Comprehensive analysis of adeno-associated virus quality using 3 µm monodisperse Strong Anion Exchange and Size Exclusion chromatography columns

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Abstract

Purpose: Demonstrate robust and easy-to-run methods for accurate and precise measurement of AAV fill state, titer and aggregates using novel 3 µm monodisperse Strong Anion Exchange (SAX) and Size Exclusion (SEC) HPLC columns.

Methods: A Thermo Scientific™ Vanquish™ Flex UHPLC system coupled with Thermo Scientific™ Vanquish™ fluorescence detector is used for analyzing AAV empty and full capsids with a Thermo Scientific™ ProPac™ 3R SAX column. The Thermo Scientific™ SurePac™ Bio SEC 550 MDi™ column is used for SEC-MALS analysis of an in-house AAV5 sample.

Results: Excellent separation of AAV empty and full capsids and other impurities using both a linear salt gradient and linear salt gradient with isocratic hold. Rapid SEC analysis with high precision and increased aggregate resolution compared to other columns on the market.

Introduction

Adeno-associated viruses (AAVs) are small, non-enveloped viruses used as vectors in gene therapy due to their low immunogenicity, safety, and long-term transient expression. The quality of AAV therapeutics depends on the accurate separation and quantitation of empty and full capsids and the identification of aggregates. Strong Anion Exchange (SAX) Chromatography, which separates AAV capsids based on surface charge differences, and Size Exclusion Chromatography (SEC), which measures AAV titer and quantifies aggregates based on hydrodynamic volume, are robust methods for AAV analysis. SAX uses a charged quaternary amine anion exchange group to facilitate AAV separation with a salt gradient, while SEC, coupled with Multi-Angle Light Scattering (MALS) detectors (SEC-MALS), allows for simultaneous measurement of AAV titer and aggregate characterization, ensuring high-quality and efficacious gene therapy vectors.

Materials and methods

Samples

AAV1, 6, and 8 empty and full samples are used as received (2 × 10^{13} vg/mL) from Virovek.

For linear salt gradient methods, AAV empty and full samples are mixed with a 1:10 ratio.

For linear salt gradient with isocratic hold methods, AAV6 full sample is used directly without further modification.

AAV5 samples used for aggregate analysis are generated in house at Thermo Fisher Scientific (Alachua, FL, US).

Columns

ProPac 3R SAX 3 µm Column, 2 X 50 mm (P/N 43203-052068)

SurePac Bio 550 SEC MDi 3 µm Column, 4.6 X 150 mm (P/N 43903-154631)
Conventional SEC column, 5 µm, 500 Å, 4.6 X 300 mm

Data Analysis

Thermo Scientific™ Chromeleon™ 7.2.10 Chromatography Data System (CDS) is used for data acquisition and analysis.

Wyatt Technology ASTRA™ 8.1.2 is used for SEC-MALS data acquisition and analysis.

Results

Monodisperse vs. polydisperse particles

The monodisperse 3 µm non-porous particles of the ProPac 3R columns (Figure 1) are based on a highly cross-linked divinylbenzene (DVB) polymer to produce a non-porous particle that is both mechanically and chemically robust. To mask the DVB and reduce hydrophobic secondary interactions, all particles were initially coated with a conformal, cross-linked hydrophilic polymer layer possessing ethoxy-hydroxyl functionality before functionalizing with SAX ligands. The monodispersity of the particle is providing the foundation to consistently manufacture highly efficient 3 µm particles without the presence of fine particles as observed in polydisperse alternatives, which lead to unwanted high backpressures (Figure 1, right image).

The SurePac Bio 550 SEC MDi 3 µm Column monodispersed silica particles are covalently modified with a proprietary diol hydrophilic layer. This proprietary process brings an extremely low level of non-desired interaction sites. Compared to traditional polydisperse particles (right image, Figure 2), the consistent size distribution of the monodisperse particles (left image, Figure 2) not only facilitates precise control over media synthesis and column packing, but also significantly improves column-to-column and lot-to-lot reproducibility. The stationary phase is housed in state-of-the-art hydrophilic-coated stainless-steel hardware. The hydrophilic coating reduces secondary interactions, ensuring optimal performance during the initial injection.

Figure 1. SEM images of monodisperse (left) and polydisperse (right) 3µm polymer particle distributions as measured by a Coulter Counter. Red arrows indicate the examples of particle "fines" observed in the polydisperse particle distribution. Scale bars are 10µm in length.

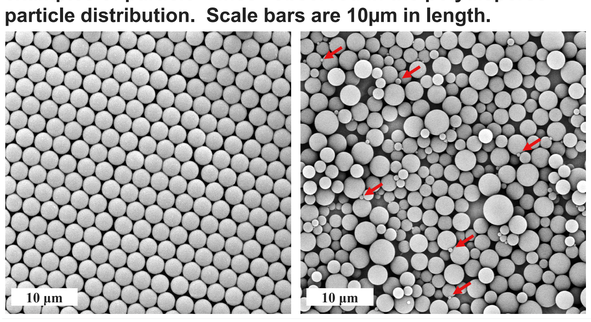
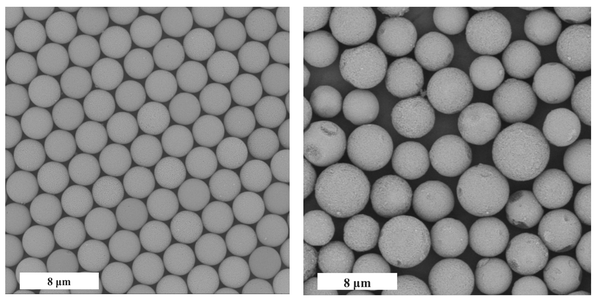


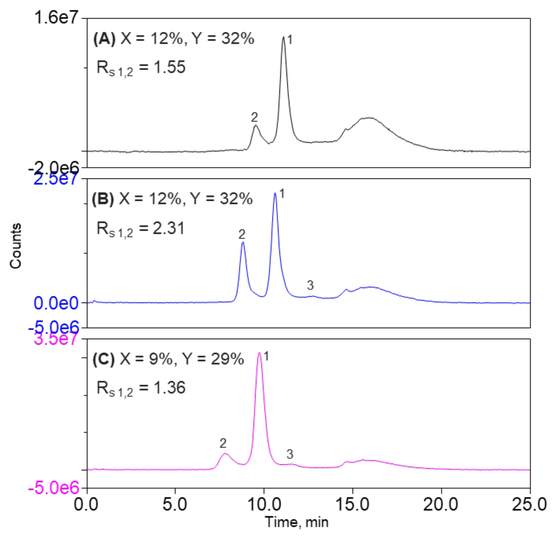
Figure 2. SEM image of 3 µm monodisperse silica particles (left) vs. 3 µm polydisperse particles (right). Scale bars are 8 µm in length.



SAX Linear salt gradient analysis of AAV fill state

Figure 3 shows the analysis of AAV1, AAV6, and AAV8 samples using a simple linear salt gradient. For each of the AAV samples tested, empty (peak 2) and full (peak 1) capsid peaks are baseline resolved using the linear gradient indicating that a simple linear salt gradient is often sufficient for separating these peaks for the purposes of quantitation. For AAV6 and AAV8 samples, an impurity peak (peak 3) is observed to elute after the full capsid peak. For the AAV separations, the relative peak areas for empty capsids (peak 2) compared to full capsids (peak 1) are visually greater than 10% as would be expected from the 1:10 mixing of empty:full standards. This is particularly obvious for AAV6 and may be due in part to the accuracy of sample concentrations from the supplier. However, we note that the fluorescence signal response for empty and full capsids is not equivalent, which will contribute to the differences in relative peak areas measured.[1] Calibration curves for both empty and full capsids would be needed to accurately measure the exact amounts of each capsid. For simplicity in this study, we report the relative peak areas for empty and full capsids in subsequent analyses.

Figure 3. Linear salt gradient separation of full capsid AAV samples spiked with empty capsid to give a 1: 10 Empty:Full ratio: (A): AAV1 sample, (B): AAV6 sample, and (C): AAV8 sample (X and Y in the figure is the starting and ending %MPB of the method)



The simple linear salt gradient method is straightforward to implement and provides good separation for each of the AAV samples evaluated; however, further improvements to the separation between the AAV empty and full capsid may be required for more difficult to separate samples to either increase resolution or improve detection sensitivity. In this section, we demonstrate a method to improve the separation of AAV6 using a linear gradient by incorporating an isocratic hold midway through the method to increase the separation of the empty capsid from the full capsid. The isocratic hold is used to elute the empty capsid at a constant salt concentration that is insufficient to promote elution of the full capsid, which remains bound to the column stationary phase. After elution of the empty capsid, a linear gradient of increasing mobile phase B concentration is performed to elute the bound full capsid, resulting in significantly improved resolution between the AAV empty and full capsid.[2,3]

Figure 4. Salt gradient separation of AAV6 separation using an isocratic hold at different %MPB (labeled as X in the figure): (A): 16.0%, (B): 16.5%, (C): 17.0%, (D): 17.5%, and (E): 18.0%

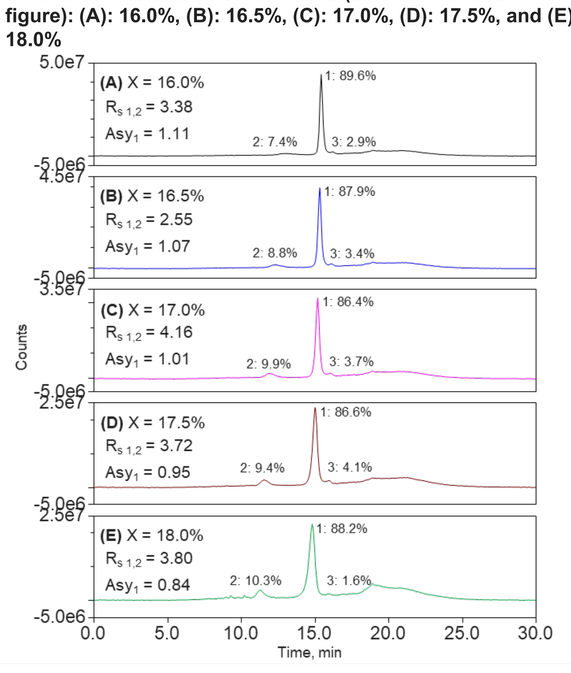


Figure 4 shows the results of different %MPB from 16.0% to 18.0% for the isocratic elution stage. Baseline separation of empty and full capsid are achieved for all the choices of isocratic %MPB tested; however, differences are observed in the quality of the separation. 17.0% MPB is considered for further optimization of this method since the empty and full capsid are baseline separated, and the full capsid peak asymmetry is close to 1 (Asy1 = 1.01).

Figure 5. AAV6 analysis using a linear salt gradient with isocratic hold with different isocratic stage holding times (labeled as X in the figure): (A): 4 min, (B): 6 min, (C): 8 min, and (D): 10 min

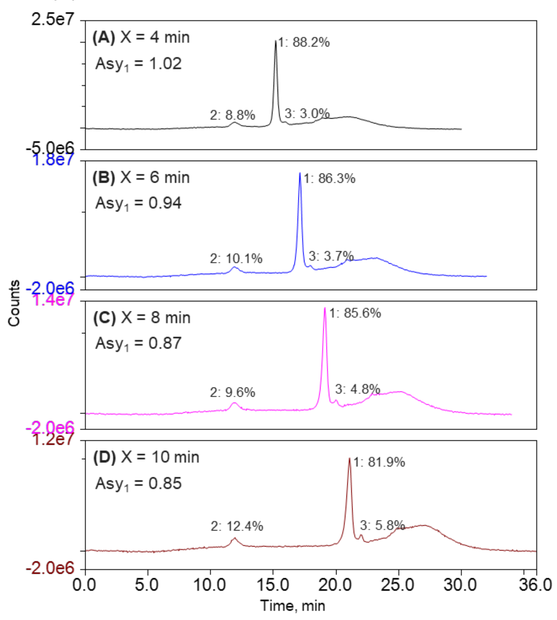
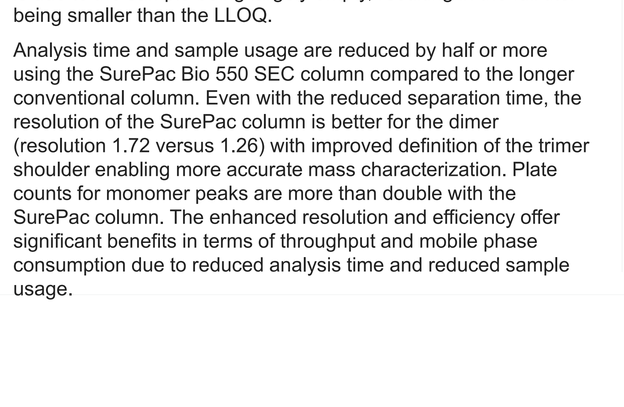


Figure 5 shows the method used in Figure 3 with an isocratic hold at 17% MBP with holds ranging in time from 4 min to 10 min. This shows separation of the empty (peak 2) and full (peak 1) capsid peaks changing due to the later elution of the full capsid peak with increasing isocratic holding time. However, as the isocratic stage holding time prolongs, there is a decrease in the intensity and relative peak area of the full capsid peak due to the full capsid also elutes slowly during the 17% MPB isocratic stage, which may lead to inaccurate quantitation of empty and full capsid if holding time is too long..

The user can employ orthogonal methods such as AUC or cryo-EM[3] in combination with AAV standards to determine which isocratic hold time method will provide the most accurate characterization of the empty/full ratio.

Analysis of AAV aggregation using SEC

Figure 6. Repeatability and intermediate results of the AAV5 aggregates peaks



	Repeatability						PASS/FAIL
	Mw	rz	Total Titer	Full Titer	Empty Titer	Acceptance criteria	
Dimer	0.9%	1.6%	3.2%	49.8%	3.9%	%CV ≤ 20%	PASS
Trimer	1.5%	2.4%	7.1%	79.6%	9.3%		
Tetramer	3.6%	4.6%	9.8%	68.3%	13.0%		

Figures 6 demonstrate the excellent repeatability of SEC-MALS data for AAV5 monomer and aggregate peaks, all meeting the acceptance criteria. In Figure 6, the higher %CV for full particles is due to the sample being largely empty, resulting in the full titer being smaller than the LLOQ.

Analysis time and sample usage are reduced by half or more using the SurePac Bio 550 SEC column compared to the longer conventional column. Even with the reduced separation time, the resolution of the SurePac column is better for the dimer (resolution 1.72 versus 1.26) with improved definition of the trimer shoulder enabling more accurate mass characterization. Plate counts for monomer peaks are more than double with the SurePac column. The enhanced resolution and efficiency offer significant benefits in terms of throughput and mobile phase consumption due to reduced analysis time and reduced sample usage.

Figure 7. (a) Separation of AAV5 with the SurePac Bio 550 SEC MDi column (Red) and a column from a different vendor (Blue). (b) Molecular mass data using the SurePac Bio 550 SEC MDi column

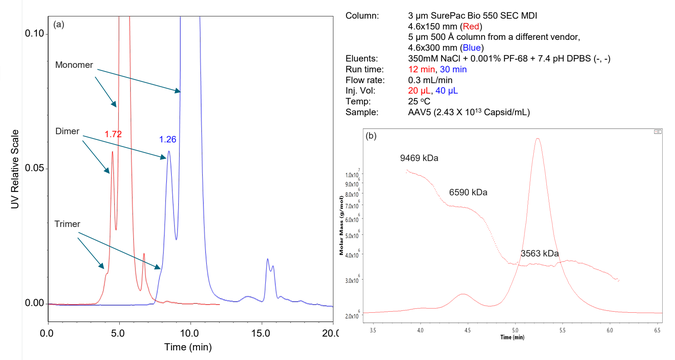


Figure 8. Agreement results of the AAV5 monomer peak between the SurePac and the conventional column

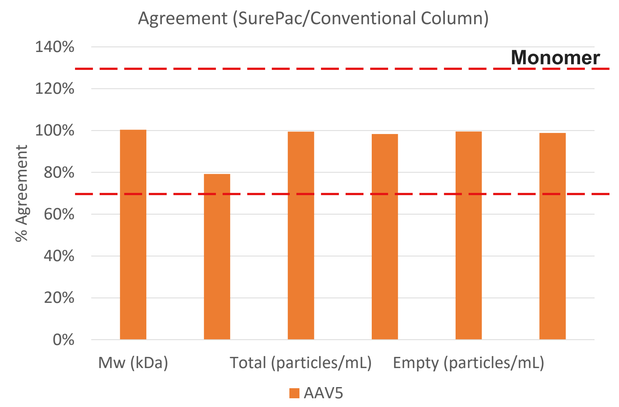


Figure 9. Agreement results of the AAV5 aggregates peaks between the SurePac and the conventional column

All monomer metrics show good agreement between the two columns. However, the agreement for aggregates is lower, likely due to the conventional column's limited separation compared to the SurePac column.

Conclusions

- The ProPac 3R SAX 3 µm column provides excellent separation of AAV empty and full capsids and other impurities using both a linear salt gradient and linear salt gradient with isocratic hold. The unique column design provides high resolution, robust performance, and lot-to-lot reproducibility needed for AAV analysis.
- Design of a linear salt gradient with an isocratic hold at an appropriate isocratic elution salt concentration and holding time can provide optimized methods for the separation and quantitation of AAV empty and full capsid.
- The SurePac column reduces analysis time and sample usage by half or more compared to conventional column
- The SurePac column has more than double the plate counts compared to the conventional column
- SEC-MALS is the gold standard for simultaneous measurement of AAV titer, aggregate quantitation (including monomers, dimers, trimers, etc.), and aggregate molecular weight.

References

1. Wang, C.; Mulagapati, S. H. S.; Chen, Z.; Du, J.; Zhao, X.; Xi, G.; Chen, L.; Linke, T.; Gao, C.; Schmelzer, A. E.; Liu, D., Developing an Anion Exchange Chromatography Assay for Determining Empty and Full Capsid Contents in AAV6.2. Molecular Therapy – Methods & Clinical Development 2019, 15, 257–263.
2. Aebischer, M. K.; Gizardin-Fredon, H.; Lardeux, H.; Kochardt, D.; Elger, C.; Haindl, M.; Ruppert, R.; Guillarme, D.; D'Atri V., Anion-Exchange Chromatography at the Service of Gene Therapy: Baseline Separation of Full/Empty Adeno-Associated Virus Capsid by Screening of Conditions and Step Gradient Elution Mode. International Journal of Molecular Sciences 2022, 23(20), 12332.
3. Khatwani, S.L.; Pavlova, A.; Pirot, Zhu., Anion-exchange HPLC assay for separation and quantification of empty and full capsid in multiple adeno-associated virus serotypes. Molecular Therapy – Methods & Clinical Development 2021, 21, 548–558.

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