Development of HPLC-based methods for the systematic quantification of multiple quality attributes of AAV9 preparations

A. Martorana, D. Dey, L. Li, H. Cumar, M. MacDonald, I. Blount, P. Boyce, A. Bloom

AviadoBio Ltd, London, UK

AVIADOBIO

P0027

OBJECTIVE

To develop size exclusion chromatography with multi-angle light scattering (SEC-MALS) and anion-exchange chromatography using fluorescence detector (AEX-FLD) methods for the high-throughput quantification of full and empty capsids in AAV9 preparations, including in-process samples during manufacture.

INTRODUCTION

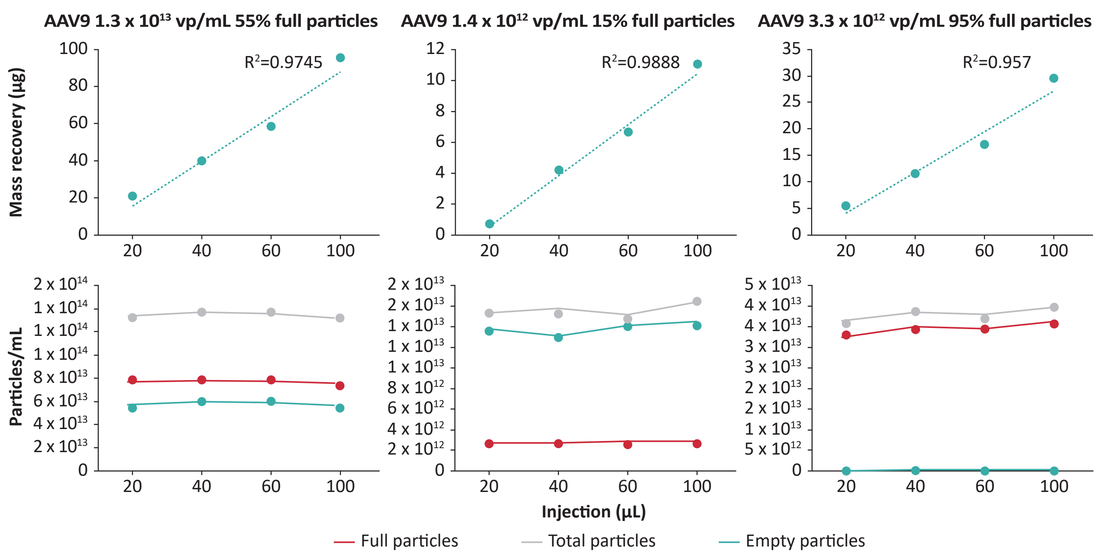
- There are many critical quality attributes (CQAs) that need to be monitored to ensure consistent adeno-associated virus (AAV) product quality, such as purity/identity, titre, structure, impurities, and full/empty capsid ratio.
- Due to their structural complexity, heterogeneity, potential instability, and limited sample availability, characterization of AAV products remains challenging and typically requires a plethora of analytical techniques.
- In addressing some of these challenges, high-performance liquid chromatography (HPLC)-based methods have become an integral part of the analytical toolbox.
- HPLC offers the reproducibility and throughput that other techniques struggle to reach, and when combined with multiple detectors (FLD and MALS), offers excellent sensitivity.
- In this work, we developed AEX-FLD and SEC-MALS methods both used for the quantification of full and empty capsids in AAV9 preparations.

METHODS

Size-exclusion chromatography with multi-angle light scattering

- SEC-MALS detection and differential refractive index detection is a powerful method for determining important CQAs of AAV preparations.
- We developed a SEC-MALS method that allowed accurate determination of genome titre, full/empty ratio, aggregation, and molar mass of AAV9 samples.
- The method showed an excellent level of accuracy and linearity (**Figure 1**), and in only 1-hour running time, provided orthogonal results to droplet-digital polymerase chain reaction (ddPCR), analytical ultracentrifugation (AUC), and transmission electron cryomicroscopy (cryoTEM).

Figure 1: Linearity and mass recovery of SEC-MALS



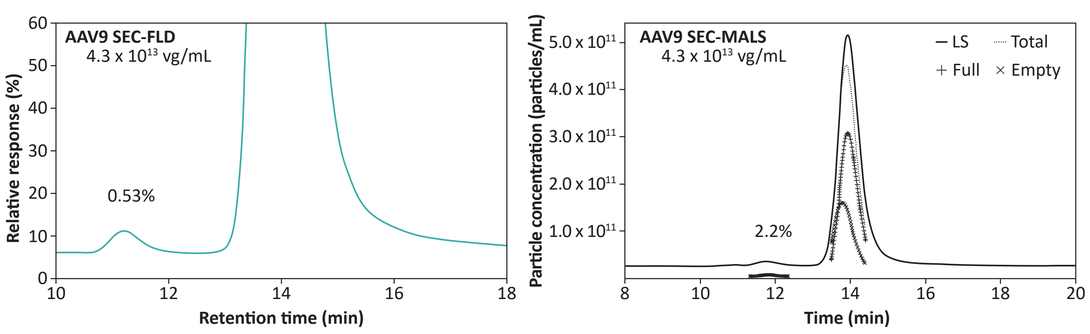
- SEC separates AAV from contaminants present in the sample, hence the technique can easily be adopted for measuring the titre of in-process samples. The results proved to be consistent with titres obtained by ddPCR (**Figure 2**).

Figure 2: In-process sample viral titre comparison

Sample	ddPCR (vg/mL)	SEC-MALS (vg/mL)	Δ (%)
Filtered Lysate	1.28×10^{11}	-	-
Clarified	9.43×10^{10}	-	-
Post-Capture	1.76×10^{13}	1.52×10^{13}	16
Post-Polish	1.09×10^{13}	1.22×10^{13}	11
Drug Substance 1	1.69×10^{13}	1.82×10^{13}	7
Drug Product 1	1.66×10^{13}	1.76×10^{13}	6
Drug Substance 2	1.78×10^{13}	1.86×10^{13}	4
Drug Product 2	1.53×10^{13}	1.65×10^{13}	7

- Aggregation is an important CQA in AAV preparations. Although aggregation is usually measured by SEC-FLD, in line with the principle that bigger particles scatter more light, we found that SEC-MALS detection provided a more accurate determination of the aggregation level (**Figure 3**).

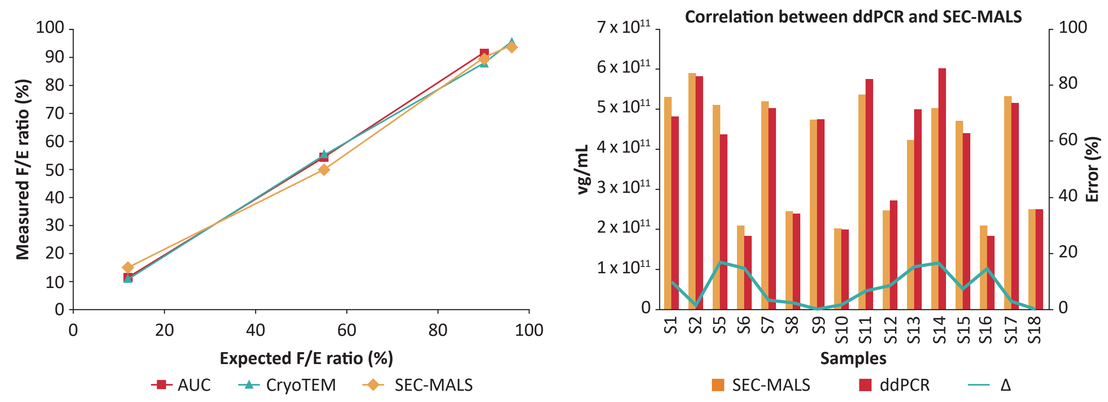
Figure 3: Aggregation detection by SEC-FLD vs SEC-MALS comparison



SEC column: Xbridge Premier GTx BEH 7.8 x 300 mm 450 Å 2.5µm (Waters). Flow rate: 0.8 mL/min.

- Regardless of the concentration of full capsids in the final preparation, SEC-MALS is an orthogonal method to AUC, CryoTEM, and ddPCR (**Figure 4**). The biggest advantages are no sample preparation, high-throughput, and quick turnaround analysis.

Figure 4: SEC-MALS correlation to AUC, CryoTEM, and ddPCR



Anion-exchange chromatography using fluorescence detector

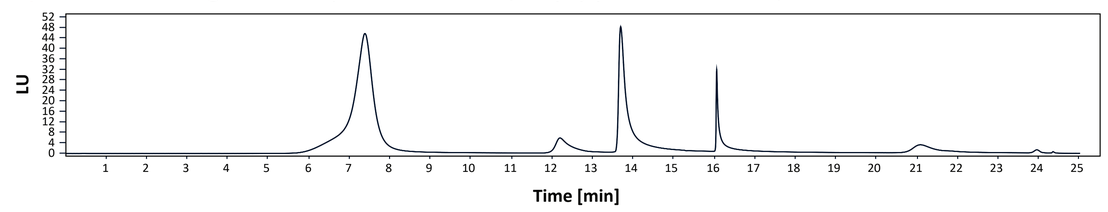
- AEX-FLD is another powerful method for determining the full/empty ratio of AAV9 preparations.
- Due to the presence of negatively charged DNA inside the capsids, the isoelectric point (pI) of full AAV capsids is slightly lower than empty capsids.
- We developed a pH gradient separation that makes use of the differences in isoelectric point (pI) between full (pI 5.9) and empty (pI 6.3) capsids, which allowed full baseline resolution of chromatographic peaks (**Table 1**).
- The sensitivity of this method clearly demonstrates the well-known heterogeneity of AAV preparations. (**Figure 5**, **Table 2**).

Table 1: Experimental conditions for AEX-FLD preparation

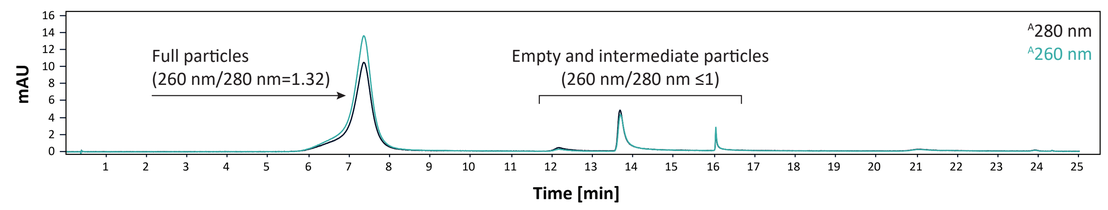
Column: strong anion exchange (-NH_4^+)	Flow rate: 0.8 mL/min
Mobile phase A: pH 9.1	Absorbance: 280 nm Emission: 330 nm
Mobile phase B: pH 2.8	Injection: 4 µL
Sample: ~5 x 10^{13} vp/mL	Column T: 30°C

Figure 5: AEX-FLD response in full/empty capsids

A) Fluorescence signal of sample with 50% full/empty particles. Ex=280, Em=330



B) UV signals of sample with 50% full/empty particles. Sig=260,4 Ref=380,100 Sig=280,4 Ref=380,100



C) Comparison of fluorescence signals between three AAV9 samples. Ex=280, Em=330

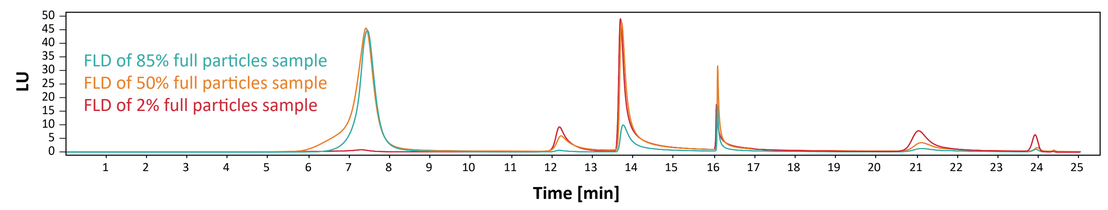


Table 2: SEC-MALS and AEX-FLD comparison

Sample	Full/empty ration by SEC-MALS (%)	Full/empty ration by AEX-FLD (%)	Δ (%)
AAV9 sample 1	55	49	11
AAV9 sample 2	85	79	10
AAV9 sample 3	51	46	7
AAV9 sample 4	3	2	17

CONCLUSIONS

- **In this work, we developed two methods for quantifying full and empty capsids in AAV9 preparations: SEC-MALS and AEX-FLD.**
- **Both techniques have been shown to be precise, linear, robust, and accurate, correlating well with orthogonal methods, such as AUC and ddPCR.**
- **We demonstrated that both SEC-MALS and AEX-FLD methods can be applied during manufacturing to determine full and empty particle ratios in in-process samples.**
- **SEC-MALS offers simultaneous measurement of multiple parameters with minimal sample preparation, while AEX-FLD provides deeper insight into the heterogeneity of AAV preparations.**

ABBREVIATIONS: **AAV:** adeno-associated virus; **AEX-FLD:** anion-exchange chromatography using fluorescence detector; **AUC:** analytical ultracentrifugation; **cp:** copy; **CQA:** critical quality attribute; **Cryo-TEM:** cryogenic transmission electron microscopy; **ddPCR:** droplet digital polymerase chain reaction; **Em:** emission; **Ex:** excitation; **HPLC:** high-performance liquid chromatography; **LU:** luminescence units; **mAU:** milli-absorbance unit; **PCR:** polymerase chain reaction; **pI:** isoelectric point; **SEC-MALS:** size-exclusion chromatography with multi-angle light scattering; **UF/DF:** ultrafiltration/diafiltration; **vg:** viral genome; **vp:** viral particle.

ACKNOWLEDGMENTS & DISCLOSURES: This study was funded by AviadoBio Ltd. **AM**, **DD**, **LL**, **HC**, **MM**, **IB**, **PB**, and **AB** are employees and/or shareholders of AviadoBio Ltd. The authors meet criteria for authorship as recommended by the International Committee of Medical Journal Editors (ICMJE). Medical writing and editorial support was provided by Calum Suggett, MSc, of Costello Medical, UK, and funded by AviadoBio Ltd.

Presented at the 31st European Society of Gene and Cell Therapy (ESGCT) Congress | 22–25 October 2024 | Rome, Italy