Optimum Pore Size for Characterizing Biomolecules with Agilent Bio SEC Columns

Application Note

BioPharma

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Introduction

Size exclusion chromatography (SEC) is a technique for separating proteins, oligonucleotides, and other complex biopolymers in their native forms by size using aqueous eluents. For molecules of discreet molecular weight, such as proteins, SEC can be used to detect and quantitate monomers, dimers, aggregates and fragments.

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Pore size choice

The choice of media pore size will influence the resolution in SEC. As the separation is based on differences in molecular size in solution, the sample must be able to permeate the porous structure of the particles. If the pore size is too small the samples will be excluded from the pores and elute in the void volume of the column, and if too large, all will be able to fully permeate the particles and there will be very little separation. This application note demonstrates the effect of pore size on separations of proteins and antibody using Agilent Bio SEC-3 HPLC columns. These columns are available in 100Å, 150Å, and 300Å pore sizes to accommodate most peptide and protein size exclusion separations.

Protein separation

For this experiment, we used a standard protein mix that covers the typical range of characteristics of recombinant biopharmaceuticals.

Conditions

Columns	Agilent BioSEC-3 100Å, 4.6 x 300 mm, 3 µm (p/n 5190-2503) Agilent BioSEC-3 150Å, 4.6 x 300 mm, 3 µm (p/n 5190-2508) Agilent BioSEC-3 300Å, 4.6 x 300 mm, 3 µm (p/n 5190-2513)
Sample	Bio-Rad Protein Standards Mix
Eluent	100 mM Sodium phosphate buffer + 0.15 M NaCl, pH 6.8
Flow rate	0.35 mL/min
Detector	UV, 220 nm
System	Agilent 1260 Bio-inert Quaternary LC

Table 1 lists the components of the protein test mix. Figure 1 shows the resulting separation achieved for each of the three different pore size columns. The 100Å column excludes the larger globular proteins (thyroglobulin, its aggregates, and γ-globulin), and so is only suitable for proteins with molecular weights less than 100,000.

Table 1. Peak Identification and Molecular Weight of a Standard Protein Mix

Peak number	Name	Molecular weight
1	Thyroglobulin aggregates	
2	Thyroglobulin	670,000
3	IgA	320,000
4	IgG (γ-globulin)	158,000
5	Ovalbumin	44,000
6	Myoglobin	17,000
7	Vitamin B12	1,355

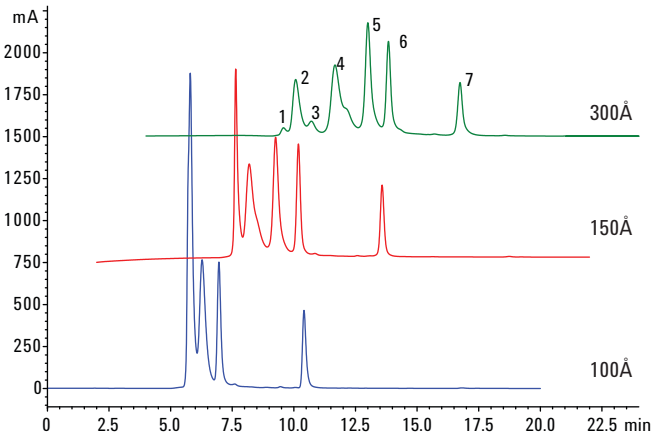

Figure 1. Separation of the standard protein mix on Agilent BioSEC-3 columns with three different pore sizes. The 100Å and 150Å columns demonstrate exclusion of the larger proteins. Note how the 300Å pore size gives definition between the thyroglobulin and its aggregates, also the resolution between the IgA and IgG peaks.

The 150Å column shows very good resolution between the ovalbumin and myoglobin (Table 2) and the only protein excluded is the high molecular weight thyroglobulin. The 300Å column is able to resolve proteins across the whole range.

Table 2 shows the resolution factors between the proteins for each pore size. From this, it is possible to make an informed decision on the most suitable column to choose for your application.

Table 2. Resolution Factors Achieved with the Separation of the Protein Mix

Media	Rs Factor Thyro/Aggs	Rs IgA/Thyro	Rs IgG/IgA	Rs Ovalb/IgG	Rs Myo/Ovalb	Rs Vit B12/Myo
100Å	*	*	*	*1.17*	1.87	12.94
150Å	*	*	*1.28*	2.02	2.59	11.66
300Å	0.93	1.06	1.38	2.32	2.19	8.24

* no resolution, excluded
Numbers in italics are achieved using the retention time of the excluded peaks

Antibody separation

In this trial, we used a mouse IgG sample to evaluate the suitability of the pore sizes for the analysis of the monomer and its dimer components. The size, type, and content of aggregates present in protein biopharmaceuticals can affect both efficacy and formulation, or worse, induce an immunogenic response. Aggregation formations occur through a variety of mechanisms, including disulfide bond formation and non-covalent interactions.

Conditions

Columns	Agilent BioSEC-3 100Å, 4.6 x 300 mm, 3 µm (p/n 5190-2503) Agilent BioSEC-3 150Å, 4.6 x 300 mm, 3 µm (p/n 5190-2508) Agilent BioSEC-3 300Å, 4.6 x 300 mm, 3 µm (p/n 5190-2513)
Sample	Mouse IgG
Eluent	100 mM Sodium phosphate buffer + 0.15 M NaCl, pH 6.8
Flow rate	0.35 mL/min
Detector	UV, 220 nm
System	Agilent 1260 Bio-inert Quaternary LC

Figure 2 shows the separation using Agilent BioSEC 3 µm columns with different pore sizes, demonstrating that the 100Å pore size column excludes the IgG and so no definition is achieved. However, both the 150Å and 300Å columns are able to resolve the monomer and dimer. Figure 3 shows the separation achieved on the 300Å column magnified and shows the detail seen by the 300Å column, making it a suitable choice for analysis of monoclonal antibodies and their aggregates.

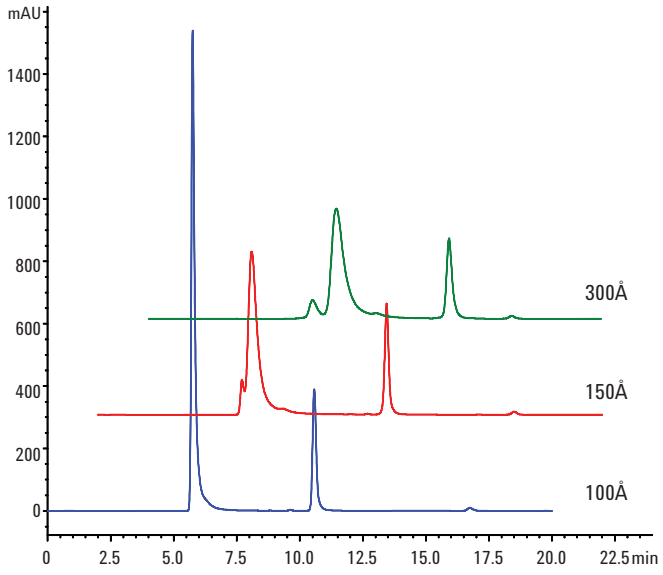

Figure 2. Separation of mouse IgG on Agilent BioSEC-3 columns with three different pore sizes. Again, resolution increases with pore size.

Conclusions

Because the size of protein aggregates, including dimers, is sufficiently different from the protein monomer, it is possible to separate the various forms using SEC. In fact, SEC with UV or light scattering is a standard technique for quantifying protein aggregation and molecular weight. However, because the choice of media pore size influences the resolution obtained when using SEC, it is worthwhile testing a range of pore sizes to match the pore size to the analyte. This approach will identify the best choice before conducting in-depth investigations, and reduce the risk of missing valuable information through the choice of media with an inappropriate pore size.

Agilent Bio SEC-3 HPLC columns are available in 100Å, 150Å, and 300Å pore sizes to accommodate most peptide and protein size exclusion separations.

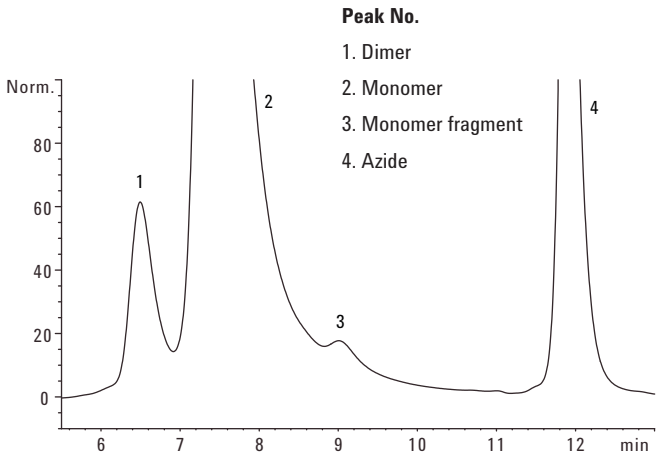

Figure 3. Magnification of the separation of mouse IgG using an Agilent BioSEC-3 300Å column – the best choice for analyte.

For More Information

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