

Nota de aplicación

## Heightened Characterization of AAVs by SEC-MALS with XBridge™ Premier GTx BEH™ SEC 450 Å 2.5 µm Columns

---

Lavelay Kizekai, Balasubrahmanyam Addepalli, Matthew A.  
Lauber

Waters Corporation

For *in vitro* diagnostic use. Not available in all countries.

---

### Abstract

Gene therapy (GTx) products comprised of adeno-associated viral vectors (AAVs) require detailed characterization to ensure their safety and efficacy. In this application note, we evaluate the utility of XBridge Premier GTx BEH SEC 450 Å 2.5 µm columns for size exclusion chromatography coupled with multiangle light scattering (SEC-MALS) and the biophysical analysis of AAVs. High

---

throughput size variant analysis is made possible by a combination of new column hardware manufactured with hydrophilic MaxPeak High Performance Surfaces (h-HPS) and high efficiency packed beds consisting of 2.5  $\mu\text{m}$  diol bonded 450  $\text{\AA}$  BEH particles. These SEC columns exhibit enhanced sensitivity for low volume samples, low MALS noise and little to no particle shedding. Accurate molar mass and size measurements are thus facilitated for sample components. This SEC-MALS technique can generate detailed information about process and product related impurities that is critical to process development decisions and the preparation of comprehensive regulatory submissions.

## Benefits

- AAV SEC separations with a well-matched fractionation range (450  $\text{\AA}$  pores) and high efficiency 2.5  $\mu\text{m}$  packing material
- Low MALS noise suitable for high confidence measurements of intact AAV
- Chromatographic resolution to quickly distinguish aggregates from monomer for robust relative quantitation
- Enhanced sensitivity and recovery for low volume samples across multiple AAV serotypes

---

## Introduction

Adeno-associated virus (AAV) is a leading gene therapy modality for *in vivo* delivery of transgenes to correct genetic

disorders in human cells. Currently, there are three AAV drug products approved by the FDA to treat hemophilia B, spinal muscular atrophy, and retinal diseases.<sup>1</sup> There are also more than 200 AAV-based gene therapy drugs in various stages of development and clinical trials.<sup>2</sup> The titer, potency and purity of these drug products must be precisely controlled because reaching a safe and efficacious dosing can be difficult. Higher doses can cause toxicity and deaths.<sup>3</sup> Precise knowledge of the immunogenic components such as the presence of empty and aggregated capsid forms is also necessary. Viral capsids in general act as colloidal particles with defined densities and tendency to aggregate. Aggregation slows diffusion, decreases the surface-to-volume ratio and the number of effective viral particles, which means aggregate levels are a critical quality attribute and they must be quantified.<sup>4</sup>

Size exclusion chromatography in combination with multiangle light scattering (MALS) is a direct approach to characterizing AAV sample components including size-distribution, molar mass of capsid and DNA, aggregation profile and titer.<sup>5</sup> In this application note, we show that the XBridge Premier GTx BEH SEC 450 Å 2.5 µm Column provides high sensitivity and efficiency for molar mass, size measurements, aggregate detection, and empty and full capsid ratio monitoring. It is also shown that excellent results can be achieved with both 4.6- and 7.8-mm diameter columns. This type of comprehensive characterization will help drug developers make quick multiattribute measurements.

---

## Experimental

2x PBS Preparation: Phosphate buffered saline salt mixture (Sigma (p/n: P-3583) from four packets were dissolved in 2 L of MΩ water to make 20 mM Phosphate, 276 mM NaCl, 5.4 mM KCl pH 7.4. The solution was filtered with a 1000 mL Nalgene™ Rapid-Flow™ Sterile Disposable PES filter with 0.1 μm pore size unit (p/n: 567-0010).

Bovine serum albumin (BSA) (heat shock fraction, Sigma, p/n: A8022-10g) was prepared by gentle dissolving in PBS at 2 mg/mL and 4.5 mg/mL concentration. Dissolved protein solutions were filtered using 0.02 μm pore size CYTIVA Whatman 10 mm diameter syringe filter (p/n: 6809-1002).

Bovine thyroglobulin (BTG) (Sigma, p/n: T1001-100mg) was prepared by dissolving in PBS at 4.5 mg/mL concentration and filtered through Low Protein binding Millex-HV Filter with 0.22 μm pore size (EMD Millipore, p/n: SLHVR13SL).

AAV2-EMPTY 2E+13 vg/mL, AAV2-CMV-GFP 2E+13 vg/mL, AAV9-EMPTY 2E+13 vg/mL, and AAV9- CMV-GFP 2E+13 vg/mL were obtained from Virovek and stored at -80 °C. Prior to use, samples were thawed to room temperature, mixed gently with a pipette, and centrifuged using a Benchmark Scientific Mini Centrifuge for 1 min at 15,000 rpm.

AAV9-Empty and AAV9-CMV-GFP (full) sample preparation: After thawing and mixing samples as described above, Empty and Full capsids were prepared at 1:0, 1:1, 1:2, 1:5, 1:10, and 0:1 ratio, mixed gently and centrifuged for 1 min. The CMV-GFP genome in the above capsids is estimated to be

2.5 kb. It is possible that these full AAV samples have some population of overfilled (2x genome) capsids.

## LC Conditions

LC system:	ACQUITY™ UPLC H-Class Bio
Detection:	ACQUITY TUV Detector (Titanium Flow Cell, 5 mm, 1500 nL)
Wavelength:	280 nm
Detection:	ACQUITY RI Detector
Data acquisition	Empower™ Pro 3 Feature Release 3
Vials:	Max Recovery Vials and Caps (p/n: 186000327C) and 300 µL polypropylene screw neck vial (Waters p/n: 186004112)
Column(s):	XBridge Premier GTx BEH SEC 450 Å 2.5 µm Column, 4.6 x 150 mm (p/n: 186010584)  XBridge Premier GTx BEH SEC 450 Å 2.5 µm Column, 4.6 x 300 mm

(p/n: 186010585)

XBridge Premier GTx

BEH SEC 450 Å 2.5 µm

Column, 7.8 x 300 mm

(p/n: 186010586)

Column temperature.:	30 °C
Sample temperature.:	6 °C
Sample manager washes:	18.2 MΩ water
Seal wash:	10% HPLC grade Methanol/90% 18.2 MΩ water (v/v)
Injection volume:	BSA (10 µL of 2 µg/µL); AAVs (5 µL to 30µL)
Flow rate:	0.20 mL/min
Mobile phase A:	2X PBS: Phosphate Buffered Saline (20 mM Phosphate, 276 mM NaCl, 5.4 mM KCl pH 7.4)
Sample(s):	BSA 2 µg/µL; BSA 4.5 µg/µL; Thyroglobulin 4.5 µg/µL; AAV2- EMPTY 2E+13 vg/mL,

AAV2-CMV-GFP 2E+13  
vg/mL, AAV9-EMPTY  
2E+13 vg/mL, AAV9-  
CMV-GFP 2E+13 vg/mL

Gradient: Isocratic

## SEC-MALS

System: WYATT DAWN™ (Neon)  
with a Wyatt QELS™  
embedded online  
dynamic light scattering  
(DLS) module

Detection: 18-Angles Light  
Scattering with QELS

Wavelength: 659 nm

Data acquisition ASTRA™ 8

---

## Results and Discussion

SEC separates sample components in solution by size. Therefore, monomeric AAV capsids can be easily distinguished from aggregates and extrinsic impurities. When paired with MALS, ultraviolet (UV), and refractive index (RI) detectors, SEC can provide detailed measurements of an AAV sample without a standard curve

through the simultaneous use of absorbance, light scattering, and refractive index data. During MALS detection, component species will scatter light depending on their size and concentration in solution. The angular dependence of scattered light intensity determines the radius of gyration ( $R_g$ , also called root mean square radius), which can be readily measured by the multiple angles (up to 18 angles) by the DAWN instrument. Intensity of the scattered light depends on the concentration and the molar mass of the materials. By combining MALS data with on-line concentration detector, such as UV or RI, the absolute molar mass can be determined. And the hydrodynamic radius ( $R_h$ ) is derived from fluctuations of light scattering intensity due to the diffusion of molecules (Brownian motion).<sup>6</sup> Dynamic light scattering (DLS) through a QELS (quasi-elastic light scattering) module measures the fluctuations in the scattered light intensity at single angles in a continuous flow mode without perturbing the system to measure the hydrodynamic radius. Wyatt ASTRA software leverages the combined UV, RI, MALS, DLS data to determine the physical attributes of an analyte, including its particle size, molar mass, and compositions (such as  $V_g/C_p$  for AAV).

An XBridge Premier GTx BEH SEC 450 Å 2.5 µm SEC 4.6 x 150 mm Column was first used to analyze BSA (bovine serum albumin), bovine thyroglobulin (BTG) and AAV2 samples. Figure 1a shows the resulting chromatograms, and Table 1 denotes the absolute molar masses observed for various components within these samples. As predicted, the AAV2 monomer eluted (12.2 minutes) earlier than the BTG monomer (13.7 minutes) and the BSA monomer (17.0 minutes). However, the soluble aggregates within these



samples presented interesting behavior. While the BSA dimer, trimer and other potential higher order aggregates eluted much later (after 14 minutes), the BTG dimer (~1,300 kDa) exhibited overlapping elution with the AAV2 monomer (~3,700 kDa). Similarly, the BTG trimer overlapped with the AAV2 dimer- trimer and BTG tetramer with the higher order aggregates of the AAV2 sample. Wyatt's ASTRA software generated biophysical properties for all components as detailed in Table 1.

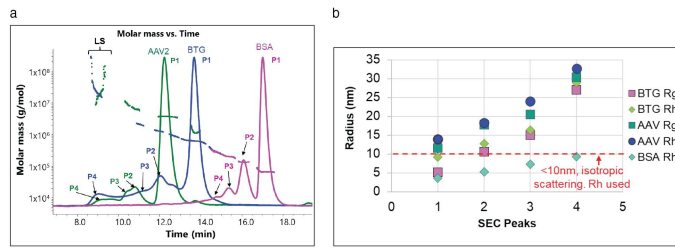


Figure 1a. SEC chromatograms and molar mass vs time data for BSA (pink 2 mg/mL), BTG (4.5 mg/mL, blue), and AAV2-empty capsid ( $2E+13$  vg/mL, green). Sample traces are provided as obtained with  $90^\circ$  angle LS (color coded for each sample) and the use of an XBridge Premier GTx BEH SEC 450 Å 2.5  $\mu$ m, 4.6 x 150 mm Column. Note the elution time differences between three different proteins and aggregates. Although the AAV2 monomer and BTG dimer exhibit similar retention times, MALS provides an accurate molar mass determination regardless of elution time.

b. Plot depicting the measured radius of gyration ( $R_g$ ) and hydrodynamic radius ( $R_h$ ) according to the peaks detected during the SEC-MALS analysis. Comparisons of the measured radii ( $R_g$  and  $R_h$ ) is provided for various protein components. A MALS threshold of <10 nm size is indicated by the red line for where isotropic scattering is predicted to occur.

Protein	Monomer (Peak 1)				Dimer (Peak 2)				Trimer (Peak 3)				Higher order aggregate (Peak 4)			
	Elution time (min)	Molar mass (kDa)	Rg (nm)	Rh (nm)	Elution time (min)	Molar mass (kDa)	Rg (nm)	Rh (nm)	Elution time (min)	Molar mass (kDa)	Rg (nm)	Rh (nm)	Elution time (min)	Molar mass (kDa)	Rg (nm)	Rh (nm)
BSA	17.0	65.4	N/D	3.6	16.1	131.4	N/D	5.22	15.4	199.6	N/D	7.3	14.8	283.7	N/D	9.3
BTG	13.7	616.1	5.1	9.2	12.0	1277.1	10.6	12.7	11.1	2275.6	15.0	16.3	9.0	22611.7	27.0	28.9
AAV2	12.2	3678.4	11.6	13.9	10.8	6437.2	17.7	18.3	10.4	9239.4	20.5	24.0	9.1	20827.4	30.4	32.7

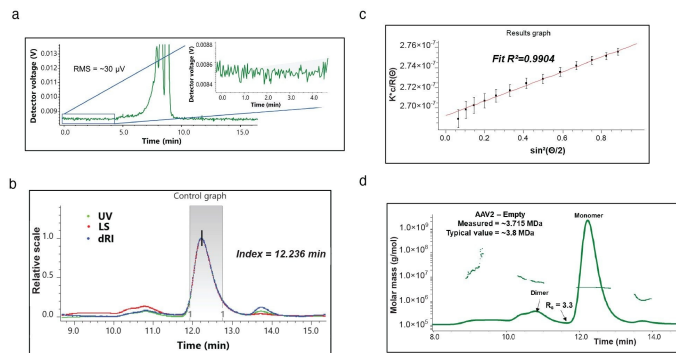
*Table 1. Biophysical properties of AAV2, BTG, and BSA determined by SEC-MALS using XBridge Premier GTx BEH SEC 450 Å 2.5 µm, 4.6 x 150 mm Column. The SEC peaks labeled as P1-P4 for each chromatogram in Figure 1 correspond to the monomer, dimer, trimer, and higher order aggregates (HOA) of each protein based on the molar mass measurements.*

Overlapping elution profiles indicate similar hydrodynamic radii. Indeed, the AAV2 monomer exhibited 11.6 nm and 13.9 nm for Rg and Rh values, which are very close to the measurements reported for AAV9.<sup>7</sup> Interestingly, the BTG dimer (peak 3) showed 10.6 and 12.7 nm values for Rg and Rh, respectively, which are close to the AAV2 monomer (Figure 1b). The AAV dimers (Rg:17.7 nm and Rh:18.3 nm) and higher order aggregates exhibited proportionally larger Rg and Rh measurements. Although Rg measurements for BSA (an isotropic scatterer) were below the MALS measurement limit of 10 nm, QELS based detection allowed the determination of Rh values, as shown in Figure 1b.

In some cases, it is of interest to interpret Rg/Rh ratios, because it is indicative of conformational information. The Rg/Rh ratio for globular, spherically folded proteins is expected to be around ~ 0.78.<sup>8</sup> The calculated Rg/Rh for the AAV monomer (Peak 1), dimer (Peak 2), and trimer (Peak 3) species were approximately 0.81. This value was slightly higher at 0.91 for the higher order aggregates of the

AAV (Peak 4), suggesting that they might have extended conformational arrangements. Furthermore, the polydispersity value (the Mw/Mn ratio) for AAV Peak 4 was found to be 1.30, indicating there to be significant polydispersity.

These measurements could be made with high confidence because the XBridge Premier GTx BEH SEC 450 Å 2.5 µm Column exhibits low MALS noise and high sensitivity MALS signal detection (Figure 2) even under low flow rate (<0.3 mL) conditions. Moreover, the extent of resolution provided by these columns ensures accurate extrapolation of data for the exact peak of interest. The measured molar mass for the AAV monomer at the indicated 12.2 minutes elution time was approximately 3.7 MDa, which is close to the average theoretical value of ~ 3.8 MDa for AAVs. The fully resolved dimeric form (Rs=3.3) at 10.5 minutes exhibited a measured molar mass of 6.4 MDa. The shoulder to the left of the dimer at 10.8 minutes showed a molar mass of 9.1 MDa, which matches to the aggregation of approximately two monomers and half monomeric AAV species. Fragmented forms of the AAV or potential process related impurities was observed at 13.7 min with a molar mass of ~1.2 MDa.

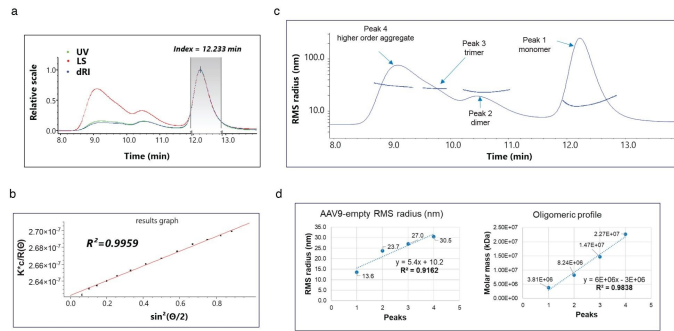


*Figure 2. Features of AAV2-EMPTY capsids following SEC-MALS analysis with an XBridge Premier GTx BEH SEC 450 Å 2.5 µm 4.6 x 300 mm Column. A. RMS noise (0-4.5 minutes) for MALS detection is shown. Note the noise value (30 µV) is much less than the standardized noise threshold (100 µV).  
 b. Superimposition of the UV absorbance, LS and dRI signal for the AAV2 capsids. The chromatographic peak selected for angular dependence is shown with the shaded box.  
 C. Debye plot ( $K \cdot c / R_{\theta}$  vs  $\sin^2 \theta$ ) of the shaded peak from panel b. This plot drops the lower and higher angle contributions (front 1, 2, and back 17 & 18 detectors). Notice the linearity of angular dependence of scattered light detected by the multiple LS detectors.  
 d. Molar mass measurements of AAV2 components.*

It should be noted that these AAV samples were expressed with a relatively small CMV-GFP genome. As such, it is possible for this sample to contain a population of both single and double genome containing AAVs (*i.e.* 2.5 kb and 5 kb packaged capsids).

AAVs exist as multiple serotypes and not all of them exhibit similar component profiles. To probe this possibility, an

AAV9 serotype (empty capsid) was evaluated by SEC-MALS (Figure 3). The XBridge Premier GTx BEH SEC 450 Å 2.5 µm 4.6 x 300 mm Column effectively resolved aggregates from the monomer for AAV9 as it did for the AAV2 sample.

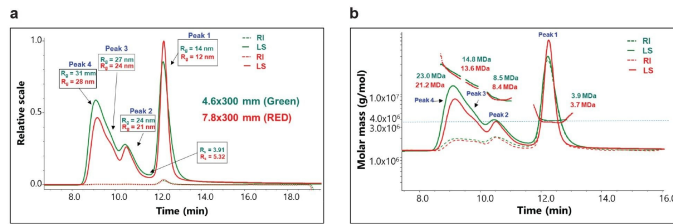


*Figure 3. SEC-MALS analysis of AAV9-Empty capsids using an XBridge Premier GTx BEH 450 Å 2.5 µm SEC 4.6 x 300 mm Column. a. Overlay of the UV absorbance (green), Refractive Index (blue), and the light scattering (red) traces with a monomer peak apex selection as shown. b. Linearity of angular dependence as demonstrated with a Debye plot. c. RMS radius (R<sub>g</sub>) plot of AAV9 component peaks. d. Linear relationship of the AAV9 component peaks for the RMS radius (R<sub>g</sub>) and the molar mass.*

Interestingly, AAV9 exhibited higher amounts of higher order aggregates compared to AAV2 (Figures 1–2). The measured molar mass of monomeric AAV9 eluting at 12.2 minutes (Peak 1) was found to be 3.8 MDa. The well resolved dimer (Peak 2) eluting at 10.50 minutes (R<sub>s</sub>=3.81) exhibited a molar mass of 8.2 MDa. Furthermore, the RMS radius (or R<sub>g</sub>) and molar mass of the aggregates showed

good correlation (Figure 3c–3d).

The three detectors employed in this SEC-MALS work differed in their flow cell volumes (UV=5  $\mu$ L, MALS=70  $\mu$ L, and RI=1.3  $\mu$ L). The large volume of the MALS flow cell meant that peak broadening could be problematic under specific situations. To understand this influence, two column formats that differ in their diameter (4.6 mm or 7.8 mm) but not length (300 mm) were employed to analyze the AAV9 sample. The injection volume and flow rate were scaled up as recommended by Empower software (15  $\mu$ L for 4.6 mm, 43.1  $\mu$ L for 7.8 mm, and the flow rate from 0.2 mL to 0.575 mL, respectively) to account for the increase in column diameter. Overlaid SEC-MALS traces from these columns (Figure 4) showed excellent agreement in terms of the peak profiles. As predicted, the 7.8 mm column provided slightly higher resolution, and that might be reflected in the slight variation observed for molar mass and  $R_g$  measurements between the two columns.



*Figure 4. Comparison of SEC-MALS data from XBridge Premier GTX BEH SEC 450 Å 2.5 µm columns with two different dimensions (4.6 x 300 mm and 7.8 x 300 mm). Overlay of AAV9 SEC-MALS traces acquired on a 4.6 x 300 mm column (green) versus 7.8 x 300 mm column (red) and the values observed for capsid radius (a) and molar mass (b).*

Total capsid concentration (C<sub>p</sub>) and viral genome concentration (V<sub>g</sub>) titers are most often measured by ELISA and qPCR, but those techniques can be time-intensive and highly variable.<sup>5</sup> The Wyatt ASTRA software (version 8) can combine UV absorbance at 280 nm and RI information with the MALS data to provide the protein and nucleic acid information, independently. This can be most valuable for determining empty-full ratios. To that end, fixed ratios of empty and full AAV capsids were injected and subjected to SEC-MALS analysis to determine the linearity of this measurement. Figure 5 illustrates the measurements of the absolute molar masses across the ratios of empty to full AAV9 capsids. SEC separates molecules based on their size in solution. Since the empty and full AAV share the same hydrodynamic radius, both full and empty capsids coelute in the SEC monomer peak. However, with the above-mentioned data analysis, protein mass, nucleic acid mass, and total mass can be differentiated. Upon analysis of the



varying empty-full ratios, a linear increase in nucleic acid mass was achieved with an  $R^2$  value of 0.9998. It should be noted that these AAV samples were expressed with a relatively small CMV-GFP genome. As such, it is possible for this sample to contain a population of both single and double genome containing AAVs (*i.e.* 2.5 kb and 5 kb packaged capsids).

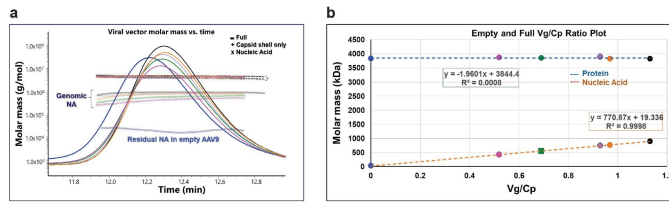


Figure 5. SEC-MALS based differentiation of Full and empty AAV capsids. a. Defined ratios of empty and full capsids of AAV9 (1:0 (blue), 1:1 (pink), 1:2 (green), 1:5 (purple), 1:10 (orange), and 0:1 (black)) were mixed and analyzed. The ASTRA software differentiates full capsids (-) of AAV9 (capsid shell and genome), capsid shell alone (+), and nucleic acid (NA) (x) in terms of their molar mass. The measured values were 3,851 kDa and 4,709 kDa for empty and full capsids, respectively. The residual nucleic acid in empty capsids and genomic nucleic acids in full capsids exhibited a mass of ~20 kDa and ~891 kDa, respectively.<sup>5</sup> Again, the presence of some overfilled capsids might have produced a slightly increased, average genome mass versus the value predicted for a single, 2.5 kb genome containing capsid.

b. Plot between molar mass and vg/cp ratio of the full and empty capsid AAV9 mixtures. Note the constant values of the measured mass for capsid protein shell (blue dashed line) across all ratios, and the empty vs full capsid ratio dependent values for vg/cp (viral genome/coat protein). The Vg/Cp ratios of 0.00, 0.52, 0.69, 0.93, 0.97, and 1.13, correspond well with the 1:0, 1:1, 1:2, 1:5, 1:10, and 0:1 ratios, respectively.

---

## Conclusion

In this application note, we illustrate the ability of XBridge Premier GTx BEH SEC 450 Å 2.5 µm columns to give excellent resolution and efficient MALS analysis of different AAV serotypes. Sample components were separated with high resolution which made it possible to readily determine their biophysical properties, including molar mass, size, and aggregate composition. These studies also demonstrate that the column pore size is appropriately matched to give efficient fractionation of the aggregates, monomers, and low molecular weight impurities of various AAV samples. Furthermore, this work shows that a 4.6 mm diameter SEC column can be paired with a large flow cell MALS detector, though resolution and size measurements seem to be best optimized with a wide diameter (7.8 mm ID) column. Finally, we have shown that SEC-MALS provides a powerful multiattribute analysis as demonstrated with the determination of empty and full capsid ratios along with quick absolute molar mass and size measurements.

---

## References

1. Fekete, S., *et al.*, Chromatographic Strategies for the Analytical Characterization of Adeno-Associated Virus Vector-Based Gene Therapy Products. *TrAC Trends in Analytical Chemistry*, 2023. 164: p. 117088.
2. AAV Vectors in Gene Therapy: How Recent Clinical Advances are Unraveling New Potentials? *DelveInsight Blog*, 2022.

3. High-dose AAV gene therapy deaths. *Nature Biotechnology*, 2020. 38(8): p. 910–910.
4. Pradhan, S., *et al.*, Viral Aggregation: The Knowns and Unknowns. *Viruses*, 2022. 14(2).
5. McIntosh, N.L., *et al.*, Comprehensive Characterization and Quantification of Adeno Associated Vectors by Size Exclusion Chromatography and Multi Angle Light Scattering. *Scientific Reports*, 2021. 11(1): p. 3012.
6. Online MALS-QELS (Quasi-Elastic Light Scattering). Wyatt Technology. AN7301.
7. Michelle Chen, A.P., Quantifying quality attributes of AAV gene therapy vectors by SEC-UV-MALS-dRI. Wyatt Technology. AN1617.
8. Tande, B.M., *et al.*, Viscosimetric, Hydrodynamic, and Conformational Properties of Dendrimers and Dendrons. *Macromolecules*, 2001. 34(24): p. 8580–8585.

---

## Featured Products

[ACQUITY UPLC H-Class PLUS Bio System <](https://www.waters.com/10166246)

[https://www.waters.com/10166246>](https://www.waters.com/10166246)

[ACQUITY Arc Bio System <](https://www.waters.com/waters/nav.htm?cid=134966135)

[https://www.waters.com/waters/nav.htm?cid=134966135>](https://www.waters.com/waters/nav.htm?cid=134966135)

[ACQUITY UPLC Tunable UV Detector <](https://www.waters.com/514228)

[https://www.waters.com/514228>](https://www.waters.com/514228)

[2414 Refractive Index \(RI\) Detector </nextgen/es/en/products/chromatography/chromatography-detectors/2414-refractive-index-ri-detector.html>](https://www.waters.com/nextgen/es/en/products/chromatography/chromatography-detectors/2414-refractive-index-ri-detector.html)

Empower Chromatography Data System <  
<https://www.waters.com/10190669>>

720007969, June 2023

© 2023 Waters Corporation. All Rights Reserved.

[Condiciones de uso](#) [Privacidad](#) [Marcas comerciales](#) [Empleo](#) [Cookies](#) [Preferencias de cookies](#)