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Separation of Empty and Full Adeno-Associated Virus Capsids from a Weak Anion Exchanger by Elution with an Ascending pH Gradient at Low Ionic Strength

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ABSTRACT

Separation of empty and full AAV8 capsids was achieved during their elution from a weak anion exchanger with an ascending pH gradient at low conductivity. Experimental data suggest elution was mediated by loss of positive charge from the exchanger. The method produced a full capsid peak with fewer empty capsids than elution of a strong anion exchanger with a salt gradient. Elution of the weak exchanger by sodium chloride gradients or by pH gradients in the presence of sodium chloride gave inferior separation performance. Pre-elution of empty capsids with a pH step allowed full capsids to be eluted by salt without compromising separation. Loading at intermediate pH prevented empty capsid binding and enabled step elution of full capsids in a physiological buffer environment.

1.0 INTRODUCTION

Anion exchange chromatography (AEC) is documented to support varying degrees of separation between empty and full capsids for the majority of adeno-associated virus (AAV) serotypes[1–10]. The technique is performed most commonly on strong quaternary amine (QA) anion exchangers eluted with salt gradients at alkaline pH. In the presence of excess magnesium ions, the first of two major peaks is populated by empty capsids and the second is populated either exclusively by full capsids, or by full capsids with a lesser proportion of empty capsids[1–4,11]. Elution in the absence of magnesium ions similarly produces an early-eluting empty capsid peak followed by a full capsid peak, but the empty capsid peak is usually smaller than in the presence of magnesium, and the full capsid peak is usually followed by later-eluting empty capsids and apparent capsid debris. The full capsid peak often contains a subset of empty capsids[11].

Elution of proteins from anion exchangers with descending pH gradients at low conductivity has been known for more than 40 years[12–16]. As proteins become more electropositive with descending pH, they become more repellant to the exchanger surface, which eventually overcomes the attraction mediated by their negatively charged residues. Selectivity is described frequently as correlating with protein isoelectric (pI) point[12–16]. Differences in pI have been documented among full and empty capsids for several parvoviruses, including AAV[17–21] but descending pH gradient elution has not proven effective for separating their empty and full capsids. A comprehensive study with structurally-similar minute virus of mice (MVM) showed that selectivity was dominated by the distribution of charges on capsid surfaces rather than their net charge[20].

Elution of anion exchangers with ascending pH gradients at low conductivity hypothetically provides a third option, based on the idea that elution should be promoted by reducing the positive charge on the anion exchanger. Weakly retained proteins should elute earlier than more strongly retained proteins, but there are secondary restrictions. Proteins become more electronegative at higher pH values, which should favor stronger retention and counteract the loss of charge from the exchanger. Stability of proteins and complex noncovalent assemblages like virus particles is also a concern. Strong anion exchangers employ QA ligands that maintain their full charge up to about pH 11.0, and half their charge at their pK_a of about pH 13.0.

Weak anion exchangers offer more potential for ascending pH gradient elution because they begin to lose charge in a lower range of pH. DEAE (diethylaminoethyl) is a tertiary amine ligand with a pK_a of about 11.5[22,23]. It begins to lose charge at pH values above 9.5, embodies half its charge at 11.5, and loses essentially all of its charge by pH 13.5. This represents a step in the right direction, but the high pH remains a concern. Weaker AEC ligands are fortunately known and they offer potential for

elution at more moderate pH values. Examples include primary amines[24,25]; ligands with combined primary and secondary amine functionalities[26]; combined primary, secondary, and tertiary amine functionalities[27–30]; and ligands combining primary, secondary, tertiary, and QA functionalities[13].

In the present study, we evaluate a novel weak anion exchanger developed for ascending pH gradient separation of empty and full AAV8 capsids. The content of empty and full capsids in eluted AAV peaks is estimated in some experiments by calculating the ratio of UV absorbance at 260 and 280 nm[31,32]. Empty and full capsid content is estimated in other experiments by cesium chloride density gradient ultracentrifugation (DGUC), followed by stratigraphic evacuation of the centrifuge tubes through a high-performance liquid chromatography (HPLC) detector array including UV, intrinsic fluorescence, and light scattering[11].

2. MATERIALS AND METHODS

2.1 Samples and Sample Preparation

Triton X-100 lysates of HEK293 cells producing AAV8 were obtained from The University of Nantes, Center for Translational Therapy for Genetic Diseases, INSERM UMR 1089, Nantes, France. All lysates were membrane filtered to 0.45 µm prior to chromatography. Host cell DNA (chromatin) contamination was reduced in some cases by tangential flow filtration (TFF, 300 kDa membrane) into 20 mM tris, 500 mM sodium chloride, 5 mM magnesium chloride, 1% sucrose, 0.1% poloxamer 188, pH 8.0; then digested with 50 unit/mL of salt-tolerant nuclease enzyme (Kryptonase, BIA Separations) at ambient temperature for 16 h in the TFF unit. The retentate was then concentrated and diafiltered into 20 mM tris, 200 mM NaCl, 1% sucrose, 0.1% poloxamer 188, pH 7.5 to remove nucleoproteins liberated by DNA lysis, and to reduce conductivity prior to the cation exchange chromatography (CEC) capture step. Chromatin contamination was alternatively reduced by the addition of prototype positively charged particles (CIMasphere H-Bond, BIA Separations) at a proportion of 2% (v:v), incubated mixing for 60 min, then filtered through a 0.45 µm membrane to remove solids. Chromatin reduction is discussed further in[3,4].

Initial AAV8 purification was performed by CEC on 1 mL or 8 mL CIMmultus SO_3 monoliths (BIA Separations), run on an ÄKTA Pure 25M1 chromatograph with a 10 mm flow cell (Cytiva). The sample was acidified to pH 3.5 and then filtered through a 0.45 µm membrane, if necessary, to remove turbidity. The column was equilibrated to 50 mM formic acid, 200 mM sodium chloride, 1% sucrose, 0.1% poloxamer 188, pH 3.5, eluted with a linear gradient to 50 mM formic acid, 2 M sodium chloride, pH 3.5, then cleaned with 2 M sodium chloride plus 1 M sodium hydroxide. Volumetric flow rate on 1 mL monoliths was 5 mL/min (5 column volumes (CV)/min). Volumetric flow rate for 8 mL monoliths was 20 mL/min (2.5 CV/min).

2.2. Anion Exchange Fractionation of Empty and Full AAV8 Capsids

The study was conducted with two different anion exchange ligands. QA-based strong anion exchangers were used as experimental controls (100 µL CIMac AAV or 1 mL CIMmultus QA, BIA Separations). They were equilibrated with 50 mM bis-tris-propane, 2 mM magnesium chloride, 1% sucrose, 0.1% poloxamer 188, pH 9.0. They were eluted with a linear salt gradient to 50 mM bis-tris-propane, 2 mM magnesium chloride, 200 mM sodium chloride, 1% sucrose, 0.1% poloxamer 188, pH 9.0, rinsed with water, then cleaned with 2 M sodium chloride plus 1 M sodium hydroxide. Volumetric flow rate was 10 CV/min. Other experiments were conducted under identical conditions except magnesium chloride was omitted from the buffers.

A novel weak anion exchange ligand, PrimaS (BIA Separations), was used to evaluate potential for elution by ascending pH (100 µL CIMac or 1 mL CIMmultus). For pH gradient experiments, the column was equilibrated with 10 mM bis-tris-propane, 10 mM tris, 2 mM magnesium chloride, 1% sucrose, 0.1% poloxamer 188, pH 8.0 (conductivity ~1 mS/cm). After sample application and washing the column with equilibration buffer, virus was eluted with a linear gradient to 10 mM bis-tris-propane, 10 mM tris, 2 mM magnesium chloride, 1% sucrose, 0.1% poloxamer 188, pH 10.0 (conductivity ~2 mS/cm). The column was rinsed with 1 CV water then cleaned with 1 CV of 50 mM sodium hydroxide plus 1 M sodium chloride. The water rinse is important to prevent formation of insoluble metal hydroxides at the interface between metal-containing buffers and sodium hydroxide. Flow rate was 10 CV/min. In other experiments, the column was eluted under identical conditions, except the magnesium chloride was omitted. Experiments with salt gradient elution were performed under conditions identical to the strong anion exchanger. Variations from any of the above-discussed conditions are noted in the discussion (section 3).

Experiments with 1 mL CIMmultus monoliths were performed on the ÄKTA chromatograph described above. Experiments with 100 µL CIMac monoliths were performed on a PATfix LPG HPLC system (BIA Separations) with integrated multiple wavelength detector (MWD)

UV-vis detector (190–700 nm, 8-channel deuterium lamp, 50 mm flow cell path length), conductivity, and pH monitor. It was further equipped with a Prominence RF-20A (200–650 nm) dual wavelength fluorescence detector (Shimadzu) and a DAWN HELEOS II multi-angle light scattering detector (Wyatt Technology). UV absorbance was monitored at 260 and 280 nm. Light scattering was measured at an angle of 90°. Intrinsic fluorescence was monitored at an excitation wavelength of 280 nm and an emission wavelength of 348 nm.

2.3. Analysis by Density Gradient Ultracentrifugation

Cesium chloride DGUC was performed on selected chromatography fractions as described in[11]. In brief, samples containing about 1E+11 vector genomes were mixed with concentrated cesium chloride to obtain an AAV sample in 3.0 M cesium chloride. Centrifugation was performed with a Sorvall WX 90+ ultracentrifuge (Thermo Fisher Scientific) using 11.5 mL polyethylene centrifuge tubes in a T890 fixed-angle rotor, at 53,500 rpm for 24 h at room temperature. Stratigraphic analysis was performed by piercing the top of the tube with a hypodermic needle extending to the bottom, and venting the tube with another hypodermic needle at the top. Contents were pumped at 1 mL/min into a PATfix LPG HPLC system with the same monitors and settings used for HPLC. Cesium chloride density was measured indirectly by conductivity. The higher the conductivity, the higher the density of the cesium chloride in the corresponding strata.

2.4. Polymerase Chain Reaction

Vector DNA content of capsids for selected fractions was measured using the methods of Dobnik *et al.*[33] on a QX200 digital droplet PCR (ddPCR) equipped with QuantaSoft version 1.7.4 software (Bio-Rad Laboratories).

3. RESULTS AND DISCUSSION

3.1. Reference Separation of Empty and Full Capsids on a Strong Anion Exchanger

Figure 1 illustrates AAV8 elution profiles on a strong anion exchanger (CIMac AAV) eluted with a sodium chloride gradient. Profile A was prepared without magnesium in the equilibration and gradient buffers. Profile B was prepared with 2 mM magnesium sulfate in the buffers. A peak dominated by empty capsids eluted first in both experiments. The ratio of UV absorbance at 260 and 280 nm was 0.62, which placed it in the typical range of 0.6–0.7 for empty capsids. A peak dominated by full capsids followed. Its 260/280 ratio of 1.32 was in the typical range of 1.3–1.4 for full capsids.

The influence of metal ions on AEC of AAV capsids has not yet been fully characterized, but **Figure 1** shows that they weaken retention, causing both empty and full capsid peaks to elute at lower conductivity while approximately maintaining the separation between them. This seems likely to reflect charge modification of metal-binding sites on the capsid proteins[34]. Association of positively charged metal ions would logically reduce the magnitude of local negative charge. Reduced negative charge would be expected to reduce the attraction to positively charged anion exchange surfaces. Metal affinity interactions are much stronger than electrostatic interactions. This is indicated by strong protein and DNA binding to metal affinity columns at salt concentrations ranging from 1 M to saturation[35,36]. The effects of magnesium binding on net surface charge of AAV capsids would therefore be expected to persist across salt gradients.

Figure 1 also shows a metal-mediated redistribution of capsids that points to a separate effect. A smaller empty capsid peak and a higher proportion of late-eluting debris

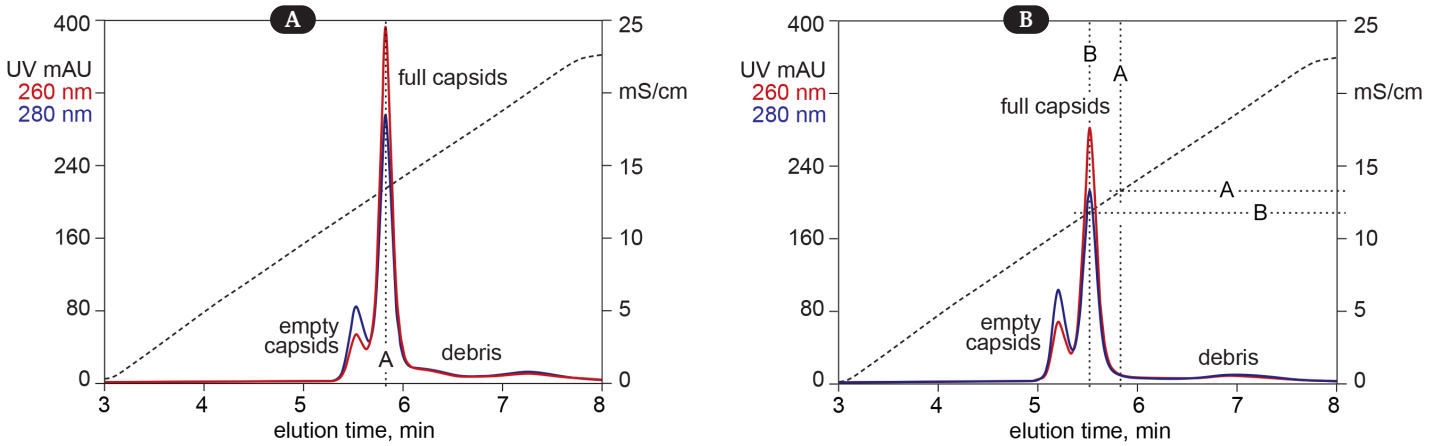

FIGURE 1. Separation of empty and full AAV8 capsids by a sodium chloride gradient on a strong anion exchanger (CIMac AAV) at pH 9.0: **(A)** in the absence of magnesium ions; and **(B)** in the presence of 2 mM magnesium ions.

was observed in the absence of magnesium (profile A). Given that magnesium and calcium ions both stabilize full AAV capsids[9,37,38], the same might be expected for empty capsids, with the further expectation that a deficiency of metal ions would leave them less stable. This hypothesis was supported by the ratio of UV absorbance at 260 and 280 nm in the later-eluting debris population being atypical of either empty or full capsids. Nearly equivalent UV absorption at both wavelengths showed a higher proportion of DNA than empty capsids, but a higher proportion of protein than full capsids. Dissociation of only empty capsids would be expected to produce a wavelength ratio similar to intact empty capsids. A lesser debris field in the presence of magnesium (profile B) may have been resident in the original sample or it may have resulted from the stress of chromatography at pH 9.0.

3.2. Separation of Empty and Full Capsids on the Weak Anion Exchanger

Similar results were produced by elution of the weak exchanger (CIMac PrimaS) with an identical salt gradient, except that the capsids eluted at lower conductivity. This fit with an expectation that the weak exchanger would become less positively charged at alkaline pH. Separation between empty and full capsids was slightly inferior to the strong anion exchanger. Gradient optimization on the weak exchanger might have improved the separation. A 2007 study reported that DEAE eluted with a salt gradient produced better separation of empty and full capsids than a strong anion exchanger[10]. This could be interpreted to imply that other weak exchangers should do the same. No trend has emerged to suggest that this is generally the case, but the notion that different exchanger chemistries require individual optimization still makes sense.

Figure 2 illustrates ascending pH gradient elution profiles from the weak anion exchanger (CIMac PrimaS) without magnesium (profile A), and with 2 mM magnesium (profile B). Results in the absence of magnesium were distinct from salt gradient elution in several respects. The first peak was much smaller and the UV absorption ratio showed a higher proportion of DNA than is usual for empty capsids. The full capsid peak was sharper and more concentrated but its resolution from later-eluting debris was reduced. Results in the presence of magnesium were similar to salt gradient elution. The elution order of empty and full capsids was the same. The presence of magnesium had the expected effect of causing earlier elution. The UV absorption ratios of empty and full peaks were the same as obtained with salt gradient elution of the strong exchanger.

Figure 3 illustrates pH gradient elution of a different AAV8 in the presence of magnesium, monitored for intrinsic fluorescence and light scattering, in addition to UV. Light scattering revealed that the peak at 7 min contained either no intact capsids or very few. This supports the hypothesis that species eluting after the full capsid peak represent capsid debris. Its 260/280 ratio indicates a higher relative protein content than the debris peak observed with salt gradient elution in the presence of magnesium (**Figure 1B**). This suggests that there was more debris from empty capsids than from full capsids. Together with **Figure 2**, these results suggest two preliminary conclusions: First, that low conductivity exposure at alkaline pH reduces capsid stability to a greater extent than exposure to moderate conductivity at alkaline pH. This is consistent with formulation studies showing that low conductivity adversely affects capsid stability[39–41]. Second, the results further suggest that empty capsids are less stable than full capsids. This merits further

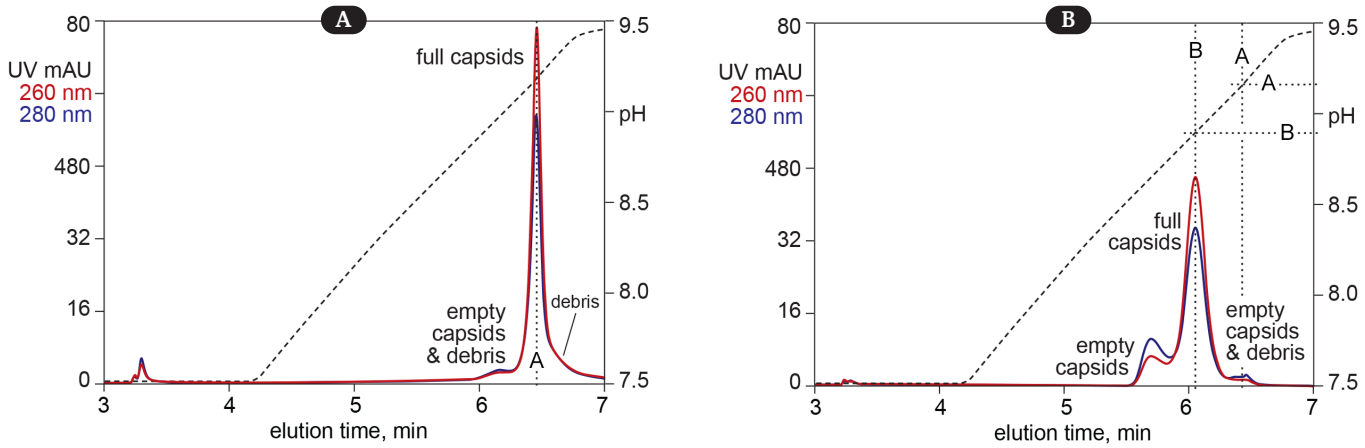

FIGURE 2. Separation of empty and full AAV8 capsids by an ascending pH gradient on a weak anion exchanger (CIMac PrimaS): **(A)** in the absence of magnesium ions; and **(B)** in the presence of 2 mM magnesium ions.

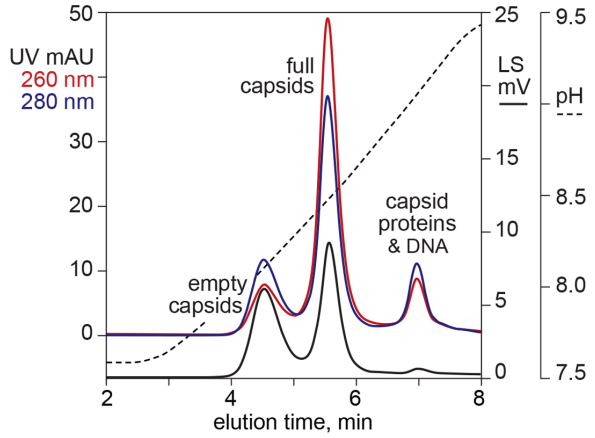

FIGURE 3. Separation of empty and full AAV8 capsids by an ascending pH gradient on a weak anion exchanger (CIMac PrimaS) in the presence of 2 mM magnesium ions with simultaneous monitoring by UV, intrinsic fluorescence, light scattering (LS), and pH.

investigation, but it is consistent with the idea that empty capsids are empty because they are deficient or aberrant in some way.

These results also raise a practical question: If capsids are less stable at low conductivity, then why elute them in a low conductivity gradient? The practical answer is illustrated by **Figure 4**, which shows that pH gradient elution reduces empty capsid content of the full capsid peak to a greater extent than salt gradient elution. Given that the pH range of capsid elution is close to 9.0 in both cases, it seems unlikely that pH gradient separation removes empty capsids more effectively because a subset of them has different charge characteristics. It seems more likely that the stress created by the combination of low conductivity and alkaline pH dissociates a subset of empty capsids that elute later as debris.

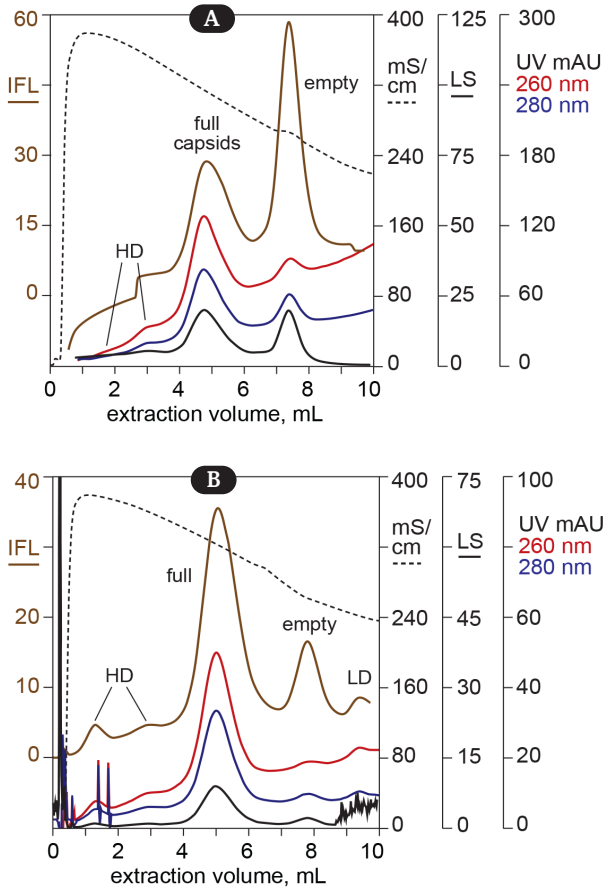

FIGURE 4. Comparison of full capsid peak contents by cesium chloride DGUC analysis: **(A)** contents of the full AAV8 capsid peak from CIMmultus QA eluted with sodium chloride; and **(B)** contents of the full AAV8 capsid peak from CIMmultus PrimaS eluted with an ascending pH gradient.
NOTES: IFL indicates intrinsic fluorescence; LS indicates light scattering; conductivity (mS/cm) is a surrogate indicator of cesium chloride density; HD indicates populations of higher density than full capsids; and LD indicates a population of lower density than empty capsids.

3.3. Elution Mechanism

Figure 5 shows results from a series of experiments in which CIMmultus PrimaS was equilibrated at various pH values in the absence of excess salt. Then a buffer was introduced with the same buffering species and pH but also including 1 M sodium chloride. Introduction of salts to ion exchange columns is known to produce so-called pH transients[42–46]. Chloride ions displace hydroxide counterions that were previously associated with the solid phase. This temporarily elevates pH. The amount of charge on the exchanger, which is a function of pH, determines the amount of hydroxide counterion it binds during equilibration. The magnitude of the pH transient can be used as an indirect measurement of the amount of charge on the exchanger at any given pH value. A large pH transient was observed at pH 7.5, less at pH 8.0, more

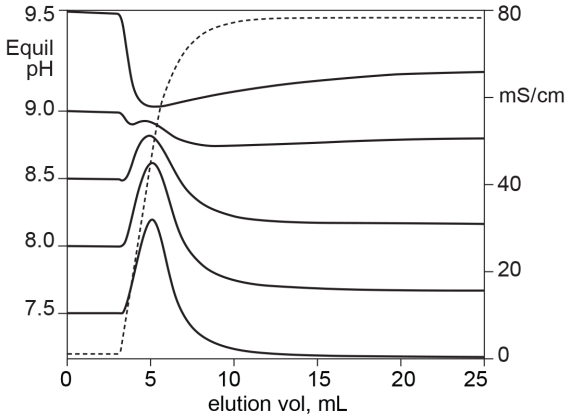

FIGURE 5. Magnitude of pH transients produced by introducing 1 M salt to CIMmultus PrimaS at the indicated equilibration pH values. The 1 M salt was administered in a buffer with the same buffering agent and pH as the equilibration step.

dramatic reduction at pH 8.5 and 9.0, with no apparent transient at pH 9.5. Taking pH 9.5 as the point at which the positive charge on the exchanger becomes negligible, this would put the apparent pK_a at about pH 7.5.

Elution by loss of charge from the exchanger should produce overall selectivity similar to salt gradient elution, with weaker-binding species eluting first. This creates an expectation that pH and salt concentration should work cooperatively to improve the separation. This proved not to be the case. Inclusion of fixed sodium chloride concentrations during pH gradient elution caused capsids to elute at lower pH values as expected, but the quality of the separation was reduced. Separation with salt gradients was compromised when operating pH was reduced from pH 9.0 to 8.0, and compromised more at pH 7.0. A combined ascending pH-salt gradient eluted all capsids in a single peak. These results provide a worthy reminder that separations are not purely functions of buffer conditions. They also depend on the charge characteristics of the species being separated which, as demonstrated by MVM[20], may defy simple predictions. For AAV, present results suggest that alkaline pH is necessary to support AEC-based separation of empty and full capsids, no matter what the elution mechanism.

Virtually all reported AEC separations of empty and full capsids are performed at alkaline pH. Most are in the range of pH 8.5 to 9.0, but some serotypes benefit from elution at pH 10 or above[7]. Remarkably, the same pattern is seen with AAV on cation exchangers. Most serotypes do not bind cation exchangers sufficiently at alkaline pH to support salt gradient elution, but a single exception was reported with a recombinant serotype that bound at pH 8.5[8]. It was eluted in a sodium acetate gradient. Elution order was reversed from anion exchange as expected, but good separation of empty and full capsid peaks was obtained. All of these results are consistent with a hypothesis that full capsids have a higher net negative charge than empty capsids at alkaline pH. They do not point to an intuitive explanation for that differential. Neither do they explain why a different subset of empty capsids has the same AEC retention behavior as full capsids. However, they do focus interest on a need to better understand the origins of charge differentiation between empty and full capsids.

Data from salt gradients at different pH values revealed an additional point of interest. Capsids bound and eluted from the weak exchanger at pH 8.0 required more salt for elution than capsids bound and eluted at pH 9.0. Capsids bound and eluted at 7.0 required more salt for elution than capsids bound at pH 8.0. These findings are consistent with **Figure 5**. They indicate that more salt is required to overcome stronger electrostatic interactions. They also imply that binding capacity of the weak exchanger should be greater at pH 7.0 and lower with increasing pH.

3.4. Method Development

The potential issue of capsid stability at alkaline pH and low conductivity points to a solution where pH is elevated sufficiently to remove empty capsids, then full capsids are eluted in the presence of salt to minimize stress. **Figure 6** illustrates a series of chromatograms where PrimaS was loaded at pH 8.0, then washed at pH 8.5 (profile A), 8.6 (profile B), and 8.7 (profile C). At pH 8.5, a minority of empty capsids eluted in the wash. The majority eluted at the beginning of the elution gradient, poorly separated from the full capsid peak. At pH 8.6, the majority of empty capsids eluted

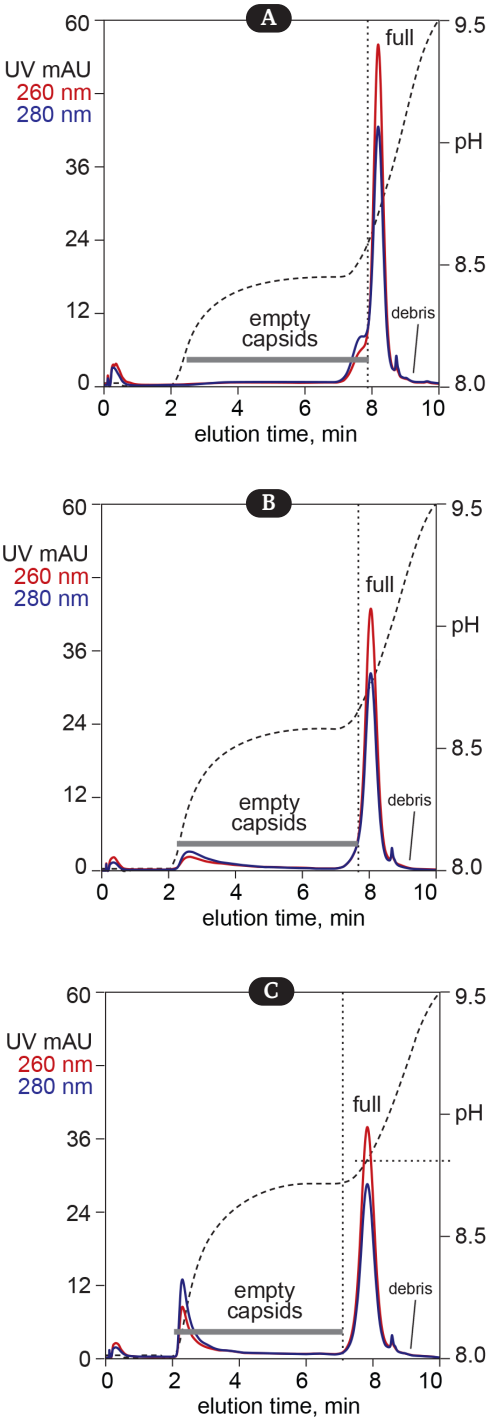

FIGURE 6. The influence of wash pH on the elimination of empty AAV8 capsids before elution of full capsids by an ascending pH gradient (CIMac PrimaS). All columns equilibrated and loaded at pH 8.0: **(A)** washed at pH 8.5; **(B)** washed at pH 8.6; and **(C)** washed at pH 8.7.

NOTE: The dotted vertical markers indicate that point at which UV dominance shifts between 260 and 280 nm.

in the wash. Only a minority remained and were eluted at the beginning of the pH gradient. At pH 8.7, the empty capsid peak was eliminated entirely by the wash. When washed at pH 8.8 (not shown), a large proportion of full capsids was eliminated with the empty capsids.

Figure 7 demonstrates two extensions of the same basic rationale. Instead of washing at elevated pH values, PrimaS was equilibrated at pH 8.6 to prevent empty capsids from binding. Full capsids were then eluted with a step at physiological conditions to prevent exposure of the full capsids to higher pH values and provide stabilization through higher conductivity. **Figure 5** instructs that the elution buffer should have excess buffer capacity to compensate for the pH transient that would be formed by the increase in salt concentration. The elution step could be replaced with a salt gradient, at least during method development, to help identify the most favorable conditions. Otherwise, step elution would provide the highest concentration of full capsids.

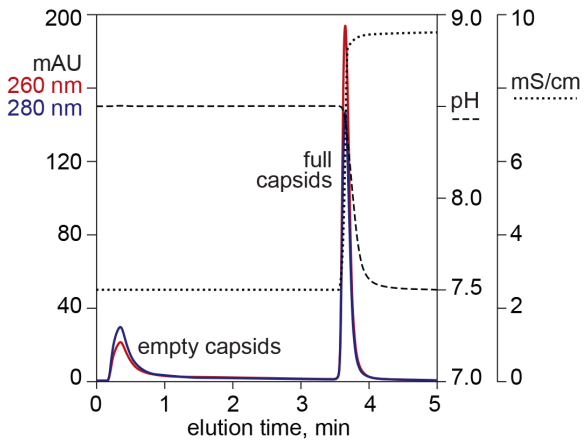

FIGURE 7. Loading AAV8 capsids at pH 8.5 to prevent binding of empty capsids, then elution with a step to physiological conditions (CIMac PrimaS).

Overall, the approach of elevating pH to minimize empty capsid content and then elute full capsids in the presence of salt represents the most gentle of all anion exchange options. Strong anion exchangers eluted with salts elevate conductivity during elution, but the capsids are exposed to high pH and low conductivity from the time of sample equilibration and throughout the duration of sample loading. Samples can be loaded onto PrimaS at lower pH values, and then exposed to relatively moderate pH to remove empty capsids before eluting full capsids in the presence of salt.

4. CONCLUSIONS

A weak anion exchange with an estimated pK_a of about pH 7.5 was evaluated for separation of empty and full AAV8 capsids. Elution with an ascending pH gradient at low conductivity permitted more effective reduction of empty AAV8 capsids than salt gradient elution of the weak exchanger or salt gradient elution of strong anion exchangers. Ascending pH gradient elution of capsids from the weak exchanger appeared to result from reduction of the positive charge of the anion exchange groups. Indications of reduced capsid instability in the combined presence of alkaline pH and low conductivity encouraged development of methods to avoid those conditions. In one experiment, empty capsids were eluted with a low conductivity step to a pH of about 8.5, after which full capsids could be eluted at pH 8.5 or less in the presence of salt. In a related format, the column was equilibrated to pH 8.5. Empty capsids failed to bind. Full capsids were eluted with a step to physiological conditions.

AUTHORSHIP CONTRIBUTIONS

Pete Gagnon: Conceptualization, writing, review and editing
Blaž Goričar: Conceptualization, methodology
Sara Drmota Prebil: Methodology
Hana Jug: Methodology
Maja Leskovec: Conceptualization
Aleš Štrancar: Conceptualization

All authors have read and agreed to submission of the manuscript and declare no conflict of interest.

REFERENCES

[1] Fu X *et al*. Analytical strategies for quantification of adeno-associated virus empty capsids to support process development. *Hum Gene Ther Met*, 2019; 30(4): 144–52. https://doi.org/10.1089/hgtb.2019.088

[2] Dickerson R, Argento C, Pieracci J, Bakhshayeshi M. Separating empty and full recombinant adeno-associated virus particles using isocratic anion exchange chromatography. *Biotechnol J*, 2021; 16: e2000015. https://doi.org/10.1002/biot.202000015

[3] Gagnon P, Leskovec M, Goricar B, Strancar A. Streamlining industrial purification of adeno-associated virus. *BioProcess Intl*, 2020; 18: S14–20. https://bioprocessintl.com/downstream-processing/separation-purification/streamlining-industrial-purification-of-aav/

[4] Gagnon P, Goricar B, Mencin N, Zvanut T, Peljhan S, Leskovec M, Štrancar A. Multiple-monitor HPLC assays for rapid process development, in-process monitoring, and validation of AAV production and purification. *Pharmaceutics*, 2021; 13(1): 113. https://doi.org/10.3390/pharmaceutics13010113

[5] Joshi PRH, Bernier A, Moco PD, Schrag J, Chahal PS, Kamen A. Development of a scalable and robust AEX method for enriched rAAV preparations in genome-containing VCs of serotypes 5, 6, 8, and 9. *Mol Ther Met Clin Dev*, 2021; 21: 341–56. https://doi.org/10.1016/j.omtm.2021.03.016

[6] Nass SA *et al*. Universal method for the purification of recombinant AAV vectors of differing

serotypes. *Mol Ther Met Clin Dev*, 2018; 9: 33–46. https://doi.org/10.1016/j.omtm.2017.12.004

[7] Lock M, Alvira M. World Patent Application WO2017160360A9. *Scalable purification method for AAV9*. 2018. https://patents.google.com/patent/WO2017160360A9/en

[8] Qu G *et al*. Separation of adeno-associated virus type 2 empty particles from genome containing vectors by anion-exchange column chromatography. *J Virol Met*, 2007; 140(1–2): 183–92. https://doi.org/10.1016/j.jviromet.2006.11.019

[9] Urabe M, Xin KQ, Obara Y, Nakakura T, Mizukami H, Kume A, Okuda K, Ozawa K. Removal of empty capsids from type 1 adeno-associated virus vector stocks by anion exchange chromatography potentiates transgene expression. *Mol Ther*, 2006; 13(4): 823–8. https://doi.org/10.1016/j.ymthe.2005.11.024

[10] Zhou J, Hauck B, Wright JF, High KA, Qu G. Weak anion exchange column chromatography enhances the resolution of separation of AAV empty capsid and full vectors. *Mol Ther*, 2007; 15(S1): S36. https://doi.org/10.1016/S1525-0016(16)44297-8

[11] Peljhan S, Štokelj M, Prebil SD, Gagnon P, Štrancar A. Multiple-parameter profiling of density gradient ultracentrifugation for characterization of empty and full capsid distribution in AAV preparations. *Cell Gene Ther Insights*, 2021; 7(2): 161–9. https://doi.org/10.18609/cgti.2021.039

[12] Sluyterman LAAE, Elgersma O. Chromatofocusing: isoelectric focusing on ion-exchange columns: I. general principles. *J Chromatogr A*, 1978; 150(1): 17–30. https://doi.org/10.1016/S0021-9673(01)92092-8

[13] Sluyterman LAAE, Wijdenes J. Chromatofocusing: IV. Properties of an agarose polyethyleneimine ion exchanger and its suitability for protein separations. *J Chromatogr A*, 1981; 206(3): 441–7. https://doi.org/10.1016/S0021-9673(00)88913-X

[14] Brorson K, Shen H, Lute S, Perez JS, Frey DD. Characterization and purification of bacteriophages using chromatofocusing. *J Chromatogr A*, 2008; 1207(1–2): 110–21. https://doi.org/10.1016/j.chroma.2008.08.037

[15] Kröner F, Hubbuch J. Systematic generation of buffer systems for pH gradient ion exchange chromatography and their application. *J Chromatogr A*, 2013; 1285: 78–87. https://doi.org/10.1016/j.chroma.2013.02.017

[16] Ahamed T, Nfor BK, Verhaert PDEM, van Dedem GWK, van der Wielen LAM, Eppink MHM, van de Sandt EJAX, Ottens M. pH-gradient ion-exchange chromatography: an analytical tool for design and optimization of protein separations. *J Chromatogr A*, 2007; 1164(1–2): 181–8. https://doi.org/10.1016/j.chroma.2007.07.010

[17] Li T, Gao T, Chen H, Pekker P, Menyhart A, Guttman A. Rapid determination of full and empty adeno-associated virus capsid ratio by capillary isoelectric focusing. *Curr Mol Med*, 2020; 20(10): 814–20. https://doi.org/10.2174/1566524020666200915105456

[18] Qu W, Wang M, Yu Y, Xu R. Scalable downstream strategies for purification of recombinant adeno-associated virus vectors in light of the properties. *Curr Pharm Biotechnol*, 2015; 16(8): 684–95. https://doi.org/10.2174/1389201016666150505122228

[19] Venkatakrishnan B *et al*. Structure and dynamics of adeno-associated virus serotype 1 VP1-unique N-terminal domain and its role in capsid trafficking. *J Virol*, 2013; 87(9): 4974–84. https://doi.org/10.1128/JVI.02524-12

[20] Leisi R, Wolfisberg R, Nowak T, Caliaro O, Hemmerle A, Roth NJ, Ros C. Impact of the isoelectric point of model parvoviruses on viral retention in anion-exchange chromatography. *Biotechnol Bioeng*, 2021; 118: 116–29. https://doi.org/10.1002/bit.27555

[21] Leuchs B, Frehtman V, Riese M, Müller M, Rommelaere J. A novel scalable, robust downstream process for oncolytic rat parvovirus: isoelectric point-based elimination of empty particles. *Appl Microbiol Biotechnol*, 2017; 101; 3143–52. https://doi.org/10.1007/s00253-016-8071-x

[22] The Metabolomics Innovation Centre. *Showing compound DEAE-cellulose (FDB011396)*. Accessed March 10, 2021. https://foodb.ca/compounds/FDB011396

[23] Tosoh BioScience LLC. *Ion exchange chromatography. Toyopearl resins for IEC*. Accessed March 11, 2021. http://wolfson.huji.ac.il/purification/pdf/ionexchange/tosoh_iec.pdf

[24] Li M, Li Y, Yu L, Sun Y. Characterization of poly(allylamine) as a polymeric ligand for ion-exchange protein chromatography. *J Chromatogr A*, 2017; 1486: 103–9. https://doi.org/10.1016/j.chroma.2016.11.012

[25] Woo M *et al*. A novel primary amine-based anion exchange membrane adsorber. *J Chromatogr A*, 2011; 1218(32): 5386–92. https://doi.org/10.1016/j.chroma.2011.03.068

[26] Bicho D, Santos BF, Caramelo-Nunes C, Sousa A, Sousa F, Queiroz JA, Tomas CT. Application of ethylenediamine monolith to purify a hemagglutinin influenza deoxyribonucleic acid-based vaccine. *Sep Purif Technol*, 2015; 154(5); 320–7. https://doi.org/10.1016/J.SEPPUR.2015.09.046

[27] Johansson BL, Belew M, Eriksson S, Glad G, Lind O, Maloisel JL, Norrman N. Preparation and characterization of prototypes for multi-modal separation media aimed for capture of negatively charged biomolecules at high salt conditions. *J Chromatogr A*, 2003; 2016: 21–33. https://doi.org/10.1016/s0021-9673(03)01140-3

[28] Flashner M, Ramsden H, Crane LJ. Separation of proteins by high-performance anion-exchange chromatography. *Anal Biochem*, 1983; 135(2); 340–4. https://doi.org/10.1016/0003-2697(83)90693-0

[29] Yu LL, Tao SP, Dong XY, Sun Y. Protein adsorption to poly(*ethylenimine*)-modified Sepharose FF: I. A critical ionic capacity for drastically enhanced capacity and uptake kinetics. *J Chromatogr A*, 2013; 1305(30): 76–84. https://doi.org/10.1016/j.chroma.2013.07.014

[30] Torres R, Pessela BCC, Fuentes M, Mateo C, Munilla R, Fernandez-Lafuente R, Guisan JM. Supports coated with PEI as a new tool in chromatography. *Enz Microbiol Technol*, 2006; 39(4): 711–6. https://doi.org/10.1016/j.enzmictec.2005.12.006

[31] Porterfield JZ, Zlotnick A. A simple and general method for determining the protein and nucleic acid content of viruses by UV absorbance. *Virology*, 2010; 407(2): 281–8. https://doi.org/10.1016/j.virol.2010.08.015

[32] Sommer JM *et al*. Quantification of adeno-associated virus particles and empty capsids by optical density measurement. *Mol Ther*, 2003; 7(1): 122–8. https://doi.org/10.1016/s1525-0016(02)00019-9

[33] Dobnik D *et al*. Accurate quantification and characterization of adeno-associated viral vectors. *Front Microbiol*, 2019; 10: 1570. https://doi.org/10.3389/fmicb.2019.01570

[34] Gagnon P, Leskovec M, Prebil SD, Žigon R, Štokelj M, Raspor A, Peljhan S, Štrancar A. Removal of empty capsids from adeno-associated virus preparations by multimodal metal affinity chromatography. *J Chromatogr A*, 2021; 1649: 462210. https://doi.org/10.1016/j.chroma.2021.462210

[35] Block H, Maertens B, Spriestersbach A, Brinker N, Kubicek J, Fabis R, Labahn J, Schäfer F. Immobilized-metal affinity chromatography (IMAC): a review. *Met Enzymol*, 2009; 463: 439–73. https://doi.org/10.1016/S0076-6879(09)63027-5

[36] Gagnon P. Monoclonal antibody purification with hydroxyapatite. *N Biotechnol*, 2009; 25(5): 287–93. https://doi.org/10.1016/j.nbt.2009.03.017

[37] Cotmore SF, Hafenstein S, Tattersall P. Depletion of virion-associated divalent cations induces parvovirus minute virus of mice to eject its genome in a 3'-to-5' direction from an otherwise intact viral particle. *J Virol*, 2020; 84(4): 1945–56. https://doi.org/10.1128/JVI.01563-09

[38] Turnbull AE, Skulimowski A, Smythe JA, Alexander IE. Adeno-associated virus vectors show variable dependence on divalent cations for thermostability: implications for purification and handling. *Hum Gene Ther*, 2004; 11(4): 629–35. https://doi.org/10.1089/10430340050015815

[39] Bennett A *et al*. Thermal stability as a determinant of AAV serotype identity. *Mol Ther Met Clin Dev*, 2017; 6: 171–82. https://doi.org/10.1016/j.omtm.2017.07.003

[40] Croyle MA, Cheng X, Wilson JM. Development of formulations that enhance physical stability of viral vectors for gene therapy. *Gene Ther*, 2001; 8: 1281–90. https://doi.org/10.1038/sj.gt.3301527

[41] Rayaprolu V *et al*. Comparative analysis of adeno-associated virus capsid stability and dynamics. *J Virol*, 2013; 87(24): 13150–60. https://doi.org/10.1128/JVI.01415-13

[42] Ghose S, McNerney TM, Hubbard B. pH transitions in ion-exchange systems: role in the development of a cation-exchange process for a recombinant protein. *Biotechnol Progr*, 2002; 18(3): 530–7. https://doi.org/10.1021/bp020002i

[43] Perez JS, Frey DD. Behavior of the inadvertent pH transient formed by a salt gradient in the ion-exchange chromatography of proteins. *Biotechnol Progr*, 2005; 21(3): 902–10. https://doi.org/10.1021/bp040025s

[44] Lendero N, Vidic J, Brne P, Francovic V, Štrancar A, Podgornik A. Characterization of ion exchange stationary phases via pH transition profiles. *J Chromatogr A*, 2008; 1185(1): 59–70. https://doi.org/10.1016/j.chroma.2008.01.023

[45] Lendero N, Vidic J, Brne P, Podgornik A, Štrancar A. Simple method for determining the amount of ion-exchange groups on chromatographic supports. *J Chromatogr A*, 2005; 1065(1): 29–38. https://doi.org/10.1016/j.chroma.2004.10.072

[46] Reijenga J, van Hoof A, van Loon A, Teunissen B. Development of methods for the determination of pKa values. *Anal Chem Insights*, 2013; 8: 53–71. PMid: 23997574; PMCid: PMC3747999 https://doi.org/10.4137/ACI.S12304

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