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Characterization of Various AAV Subpopulations Separated by the Multimodal Column CIMmultus® PrimaT

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Introduction

In each downstream adeno-associated virus (AAV) process, one of the key steps is the enrichment of full capsids. This can be achieved by density gradient ultracentrifugation; however, a main drawback is its scalability. A more common approach is liquid chromatography using ion exchange chemistries which, based on particles' charge differences, enables the separation of full (F) capsids and product-related impurities including non-functioning AAV capsids (empty, partially filled, misfolded, and wrongly packaged genome or other DNA -containing subspecies).

Sample heterogeneity presents a significant challenge for the downstream process, which aims to isolate and purify only the active drug substance. Relying solely on vector genome estimation can be misleading and may result in the inadvertent pooling of not only potent, intact full AAV capsids but also product-related impurities.

Various factors such as AAV serotype, the expression system (which will produce a specific combination of AAV subpopulation, as well as glycosylation and phosphorylation of capsids, all contribute to slight charge variations. However, relying only on charge differences, using ion exchange columns) results in diminished resolution or a complete lack of separation between the subspecies.

To overcome limitations posed by charge separation, we present a novel multimodal CIMmultus® PrimaT column that enables AAV subspecies separation based on its weak anion-exchange and hydrogen bonding properties withmetal affinity coordination effect.

Materials and Methods

Sample Preparation

An AAV 2 | 8 serotype sample, previously captured by cation exchange chromatography (CIMmultus® SO3), was buffer exchanged in PrimaT loading buffer and loaded on a CIMmultus® PrimaT column at 1.5E+14 viral genomes (vg)/mL of column. The sample (SO3 eluate) consisted of 47% full AAV capsids before any enrichment step. Purification was done using Cytiva ÄKTA pure™ chromatography system.

Characterization of AAV Subpopulations

Collected fractions fromthe CIMmultus® PrimaT column were further analyzed by four orthogonal analytics:

- CsCl density gradient ultracentrifugation coupled with HPLC (UC-HPLC)
- HPLC analytics anion exchange chromatography (AEX) and size exclusion chromatography with PicoGreen (SEC-PG) using PATfix® Analytical System
- Cryo–TEM
- Mass photometry using a SamuxMP from Refeyn

Comparison run

A comparison run was performed using CIMmultus® QA column as a polishing step, both columns, including PrimaT, were loaded at 1E+13 vg/mL column.

Buffering system andmethod

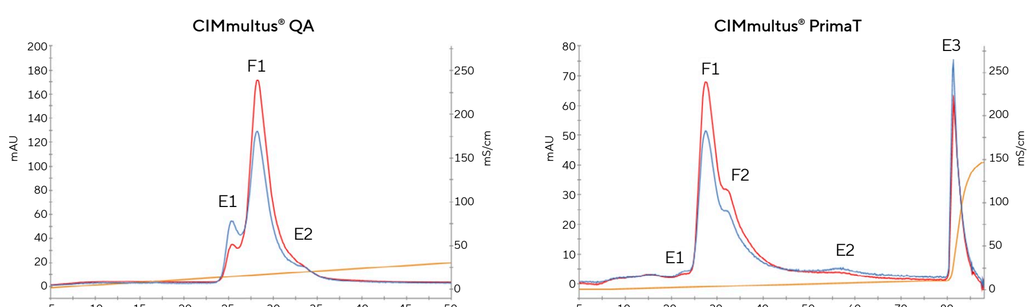
Table 1: *Buffer composition and elution conditions for CIMmultus® QA and PrimaT runs*

	CIMmultus® QA	CIMmultus® PrimaT
A	25 mM BTP, 1% sucrose, 0.1% poloxamer 188, pH 9.0	25 mM HEPES, 1% sucrose, 0.1% poloxamer pH 7.0
B	25 mM BTP, 0.5 M KCl, 1% sucrose, 0.1% poloxamer 188, pH 9.0	50 mM Tris, 13.6 mM borate, 1% sucrose, 0.1 % poloxamer pH 9.0
C	-	50 mM Tris, 9.6 mM borate, 50 mM $MgCl_2$, 1% sucrose, 0.1% poloxamer pH 9.0
D	-	50 mM Tris, 12 mM borate, 2 M NaCl, 1% sucrose, 0.1% poloxamer pH 9.0
Elution	LG, 0-50% B over 50CVs	LG, 0-100% C over 50 CVs (gradient is formed between B and C), then 100% D for 10 CVs

Results - Comparison Run

The landscape of AAV vector-based drug development is marked by significant research and development expenses, estimated to range from US$318 million to US$3 billion per gene therapy [2]. Additionally, the manufacturing capacity for AAV vector-based drugs faces substantial challenges, struggling to keep pace with the growing demands from clinical studies and licensed products [3]. This underscores an urgent need for the development and optimization of systems that streamline both research and development and manufacturing processes.

Figure 1: *Comparison of AAV Subspecies Separation Abilities of CIMmultus® QA and CIMmultus® PrimaT Column (1 mL Bed Volume)*



Note. AAV 2 | 8 sample with concentration 1E+13 vg/mL column was loaded. Methods differ and were optimized for each column, see Table 1. Empty capsids (E) and full capsids (F) are labeled with numbers which indicate individual subspecies eluted from the corresponding column. Legend: A260 (red), A280 (blue), conductivity (orange).

Figure 1 compares the capabilities of strong anion exchanger (CIMmultus® QA) and multimodal column (CIMmultus® PrimaT) for separating AAV subspecies.CIMmultus® QA columns retain their charge over the entire pH range. Therefore altering physicochemical properties, such as pH and conductivity, will affect only the analyte, in our case AAV. CIMmultus® PrimaT goes beyond this; as a weak anion exchanger, it holds charge only in a specific pH range, thereby enabling control over both column and AAV charging. It also uses hydrogen bonding and metal affinity properties. Using these three properties, CIMmultus® PrimaT resolves five individual peaks: three belong to empty and two belonging to full AAV capsid subpopulations.

Results — Characterization of AAV Subspecies

Figure 2: *A) Preparative chromatogram for CIMmultus® PrimaT, loaded at 1.5E+14 vg/mL column. E1 to E5 represent fractions taken, later assessed by orthogonal analytics. Legend: A260 (red), A280 (blue), conductivity (orange). B) Cryo-TEM micrographs for fractions E2, E3, and E5 taken by JEM-2200FS microscope.*

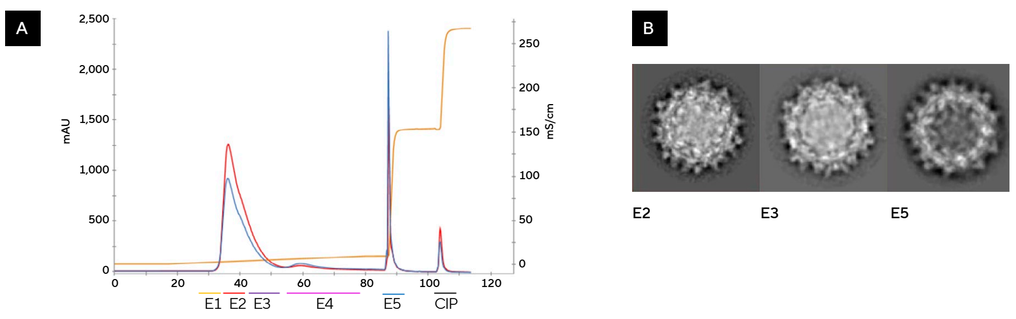
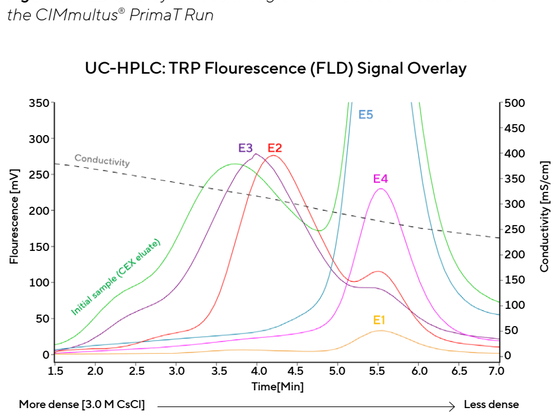
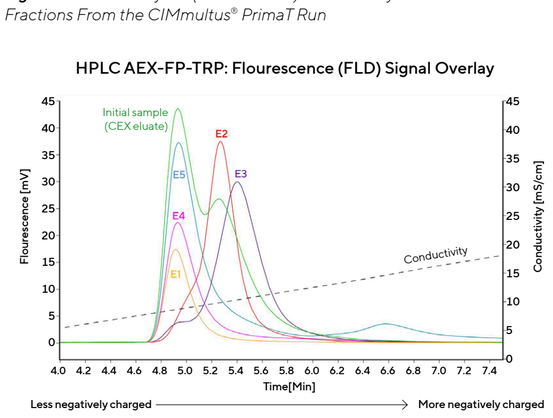


Figure 3: *FLD Overlay Ultracentrifugram of Individual Fractions From the CIMmultus® PrimaT Run*



Note. Fractions were formulated in a solution of 3.0M CsCl and loaded at 3E+12 vg/vial (applies to genome containing fractions). Ultracentrifugation was performed on Sorvall WX90+ system at 245,000 g for 24h. After ultracentrifugation, the content of the vial was ran through PATfix® Analytical System detectors (UV, FLD, MALS), only FLD is shown.

Figure 4: *HPLC Analytics (AEX-FP-TRP) FLD Overlay of Individual Fractions From the CIMmultus® PrimaT Run*



Note. Fractions were applied to a CIMac™ AAV column which, under an ascending salt gradient, separates particles based on their electro-negative charge. This assay enables direct estimation of E/F ratio.

Figure 5: *HPLC Analytics (SEC-FP-PG) For E2 and E3 CIMmultus® PrimaT Fractions Post-Ultracentrifuge*

Note. Fractions were stained with PicoGreen® incubated for 24h and applied to a TSKgel G4000SWXL column, which enabled separation based on size differences and at the same time characterization of dsDNA presence.

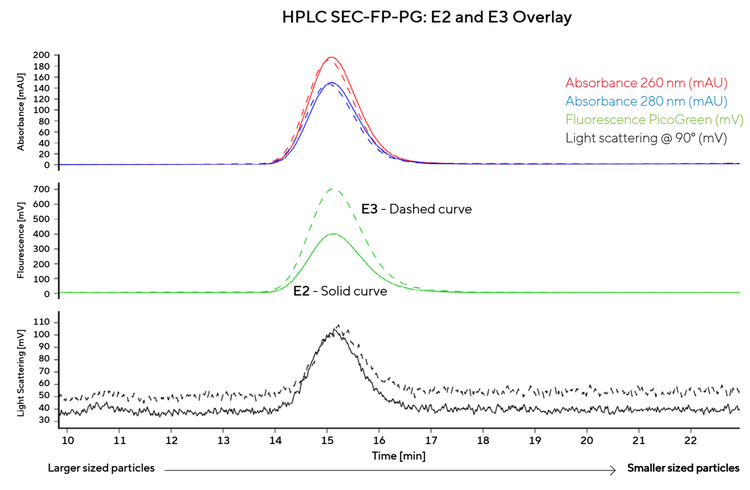
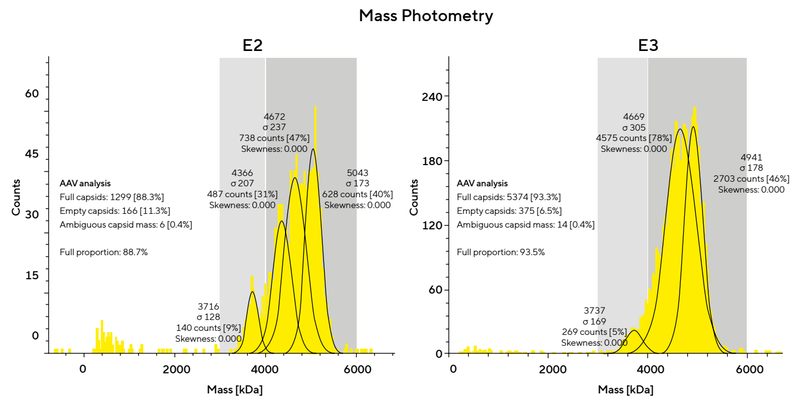


Figure 6: *Mass Photometry Analytics For E2 and E3 CIMmultus® PrimaT Fractions*

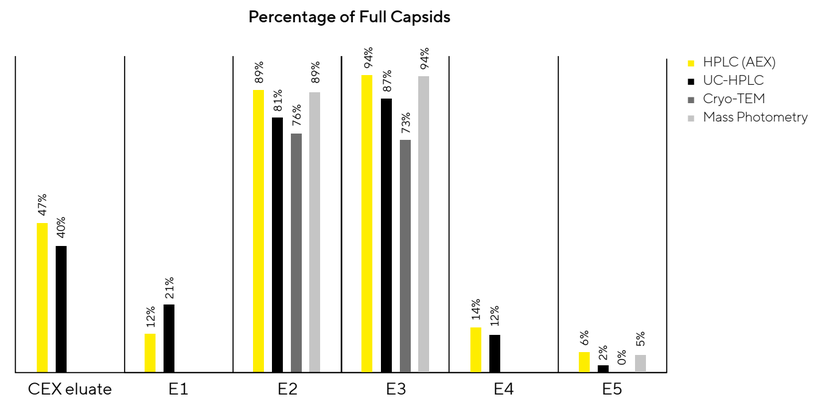
Note. Fractions were analyzed by SamuxMP system from Refeyn. The percentage of E/F capsids and particle mass is presented. Empty capsids have mass of approximately 3.7 MDa where full AAV capsids are found in 4.1- 5.3 MDa range.



The characterization of individual CIMmultus® PrimaT fractions by orthogonal analytics revealed that the complexity of the AAV sample extends beyond empty and full AAV species. At least three subpopulations of empty and two subpopulations of full AAV capsids were characterized. Where as subspecies of empty AAV capsids did not exhibit any significant differences in either UC-HPLC or anion-exchange chromatography, finger print, tryptophan (AEX-FP-TRP) assays, differences were observed for E2 and E3 full AAV subspecies. Figures 3-5 show that the E3 fraction is more dense and has a higher electro-negative charge than the E2 fraction.Moreover, although they possess the same concentration of capsids, as measured byMALS and UV signal, the SEC-FP-PG assay points to a higher presence of dsDNA in the E3 fraction. Mass photometry indicates a higher mass of full AAV capsids in E3, with an average value of 4.69MDa (E2) and 4.81MDa (E3).

Figure 7 demonstrates that the estimations of full AAV capsids from the PrimaT column correlate well with those obtained by other methods. Average values of 83% ± 7%, 87% ± 10%, and 3% ± 78%, are found in E2, E3 and E5 fractions respectively.

Figure 7: *Graph Representing Percentage of Full AAV Capsids Based on HPLC AEX-FP-TRP, UC-HPLC, Cryo-TEM and Mass Photometry Assays*



Note. In case of cryo-TEM, only distinctive full capsids are shown not accounting uncertain species which represent additional 11.63% (E2) and 15.93% (E3).

Conclusion

- A novel column CIMmultus® PrimaT provided significant evidence for ability of AAV subspecies separation.
- There are at least three empty AAV capsid subspecies which are similar in both charge and density.
- In contrast to strong anion exchangers, separation of DNA containing AAV subpopulations was obtained.
- Later eluting fraction (E3) is higher in density, is more electro-negative in charge, containsmore dsDNA and has a higher mass, compared to E2
- HPLC analytics for full capsids estimation using PATfix® correlates well with mass photometry, cryo-TEM and UC results.