

# AN2003: Quantifying AAV aggregation and quality attributes by FFF-MALS

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## Summary

Analysis of adeno-associated virus (AAV) with size exclusion chromatography and multi-angle light scattering (SEC-MALS) quantifies critical quality attributes (CQAs), such as particle concentration and capsid content. However, SEC-MALS is limited in analyzing large AAV aggregates. Field-flow fractionation combined with MALS (FFF-MALS) is an ideal technique for resolving and analyzing large AAV aggregates. Using Wyatt Technology's Eclipse FFF-MALS system, both SEC-MALS and FFF-MALS may be performed with a single set of detectors, providing valuable orthogonal characterization of these gene therapy products.

## Introduction

Gene delivery is one of the most promising therapeutic technologies in active development and commercialization. Adeno-associated virus, or AAV, is one of several carrier vehicles that can deliver the nucleic acids to their intended targets. At approximately 22 nm in diameter, these viruses are smaller than other viral vectors and so deliver a smaller nucleic acid payload than larger viruses, typically around 4.7 kilobases<sup>1</sup>. On the other hand, AAVs produce stable gene expression and elicit low immunogenic response<sup>2</sup>. AAV-based therapeutics have already been approved by the FDA for genetic diseases such as Luxturna® by Spark Therapeutics and Zolgensma® by Novartis.

The emergence of novel AAV therapies necessitates full characterization of their properties during process development and production. Critical quality attributes (CQAs) are quantified to ensure that the final product meets requirements for efficacy and safety. Several of the CQAs for AAVs, including physical viral titer, capsid content, and product stability [have been quantified successfully with size-exclusion chromatography coupled with multi-angle](#)

[light scattering](#) (SEC-MALS) using Wyatt MALS and refractive index detectors<sup>3</sup>. In that analysis, a UV spectrophotometer and an [Optilab® differential refractometer](#) were used in conjunction with a [DAWN® MALS instrument](#) to determine the total AAV concentration, fraction of full and empty AAV, and the relative capsid content ( $c_{vg}/c_{cp}$ ).

While [SEC-MALS](#) measures these CQAs accurately and precisely, the technique is limited with respect to large AAV aggregates. SEC columns may disrupt or filter out large aggregates or, should they pass the column without modification, be unable to resolve them by size, precluding detailed measurement by the downstream MALS detector. Since large aggregates may pose a danger of provoking immunogenic responses, an alternative separation technique is required to provide complete aggregate characterization.

[Asymmetric-flow field-flow fractionation](#) (AF4 or FFF) utilizes an open channel for separation. This removes the possibility of the sample being filtered or sheared, as occurs in SEC columns. Furthermore, FFF coupled with MALS quantifies CQAs in the same manner as SEC-MALS, and additionally provides extended characterization of aggregates and other components.

## Mechanics of FFF separation

FFF separates samples in a channel with an open interior volume. Prior to separation, samples are simultaneously focused into a narrow band by opposing flows, and also depressed against the bottom semipermeable membrane by solvent flowing through the membrane (cross flow). Due to a balance between cross flow and diffusion, each component in the sample equilibrates to a certain height based on its hydrodynamic volume.

Smaller particles diffuse higher into the channel than larger particles and experience faster lateral velocity, due to the parabolic profile of the channel flow, illustrated in

Figure 1. So, like SEC, the elution mechanism is based on hydrodynamic size, but in FFF smaller particles elute first whereas larger particles elute first in SEC.

A MALS instrument and other detectors are required downstream to quantify molar mass, radius, and other key physical properties.

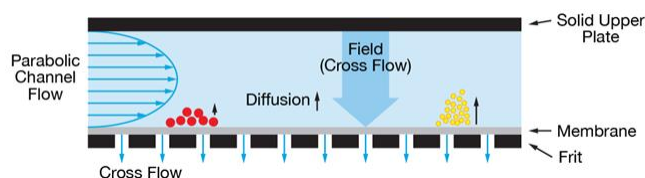


Figure 1. Side view of an FFF channel. The smaller particles (yellow) diffuse higher into the channel against the cross flow than the larger particles (red), thus experiencing a faster channel flow and eluting earlier.

## Materials and methods

Samples consisted of empty, partially-filled, and filled AAVs from [Virovek Inc.](#) The setup, shown in Figure 2, included an Agilent 1260 Infinity™ pump, autosampler, and VWD UV detector. Phosphate-buffered saline (PBS) was used as the mobile phase. Separation was achieved using the Eclipse short channel with 350 μm spacer and 30 kDa cutoff regenerated cellulose membrane. FFF flows were controlled by an Eclipse™ AF4 instrument.

Size, molar mass, and particle concentration were measured using the DAWN® multi-angle light scattering detector and Optilab® differential refractive index detector. Flow was controlled by the VISION® program, and analysis was performed in ASTRA® software.

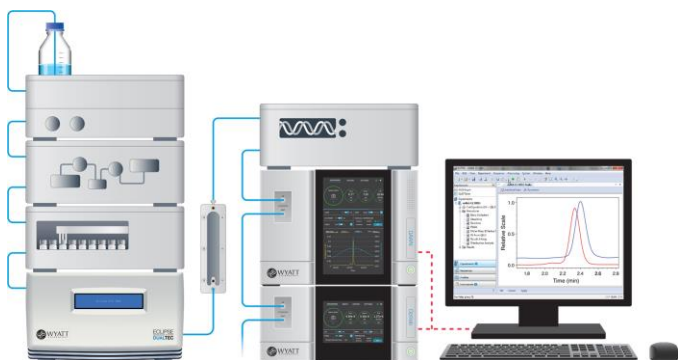


Figure 2. An FFF-MALS system comprises HPLC components together with an Eclipse FFF flow controller and separation channel, a DAWN MALS instrument and Optilab differential refractometer, plus VISION and ASTRA software for control, data acquisition and analysis.

## Results and discussion

### Quantitation of AAV aggregates

SEC columns may provide poor resolution of large aggregates or may exclude them entirely. In the former case, the aggregates will co-elute in the column void volume and prevent accurate quantitation. In the latter case, the aggregates may not be seen at all.

An example of poor SEC resolution is seen in Figure 3. The rms radius  $R_g$  measured by MALS is overlaid on the light scattering chromatogram. The SEC-MALS data appear to show three discrete species: monomer (~14 minutes), oligomer (~12 minutes), and a large aggregate (~10 minutes). While there is some separation between these peaks, they are not baseline-resolved. This co-elution of larger species with the main AAV peak will skew the AAV analysis results since the peak of interest is no longer solely comprised of monomer AAVs. Additionally, the true size distribution of the aggregate peak is obscured since the majority of the particles are co-eluting in the void volume.

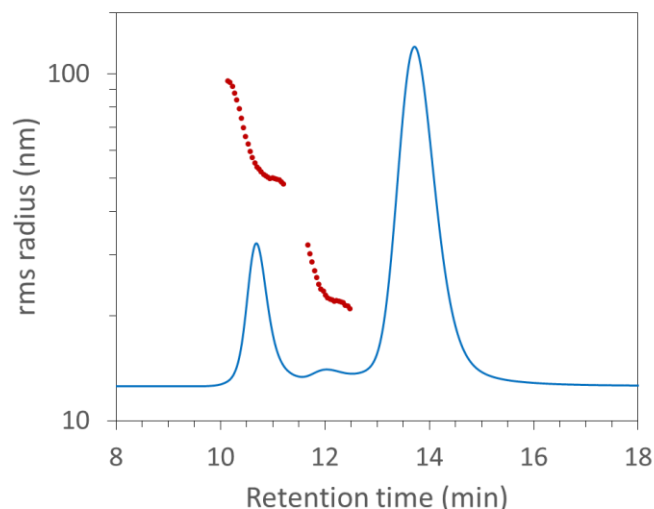


Figure 3. Overlay of rms radius (red) on the light scattering chromatogram (blue) for a full AAV, analyzed by SEC-MALS. The aggregate, oligomeric, and monomer peaks are not well separated.

In contrast, the FFF fractogram plot (Figure 4) shows clear baseline separation between the AAV monomer/oligomer and aggregates. The polydispersity in the aggregates is clearly evident from the slope in rms radius from ~22 minutes elution time to ~30 minutes elution time. This level of detail and ability to

characterize the aggregate size distribution is lost in the SEC-MALS data.

There is also evidence that the SEC column is filtering out a large portion of aggregates. Total particle concentration can be determined from MALS data alone<sup>4,5</sup>. Using this technique, the total number of eluting aggregate particles was determined for the FFF-MALS and SEC-MALS data. The aggregate peak measured by FFF-MALS contained  $1.89 \times 10^{14}$  particles whereas the SEC aggregate peak contained  $1.08 \times 10^7$  particles. This implies that  $<0.00001\%$  of the aggregate particles eluted from the SEC column, and the rest were removed by the stationary phase. Such a large exclusion of aggregates by SEC-MALS illustrates the necessity for FFF-MALS in aggregate assessment.

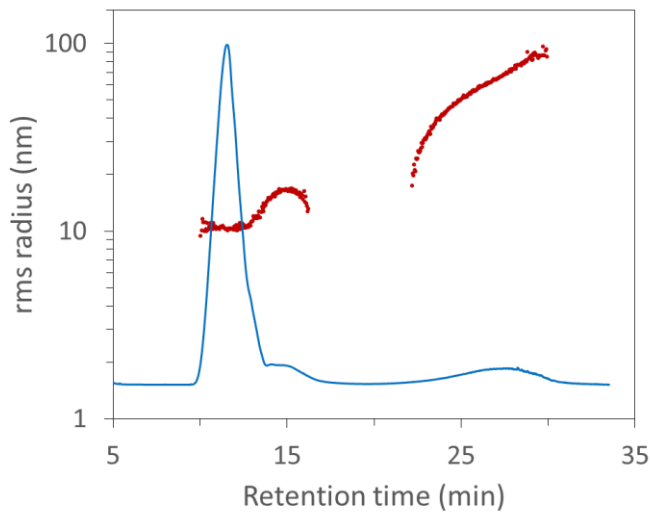


Figure 4. Plot of rms radius (red) vs. elution for a full AAV, analyzed by FFF-MALS. The separation by FFF between the large aggregates and the monomer/dimer is drastically improved over SEC.

FFF is capable of handling even extreme examples where an AAV sample has an inordinate number of very large aggregates. This is seen in Figure 5, where the peak area for the aggregate is three orders of magnitude larger than that of the monomer. In this particular sample, the buffer was formulated with an additive to determine its effect on aggregation. Screening these formulations with SEC-MALS alone would miss these valuable data. In addition, the aggregate load would put the integrity of column at risk; on the other hand, the FFF channel would not encounter any degradation, even with repeated injections.

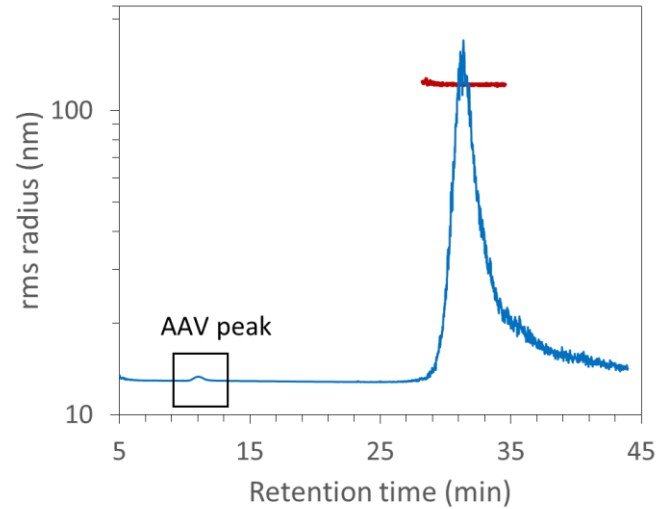


Figure 5. An extreme example of AAV aggregates is shown in this plot of RMS radius (red) vs FFF elution. The light scattering (LS, blue) signal of the  $\sim 120$  nm  $R_g$  aggregates dwarfs the AAV monomer peak.

### Comparison of CQAs from FFF and SEC

Although the primary advantage of FFF-MALS for AAV analysis is in characterizing very large aggregates, the same quantification of CQAs that SEC-MALS provides is also available by FFF-MALS. In Table 1, CQA data derived from FFF-MALS and SEC-MALS of the full AAV in Figure 3 and Figure 4 are compared.

Table 1. Comparison of AAV particle concentrations and  $c_{vg}/c_{cp}$  ratio derived from FFF-MALS and SEC-MALS

	Total particles $c_{cp}$ ( $\text{mL}^{-1}$ )	Full particles $c_{vg}$ ( $\text{mL}^{-1}$ )	$c_{vg}/c_{cp}$
FFF	$3.46 \times 10^{13}$	$2.80 \times 10^{13}$	0.83
SEC	$3.36 \times 10^{13}$	$3.34 \times 10^{13}$	0.97

The total particle concentration  $c_{cp}$  measured by FFF-MALS is within 3% of the total concentration by SEC-MALS. However, FFF-MALS underestimates the amount of DNA payload, as indicated by the lower measured concentration of full particles  $c_{vg}$  and the ratio  $c_{vg}/c_{cp}$ . This discrepancy is primarily due to the higher level of dilution of FFF relative to SEC, which lowers the signal-to-noise ratio (SNR) of the differential refractive index (dRI) signal used in the AAV analysis. Furthermore, the dRI signal can exhibit drift due to channel pressure changes during the FFF method.

Both of these issues—lower SNR and dRI drift—can be overcome by various measures, including injecting more sample, analyzing the protein-RNA conjugate using two UV wavelengths rather than via the combination of a single UV wavelength with dRI, and adjusting FFF experimental conditions to produce less drift. While SEC-MALS remains the primary choice for the analysis of AAV monomers due to its robustness and ease of use, FFF-MALS is an acceptable alternative.

## Conclusions

The emergence of AAVs as a critical therapeutic vehicle has created a need for relevant characterization tools. Here, we showcase FFF-MALS for complete characterization of AAV aggregates, which are typically excluded or poorly separated by SEC columns. In addition, we show orthogonal characterization of AAV CQAs by FFF-MALS. Moreover, both SEC and FFF separation can be controlled by the same HPLC and share MALS and concentration detectors for complete characterization in a single system.

## Acknowledgements

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## References

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