Multiple Detector Approaches to Protein Aggregation by SEC

Application Note

BioPharma

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Abstract

Protein aggregation, where molecules assemble into dimers, trimers, tetramers, or larger multimers, is a common occurrence that can have devastating consequences. Biotherapeutic proteins, such as monoclonal antibodies, can give rise to severe immunological events as a result of aggregation. Therefore, quantitation and monitoring using size exclusion chromatography (SEC) are paramount during manufacture and lot-release QC. This application note considers some of the factors that can influence the degree of aggregation and the importance of optimizing methodology.

Introduction

Biotherapeutic proteins have increasing clinical importance and are used to treat a variety of serious illnesses and chronic diseases, such as multiple sclerosis, heart disease, anemia, rheumatoid arthritis, and cancer. Like many other proteins, they are able to form aggregates, particularly under conditions of stress. The presence of aggregates is a potentially serious problem and can cause a variety of undesirable effects, ranging from reduced shelf life or poor bioavailability through to severe immunological events such as anaphylactic shock. Aggregation from simple dimerization to formation of insoluble agglomerates can occur both during manufacture, formulation, or storage. It is a complex, poorly understood process that can develop from thermal or shear stress or be due to the presence of minor contaminants. Aggregation can include self-association following partial unfolding or covalent aggregation.

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SEC has long been recognized as the ideal tool for separating and quantifying aggregation. By combining SEC with light scattering detection, this tool becomes even more powerful. To generate good results, it is important that the chromatographic process is fully understood and that the method does not influence or change the analysis.

Clearly, the choice of column (pore size and particle size) and the mobile phase pH or ionic strength could affect the analysis. Other factors, such as sample storage and preparation method including concentration, can also play a role.

This application note investigates the effect of sample preparation parameters, including concentration, storage temperature, and sonication, on the level of aggregation of a bovine immunoglobulin protein.

Materials and Methods

To choose the most appropriate pore size column for this investigation, a mixture of protein standards was analyzed using Agilent Bio SEC-5 300Å, 7.8 × 300 mm columns and Agilent Bio SEC-5 500Å, 7.8 × 300 mm columns (Figure 1). The mixture contained γ-globulin, bovine serum albumin, ovalbumin, β-lactoglobulin, myoglobin, and cytochrome c, covering a wide molecular weight range.

Bovine γ-globulin was chosen as a model protein as it contains a significant amount of dimer and higher aggregates and is representative of an IgG monoclonal antibody. With a monomer mass of 150 kDa (and hence 300 kDa for dimer and considerably higher for larger aggregates), to avoid the risk of exclusion of high molecular weight species, the most appropriate pore size was 500Å. Therefore, the Bio SEC-5 500Å, 7.8 × 300 mm column was selected for the rest of the work.

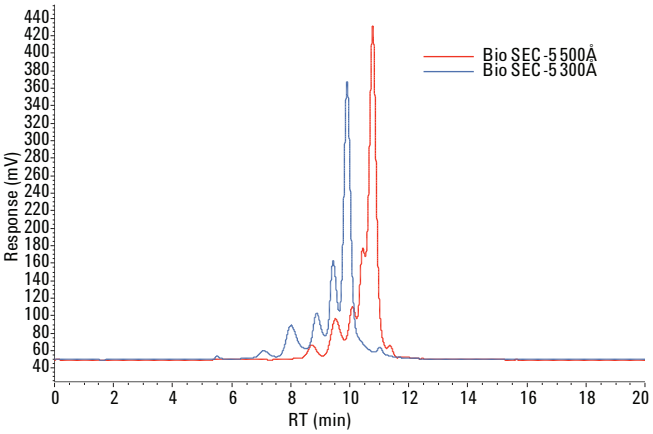

Figure 1. Protein standards by size exclusion chromatography on 300Å (blue trace) and 500Å (red trace) Agilent Bio-SEC columns.

The use of a light scattering detector in combination with a concentration detector, such as UV or RI, can provide absolute molecular weight information. Figure 2 shows three regions of the chromatogram were chosen, and the molecular weight information derived from software. This clearly illustrates the monomer, dimer, and trimer regions of the chromatogram. Furthermore, the signal from the light scattering detector was noticeably more responsive to higher molecular weight material, as expected.

Conditions

Columns:	Agilent Bio SEC-5 500Å, 7.8 × 300 mm, stainless steel (p/n 5190-2531)
Mobile phase:	50 mM sodium phosphate, 250 mM NaCl, pH 7.0
Injection volume:	50 µL
Flow rate:	1.0 mL/min
Gradient:	Isocratic
Temperature:	30 °C
Sample:	Bovine γ-globulin
Sample concentration:	1.0, 2.0 and 4.0 mg/mL
Detection:	UV, 280 nm
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC System with Agilent 1260 Infinity GPC/SEC Multi Detector Suite

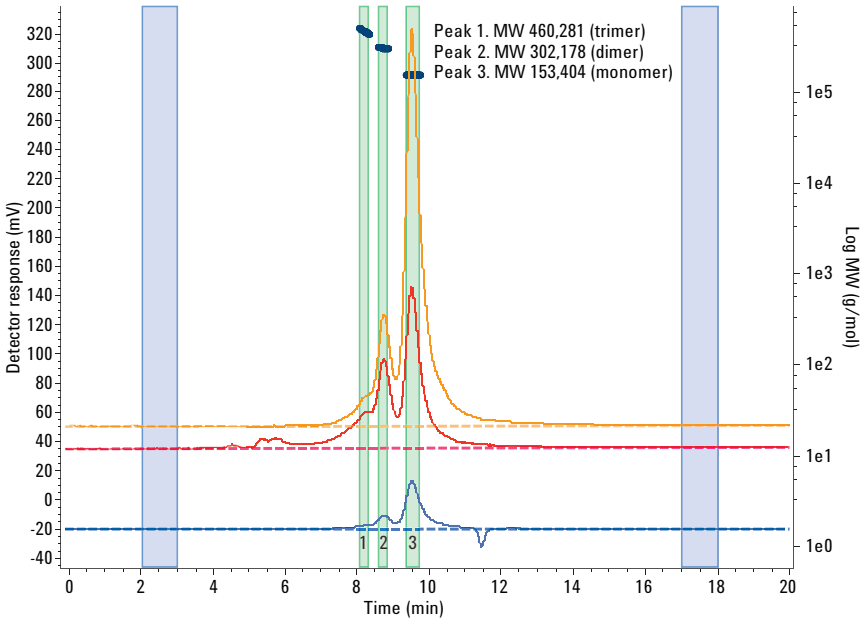

Figure 2. Detector signals from monomer (region 3), dimer (region 2), and trimer (region 1) of bovine IgG; UV 280 nm (orange), light scattering 90° (red), and refractive index (blue), run length 20 minutes. Blue columns indicate the beginning and end of the baseline. Blue bars indicate the molecular weights of the peaks.

Results and Discussion

To ensure reproducibility, each experiment was carried out in duplicate and the average taken. Integration of peak area was established by using a template that consistently applied the same criteria to each chromatogram (Figure 3).

Initially, the sample was prepared at four different solution strengths to determine the effect of concentration on aggregation. Results were very similar, with slightly higher levels of aggregation at higher sample concentrations, as expected (Table 1).

Previously, we demonstrated the effect of column temperature on analysis of protein aggregation with very little noticeable effect observed at low to mid temperatures (5, 25, and 45 °C) but significant effects at 65 °C [1]. In this experiment, we looked at the sample storage temperature (set temperature for the autosampler) to investigate the effect of 7, 15, 25, and 35 °C on the extent of aggregation of 4 mg/mL samples (Table 2). By increasing the temperature at which the sample was stored prior to injection, the level of aggregation could be reduced.

Table 1. Quantification of protein aggregation – effect of sample concentration.

Sample concentration (mg/mL)	Monomer (%)	Dimer (%)	Higher aggregates (%)
1	77.33	16.05	6.63
2	76.71	16.43	6.87
4	76.12	16.45	7.44

Table 2. Quantification of protein aggregation – effect of autosampler temperature.

Temperature (°C)	Monomer (%)	Dimer (%)	Higher aggregates (%)
7	76.12	16.45	7.44
15	78.23	15.82	5.96
25	80.28	14.44	5.29
35	83.59	12.36	4.06

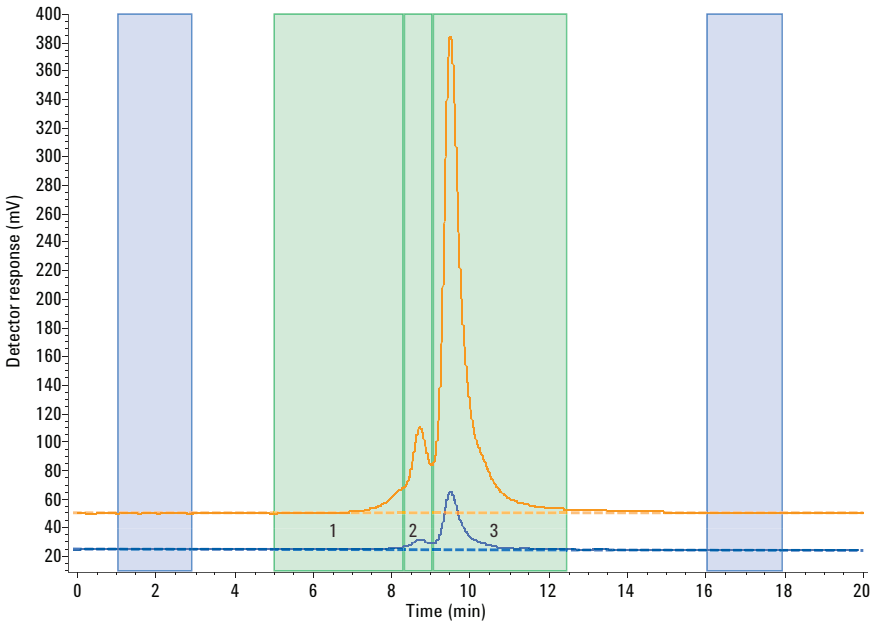

Figure 3. Peak regions of bovine IgG defined for area integration (higher aggregates region 1, dimer region 2, and monomer region 3); 4 mg/mL, sonicated at 7 °C, run length 20 minutes. Blue columns indicate the beginning and end of the baseline.

Other techniques employed in the laboratory to aid sample dissolution, such as sonication, can also affect the degree of aggregation (Figure 4 and Table 3).

Conclusions

Sample preparation is an important factor that can affect the level of aggregation in protein analysis by size exclusion chromatography. Inclusion of a light scattering detector helps to provide additional insights and valuable information. Particular care must be taken to ensure that the method itself does not change the property under investigation. In this instance, smaller aggregates (dimers and trimers) appeared to be relatively weakly associated and, with additional energy input through moderate heat or sonication, could be readily dissociated, thereby changing the outcome of the experiment.

Table 3. Quantification of protein aggregation – effect of sonication.

	Monomer (%)	Dimer (%)	Higher aggregates (%)
Before sonication	76.12	16.45	7.44
After sonication	83.80	12.25	3.95

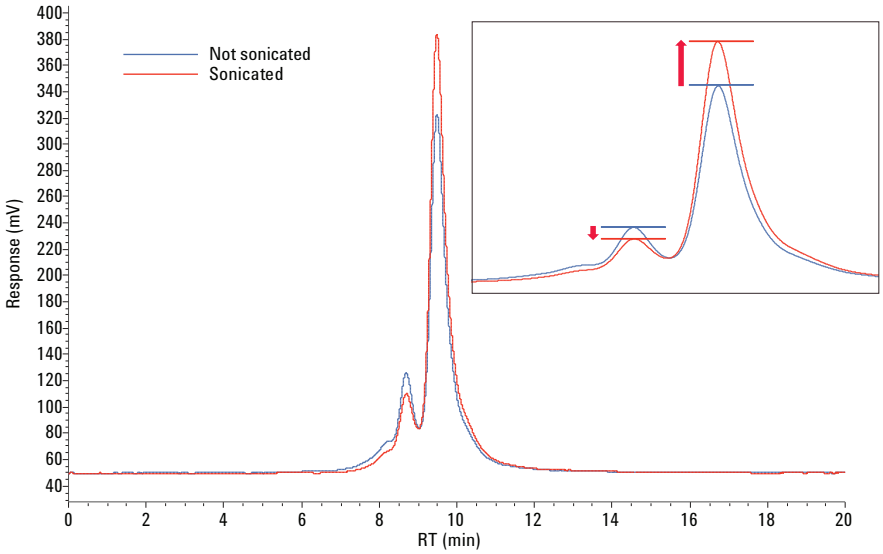

Figure 4. Effect of sonication on sample aggregation in bovine IgG; 4 mg/mL, 7 °C, sonicated (red) and not sonicated (blue).

Reference

1. Umbreen Ahmed and Greg Saunders. “The Effect of Temperature on Protein Size Exclusion Chromatography”. Application Note, Agilent Technologies, Inc., Publication Number 5990-8140EN (2012).

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