collectively, these findings advance the mechanistic understanding of AAV chromatographic behavior and provide a framework for developing reliable analytical methods while informing future optimization of preparative-scale purification workflows under stability-preserving conditions.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/pharmaceutics18020263/s1>, Supplementary Methods. 1.1. Recovery Determination Using Cation-Exchange Chromatography (CEX). Supplementary Results and Discussion, including: 2.1. Additional Method Optimization on AEX[64]. 2.2. Assessment of AAV8 Capsid Recovery and Empty-Full Separation Using Cation Exchange Chromatography[65–69]. Supplementary Figures and Tables: Figure S1. Minor method optimization on DEAE using magnesium acetate gradients: (A) 2–25 mM magnesium acetate gradient (pH 8.0, HCl-titrated); (B) 2–25 mM magnesium acetate gradient (pH 8.0, propionic acid-titrated). Figure S2. Empty–full (E–F) capsid separation on DEAE using pH-gradient elution. FT = flow-through. Figure S3. Capture behavior of AAV8 capsids on a strong cation-exchange (CEX) monolithic column (SO₃). Figure S4. Attempted empty–full (E–F) separation of AAV8 capsids on a strong CEX (SO₃) monolithic column. Table S1. Composition of commonly used in-house downstream process buffers evaluated for AAV8 sample dilution and in-line buffer exchange chromatographic analysis. Table S2. Recovery assessment of AAV8 capsids using PATfix CEX and orthogonal analytical methods. *dPCR is not suitable for recovery determination after QA loading due to the selective enrichment of empty and full capsids. Table S3. Summary of working buffers used in chromatographic experiments.

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