Application Note

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High Resolution Size Exclusion Chromatography Analysis of Small Therapeutic Proteins

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Abstract

Protein denaturation processes involving aggregation are among the factors impeding the development of stable protein drug formulations. The use of size exclusion chromatography (SEC) HPLC for determining purity and aggregates of these proteins is a relatively straightforward technique. Regular calibration of SEC methods ensures better reproducibility, leading to improved accuracy, and enables earlier detection of potential problems with samples and batches. Agilent AdvanceBio SEC 120 Å 1.9 µm columns are compared to columns with sub-2 µm particle technology from other vendors. Analysis of recombinant human growth hormone (hGH), granulocyte colony-stimulating factor (hG-CSF), and interferon α-2b (INF α-2b) proteins demonstrate the superior performance of the AdvanceBio column for small protein therapeutic applications

Introduction

In recent years, there has been a large increase in the development of biologically derived therapeutics, known as biologics, to treat a myriad of diseases. Some of the biologic drugs include small protein therapeutic agents such as growth factors and cytokines because of their key roles in regulating the production, maturation and activity of blood, muscle and bone cells. For example, human growth hormone (hGH) is used to stimulate growth in children and adults exhibiting slow or subnormal growth due to hormonal deficiencies.[1] Granulocyte colony-stimulating factor (hG-CSF) is employed to treat cancer patients undergoing chemotherapy, to help raise white blood cell levels that have been reduced by cytotoxic therapeutic agents.[2] Interferons are a class of glycoproteins that have multiple therapeutic uses but are known to form partially unfolded species as well as aggregates particularly when exposed to pH or thermal degradation.[3]

Protein denaturation processes involving aggregation are among the prime factors impeding the development of stable protein drug formulations. The United States Pharmacopeia monograph method recommends size exclusion chromatography (SEC) HPLC for determining purity and aggregates of these proteins. SEC is a relatively straightforward technique. SEC relies on simple diffusion into the pore structure of the stationary phase; larger molecules cannot permeate the particles, and elute first, while smaller molecules diffuse readily into the pores, and elute later. Agilent AdvanceBio SEC 120 Å 1.9 µm columns are designed for aqueous size exclusion chromatography (SEC) of biomolecules. The particles have been manufactured using proprietary technology to combine optimum pore size and pore volume for separating molecules such as smaller proteins and peptides.

Experimental

Equipment and materials

All chemicals and reagents were HPLC grade or higher and were obtained from Sigma-Aldrich (now Merck) or VWR Scientific. Water was purified using a Milli-Q A10 (Millipore).

Instrumentation

Agilent 1260 Infinity II Bio-inert LC instrument comprising:

- Agilent 1260 Infinity II Bio-inert Pump (G5654A)
- Agilent 1260 Infinity II Bio-inert Multisampler (G5668A) with sample cooler (option #100)
- Agilent 1260 Infinity II Multicolumn Thermostat (G7116A) with bio-inert heat exchanger (option #019)
- Agilent 1260 Infinity II Diode Array Detector WR (G7115A) with bio-inert flow cell (option #028)

Software

OpenLab 2.2 CDS

Method conditions

HPLC Conditions	
Column	AdvanceBio SEC 1.9 µm 120 Å, 4.6 × 300 mm (p/n PL1580-5250)
Mobile Phase	150 mM Sodium phosphate, pH 7.0
Flow Rate	0.30 or 0.35 mL/min (as shown in text)
Column Temperature	25 °C
Injection Volume	2 µL, 1 mg/mL
Samples	Low molecular weight protein standard mix Human growth hormone, rhGH Human granulocyte colony stimulating factor, rG-CSH
Total Run Time	15 or 20 minutes (depending on flow rate)

Results and discussion

Proteins are complex molecules containing numerous side chain functionalities: acidic, basic, neutral, and hydrophobic. Finding the optimum conditions to avoid secondary interactions can be challenging, however the AdvanceBio SEC product range has a polymeric surface coating applied to the silica particle that overcomes many of these issues. The mechanism of separation relies on differences in size of molecules in solution (hydrodynamic radius). Protein structures are often compact and globular in nature, and proteins often aggregate under stress conditions such as extremes of temperature, pH, or salt composition and for dimers and larger units. This is a particular issue for protein molecules, where the presence of aggregated proteins can lead to adverse effects if administered as a therapeutic molecule. SEC provides the ideal tool for quantifying and monitoring protein aggregation. Figure 1 represents the SEC separation of low molecular weight protein and peptide standards. The calibration curve of these standards based on their retention time is shown in Figure 2. One can estimate the optimal molecular range for this column to be 1 to 80 kDa.

Peak	Protein/Peptide	Molecular Weight (Da)
1	Ovalbumin	44,000
2	Myoglobin	17,000
3	Aprotinin	6,700
4	Neurotensin	1,700
5	Uridine	244

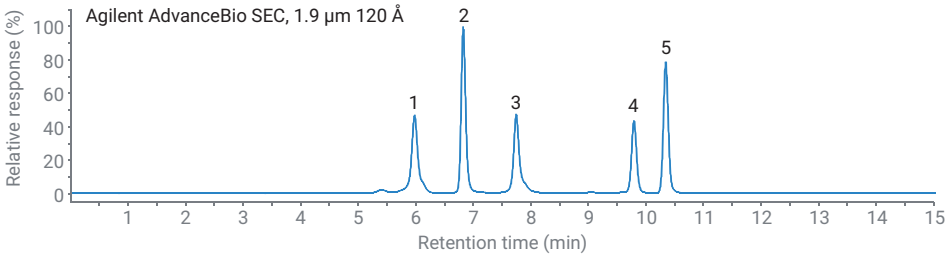

Figure 1. Size-exclusion chromatogram of low molecular weight protein and peptide mix at 0.35 mL/min.

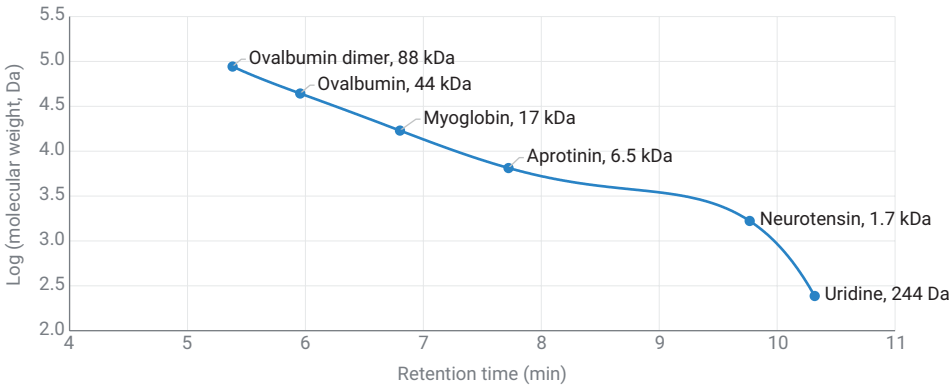

Figure 2. AdvanceBio SEC 1.9 µm 120 Å calibration curve of low molecular weight protein and peptide standards.

Even if the intention is to use the AdvanceBio SEC column for quantification of monomer and dimer content, it is still good practice to regularly perform a calibration using appropriate molecular weight standards. Regular calibration ensures better reproducibility, leading to improved accuracy, and enables earlier detection of potential problems, reducing system downtime and troubleshooting. For protein separations, the standards should be a range of well-characterized proteins covering the entire operating range of the column. The proper choice of standards provide two key aspects for the successful use of SEC: There should be minimal, secondary interactions between the analyte and the stationary phase. The pore size should be chosen to match the size of molecules being analyzed.

This application note demonstrates high resolution separation with an Agilent AdvanceBio SEC 120 Å 1.9 µm column for size-exclusion chromatography (SEC) analysis of the recombinant hGH and hG-CSF therapeutic proteins compared to current competition with sub-2 µm particle technology. By further optimizing the mobile phase conditions, the SEC separation of nondegraded and thermally degraded interferon alpha-2b (IFN α-2b) is also compared.

By comparing the retention time of the analyte of interest with the calibration curve, it is possible to determine if there are any signs of secondary interactions. Peaks that elute earlier or later than expected or have poor shape are signs that the mobile phase conditions may not be sufficiently optimized. Figure 3 shows the size-exclusion chromatogram of hG-CSF on the AdvanceBio SEC 1.9 µm 120 Å column where the retention time corresponds well to that of a protein of around 20 kDa.

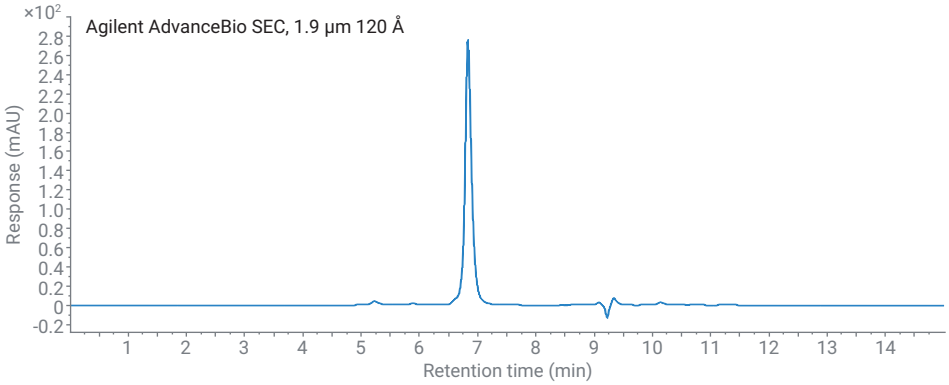

Figure 3. Size-exclusion chromatogram of hG-CSF on an Agilent AdvanceBio SEC 1.9 µm 120 Å 4.6 × 300 mm column at 0.35 mL/min.

Figure 4 shows the close up of the baseline of hG-CSF run on the AdvanceBio SEC 1.9 µm 120 Å column as well as other sub-2 µm columns from other vendors. The chromatogram at the bottom of the diagram is indicative of problems associated with secondary interactions (later than expected elution time and tailing peak).

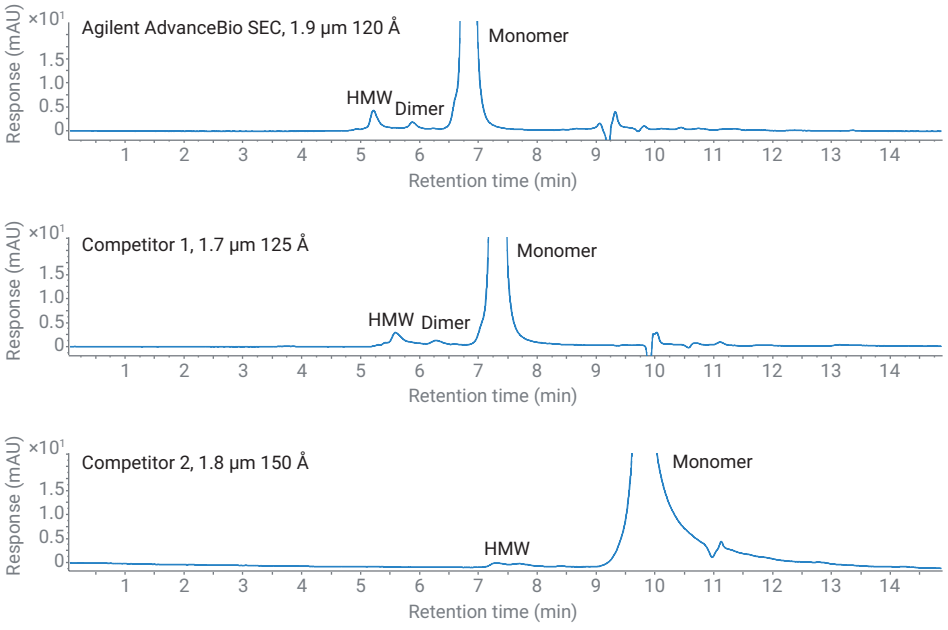

Figure 4. Close up of size-exclusion chromatograms of hG-CSF at 0.35 mL/min.

Many other biotherapeutic proteins have similar molecular weights and are therefore also suitable for analysis on the same AdvanceBio SEC 1.9 µm 120 Å column. The recombinant form of hGH, somatropin, may contain some impurities due to post-translational modification or as a result of downstream processing. Figure 5 shows the size-exclusion chromatogram of somatropin carried out under the same conditions as described previously. The inset shows the zoomed baseline region where dimer and higher molecular weight aggregates are evident.

Other proteins may require further method development to obtain the optimum peak shape and resolution. A series of experiments with different mobile phase conditions was used to determine the optimum composition for peak shape and protein recovery of IFN α-2b as shown in Table 2.

Table 1. Peak area data for high molecular weight (HMW), dimer, and monomer peaks for hG-CSF.

	AdvanceBio SEC 1.9 µm 120 Å				Competitor 1, 1.7 µm 125 Å				Competitor 2, 1.8 µm 150 Å			
	RT (min)	%Area	Rs USP	Peak Tailing	RT (min)	%Area	Rs USP	Peak Tailing	RT (min)	%Area	Rs USP	Peak Tailing
HMW	5.22	2.61		1.16	5.59	2.49		1.28	7.40	2.01		1.37
Dimer	5.88	1.02	2.41	1.11	6.27	0.83	1.68	1.26	N.D.			
Monomer	6.82	96.37	3.77	1.13	7.31	96.68	3.04	1.11	9.74	97.99		2.13

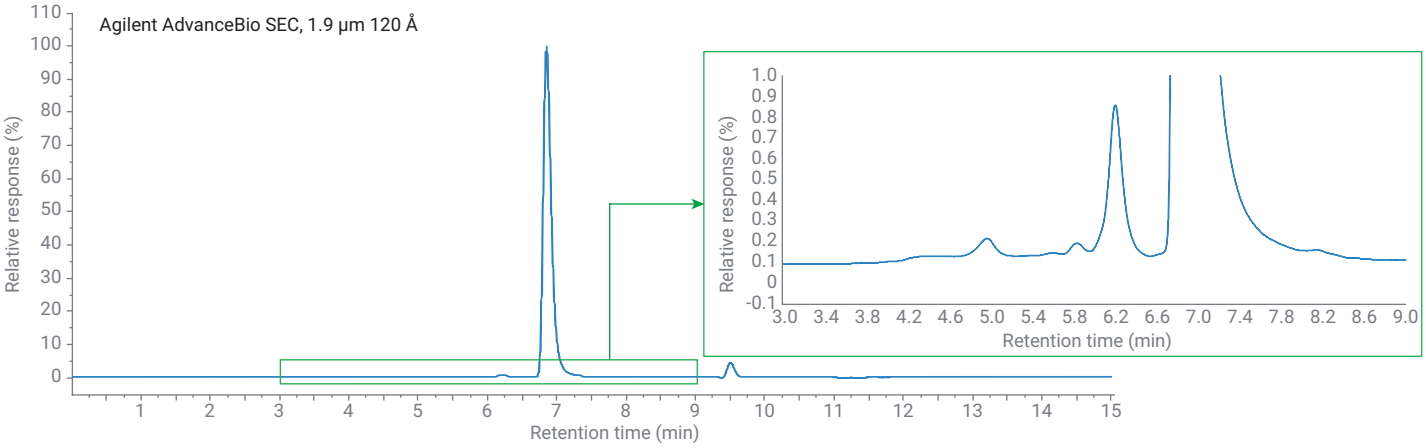

Figure 5. Size-exclusion chromatogram of somatropin (rhGH).

Table 2. Peak shape data during method optimization for IFN α-2b.

NaCl (mM)	Peak Width (min)	Tailing	Resolution HMW-Monomer	Resolution Monomer-LMW
100	0.20	2.88	1.94	1.98
150	0.18	2.65	2.25	2.31
200	0.16	2.52	2.26	2.66
250	0.15	2.39	2.84	2.86
400	0.14	2.08	3.32	3.59

The size-exclusion chromatograms of interferon alpha-2b reference material run on three different sub-2 µm SEC columns is shown in Figure 6, along with the retention time and peak tailing data. The difference in column performance may lead to a difference in resolution when separating IFN α-2b impurities by SEC therefore the experiment was repeated using a degraded sample.

In the case of interferon alpha-2b, it has been suggested that the partial unfolding of the molecule is involved in the formation of aggregates, but that the partially unfolded species are somewhat stable.[3] Furthermore, the presence of O-glycosylation can also reduce the thermal stability of these molecules4. The choice of cell line for recombinant protein manufacture is a critical parameter since *E. coli* cell lines do not introduce glycosylated variants.

Optimized HPLC Conditions for INF α-2b	
Column	Agilent AdvanceBio SEC 1.9 µm 120 Å, 4.6 × 300 mm (p/n PL1580-5250)
Mobile Phase	200 mM Sodium phosphate + 250 mM NaCl, pH 6.5
Flow Rate	0.35 mL/min
Column Temperature	25 °C
Injection Volume	2 µL, 1 mg/mL
Samples	Interferon alpha-2b (INF α-2b) Heat stressed interferon alpha-2b (INF α-2b): 60 °C for 30 min
Total Run Time	15 min

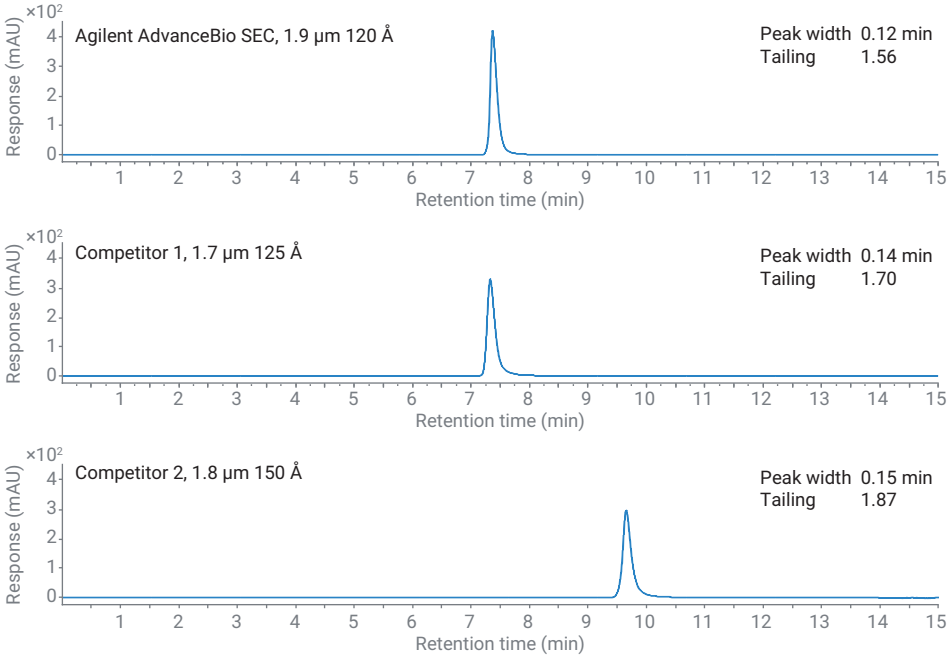

Figure 6. Size-exclusion chromatograms of interferon α-2b.

By exposing the interferon alpha-2b sample to thermal degradation (heated to 60 °C for 30 minutes), it was possible to introduce various impurities. The impurities include both early eluting high molecular weight species (HMW) as well as later eluting low molecular weight species (LMW) as seen in Figure 7. As expected, the resolution of both the HMW to monomer and monomer to LMW species is greatest on the AdvanceBio SEC 1.9 µm 120 Å column. This column had the narrowest peaks and the least amount of peak tailing in the previous separation of the nondegraded sample.

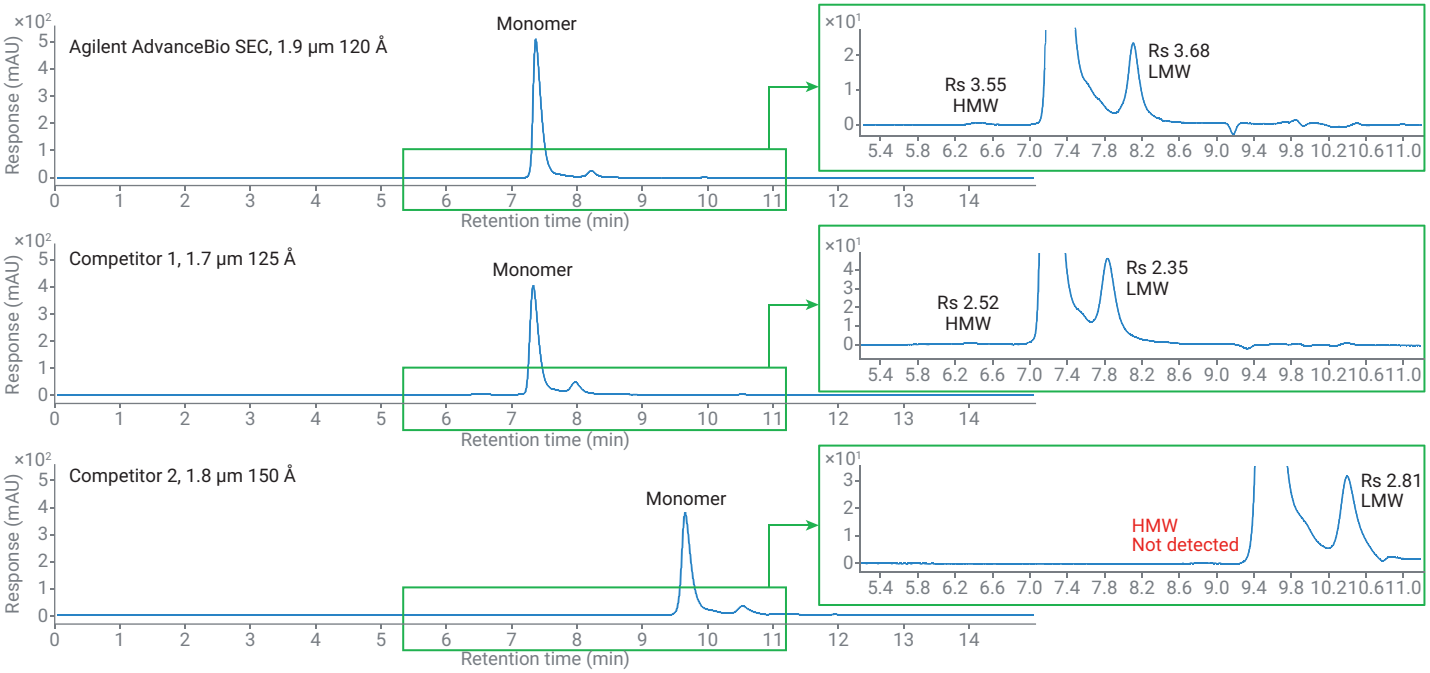

Figure 7. Size-exclusion chromatograms of heat stressed interferon α-2b.

Conclusion

Agilent AdvanceBio SEC offers a range of column dimensions and different pore sizes suitable for differently sized molecules. The featured AdvanceBio SEC 120 Å 1.9 µm column demonstrates superior performance with high resolution SEC analysis of small protein therapeutic applications when compared to columns of similar particle size and pore size characteristics from other vendors.

Calibrating your AdvanceBio SEC size exclusion column with appropriate standards ensures you understand the correct working range. These standards allow you to use calibration curves to estimate the molecular size of unknown molecules. However, regular calibration with a selection of standards is beneficial, and can be used to monitor column performance over time, allowing early detection of potential problems. In turn, corrective action can be taken, ultimately reducing system downtime and improving productivity.

References

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