SIZE EXCLUSION CHROMATOGRAPHY FOR BIOMOLECULE ANALYSIS:

A "How to" Guide

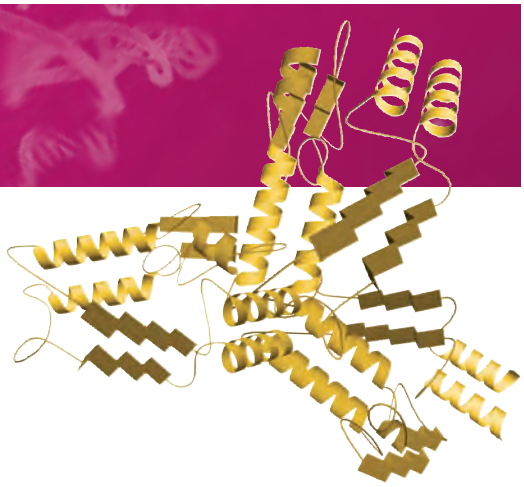
The Measure of Confidence

Agilent Technologies

INTRODUCTION

The chromatographic separation of biomolecules based on their size in solution is known as Size Exclusion Chromatography (SEC). Unlike other modes of chromatography, it relies on the *absence* of any interaction between the analyte and the stationary phase packed in the column. This provides an ideal solution for separating and analyzing intact proteins from contaminants that can include aggregates, excipients, cell debris, and other impurities arising from degradation. SEC is therefore widely used in both development and manufacture for biotherapeutic molecule characterization.

In this guide, we will discuss SEC separations, the effect of solute size and molecular weight, column selection choices, important mobile phase considerations, general rules of thumb for using SEC, and more.



Separation is straightforward and uncomplicated

With SEC, molecules are separated from largest to smallest in proportion to their molecular weight. Very large molecules are excluded from the packed bed and elute first, in the void volume. Smaller molecules will be able to penetrate the pores to various degrees depending on their size (**Figure 1**), with the smallest molecules diffusing furthest into the pore structure and eluting last.

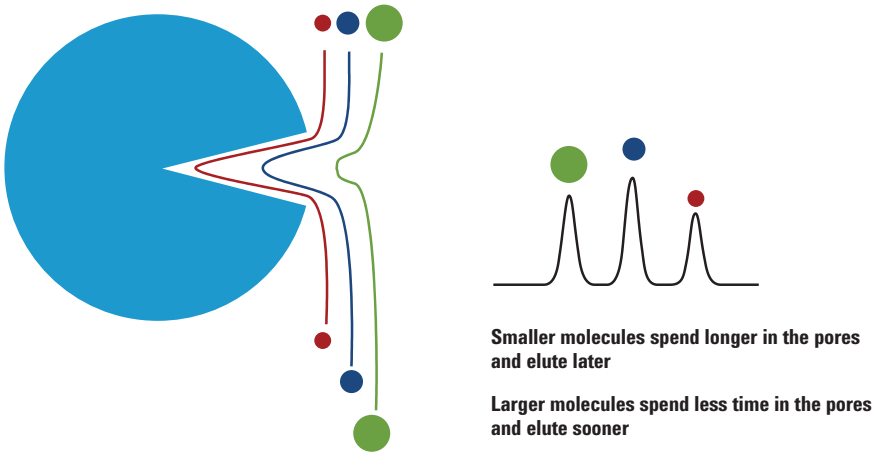


Figure 1 – Molecules permeate the pores of the stationary phase to different extents depending on their size

Learn more about Agilent biocolumns for SEC at **agilent.com/chem/bioHPLC**

Size Exclusion Chromatography is suitable for separating and quantifying protein mixtures, and is therefore a valuable technique for quality control in recombinant protein manufacture. This includes measuring aggregates (dimers, trimers, tetramers etc.) or separating low molecular excipients and impurities from larger molecular weight proteins (**Figure 2**).

Understanding and controlling aggregation in therapeutic proteins is essential as it will affect efficacy, lifetime, and could even result in a potentially serious immunogenic response. Regulations, such as ICH(Q6B) clearly state that aggregates must be resolved from the desired product and quantitated (ICH(Q6B), page 16).

Intact MAb monomer and dimer separation

Column:	**Agilent Bio SEC-3, 300Å 7.8 x 300 mm, 3 µm (p/n 5190-2511)**
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC System
Flow Rate:	1.0 mL/min
Temperature:	Ambient
Detector:	UV, 220 nm
Injection:	5 µL
Sample:	CHO-humanized MAb, 5 mg/mL – intact
Sample Conc:	150 mM sodium phosphate buffer, pH 7.0

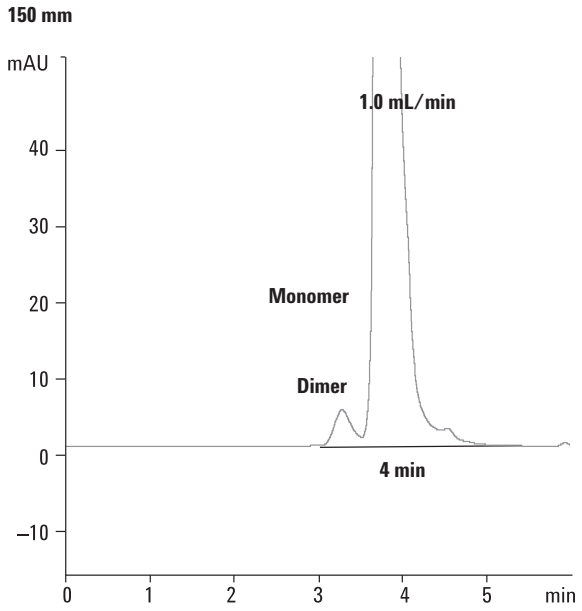


Figure 2 – Separation of IgG aggregates and excipients

Elution order typically follows molecular weight – molecules with the highest molecular weight eluting first – however the true mechanism of SEC is based on size in solution. Most proteins are compact, but some protein molecules are cylindrical, so may elute earlier than expected due to their larger hydrodynamic radius in solution (**Figure 3**). Furthermore, different mobile phases can affect the elution order because of changes in size in solution (hydrodynamic radius or radius of gyration).

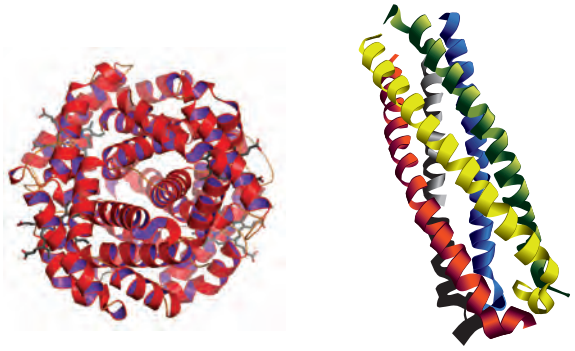


Figure 3 – Comparison of compact, globular protein versus cylindrical protein

SEC Method Development Guide

Choose initial columns and conditions for size-based separation of biomolecules, aggregation analysis – peptides, polypeptides, and proteins

Peptides, Polypeptides, Proteins, MAbs
MW >0.1-1,250 kDa

Peptides, Polypeptides, Proteins, MAbs
MW >0.1-10,000 kDa

Select column based on molecular weight range and pore size

Agilent Bio SEC-3 (3 µm)

Pore Size	MW range, kDa
100Å	0.1-100
150Å	0.5-150
300Å	5-1,250

Agilent Bio SEC-5 (5 µm)

Pore Size	MW range, kDa
100Å	0.1-100
150Å	0.5-150
300Å	5-1,250
500Å	15-5,000
1000Å	50-7,500
2000Å	>10,000

Recommended Initial Separation Conditions

Column: Agilent Bio SEC (3 µm and 5 µm)

Mobile Phase: 150 mM phosphate buffer, pH 7.0*

Gradient: Isocratic in 30-60 min range

Temperature: Recommended: 10-30 °C, Maximum: 80 °C

Flow Rate: 0.1-0.4 mL/min for 4.6 mm id columns
0.1-1.25 mL/min for 7.8 mm id columns

Sample Size: ≤ 5% of total column volume

*Other aqueous buffers with high and low salt can be used

For additional information, see application note: *Defining the Optimum Parameters for Efficient Size Separations of Proteins* (publication # 5990-8895EN)
www.agilent.com/chem/library

After the initial chromatogram, additional changes may be needed to improve the separation, maintain protein solubility, or to decrease sample interaction with the chromatographic media. The ionic strength of the mobile phase can be adjusted up or down in strength to attain an optimized separation. pH can also be adjusted usually + 0.2 units. If further optimization is necessary, the upward or downward range should be expanded. A change of temperature or addition of an organic solvent can also be used.

For protocols requiring additional salt, these buffers are typical:

100-150 mM sodium chloride in 50 mM sodium phosphate, pH 7.0
100-150 mM sodium sulfate in 50 mM sodium phosphate, pH 7.0
50-100 mM urea in 50 mM sodium phosphate, pH 7.0
Other similar salts (e.g. KCl) and guanidine hydrochloride can also be used

pH Range:

2.0-8.5

Potential organic solvent additions include:

5-10% ethanol (or other similar solvents) in 50 mM sodium phosphate, pH 7.0
5% DMSO in 50 mM sodium phosphate, pH 7.0

Temperature:

Typically, SEC separations are run at 20-30 °C. Separation of proteins and peptides may require higher temperature to improve both resolution and recovery of proteins and hydrophobic peptides.

Maximum temperature of Bio SEC columns is 80 °C.
Note that higher temperatures can denature proteins.

Learn more about Agilent biocolumns for SEC at **agilent.com/chem/bioHPLC**

Instrumentation Considerations for SEC

The SEC separation mechanism means that the elution volume, or retention time, is absolutely critical to the analysis. This requires high performance instrumentation to ensure precision and reproducibility. Isocratic pumps or gradient pumps operated in isocratic mode are suitable, so refractive index (RI) detectors – as well as the more conventional UV or DAD detector – can be employed. To ensure baseline stability – especially when using an RI detector – online degassing of the mobile phase, as well as thermostatted compartments, are highly recommended. Operating at elevated temperatures increases the diffusion coefficient, leading to better resolution, better reproducibility and reduced stress on the column. Therefore, thermostatted compartments are essential for a high performance system.

Agilent 1290 Infinity LC System

Agilent 1260 Infinity Bio-inert Quaternary LC System

Robust and reliable operation even under challenging solvent conditions

Buffers with high salt concentrations such as 2 M NaCl or 8 M urea and extreme pH values of 1-13 are commonly used in the analysis of biomolecules, posing a significant challenge for LC instruments. The dedicated design of the 1260 Infinity Bio-inert Quaternary LC handles these harsh solvent conditions with ease. Corrosion-resistant titanium in the solvent delivery system and metal free materials in the sample flow path create an extremely robust instrument – protecting not only your sample but your investment.

Protect your proteins during analysis

Heat can denature proteins, so it is important that your sample is being kept at constant temperature in the whole LC flow path. The Bio-inert autosampler with inert sample loop and ceramic needle can be cooled with an add-on thermostat. Bio-inert heat exchangers for the thermostatted column compartment keep the temperature constant. Agilent offers a number of bio-inert flow cells to enable reliable analysis of your protein under various conditions. Get more information about flow cell options at **www.agilent.com/chem/bioflowcells**.

Bio-inert flow cell with RFID tag, 10 mm, 13 µL (p/n G5615-60022)

Software solutions to provide new insights

When working with size exclusion chromatography, there are several software options available to support you:

- **HPLC software** – Agilent OpenLAB CDS Chemstation software helps you to acquire, review and organize chromatographic data and perform quantitative analysis.
- **GPC/SEC software** – Available as part of the Agilent GPC/SEC system. This software introduces more information based on molecular weight.
- **Agilent Buffer Advisor software** – Eliminates the tedious and error-prone method-development steps of buffer preparation, buffer blending and pH scouting by providing the fast and simple way to create salt and pH gradients.

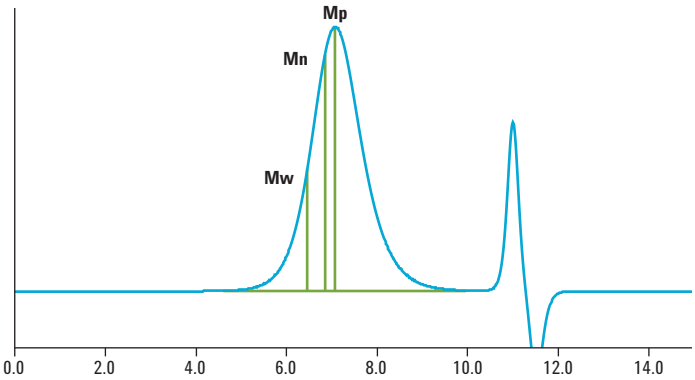


Figure 4 – SEC separation of polysaccharide showing Mw, Mn, Mp determination

Comprehensive molecular characterization

SEC can be used to determine the average molecular weight of polymeric analytes – including naturally occurring molecules (polysaccharides, starches, etc), as well as synthetic polymers (polyethyleneglycol or polyethylene oxide) (see **Figure 4**). For proteins or more complex samples, including vaccines, a more sophisticated form of data analysis with dedicated software is often required; in combination with the appropriate detectors, valuable information on conformation of the sample can be obtained. See page 16 for more information about detector choices.

Learn more about Agilent biocolumns for SEC at **agilent.com/chem/bioHPLC**

Components of Size Exclusion Characterization

Sample Preparation

Sample preparation for size exclusion chromatography is similar to that for any protein analysis for HPLC methods. The most important aspect is that the sample must be soluble in the eluent and should ideally be dissolved in the mobile phase itself. Because of the larger column dimensions and low linear velocity as a result of relatively slow flow rates compared with other forms of HPLC (see "Column size", below), sample concentrations and injection volumes may need to be larger than normal. In order to protect the column from possible damage it is recommended that samples should be filtered before use to remove particulates, but filtration should not be used as a solution to address poor sample solubility – an alternative eluent may need to be found.

For effective sample preparation, it is also important to ensure that the methods used to dissolve the sample do not change the properties of the sample itself. Some proteins may aggregate (forming dimers and higher molecular weight multimers) or dissociate (forming lower molecular weight sub-units) under stress conditions which may include: freeze-thaw cycles, extremes of temperature, sonication, or even concentration. See the method development guide on page 5 for more information.

Captiva Low Protein Binding Filters

Regardless of what sample prep you are performing, it's a good idea to filter your sample with a low protein binding filter.

Agilent PES filters provide superior and consistent low protein binding for protein-related filtration. The PES filter membranes are a better option than PVDF membranes for most LC analyses. Agilent PES has similar compatibility to PVDF filters for common LC solvents and is superior in terms of protein binding and cleanliness. Learn more at **agilent.com/chem/filtration**

Captiva PES Filters

Diameter (mm)	Pore Size (µm)	Certification	Housing	Part Number
4	0.45	LC	Polypropylene	5190-5095
4	0.2	LC/MS	Polypropylene	5190-5094
15	0.2	LC/MS	Polypropylene	5190-5096
15	0.45	LC	Polypropylene	5190-5097
25	0.2	LC/MS	Polypropylene	5190-5098
25	0.45	LC	Polypropylene	5190-5099

Column Selection

Column size

SEC columns are usually much larger than those used for other types of chromatography and are operated at comparatively low flow rates or slow linear velocities. The standard column dimension for SEC is 7.8 x 300 mm, operated at 1.0 mL/min, compared to a reversed-phase column that is likely to be 2.1 or 4.6 x 150 mm and operated at 2-3 times greater linear velocities. This is not a column size effect but due to SEC mechanism.

With SEC, there is no increase in concentration of samples typically seen with other chromatography techniques due to absorption or interaction with the stationary phase. Therefore, samples analyzed by SEC are injected in much larger volumes (20-100 µL), often at high concentrations (1-4 mg/mL). Run times are typically 10-12 minutes per column (assuming a conventional 7.8 x 300 mm column operated at 1.0 mL/min) and peaks are usually broad, so high data collection rates are not needed. For comparison or quantitation for protein aggregation, HPLC software is used. In order to obtain molecular weight distribution information for polydispersed polymers, specific SEC software is used.

Understanding the properties of your chosen column through the use of regular calibration is of paramount importance. By including a sufficiently large molecule – one that is too big to permeate any of the pores – it should be possible to determine the exclusion limit for the column. Similarly, using a very small molecule – one that is small enough to permeate all of the pore structure – it is possible to determine the total permeation limit of the column. You should then ensure that the separation you are trying to achieve occurs between these two limits. If the chromatogram of your sample includes excluded material or material that elutes at the total permeation point it is a sign that you may need to use a different pore size column for your analysis.

Increasing analytical speed with shorter columns

It is usually necessary to use columns of 300 mm in length in order to obtain the degree of resolution you need for your analysis. However, in order to improve the speed of separation, you can consider using shorter column lengths. The separation can be accomplished in half of the time by using a column 150 mm in length, however the resolution will be impaired. Where high-throughput is necessary, shorter columns can often be run at higher flow rates without risk of backpressure limits being reached, so a further reduction in analysis time can be achieved. See **Figure 5**.

Column:	**Agilent Bio SEC-3 300Å, 7.8 x 300 mm SS vs. Agilent Bio SEC-3 300Å, 7.8 x 150 mm SS**
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC System
Mobile Phase:	150 mM sodium phosphate, pH 7.0
Gradient:	Isocratic
Temperature:	30 °C
Detector:	UV, 280 nm
Injection:	20 µL
Sample:	Bovine serum albumin (BSA)
Sample Conc:	2.0 mg/mL

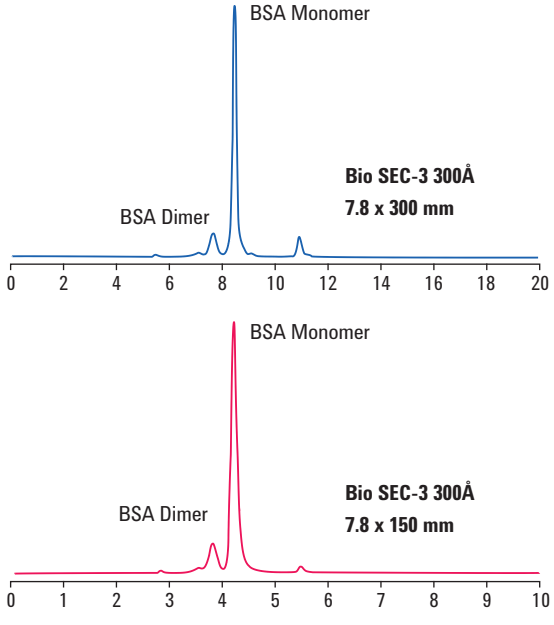


Figure 5 – Comparison of analysis using 300 mm columns vs. 150 mm columns to demonstrate time savings.

Learn more about Agilent biocolumns for SEC at **agilent.com/chem/bioHPLC**

Column media choice

Choose a size exclusion column suitable for your molecule type and size after determining the solubility of the sample and the mobile phase – water, buffer, or organic solvent – of your separation. Columns packed with polymer-based sorbents are frequently used for polymeric molecules with broad molecular weight distributions, such as heparin, starch, or cellulose. Proteins and molecules which have a discrete molecular weight, are best suited to silica-based stationary phases (**Table 1**).

It is important to remember that proteins contain numerous amino acids with differing side chain functionalities: acidic, basic, hydrophobic, and neutral/hydrophilic. In order to prevent interactions occurring with silica columns, buffers are needed in the mobile phase.

Agilent suggests the appropriate molecular weight range for its columns and ideally your column choice should fall in the middle of the operating range.

Size Exclusion Chromatography (SEC)

Application	Agilent Columns	Notes
Proteins		
Peptides, proteins, MAbs	Agilent Bio SEC-3	Higher resolution and faster separations from 3 µm particles, with 100Å, 150Å, and 300Å pore sizes.
Large biomolecules and samples with multiple molecular weight components	Agilent Bio SEC-5	More pore size options (100Å, 150Å, 300Å, 500Å, 1000Å, and 2000Å) to cover a wider range of analytes.
Globular proteins, antibodies	ProSEC 300S	Single column option for protein analysis in high salt conditions.
Proteins, globular proteins	ZORBAX GF-250/450	Higher flow rate capabilities and larger column size for SEC semi-prep and prep.
Water Soluble Analytes		
Low MW polymers and oligomers, oligosaccharides, PEGs, lignosulfonates	2 or 3 PL aquagel-OH • PL aquagel-OH 8 µm • PL aquagel-OH 20 5 µm • PL aquagel-OH MIXED-M 8 µm	The PL aquagel-OH analytical series has a pH range of 2-10, compatibility with organic solvent (up to 50% methanol), mechanical stability up to 140 bar (2030 psi), and low column operating pressures.
Polydisperse biopolymers, polysaccharides, cellulose derivatives	2 or 3 PL aquagel-OH • PL aquagel-OH MIXED-H 8 µm • PL aquagel-OH 60/50/40 8 µm	
Very high MW polymers, hyaluronic acids, starches, gums	PL aquagel-OH 60/50/40 15 µm in series	

Table 1 – Column selection choices according to application and sample size

Agilent Bio SEC columns for biomolecule separations, including protein aggregation, and Agilent GPC columns for natural polymer analysis, including polysaccharide molecular weight determination.

Pore size

Proteins are relatively small and compact compared to other biopolymers, so 300Å pore size is a good choice for an initial column selection. **Figure 6** compares the resolution of a 5 protein mix reference standard and a mouse IgG sample on columns with different pore sizes and clearly shows the effect of pore size on the resolution. With the 300Å pore size, the largest protein thyroglobulin and the IgG dimer are resolved, but as the pore size decreases, the largest proteins are excluded and there is no separation.

BioRad gel filtration standards mix

Column A: **Bio SEC-3, 300Å 4.6 x 300 mm, 3 µm (p/n 5190-2513)**

Column B: **Bio SEC-3, 150Å 4.6 x 300 mm, 3 µm (p/n 5190-2508)**

Column C: **Bio SEC-3, 100Å 4.6 x 300 mm, 3 µm (p/n 5190-2503)**

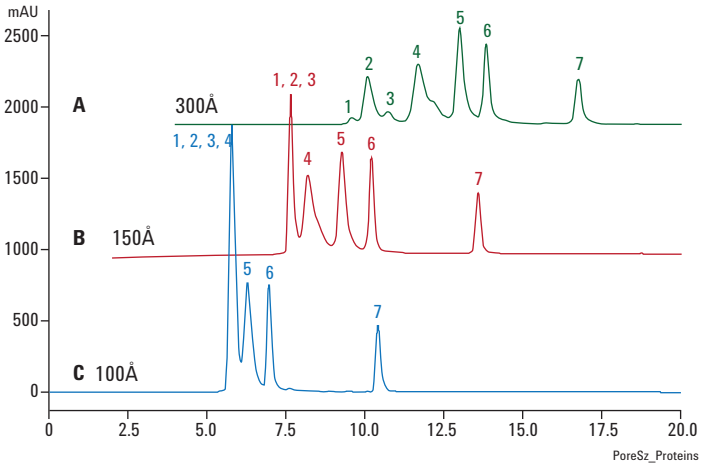
Instrument: Agilent 1260 Infinity Bio-inert Quaternary LC System

Mobile Phase: 50 mM Na_2HPO_4, 50 mM NaH_2PO_4 + 0.15 M NaCl, pH 6.8

Flow Rate: 0.35 mL/min

Detector: UV, 220 nm

Sample: BioRad gel filtration standards mix



1. Thyroglobulin Aggregates
2. Thyroglobulin
3. IgA
4. γ-globulin
5. Ovalbumin
6. Myoglobin
7. Vitamin B12

Column A: **Bio SEC-3, 300Å 4.6 x 300 mm, 3 µm (p/n 5190-2513)**

Column B: **Bio SEC-3, 150Å 4.6 x 300 mm, 3 µm (p/n 5190-2508)**

Column C: **Bio SEC-3, 100Å 4.6 x 300 mm, 3 µm (p/n 5190-2503)**

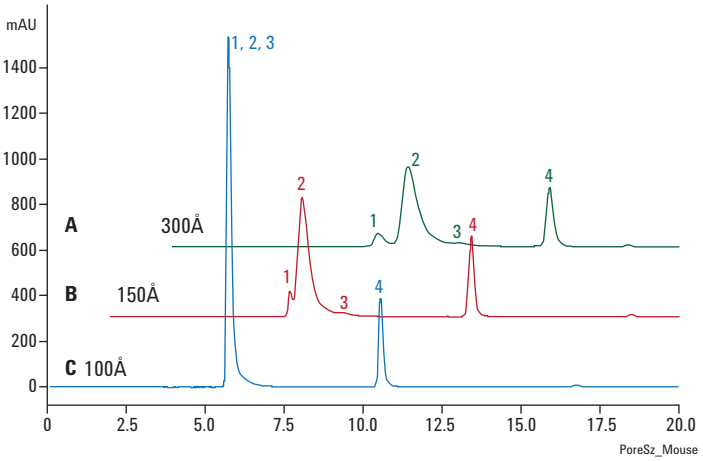
Instrument: Agilent 1260 Infinity Bio-inert Quaternary LC System

Mobile Phase: 50 mM Na_2HPO_4, 50 mM NaH_2PO_4 + 0.15 M NaCl, pH 6.8

Flow Rate: 0.35 mL/min

Detector: UV, 220 nm

Sample: Mouse IgG



1. Dimer
2. Monomer
3. Monomer Fragment
4. Azide

Figure 6 – Comparison of pore sizes on the resolution of BioRad Gel Filtration Standards and Mouse IgG

Evaluating SEC Permeation Ranges

With proteins, it is important to recognize that the SEC mechanism works by separating solutes dependant on their size in solution and not their molecular weight. This is evident when comparing the calibration plot of the proteins/peptides with the pullulan/polysaccharide and PEG/PEO curves, as shown in **Figure 7**. The pullulan/polysaccharide and PEG/PEO calibrants provided quite similar calibration curves but the protein/peptide curve is shifted and a different shape.

Proteins are composed of complex polypeptide chains that form three-dimensional structures. These structures are affected by the enviornment to which they are exposed. For example, pH or ionic strength. The changes will form the shape that is most suited to them and so the structure and size may vary.

To demonstrate that elution time is due to size rather than molecular weight, consider the retention times of calibrants with a molecular weight of approximately 50,000, in which there is significant difference (**Figure 8**). The PEG elutes just after 7 minutes, the polysaccharide elutes at just over 7.5 minutes, but the protein elutes at approximately 9.5 minutes.

This clearly demonstrates that the SEC separation mechanism is based on the actual size and not molecular weight. Therefore, when using calibration curves it is important to specify what calibrants have been used. For example, it can be stated that the sample of interest has a pullulan/polysaccharide equivalent molecular weight of 50,000. See page 16 for advanced detectors that overcome this relative nature.

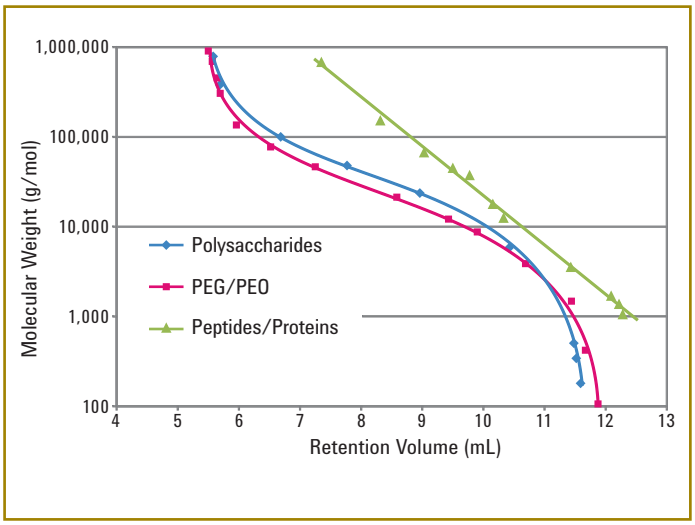


Figure 7 – Comparison of calibration plots generated for all three types of calibrant.

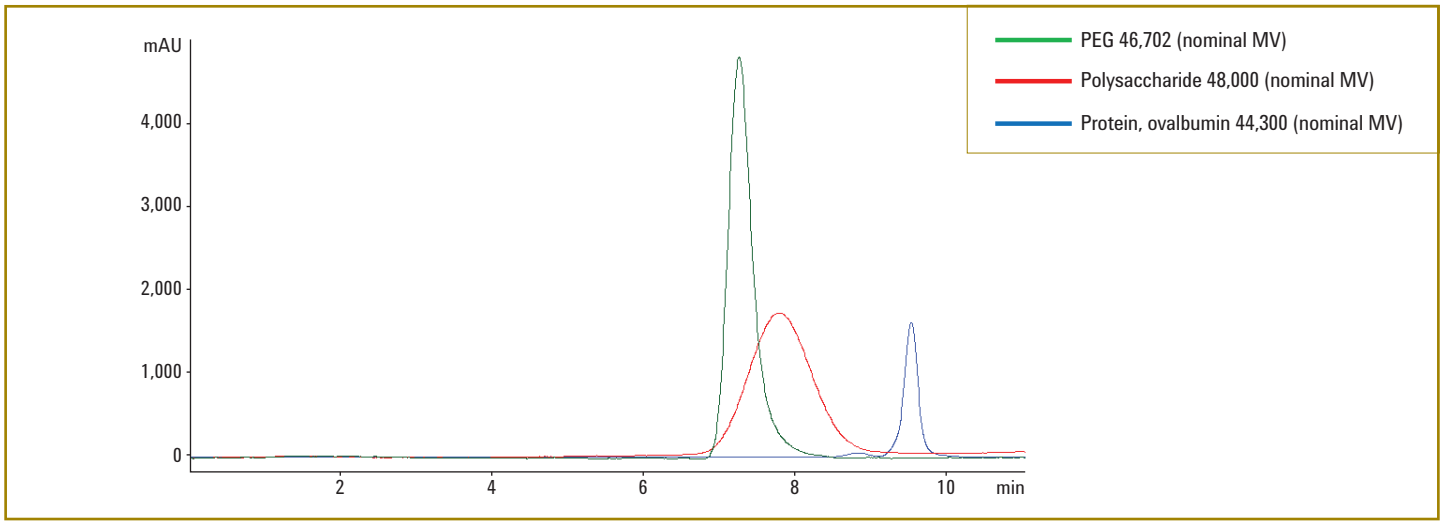


Figure 8 – Overlay of chromatograms obtained for calibrants of similar molecular weight.

Particle size

Particle size is also an important consideration in column selection. Smaller particle sizes provide more efficient separation, but at the risk of degrading (shearing/deforming) the protein. **Figure 9** shows a comparison between Agilent's 3 µm Bio SEC-3 and 5 µm Bio SEC-5 columns. There is a greater risk of higher backpressure and columns becoming blocked if samples and eluents are not prepared carefully. Filtration is recommended to remove insoluble matter and debris. The use of a guard column or in-line filter can also extend the column lifetime.

Comparison between Agilent Bio SEC-3 and Agilent Bio SEC-5

Analysis of monoclonal antibody

Column:	**Bio SEC-3, 300Å 7.8 x 300 mm, 3 µm (p/n 5190-2511)**
Column:	**Bio SEC-5, 300Å 7.8 x 300 mm, 5 µm (p/n 5190-2526)**
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC System
Mobile Phase:	150 mM sodium phosphate, pH 7
Flow Rate:	1 mL/min
Detector:	UV, 220 nm
Sample:	Humanized monoclonal antibody

The 3 µm column gives better separation.

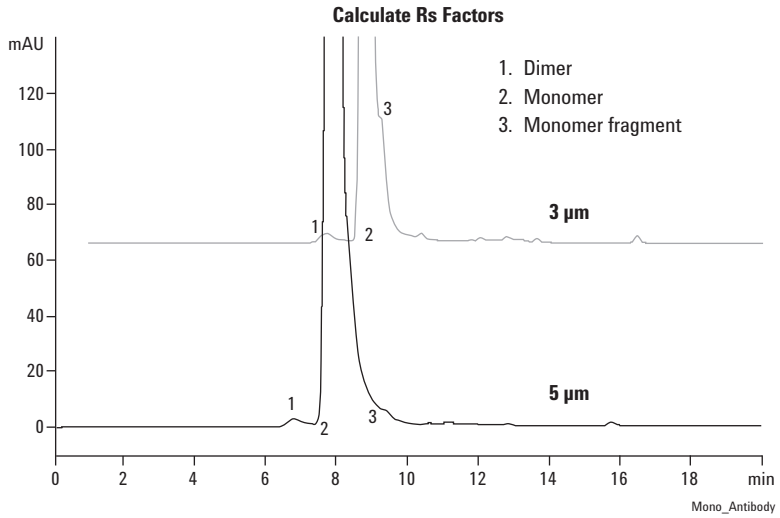


Figure 9 – Comparison of Agilent Bio SEC-3 versus Agilent Bio SEC-5

Column diameter

Column diameter can also be important depending on the amount of sample being analyzed. If only limited amounts of material are available, 4.6 mm id columns (operated at 0.35 mL/min) are useful. But it is important to minimize system volumes when using the smaller id columns to prevent excessive dispersion and loss of resolution.

SEC is considered to be a non-denaturing technique when aqueous eluents are used, so it is extremely useful for fractionation of complex samples or isolation of a sample component for further analysis. Larger diameter columns – such as 9.4 mm found in ZORBAX GF-250 and GF-450 products – mean that semi-preparative separations can be performed using analytical HPLC systems.

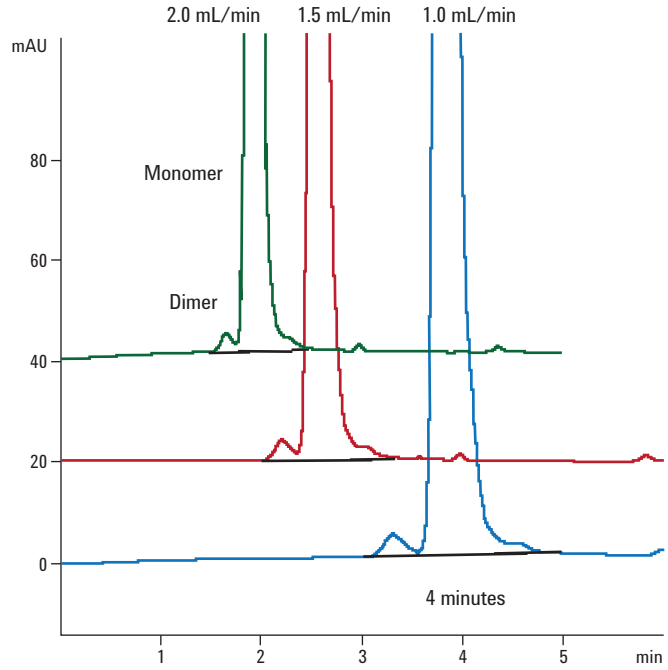
Bio SEC columns in 4.6 mm and 3.0 mm id

Learn more about Agilent biocolumns for SEC at **agilent.com/chem/bioHPLC**

Method Parameters

Flow rate

For some applications the speed of analysis is crucial. A shorter column can be used to reduce the analysis time – 150 mm vs. the conventional 300 mm – and/or flow rates can be increased. This could have a detrimental effect on resolution though, because SEC relies on diffusion into and out of a pore to create differential path lengths through the column. However, as shown in **Figure 10**, it is possible to obtain sufficient resolution to quantify an IgG dimer and monomer in under four minutes when using a 150 cm long column at a flow rate of 2 mL/min.



Column:	**Agilent Bio SEC-3 7.8 x 150 mm**
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC System
Flow Rate:	1.0, 1.5, and 2 mL/min (56 bar, 75 bar, 105 bar)
Eluent:	150 mM sodium phosphate + 100 mM Na-sulfate
Detector:	220 nm
Injection:	5 µL
Sample:	mAb (2 mg/mL)

Flow Rate	Resolution Monomer/Dimer	Monomer Efficiency	Percentage Dimer
1.0 mL/min	1.53	3,510	0.64
1.5 mL/min	1.43	2,502	0.67
2.0 mL/min	1.13	1,917	0.64

Figure 10 – Effect of flow rate on the resolution of an IgG dimer and monomer when using a 150 mm long column

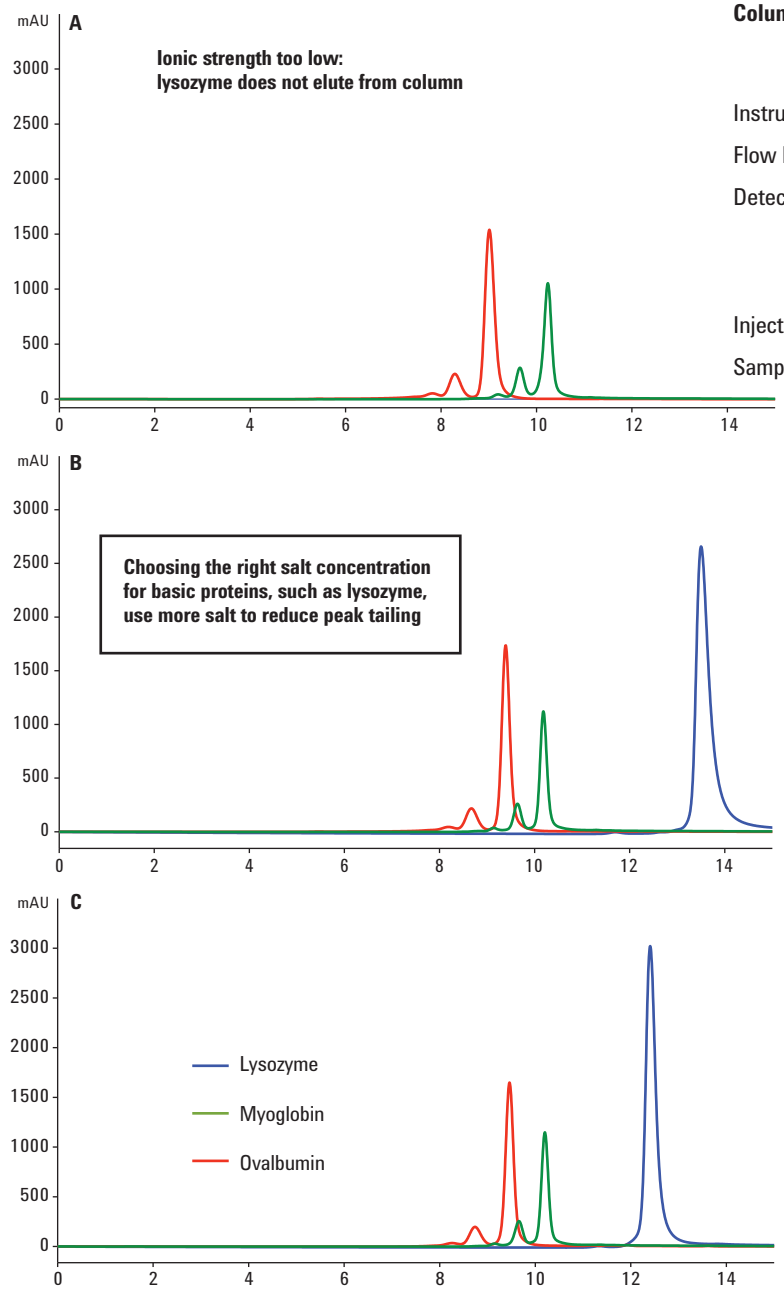
Troubleshooting your SEC method

Problem	Source	Solution
Lower than expected recovery, or a broadening of the peaks	Hydrophobic analytes	Add a small amount (10-20%) of organic modifier (acetonitrile or methanol) to mobile phase
Peaks that show up when they shouldn't, based on molecular weight, or peak tailing	Ionic interactions or basic proteins	Increase the ionic strength – salt concentration – at 50-100 mM intervals; Add to phosphate buffer
Poor peak shapes	Non-specific adsorption	Increase salt concentration or try an Agilent 1260 Infinity Bio-inert LC system
Poor retention/resolution of analytes	Insufficient pore size for molecule size	Check your pore size; See page 11 for more information

Mobile phase selection

Secondary interactions can cause difficulties

In order to overcome undesirable secondary interactions, it may be necessary to perform method optimization. Such interactions may lead to an analyte eluting later than expected and could give the appearance of a lower molecular weight. Slight adjustments in the mobile phase composition – pH, ionic strength, or organic modifiers – can help to overcome such difficulties (**Figure 11**). It may also be necessary to refine the choice of pore size, combine columns in series, reduce analysis flow rate, or change temperature to achieve the desired separation.



Column: **Agilent Bio SEC-3 300Å, 4.6 mm x 300 mm, 3 µm (p/n 5190-2513)**

Instrument: Agilent 1260 Infinity Bio-inert Quaternary LC System

Flow Rate: 0.35 mL/min

Detector: 220 nm

A: Eluent 20 mM phosphate buffer, pH 7 + 50 mM NaCl

B: Eluent 20 mM phosphate buffer, pH 7 + 100 mM NaCl

C: Eluent 20 mM phosphate buffer, pH 7 + 400 mM NaCl

Injection: 5 µL

Sample: Protein (1 mg/mL 20 mM phosphate buffer, pH 7)

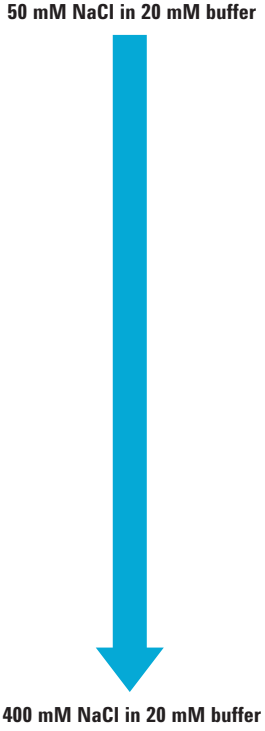


Figure 11 – Effect of too much or too little ionic strength on achieving your desired separation

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Calibration

Once you have chosen a column, it will be necessary to construct a calibration with standards of a known molecular weight. Each time you change your choice of column or make changes to the mobile phase, you will need to repeat the calibration. The calibration curve is obtained by plotting retention time against molecular weight (**Figure 12**). It is particularly important to choose standards appropriate to the molecule of interest. For a protein separation, use protein molecular weight standards. Pullulan molecular weight standards should be used for a polysaccharide separation.

Calibration curves – Bio SEC-5

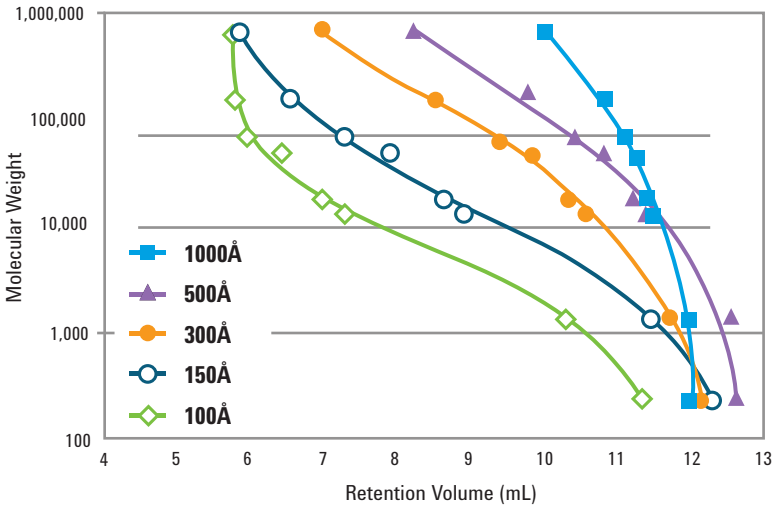
Column: **Agilent Bio SEC-5 7.8 x 300 mm, 5 µm (p/n 5190-2521)**

Instrument: Agilent 1260 Infinity Bio-inert Quaternary LC System

Mobile Phase: 150 mM Na phosphate, pH 7.0

Flow Rate: 1.0 mL/min

Detector: UV



		Retention Volume				
Proteins	**MW**	**1000Å**	**500Å**	**300Å**	**150Å**	**100Å**
Thyroglobulin	670000	10.07	8.23	7.03	5.82	5.77
Gamma globulin	158000	10.88	9.80	8.57	6.55	5.79
BSA	67000	11.13	10.44	9.44	7.29	6.00
Ovalbumin	45000	11.28	10.83	9.89	7.90	6.40
Myoglobin	17000	11.44	11.28	10.42	8.66	7.05
Ribonuclease A	12700	11.52	11.41	10.58	8.93	7.32
Vitamin B-12	1350	12.00	12.59	11.78	11.49	10.30
Uracil	112	12.08	12.68	12.21	12.13	11.41

Figure 12 – Calibration curve obtained by plotting retention time against molecular weight

Ideally the standards should be dissolved in the mobile phase and care should be taken to ensure that the sample has dissolved fully. If the solution appears cloudy, then it will be necessary to take further action. Centrifugation or filtration should be used to remove insoluble matter prior to injection. However, it may be necessary to look at alternative mobile phase conditions that will improve sample solubility since physical processes could be altering the molecular weight composition.

Advanced Detection Techniques

Further SEC considerations include choice of detector. UV or diode array (DAD) is commonly used for protein separations. Best results – highest sensitivity – for peptides and proteins will normally be obtained at 220 nm. Although some buffer solutions or organic modifiers may have too much background absorbance at low wavelengths, in which case 254 nm or 280 nm may be necessary. A drawback of UV detection is that some molecules do not possess a chromophore, but since analytes are eluted isocratically, it is possible to use an RI detector instead.
The addition of advanced light scattering detection significantly increases the performance of SEC. Static light scattering determines accurate molar masses, independent of column calibrations and unwanted interactions and is complemented with dynamic light scattering to study the molecular size. Light Scattering has increased sensitivity to large moieties allowing for discovery of aggregation at much lower quantities, **Figure 13**. It is important to select a detector with low dead volume to ensure this advanced information is obtained without sacrificing chromatographic performance.

Column:	**Agilent Bio SEC-3 300Å 7.8 mm x 300 mm SS**
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC System with Agilent 1260 Multi-Detector GPC/SEC
Mobile Phase:	300 mM sodium phosphate, pH 7.0
Gradient:	Isocratic
Temperature:	30 °C
Detector:	UV, 280 nm + RI + LS 90°
Injection:	50 µL
Sample:	Bovine gamma globulin
Sample Conc:	2.0 mg/mL

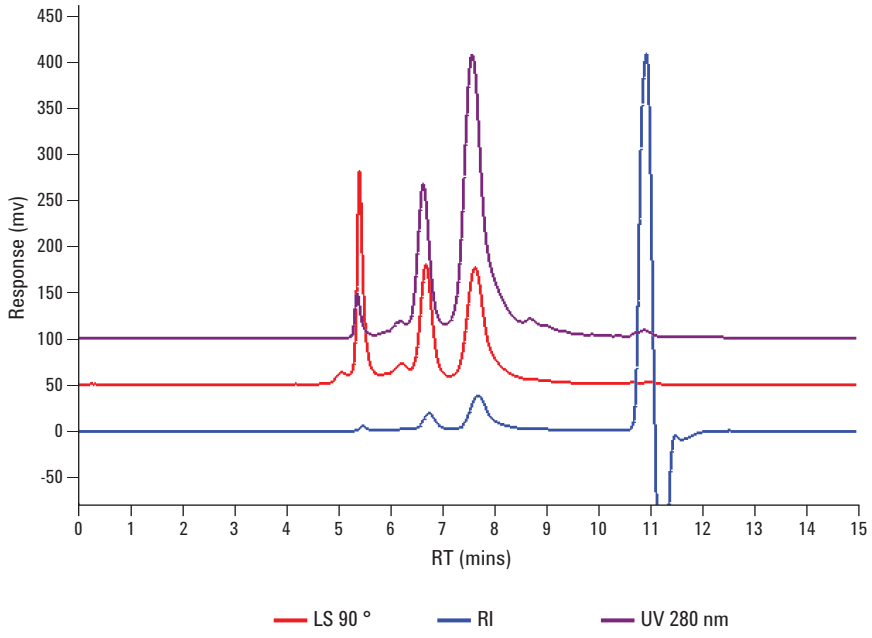


Figure 13 – Results of using different detectors on MDS protein separation

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PEGylated Proteins

Many therapeutic proteins have been conjucated with a water-soluble synthetic polymer, polyethylene glycol (PEG), to enhance their pharmacological activities. Conjugation of PEG (PEGylation) to various proteins not only increases their half-life in the blood stream, but also significantly reduces their immunogenicity.

PEG-proteins as well as other polymer-conjugates present new challenges with regard to both their preparation and purification.

Monitoring the degree of PEGylation is important to achieve a balance between retention of bioactivity, stability and immunogenicity of PEGylated proteins. SEC is routinely used for monitoring PEGylation reactions and purification. Below, a simple and sensitive SEC method for monitoring the protein PEGylation reaction and purification has been developed. The SEC HPLC analysis indicates that the purified PEG-lysozyme is homogenous.

Column:	**Agilent Bio SEC-3** **7.8 mm x 300 mm**
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC System with Agilent 1260 Multi-Detector GPC/SEC
Mobile Phase:	150 mM sodium phosphate, pH 7.0 containing 150 mM sodium chloride
Flow Rate:	0.8 mL/min
Gradient:	Isocratic
Temperature:	Ambient
Thermostat Autosampler:	5 °C
Detector:	214 nm and 280 nm
Injection:	2 µL
Peak Width:	20 Hz

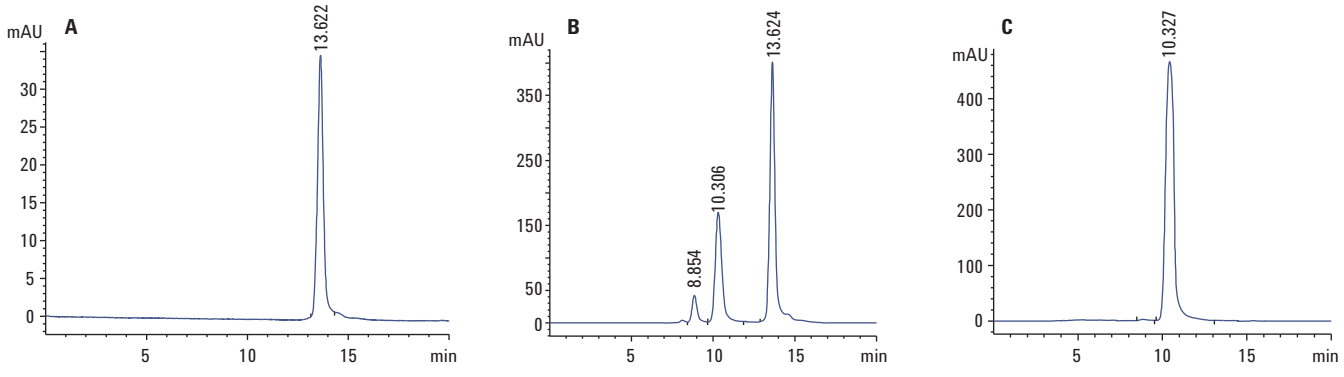

Figure 4 – SEC profile of lysozyme PEGylation and purification. (A) Native lysozyme; (B) Reaction mixture; (C) Purified PEG lysozyme

Sample preparation

- Ideally, samples should be dissolved in the mobile phase.
- If the sample is cloudy, it may be necessary to change the mobile phase conditions.
- Filtration or centrifugation can be used to clarify samples, but these processes could alter the molecular weight composition of the sample.
- In order to dissolve a sample, gentle heating, vortexing, or sonication is sometimes used, but should be applied with caution because this can alter the molecular weight composition.
- Care should also be taken to ensure the sample does not change during storage.
- Samples should be made up fresh and analyzed as soon as possible.
- Bacterial growth can develop quickly in buffer solutions.
- Samples made up at high concentration can also change over time, leading to aggregation or even precipitation.

Column selection

- To ensure sample integrity, SEC is carried out slowly on long columns.
- Column lengths are typically 250 or 300 mm.
- Normal flow rate is 1.0 mL/min on a 7.5 or 7.8 mm id column and 0.35 mL/min on a 4.6 mm id column.
- Columns are often run in series to increase resolution in biopolymer applications.
- Smaller particle sizes are used to increase resolution in protein applications.
- Separations done on 150 mm columns with smaller particle sizes can reduce analytical time.

Column media choice

- There should be no non-specific interactions between analytes and column media.
- Silica-based sorbents are used for analyzing peptides and proteins.
- Polymer-based sorbents are for analyzing biopolymers.

Column parameters

- **Pore size** – depends on the molecular weight range of the sample, avoid exclusion of sample components, and maximize volume in required separation region.
- **Particle size** – use smaller particles for higher resolution (but higher backpressure).
- **Column length** – compromise between resolution and analysis time.
- **Column id** – use smaller column for reduced solvent consumption and smaller injection volume.

Mobile phase

- Mobile phase should contain buffer/salt to overcome ionic interactions, but too much may cause hydrophobic interactions.
- Do not alter the analyte to avoid degradation/aggregation, etc.
- Make up fresh mobile phase and use promptly, as bacterial growth is rapid in dilute buffer stored at room temperature.
- Buffer shelf life is less than 7 days unless refrigerated.
- Filter before use. Particulates may be present in water (less likely) or in buffer salts (more likely).
- High pH phosphate buffers (particularly at elevated temperature) can significantly reduce column lifetime when using silica columns.

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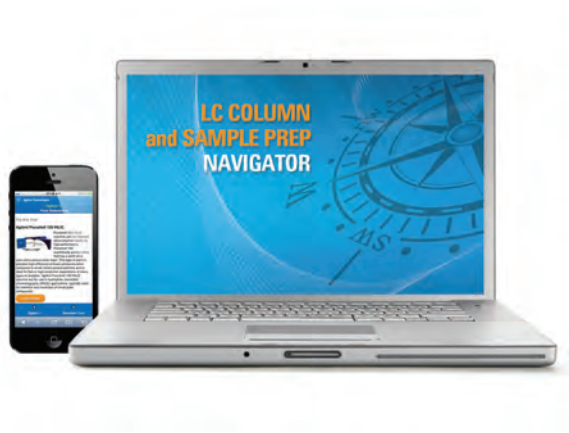

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The NAVIGATOR presents four easy search options:

- By part number – cross-reference for LC columns and sample prep products to find the best Agilent replacement
- By column – recommendations based on method
- By compound – drop down list
- By USP method

In addition, the tool offers column support to optimize chromatography, sample prep product recommendations, and quick access to technical support resources and other tools.

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