SARTORIUS

Simplifying Progress

Characterization of various AAV subpopulation species, separated by multimodal column PrimaT

Rok Žigon[1*], Janja Merkelj Koren[1], Sara Drmota Prebil[1], Andrej Mihevc[1], Maja Štokelj[1], Ažbe Žnidaršič[1], Maja Leskovec[1] and Aleš Štrancar[1]

[1] BIA Separations, now part of Sartorius, Slovenia
Corresponding author: rok.zigon@biaseparations.com

Introduction

In each adeno associated virus (AAV) downstream process one of the key steps is enrichment of full capsids. This could 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 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 poses a cumbersome challenge on downstream process in order to isolate and purify only the active drug substance. Accounting only on vector genome estimation might mislead and result in pooling not only potent intact full AAV capsids, but also product related impurities.

Various factors such as AAV serotype; expression system which produces a combination of AAV subpopulation species; as well as glycosylation and phosphorylation of capsids, all contribute to slight charge variations. Relying only on charge differences, using ion exchange columns, results in diminished or absence of resolution between the capsids subspecies.

To overcome limitations posed by charge separation, we present a novel multimodal PrimaT column that enables AAV sub-species separation based on its weak anion-exchange and H-bond properties with metal affinity coordination effect.

1. Materials and methods

Sample preparation

AAV 2/8 serotype sample, previously captured by cation exchange chromatography (CIMmultus™ SO3), was buffer exchanged in PrimaT loading buffer and loaded on CIMmultus™ PrimaT column at concentration 1.5E+14 vg/mL of column. Sample (SO3 eluate) consisted of 47% full AAV capsids before any enrichment step. Purification was done using Cytiva AKTA Pure 25 chromatography system.

Characterization of AAV subpopulation species

Collected fractions from PrimaT were further analyzed by four orthogonal analytics:

- CsCl density gradient ultracentrifugation coupled with HPLC (UC-HPLC)
- HPLC analytics (AEX and SEC-PG) using PATfix® HPLC 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 and method

	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 MgCl2, 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 50 CVs	LG, 0-100 %C over 50 CVs (gradient is formed between B and C), then 100 %D for 10 CVs

Table 1: Buffer composition and elution conditions for CIMmultus™ QA and PrimaT runs.

2. Results - Comparison run

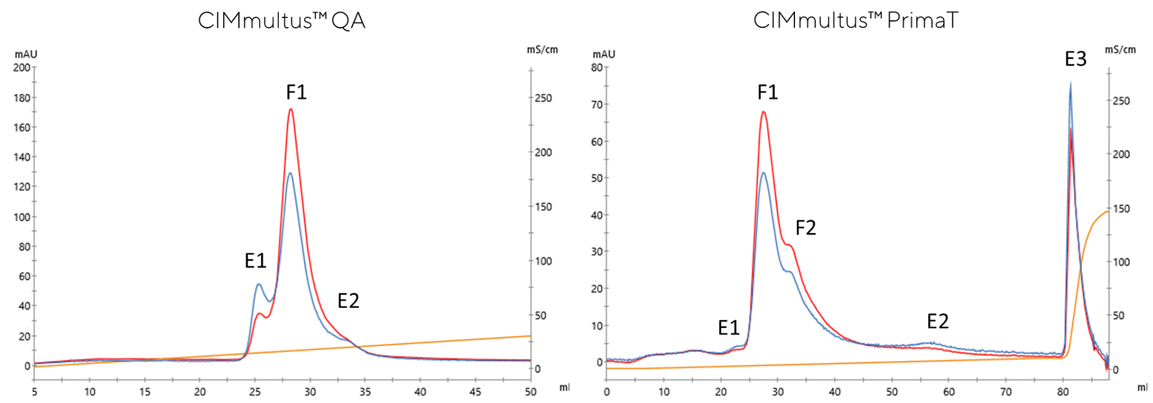

Figure 1: Comparison of AAV subspecies separation ability by CIMmultus™ QA and CIMmultus™ PrimaT column of 1 mL bed volume. AAV 2/8 sample with concentration 1E+13 vg/mL column was loaded. Methods differ and were optimized for each column, see Table 1. Empty capsids (E) and full capsids (F) are labeled with numbers which indicate individual subspecies eluted from the corresponding column. Legend: A260 (red), A280 (blue), conductivity (orange).

Figure 1 compares capabilities of AAV subspecies separation by strong anion exchanger QA and multimodal column PrimaT. In first case column retains charge over entire pH range, therefore altering physicochemical properties, such as pH and conductivity, will affect only the analyte, in our case AAV. PrimaT goes beyond this, since as a weak anion exchanger it holds charge only in a specific pH range and therefore enables control over both, column and AAV charging, as well as use of H-bond and metal affinity properties. By implementation of all three mentioned, PrimaT delivers separation of five individual peaks from which three belong to empty and two to full AAV capsid subpopulations.

3. Results - Characterization of AAV subpopulation species

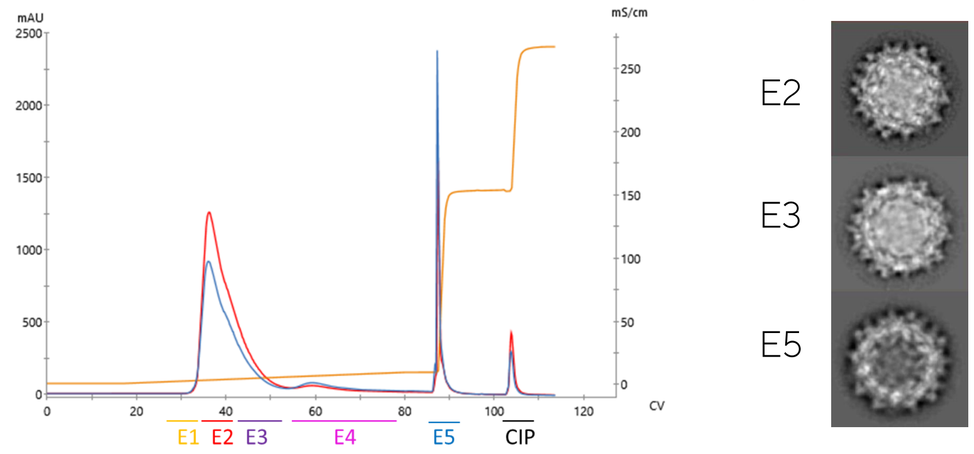

Figure 2: LEFT: Preparative chromatogram for CIMmultus™ PrimaT, loaded at 1.5E+14vg/mL column. E1 to E5 represent fractions taken, later assessed by orthogonal analytics. Legend: A260 (red), A280 (blue), conductivity (orange). RIGHT: cryo-TEM micrographs for fractions E2, E3 and E5 taken by JEM-2200FS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

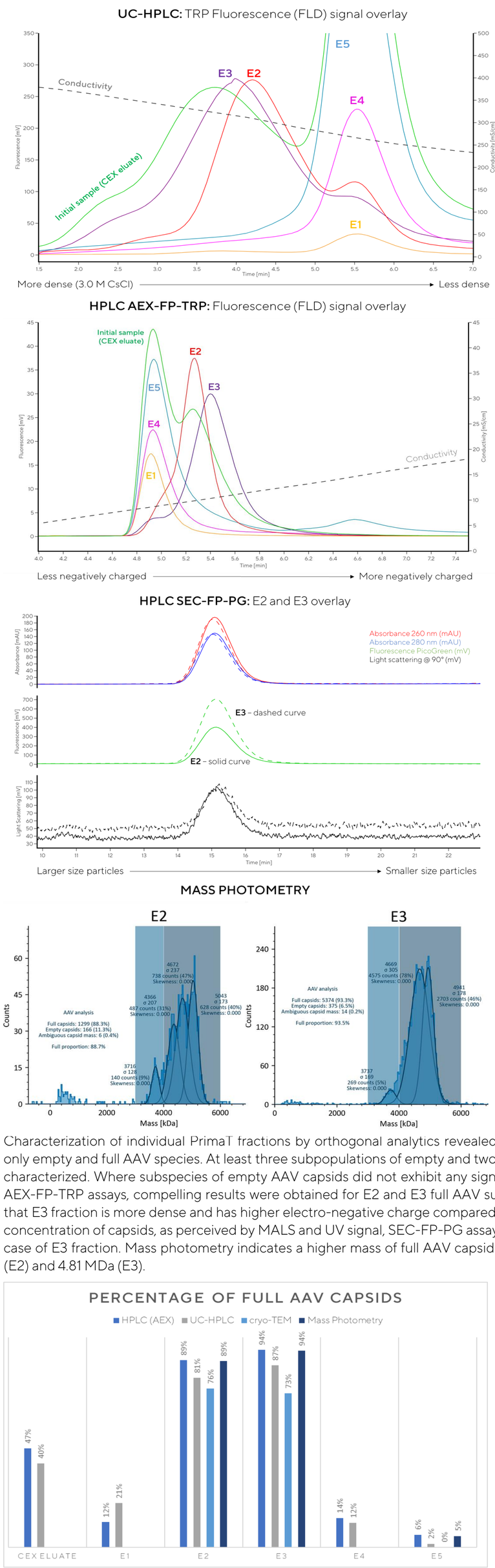

Figure 3: FLD overlay ultracentrifugram of individual fractions from PrimaT run. Fractions were formulated in solution of 3.0M CsCl and loaded at 3E+12 vg/vial (applies to genome containing fractions). Ultracentrifugation was performed on Sorvall WX90+ system at 245,000 g for 24h. After ultracentrifugation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

Figure 4: HPLC analytics (AEX-FP-TRP) FLD overlay of individual fractions from PrimaT run. Fractions were applied to CIMac™ AAV column which under ascending salt gradient separates particles based on their electro-negative charge. Assay enables direct estimation of E/F ratio.

Figure 5: HPLC analytics (SEC-FP-PG) for E2 and E3 PrimaT fraction post ultracentrifuge. Fractions were stained with PicoGreen® incubated for 24h and applied to 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 PrimaT fraction. Fractions were analyzed by SamuxMP system from Refeyn. 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.

Characterization of individual PrimaT fractions by orthogonal analytics revealed that there is more to AAV sample than only empty and full AAV species. At least three subpopulations of empty and two subpopulations of full AAV capsids were characterized. Where subspecies of empty AAV capsids did not exhibit any significant differences in either UC-HPLC or AEX-FP-TRP assays, compelling results were obtained for E2 and E3 full AAV subspecies. From Figures 3-5 one can see that E3 fraction is more dense and has higher electro-negative charge compared to E2 fraction. Moreover, although same concentration of capsids, as perceived by MALS and UV signal, SEC-FP-PG assay points to a higher presence of dsDNA in case of E3 fraction. Mass photometry indicates a higher mass of full AAV capsid in E3, with an average value of 4.69MDa (E2) and 4.81 MDa (E3).

Figure 7: Graph representing percentage of full AAV capsids based on HPLC AEX-FP-TRP, UC-HPLC, Cryo-TEM and Mass Photometry assays. 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).

Figure 7 indicates a good correlation in estimation of full AAV capsids in collected fractions from PrimaT column. Average value of 83% ± 7%, 87% ± 10%, and 3% ± 78%, is found in E2, E3 and E5 fractions respectively.

4. 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, contains more 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.