Application Note

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Robust Wide Pore Size Exclusion Columns for Virus-Like Particles (VLPs) Analysis

Using Agilent AdvanceBio SEC column technology

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Abstract

Aggregate analysis of VLPs is a key critical quality attribute (CQA) and requires careful consideration of the column parameters needed. This application note demonstrates the use of a new wide pore size stationary phase that retains the key characteristics of high pore volume and efficient particle size. The new column can separate and resolve aggregates from the monomeric VLP.

Introduction

VLPs are self-assembling structured protein particles, typically 20 to 150 nm in diameter, that contain no virus genetic material. This makes VLPs noninfectious and safer for biotherapeutic applications.

It is important to thoroughly characterize VLPs as a biotherapeutic and to determine their CQAs. One of the primary CQAs is aggregation, and it is essential to determine the amount of aggregation and monitor its level during the manufacturing process.

Size exclusion chromatography (SEC) is a well-established technique used for monitoring aggregation, along with nonchromatographic techniques such as analytical ultracentrifugation (AUC), field flow fractionation (FFF), and microscopy. As next generation biotherapeutics become increasingly larger and more complex, technology to monitor aggregation must also advance. For SEC, it is crucial to choose a suitable pore size column that can accommodate the test analyte, its aggregate, and which can provide a baseline separation with excellent sample recovery.

Previously, there have been few robust SEC column options with large enough pore sizes paired with smaller particles and high pore volumes that enable reproducible, high-resolution separations. While pore size influences what size analytes may be characterized, pore volume impacts the separation capacity within that size range, or the ability to differentiate one size species from another. Agilent AdvanceBio SEC columns meet the need for robust, high-resolution separations with this ideal combination of small particles and large pore volumes with the right size pore for the application.

In this application, a newly developed AdvanceBio SEC 1000 Å, 2.7 µm, 4.6 × 300 mm chromatographic column was used to perform aggregate analysis on a 50 nm diameter recombinant human papilloma virus type 16 L1 protein (HPV-16) VLP sample.

Experimental

Reagents and chemicals

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

Equipment and materials

HPV-16 VLP samples (1 mg/mL, 500 mM Histidine, 100 mM NaCl, 0.02% Tween 80, pH 6.0) were bought from Creative Diagnostics. The HPV-16 VLP particle size was determined by the vendor using Cryo Electron Microscopy (Cryo-EM).

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 the Method conditions section. 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, and 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

HPLC Conditions	
Column	Agilent AdvanceBio SEC 1000 Å, 4.6 × 300 mm, 2.7 µm (p/n PL1580-5302)
Mobile Phase	50 mM Sodium phosphate + 400 mM NaCl, pH 7.2
Flow Rate	0.35 mL/min
Column Temperature	Room temperature
Injection Volume	1 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 results of the chromatographic separation are shown in Figure 1. A detection wavelength of 220 nm was selected instead of 280 nm as it showed higher signal response (Figures 1A and 1B). It has been reported that fluorescence detection has higher sensitivity and when connected with the 1290 Infinity II Bio LC, it showed higher signal response for the VLP sample as shown in Figure 1C, making it an ideal choice of detector. For this case study, sample limitation was not an issue, however, when limited by sample amount and concentration, and where sensitivity is a challenge, fluorescence is often a preferred method of detection.

When connected in series, UV and fluorescence detector results are nearly identical, as shown in Figures 1B and 1C, indicating sufficient sensitivity with UV detection for this particular sample. Figure 1B shows the chromatographic separation of the HPV-16 VLP sample with UV detection, with baseline separation of dimer species from the monomer with a resolution of 1.67. The newly developed column was also able to separate higher order aggregates, clearly distinguishing them from the HPV dimer peak.

An Agilent AdvanceBio SEC 300 Å protein standard (part number 5190-9417) sample was injected as an internal QC standard on the newly developed 1000 Å SEC column. It was observed that the monomeric thyroglobulin (MW 670 kDa) peak eluted at RT 10.27 minutes with its dimer (MW 1.3 MDa) peak eluting at RT 9.74 minutes, as shown in Figure 2. The reported hydrodynamic radius of monomeric thyroglobulin is 8.6 nm[1] and, based on the monomer and dimer molecular weight and hydrodynamic radii, the tested VLP sample is expected to have an earlier retention time, which was observed at approximately 9 minutes, as shown in Figure 1.

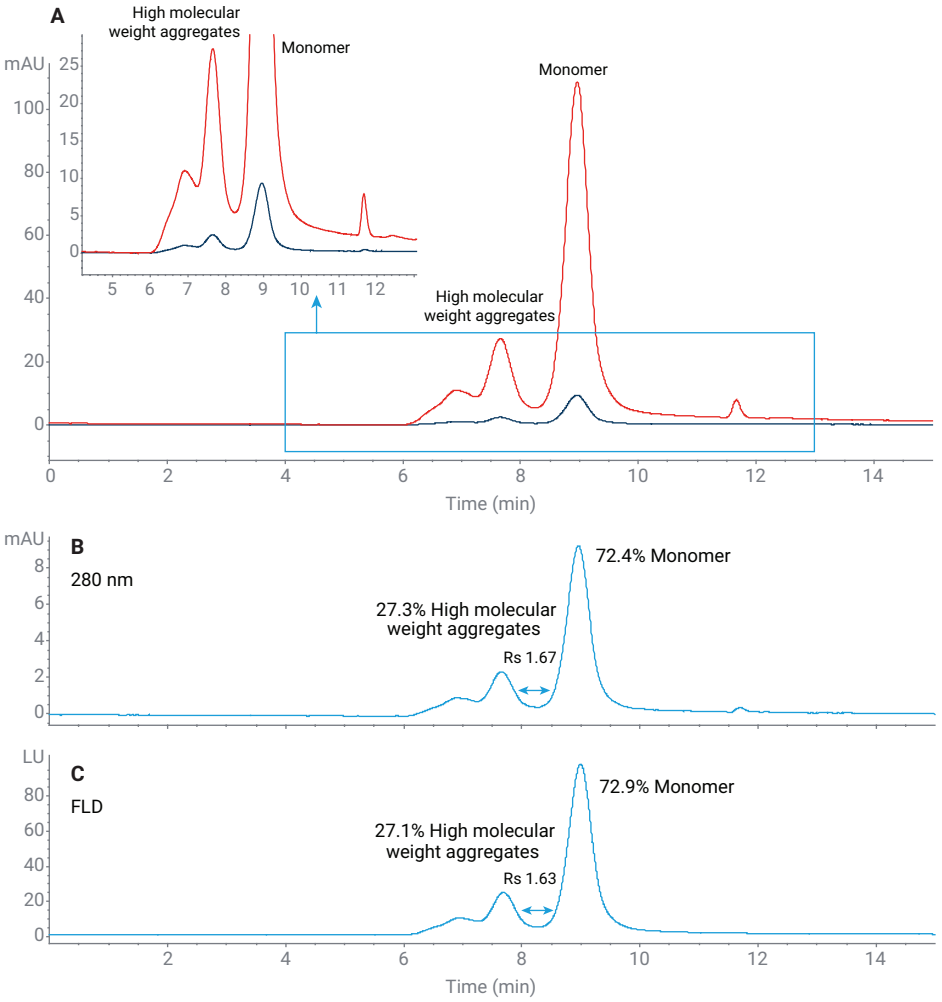

Figure 1. (A) Overlay of chromatographic trace at UV 280 nm (blue trace) and UV 220 nm (red trace) of 1 µL HPV-16 VLP sample. (B) UV chromatogram of 1 µL injection of HPV-16 VLP sample at 280 nm. (C) Fluorescence chromatogram of 1 µL injection of HPV-16 VLP sample with excitation at 280 nm and emission at 348 nm.

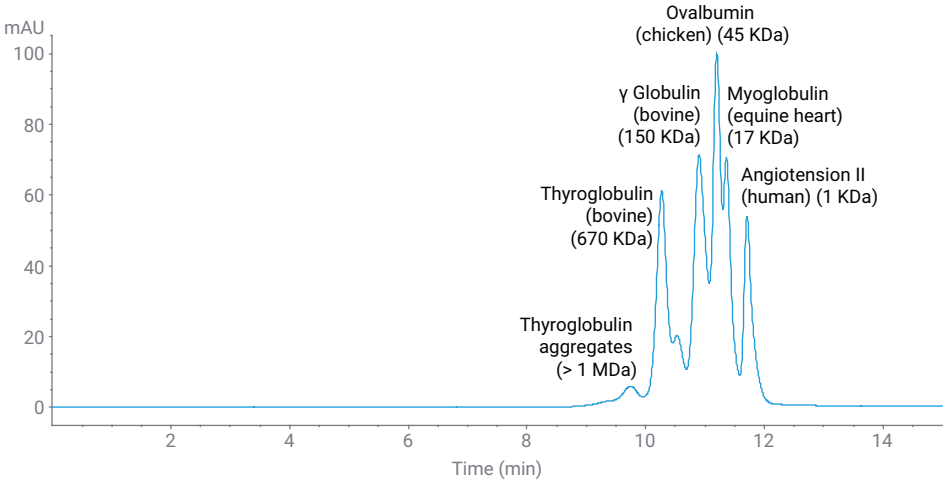

Figure 2. UV chromatogram (220 nm) of Agilent AdvanceBio SEC 300 Å protein standard. Good separation is observed between thyroglobulin monomer and its aggregates.

The HPV-16 VLP sample was injected multiple times (n = 5) on the same column to determine consistent injection-to-injection performance. It showed exceptional reproducibility as shown in Figure 3, without conditioning beyond the recommended 10 column volumes to equilibrate the column to a new mobile phase. It displayed excellent column performance with consistent retention time and resolution, with 0% relative standard deviation (RSD) and < 2% RSD for the total peak area (Table 1).

To further evaluate column performance and robustness, four different batches of 1,000 Å media were packed and assessed. As shown in Figure 4, the tested sample showed remarkably high reproducibility with < 10% RSD for different parameters such as retention time, %monomer, total recovery, resolution between monomer peak and its aggregates, and tailing factor for monomer peak (Table 2).

The chromatographic separation of HPV-16 was compared with an Agilent Bio SEC-5 1000 Å, 5 µm column (part number 5190-2538), as shown in Figure 5. The newly developed AdvanceBio SEC column with smaller particles yielded better resolution between aggregate and monomer peaks. Additionally, due to the higher pore volume, more of the higher order aggregates were included and better resolved from the dimer, indicating higher column performance. The monomer peak was also narrower on the AdvanceBio SEC column than with the Agilent Bio SEC-5 column.

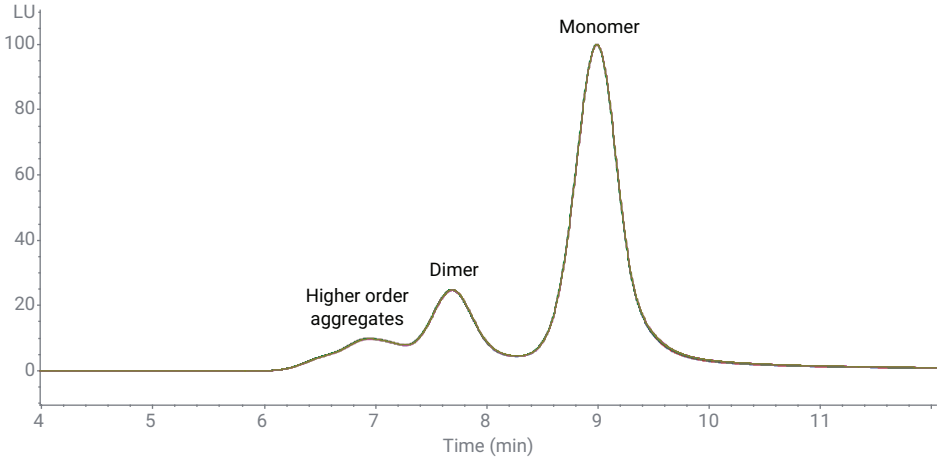

Figure 3. Overlay of fluorescence chromatogram (excitation 280 nm; emission 348 nm) of HPV-16 (five injections) showing excellent reproducibility.

Table 1. Agilent AdvanceBio SEC 1000 Å columns show excellent injection-to-injection reproducibility with %RSD < 1 for 1 µL injections of HPV-16.

Injection	RT Monomer	% HMW	% Monomer	Resolution	Tailing Factor
1	8.99	27.07	72.93	1.64	1.10
2	8.99	27.11	72.88	1.63	1.11
3	8.99	27.01	72.99	1.64	1.12
4	8.99	26.57	73.35	1.63	1.13
5	8.99	26.87	73.13	1.63	1.13
Average (n = 5)	8.99	26.93	73.06	1.63	1.12
Standard Deviation	0.00	0.20	0.17	0.01	0.01
%RSD	0.01%	0.74%	0.23%	0.38%	1.07%

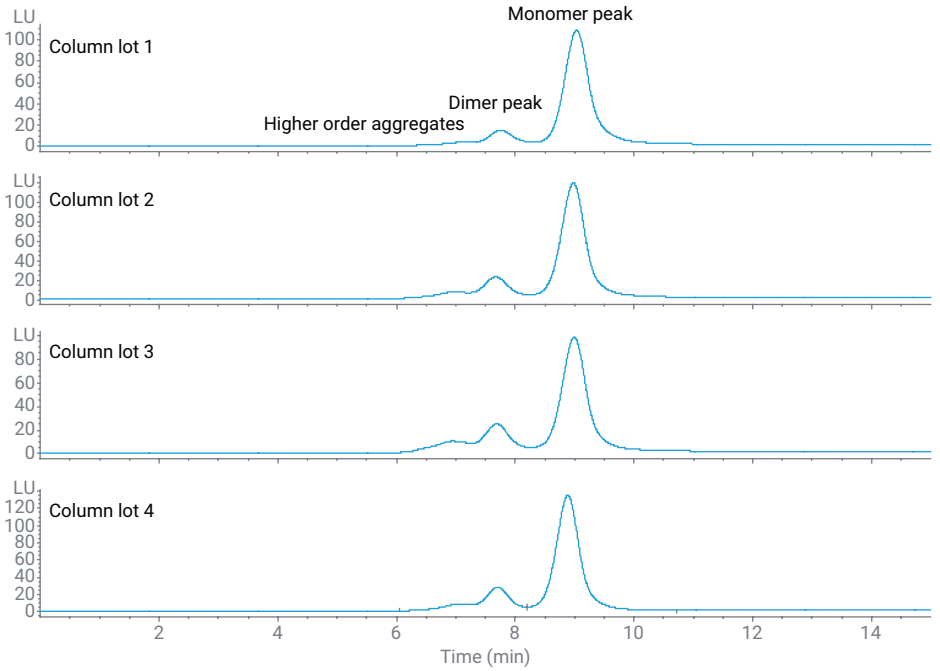

Figure 4. Overlay of the fluorescence chromatogram (excitation 280 nm, emission 348 nm) of HPV-16 injected on different batches of packed columns.

Conclusion

The newly developed Agilent AdvanceBio SEC 2.7 µm columns, with a wider pore size of 1,000 Å, are suitable for aggregate and fragment analysis of VLP samples within the size range of approximately 20 to 80 nm. It is recommended to use orthogonal methods such as dynamic light scattering (DLS) or AUC for larger insoluble aggregates in conjunction with SEC-HPLC to obtain a complete aggregation profile of a test analyte.

Reference

1. Schneider, S. Determination of Protein Molecular Weight and Size Using the Agilent 1260 Infinity Multi-Detector Bio-SEC Solution with Advanced Light Scattering Detection. *Agilent Technologies application note*, publication number 5991-3955EN, **2014**.

Table 2. Agilent AdvanceBio SEC 1000 Å columns showed high reproducibility when different column batches were tested.

	RT Monomer	Resolution	Tailing Factor
Batch 1	9.03	1.61	1.24
Batch 2	8.98	1.67	1.14
Batch 3	8.99	1.63	1.12
Batch 4	8.89	1.62	1.09
Average	8.97	1.63	1.15
Standard Deviation	0.05	0.02	0.06
%RSD	0.59%	1.45%	4.87%

A

	RT Monomer (min)	% HMW	% Monomer	Resolution	Tailing Factor
AdvanceBio SEC 1000 Å	8.89	20.86	79.14	1.62	1.09

B

	RT Monomer (min)	% HMW	% Monomer	Resolution	Tailing Factor
Bio SEC-5 1000 Å	8.10	18.40	81.60	1.15	1.0

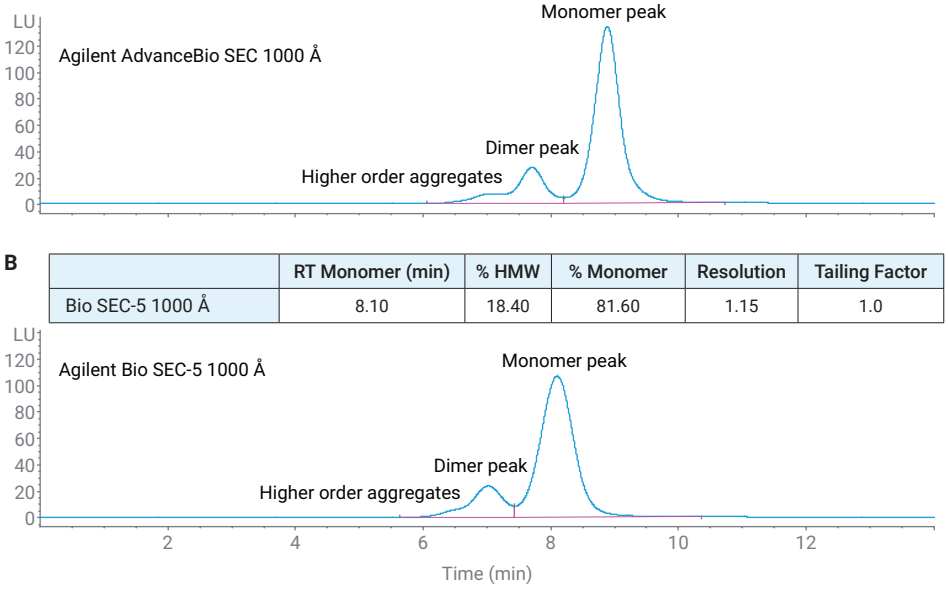

Figure 5. Fluorescence chromatogram (excitation 280 nm, emission 348 nm) of HPV-16 injected on an Agilent Bio SEC-5 1000 Å column (A) and Agilent AdvanceBio SEC 1000 Å column (B). The new AdvanceBio SEC 1000 Å column showed improved separation of aggregates from the monomer.

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