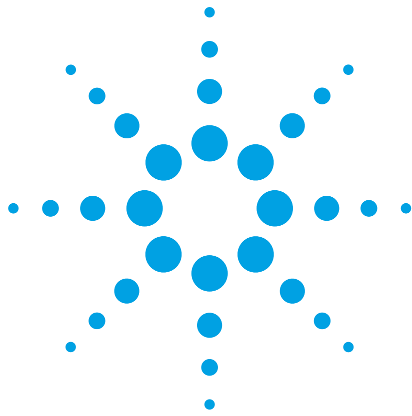

Fast, High-Resolution Size Exclusion Chromatography of Aggregates in Biotherapeutics

Application Note

Biologics and Biosimilars

Author

Andrew Coffey

Agilent Technologies, Inc.

Introduction

Protein aggregation is a critical quality attribute for biotherapeutic proteins because aggregates can have significant impact on safety, and may result in an antigenic response [1]. Aggregates may also reduce the efficacy of the biopharmaceutical, and can significantly compromise process economics. Proteins frequently aggregate when exposed to stress conditions such as changes in pH, temperature, or concentration, therefore, aggregation can occur at many different stages of the production. Size exclusion chromatography (SEC) has been identified as the method of choice for quantification of aggregates.

Monitoring aggregate formation during biotherapeutic development, for example during clone selection, or while optimizing fermentation conditions through a rigorous "design of experiments" approach, may result in a great many samples requiring size exclusion analysis.

Conditions normally used for SEC often result in run times of 20 minutes or more, greatly restricting the ability to analyze large numbers of samples. Agilent AdvanceBio SEC columns are designed with highly optimized particle size and pore size that can enable faster separations to significantly reduce this analytical bottleneck. This application note describes techniques to increase sample throughput without compromising the accuracy of analysis.

Materials and Methods

Reagents, samples and materials

Immunoglobulin G (IgG) was purchased from Sigma-Aldrich, Corp. Protein standard mix was obtained from Bio-Rad Laboratories, Inc. Components of the protein standard mix were thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B12 (1.35 kDa). All chemicals and solvents were HPLC grade, and highly purified water from a Milli-Q water purification system was used.

Instruments

A completely biocompatible Agilent 1260 Infinity Quaternary Bio-inert LC was used, consisting of the following modules:

- Agilent 1260 Infinity Bio-inert Quaternary LC Pump (G5611A)
- Agilent 1260 Infinity Bio-inert High Performance Autosampler (G5667A)
- Agilent 1200 Infinity Series Thermostat (G1330B)
- Agilent 1260 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1260 Infinity Diode Array Detector (G1315D) with bio-inert flow cell

Software was Agilent ChemStation B.04.03.

Conditions

Columns:	Agilent AdvanceBio SEC 300Å, 7.8 × 150 mm, 2.7 µm (p/n 1180-3301), Agilent AdvanceBio SEC 300Å, 7.8 × 300 mm, 2.7 µm (p/n 1180-5301), Other vendor diol column, 7.8 × 300 mm, 5 µm
Mobile phase:	150 mM sodium phosphate, pH 7.0
TCC temp:	30 °C
Inj. vol:	5 µL
Flow rate:	0.5 – 1.4 mL/min (see figure legends)
Detection:	UV at 220 nm

Results and Discussion

AdvanceBio SEC columns have 2.7 µm particles designed to give maximum efficiency without risk of shear degradation of samples, or clogging between particles. The unique method of manufacture controls pore size and structure, and also pore volume. Applying a hydrophilic polymeric coating ensures that protein peaks are sharp and resolved. Figure 1 shows the comparative SEC chromatograms of the separation of protein standards using an AdvanceBio SEC column and another vendor's column of the same dimensions (7.8 × 300 mm). The AdvanceBio SEC column showed a high efficiency separation of protein standard compared to the other column. The AdvanceBio SEC column showed an ideal chromatographic peak shape for the IgG monomer (peak 4). The ovalbumin dimer (peak 5) was well resolved compared to the other vendor column. These results demonstrate the superior performance of the AdvanceBio SEC column.

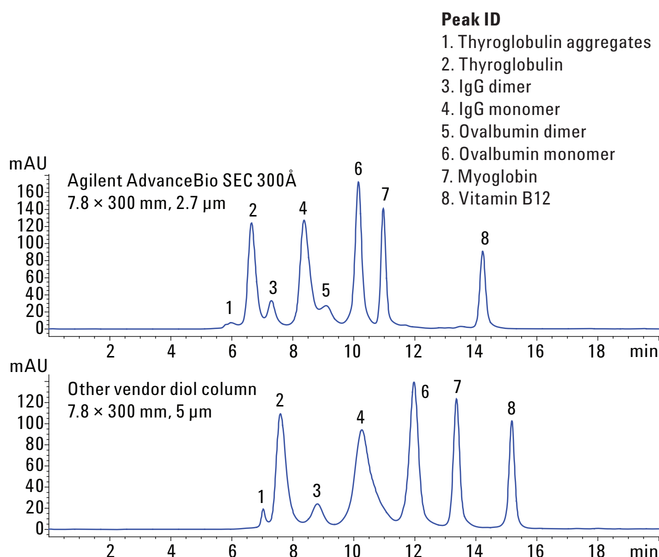

Figure 1. Size exclusion of protein standards under high-resolution conditions at 0.8 mL/min with a run time of 20 minutes, showing the superior performance of the Agilent AdvanceBio SEC column.

Under "standard" high-resolution conditions with 300 mm columns and 0.8 mL/min flow rate, the run time is 20 minutes. This gives a maximum throughput of three samples per hour, taking over 1.3 days to run 96 samples. To increase productivity, shorter 150 mm columns give correspondingly shorter run times. Figure 2 shows the separation of immunoglobulin G run on 300 mm and 150 mm AdvanceBio SEC columns operated at 0.8 mL/min. The retention time reduces by half with 150 mm column, and with a resolution factor of 1.73 between monomer and dimer peaks the quantitation would not be compromised. The shorter AdvanceBio SEC column allows the faster run time and high-resolution analysis of mAbs.

Aggregation of biotherapeutic proteins has been implicated in enhanced immunogenicity, affecting efficacy and toxicity. Accurate quantification of aggregates by SEC analysis mainly depends on resolution between monomer and aggregate peaks. Hence, resolution and quantification are two critical parameters in SEC, where linear velocity influences resolution. To evaluate these two parameters, different flow rates were tested with a 150 mm column. The excellent particle stability of the AdvanceBio SEC columns allows operation at significantly higher flow rates without undue loss in performance. At higher flow rates, the resolution factor is diminished. However, quantification of monomer peak area remains largely unchanged (Figure 3), allowing significantly more samples to be screened to determine their aggregate content.

Maximizing the sample throughput provides competitive advantages and higher confidence results, leading to economic benefits. Decreasing column length with increased flow rate is a straightforward strategy to achieve fast SEC runs. Table 1 shows the theoretical calculation on flow rate versus number of sample analyses per day. At a 1.5 mL/min flow rate with a 4.8 minute run time, 300 samples can be analyzed per day, demonstrating up to 4.2-fold higher throughput.

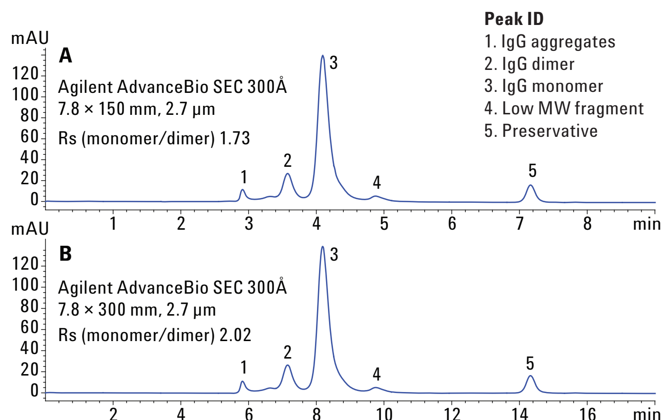

Figure 2. Size exclusion of immunoglobulin G under high-resolution conditions at 0.8 mL/min. A 150 mm column with a run time of nine minutes (A) versus a 300 mm column with a run time of 18.5 minutes (B).

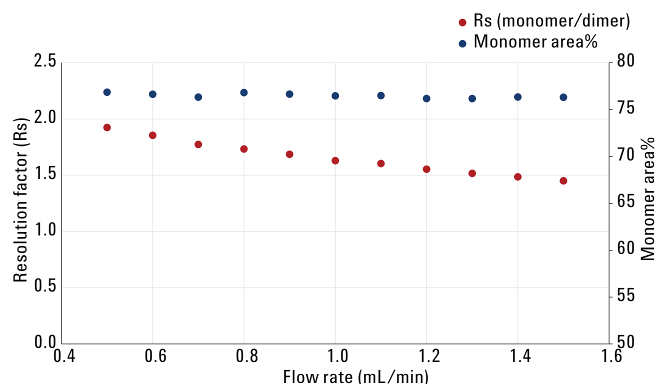

Figure 3. Effect of flow rate on resolution factor and monomer area percent determination.

Table 1. Gains in productivity by increasing flow rate to allow higher sample throughput.

Column length (mm)	Flow rate (mL/min)	Run time (min)	Samples per hour	Samples per day
300	0.8	20	3	72
150	0.5	15	4	96
150	0.6	12	4–5	120
150	0.7	10	5–6	144
150	0.8	9	6–7	160
150	0.9	8	6–7	180
150	1.0	7	7–8	205
150	1.1	6.5	8–9	220
150	1.2	6	8–9	240
150	1.3	5.5	11	260
150	1.4	5	12	288
150	1.5	4.8	12–13	300

Figure 4 shows the overlay SEC chromatograms with 300 mm and 150 mm columns at different flow rates. Maintaining accurate quantification of the monomer content, it is clear that fast SEC conditions can be achieved in one quarter of the time.

Conclusions

This study demonstrates the advantages of using shorter Agilent AdvanceBio SEC columns and higher flow rates to significantly increase throughput for the analysis of aggregates in biotherapeutic proteins. By reducing column length from 300 mm to 150 mm, and by increasing flow rate from 0.8 mL/min to 1.5 mL/min, it is possible to achieve 400% increase in sample throughput and productivity, such that the time required to analyze 96 samples can be reduced from 1.3 days to under eight hours.

Reference

1. K. D. Ratanji, J. P. Derrick, R. J. Dearman, I. Kimber. Immunogenicity of therapeutic proteins: Influence of aggregation. *J. Immunotoxicol*. **2014**, *11(2)*, 99–109.

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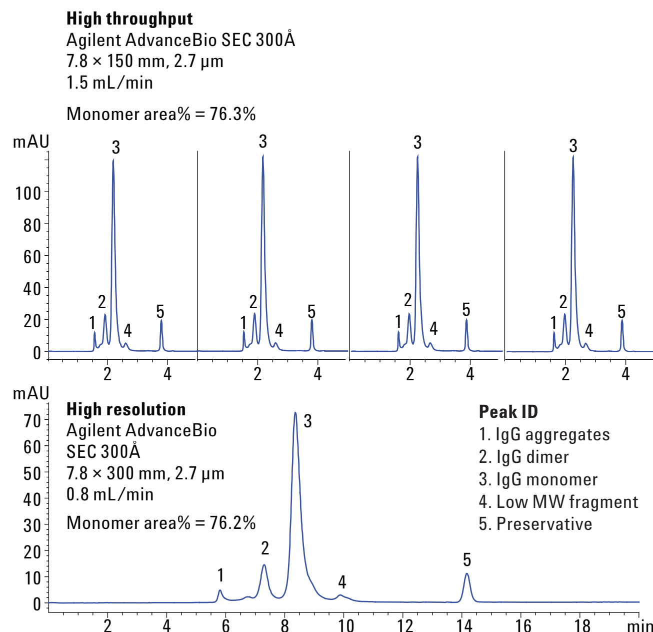

Figure 4. Comparative chromatograms of immunoglobulin G under high-throughput and high-resolution conditions.

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