Application Note

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Robust Column Performance for Wide Pore Size Exclusion Columns for Adeno-Associated Virus Particles (AAVs) Analysis

Using Agilent AdvanceBio SEC column technology

Authors

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Abstract

To ensure the safety and purity of adeno-associated virus (AAV) vectors, high-resolution size exclusion chromatography (SEC) is applied to determine the presence of aggregates in AAV samples. This application note demonstrates the superior resolution and recovery of ultrawide pore Agilent AdvanceBio SEC 500 Å columns when compared with competitor products for AAVs.

Introduction

AAVs are virus particles (~ 25 nm in diameter) used as a delivery vehicle for transferring up to ~ 4.7 kb of any gene of interest. Their icosahedral structure is made up of 60 capsid proteins. It is important to completely characterize AAVs and determine their critical quality attributes (CQAs) to ensure the safety and purity of the sample. One critical CQA is aggregation, and it is essential to determine the amount of aggregation present in formulated and in-process AAV samples. High-resolution SEC is one of the main techniques used for monitoring aggregation. Agilent AdvanceBio SEC columns with smaller particles, appropriate pore sizes, and larger pore volumes provide a robust, high-resolution baseline separation of AAV aggregates from the monomer peaks.

In this application, an Agilent AdvanceBio SEC 500 Å, 2.7 µm, 4.6 × 300 mm chromatographic column was used to perform aggregate analysis on different AAV serotype samples. The column performance was compared with competitor products.

Experimental

Reagents and chemicals

All reagents were HPLC grade or higher. All chemicals were bought from Sigma-Aldrich, unless otherwise stated.

Equipment and materials

AAV (Full-GFP) samples (AAV1 3.42×10^{11} GC/mL, AAV2 1.27×10^{13} GC/mL, AAV5 2.6×10^{11} GC/mL, AAV6 4.1×10^{11} GC/mL, AAV8 3.53×10^{11} GC/mL, and AAV9 6.07×10^{11} GC/mL) were bought from Charles River Laboratories. AAV9 empty sample (2.0×10^{13} VP/mL) was bought from Virovek.

Instrumentation

Chromatography analysis was performed on an Agilent 1290 Infinity II bio LC system connected to a diode array detector (DAD) and an Agilent 1260 Infinity II fluorescence detector (FLD) for higher sensitivity. The chromatography conditions used are shown in Table 1. Data acquisition and analysis was performed using Agilent OpenLab CDS ChemStation software.

Sample preparation

Sample aliquots were stored at –80 °C. Before being injected, samples were thawed in an ice bucket, spun at a low rpm, then transferred to an Agilent HPLC vial (part number 5188-2788).

Mobile phase preparation

The mobile phase consisted of 50 mM phosphate buffer and 400 mM NaCl, pH 7.2. The buffer was filtered using a 0.2 µm filter to remove any particulates and to reduce the risk of any microbial growth.

Method conditions

Table 1. HPLC conditions.

Parameter	Value
Column	– Agilent AdvanceBio SEC 500 Å, 2.7 µm, 4.6 × 300 mm (p/n PL1580-5325) – Agilent AdvanceBio SEC 500 Å, 2.7 µm, 4.6 × 150 mm (p/n PL1580-3325) – Competitor A 700 Å, 3 µm, 4.6 × 300 mm – Competitor B 450 Å, 2.5 µm, 4.6 × 300 mm
Mobile Phase	50 mM Sodium phosphate + 400 mM NaCl, pH 7.2
Flow Rate	0.35 mL/min
Column Temperature	Room temperature
Injection Volume	5 µL
Detection	UV: 280 nm FLD: Excitation 280 nm, emission 348 nm
Run Time	15 min
HPLC System	Agilent 1290 Infinity II bio LC system with binary high-speed pump

Results and discussion

The AdvanceBio SEC 500 Å, 2.7 µm, 4.6 × 300 mm was injected with different AAV serotypes (AAV1, AAV2, AAV5, AAV6, AAV8, and AAV9) to evaluate if the column could exhibit robust performance in separating and resolving aggregates found in different AAV samples. As shown in Figure 1, the AdvanceBio SEC 500 Å showed a baseline separation between AAV aggregates and the monomers with a resolution Rs > 2.4 observed for all the tested AAV serotype samples. The column was able to differentiate and separate higher-order aggregates, clearly separating them from the AAV dimer peak (Figure 1B). All serotypes showed exceptional recovery.

For a shorter run time, a shorter length AdvanceBio SEC, 4.6 × 150 mm column was run at 0.35 mL/min, and its performance was compared to a longer length AdvanceBio SEC 4.6 × 300 mm column. As demonstrated in Figure 3, the shorter length column was able to capture similar amounts of aggregates, and was still able to provide baseline separation of the aggregates from the AAV monomer with an Rs of 1.64. The AAV monomer eluted with a retention time (RT) of 4.14 minutes with a total run time of less than six minutes. This presents a faster high-throughput method, and can be applied when many samples need to be analyzed in a short period of time.

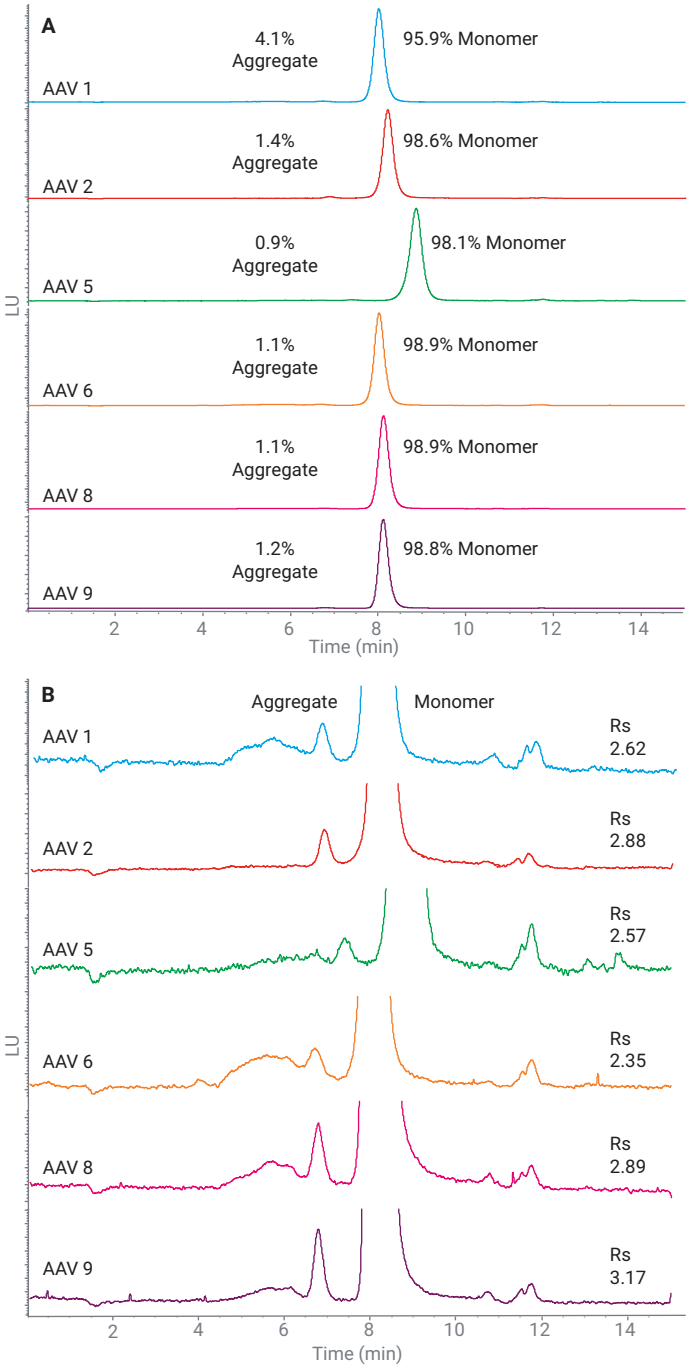

Figure 1. (A) Five-microliter injections of different AAV serotypes with fluorescence detection. (B) Expanded Y-axis view of the above chromatograms with monomer/aggregate resolution noted on each chromatogram.

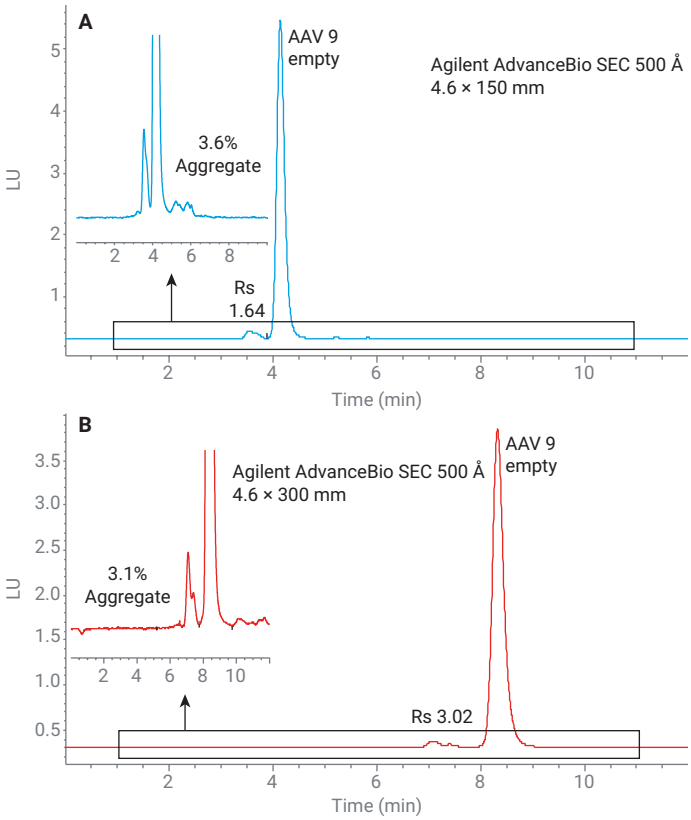

Figure 2. Fluorescence chromatogram of AAV9 empty capsid injected on an Agilent AdvanceBio SEC 500 Å 4.6 × 150 mm column (A) and an Agilent AdvanceBio SEC 500 Å 4.6 × 300 mm column (B). The shorter column gave a faster run time of < 6 minutes.

To further evaluate the column performance, the chromatographic separation of AAV2 (full) and AAV9 (empty) was compared to columns from other vendors (Competitors A and B). Each sample was injected in triplicate on each test column, and the results are presented in Table 2, showing good reproducibility. In Figure 3, the AdvanceBio SEC column, with smaller particles and larger pore volume[1], yielded superior resolution between aggregate and monomer peaks (Rs ~ 3.0). Furthermore, higher-order aggregates were observed in the AAV9 empty sample and were well-resolved (Figure 3B) with the AdvanceBio SEC column, indicating higher column performance when compared to other columns. Lower tailing factor for the monomer peak was also noticed for AAV2 (full) and AAV9 (empty) on the AdvanceBio SEC column.

Table 2. Comparison of column performance of an Agilent AdvanceBio SEC 500 Å with other vendor columns on triplicate injections of AAV2 (top) and AAV9 empty (bottom) samples.

AAV2 (n = 3) Injection Volume 3 µL		RT Monomer	Resolution	Tailing Factor	% HMW
Agilent AdvanceBio SEC	Average	8.44	2.89	1.14	1.15
	STD	0.00	0.01	0.00	0.06
	%RSD	0.01%	0.25%	0.41%	5.59%
Competitor A	Average	8.37	2.33	1.28	1.50
	STD	0.00	0.02	0.01	0.04
	%RSD	0.01%	0.73%	0.70%	2.48%
Competitor B	Average	7.50	2.10	1.46	1.16
	STD	0.00	0.04	0.01	0.04
	%RSD	0.01%	1.83%	0.54%	3.30%
AAV9 Empty (n = 3) Injection Volume 2 µL		**RT Monomer**	**Resolution**	**Tailing Factor**	**% HMW**
Agilent AdvanceBio SEC	Average	8.32	3.02	1.28	3.10
	STD	0.00	0.02	0.01	0.14
	%RSD	0.01%	0.60%	0.76%	4.51%
Competitor A	Average	8.41	1.88	1.24	2.91
	STD	0.00	0.02	0.00	0.08
	%RSD	0.01%	0.88%	0.27%	2.58%
Competitor B	Average	7.48	1.79	1.33	2.40
	STD	0.00	0.01	0.01	0.02
	%RSD	0.01%	0.71%	0.82%	0.88%

While smaller particles and inert chemistry improve SEC resolution, pore volume also plays a significant role. Higher pore volume creates more potential for analytes to differentially migrate in and out of pores, increasing the separation between them. As shown in application note 5994-7934EN[1], AdvanceBio SEC 500 Å columns have higher pore volumes than the competitor columns A and B shown in this study, which explains why columns of similar particle size have such different resolution.

Recovery was determined by comparing the total peak area of AAV2-full and AAV9-empty sample with the total peak area obtained when no column was used. It was observed that AdvanceBio SEC columns exhibited superior recovery for both AAV2-full and AAV9-empty samples when compared to the tested samples, as demonstrated in Figure 4.

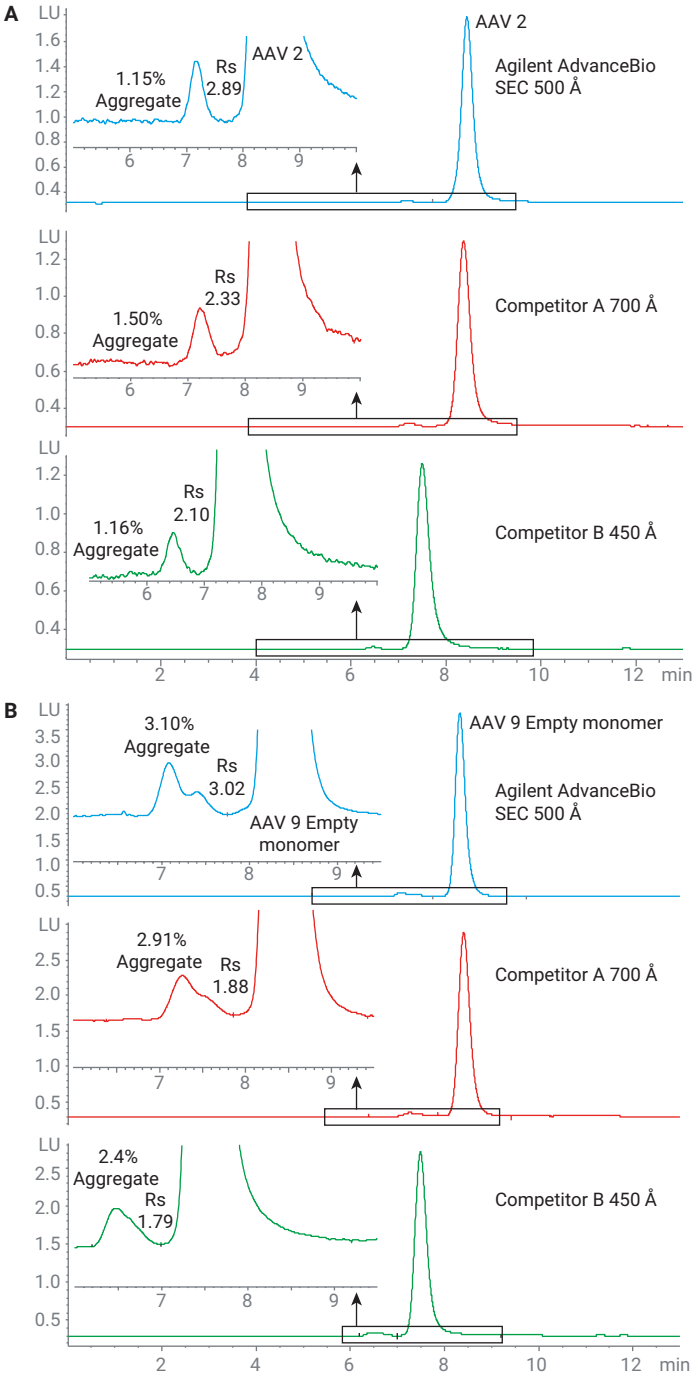

Figure 3. Comparison of the chromatographic separation of AAV2-full capsid (A) and AAV9-empty capsid (B) when injected on an Agilent AdvanceBio SEC 500 Å 4.6 × 300 mm column and other vendor columns. AdvanceBio SEC 500 Å columns shows better separation with higher resolution between aggregate and monomer peaks.

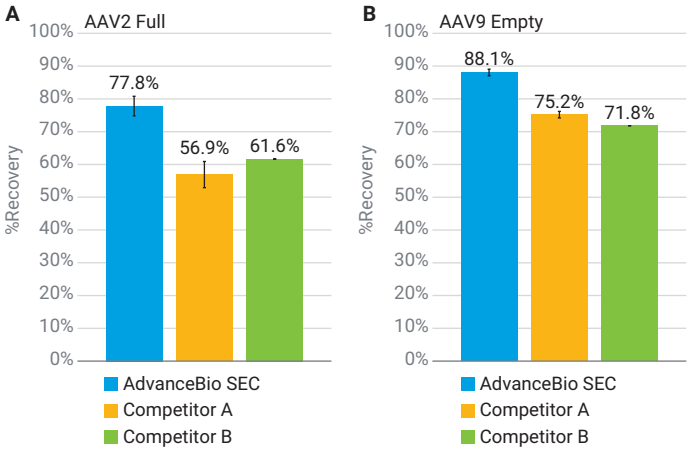

Figure 4. Total recovery comparison of AAV2-full (A) and AAV9-empty (B) samples on an Agilent AdvanceBio SEC 500 Å and other vendor columns. AdvanceBio SEC 500 Å columns have higher recovery for both samples, indicating better performance than other columns.

Conclusion

The newly developed Agilent AdvanceBio SEC 2.7 µm columns with a wider pore size of 500 Å exhibit superior efficiency in separating and resolving aggregates and fragments from AAV samples versus competitor products. These columns provide exceptional compatibility and robust performance with different AAV serotypes and can be used for high-throughput methods. AdvanceBio SEC columns also demonstrate excellent recovery when compared to SEC columns from other vendors.

References

1. Chromatographic Comparison of Wide Pore Size Exclusion Columns from Different Vendors. *Agilent Technologies application note*, publication number 5994-7934EN, **2024**.

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