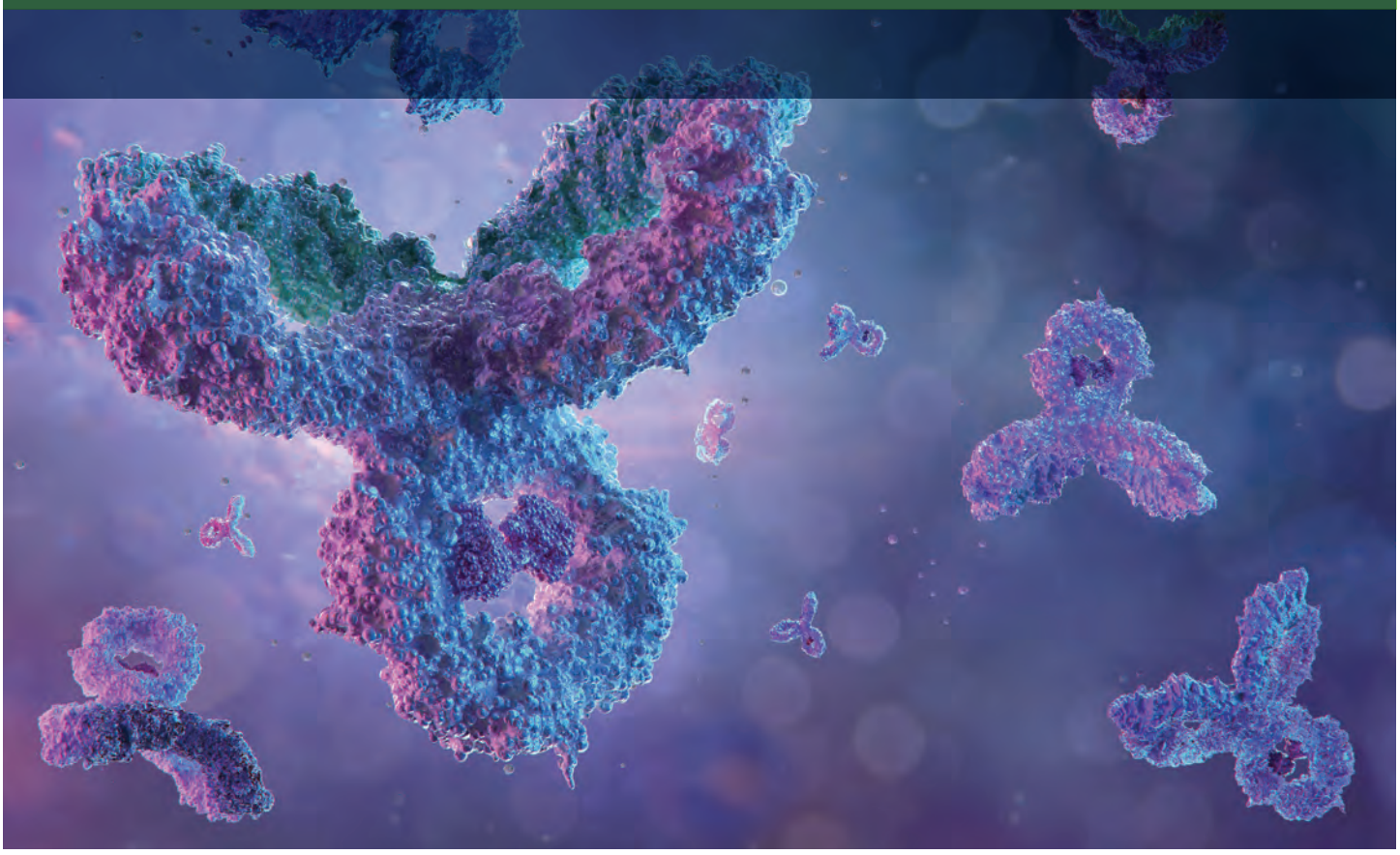
Agilent

Trusted Answers

Agilent Biocolumns

Aggregate/Fragment Analysis

Application Compendium



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Aggregate/Fragment Analysis

Background

Proteins are often susceptible to aggregation, forming dimers and larger oligomers or high order structures because of exposure to stress conditions. This is particularly problematical in biotherapeutic protein manufacture since the target protein will be subjected to a wide variety of conditions that may induce aggregation. These conditions include changes in temperature and concentration during fermentation and changes in pH and concentration during downstream processing. Even shear forces (from impeller blades, stirrers, and other engineering plant equipment) can result in stress-related aggregation. The presence of aggregates, particularly very large multimers up to subvisible particles, are potentially harmful to health. It is therefore a prerequisite that the level of aggregation is quantified and determined and that limits are put into place.

Size exclusion chromatography is one of the techniques that is particularly suited to the separation of monomer peaks from higher-order aggregates. With a suitable concentration detector such as UV or DAD quantification is relatively straightforward. More complex molecules such as antibody drug conjugates, or ADCs, may be more challenging due to the presence of hydrophobic cytotoxic drugs that can result in nonideal behavior with many size exclusion chromatography columns. To address this issue, Agilent has developed a new stationary phase that demonstrates greatly reduced risk of secondary interactions. The new AdvanceBio SEC columns are therefore ideally suited to rapid separation and quantification of aggregates.

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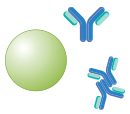

Aggregate/ Fragment Analysis

Size exclusion chromatography

Accurate, precise quantitation for a broad range of biomolecule separations

AdvanceBio SEC 1.9 and 2.7 µm

Versatile performance for routine and challenging applications

Attribute	Advantage
Hydrophilic polymer coating	Avoid secondary interactions
Increased analytical speed	Meet vital deadlines
Higher reproducibility	Reduce rework
Greater sensitivity	Quantitate aggregates, even at low levels
1.9 µm particle	Highest resolution
PEEK-lined stainless steel hardware option	Metal-free pathway for metal-sensitive samples and detection

Bio SEC-3 and Bio SEC-5

Extra wide pore and scale-up options

Attribute	Advantage
Compatibility with most aqueous buffers	Method flexibility
Wide range of pore size options, including 1000 Å and 2000 Å	Options for everything from peptides to VLPs
Analytical and semi-prep dimensions	Easy scale up or down

Getting Started

Since protein aggregation can occur as a result of external factors, one of the most important steps in aggregate analysis is sample preparation. It is necessary to ensure that the protein is fully dissolved in the mobile phase but is not subjected to factors that may alter the level of aggregation, such as sonication, temperature, pH, and excessive concentration. Size exclusion chromatography is a relatively straightforward technique relying on isocratic elution. The mobile phase conditions should ensure that there is no secondary interactions between the protein and the column stationary phase. Typically aqueous buffers such as 150 mM sodium phosphate or PBS (phosphate buffered saline) at neutral pH are used. Such mobile phase solutions are also ideal conditions for bacterial growth. It is therefore important to prepare fresh buffer regularly and to filter it through 0.2 µm filter before use. Under no circumstance should unused buffer be left on the instrument for a prolonged period.

Avoiding the temptation to add preservatives such as sodium azide to the mobile phase can allow low wavelengths (210–220 nm) to be used, greatly improving the sensitivity of the technique. Preservatives, such as 20 % ethanol, are recommended for column storage. However, care must be taken since the viscosity of the mobile phase containing organic modifiers is often significantly higher and column damage could ensue.

Size Exclusion Chromatography For Biomolecule Analysis: A "How-To" Guide

A Guide To Successful SEC

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

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Separation is straightforward and uncomplicated

With SEC, molecules are separated from largest to smallest in proportion to their molecular size in solution. 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.

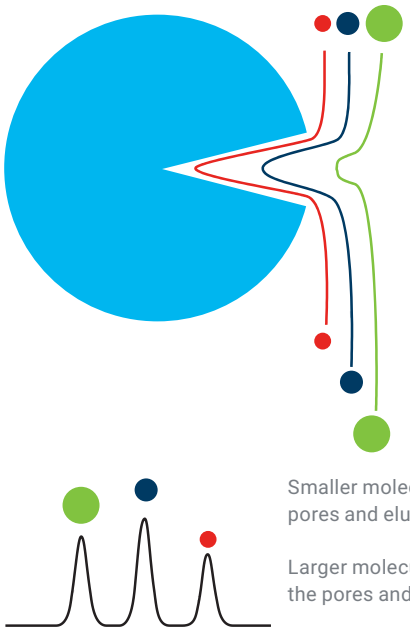

Figure 1. Molecules permeate the pores of the stationary phase to different extents depending on their size.

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 weight excipients and impurities from larger molecular weight proteins (Figure 2).

Understanding and controlling aggregation in therapeutic proteins is essential as it will affect efficacy and 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.

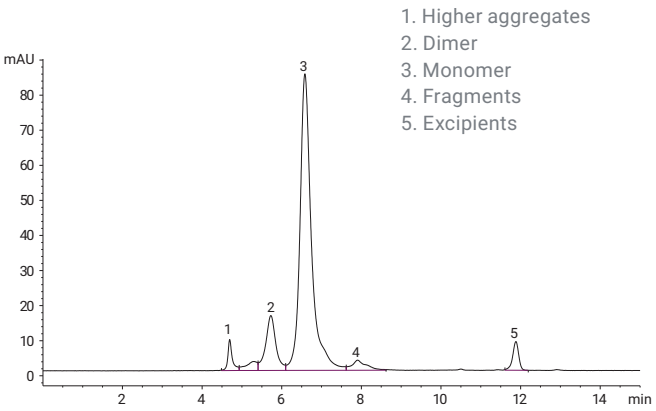

Figure 2. Separation of IgG aggregates and excipients.

Conditions, intact IgG monomer and dimer separation

Parameter	Value
Sample:	Polyclonal IgG
Sample conc:	150 mM sodium phosphate buffer
Column:	Agilent AdvanceBio SEC, 300 Å 7.8 x 300 mm,
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC System
Flow rate:	1.0 mL/min
Temp.:	Ambient
Detector:	UV, 220 nm
Injection:	5 µL

Elution order typically follows molecular weight. Molecules with the highest molecular weight elute 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).

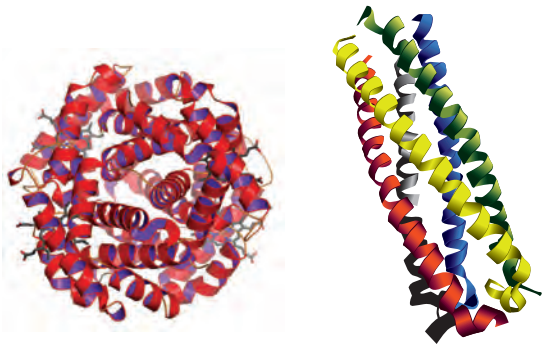

Figure 3. Comparison of compact globular protein versus cylindrical protein.

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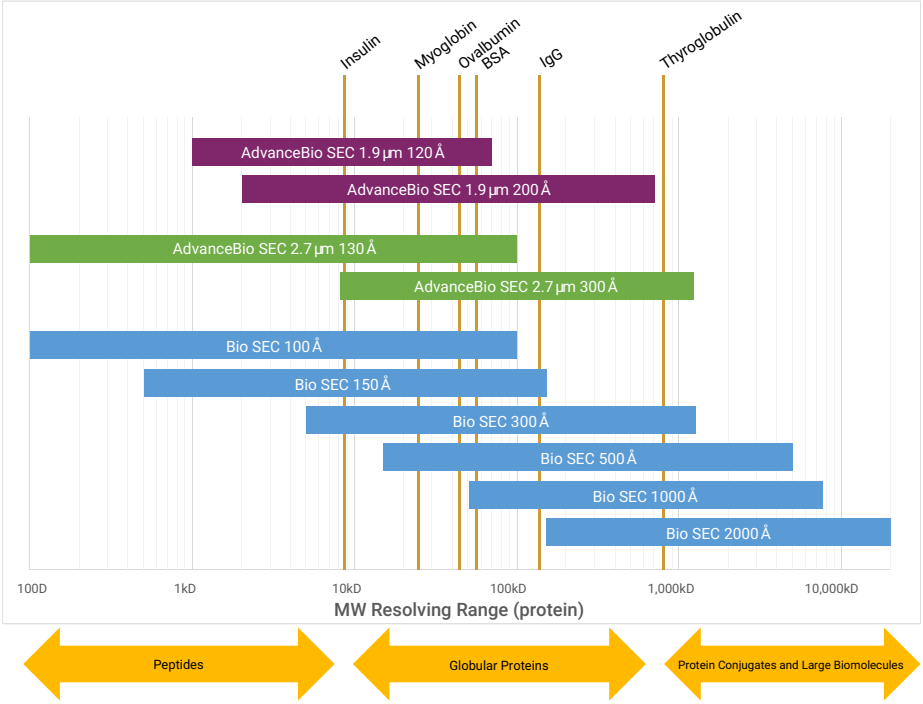
SEC-UV/DAD Method Development Guide

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



Recommended Initial Separation Conditions

Column:	AdvanceBio SEC or Agilent Bio SEC-5
Mobile phase:	150 mM phosphate buffer, pH 7.0*
Gradient:	Isocratic in 10-30 min range
Temperature:	Recommended: 10-30 °C, Maximum: 80 °C
Flow rate:	0.1-1.25 mL/min for 7.8 mm id columns 0.1-0.4 mL/min for 4.6 mm id columns 0.05-0.1 mL/min for 2.1 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 no. 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 such as methanol or acetonitrile) in 50 mM sodium phosphate, pH 7.0, 5 % DMSO in
50 mM sodium phosphate, pH 7.0. Note that it may be necessary to reduce the flow rate to keep below the maximum operating pressure when using higher viscosity mobile phases.

Temperature:
Typically, SEC separations are run at 10-30 °C. Separation of proteins and peptides may require higher temperature to improve resolution and recovery of proteins and hydrophobic peptides. SEC may be run in a cold room to maintain maximum biological activity of temperature sensitive proteins. Maximum operating temperature of Agilent Bio SEC columns is 80 °C. Note that higher temperatures can denature proteins.

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Instrumentation consideration 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, and 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 and 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 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 between 1 and 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. The detector is also designed for biomolecule separations and does not affect the analysis of proteins, peak shape, and recovery.


Bio-inert flow cell with RFID tag, 10 mm, 13 µL (p/nG5615-60022)

Protect your proteins during analysis

Heat can denature proteins, and so it is important that your sample is kept at constant temperature in the whole LC flow path. The Agilent 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 and protein-friendly flow cells to enable reliable analysis of your protein under various conditions. For DAD use, the new Max Light Cartridge Cell LSS will mitigate light intensity, ensuring sample integrity. Learn more about flow cell options at **www.agilent.com/chem/bioflowcells**

Software solutions provide new insights

When working with size exclusion chromatography, there are several software options 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, providing more information based on molecular weight.
- Buffer Advisor software: Eliminates the tedious and error-prone method-development steps of buffer preparation, buffer blending, and pH scouting by creating salt and pH gradients quickly and easily.

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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. To protect the column from possible damage, we recommend that samples be filtered or centrifuged before use to remove particulates. However, filtration should not be used 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. These 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 is 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. 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 **www.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	Polypropylene	5190-5094
15	0.2	LC	Polypropylene	5190-5096
15	0.45	LC	Polypropylene	5190-5097
25	0.2	LC	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 dimensions for SEC are 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 the 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 (5-20 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. 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 an indication 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 to obtain the degree of resolution you need for your analysis. However, to improve the speed of separation, you can consider using shorter column lengths. The separation can be accomplished in half 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, and so a further reduction in analysis time can be achieved. See Figure 5.

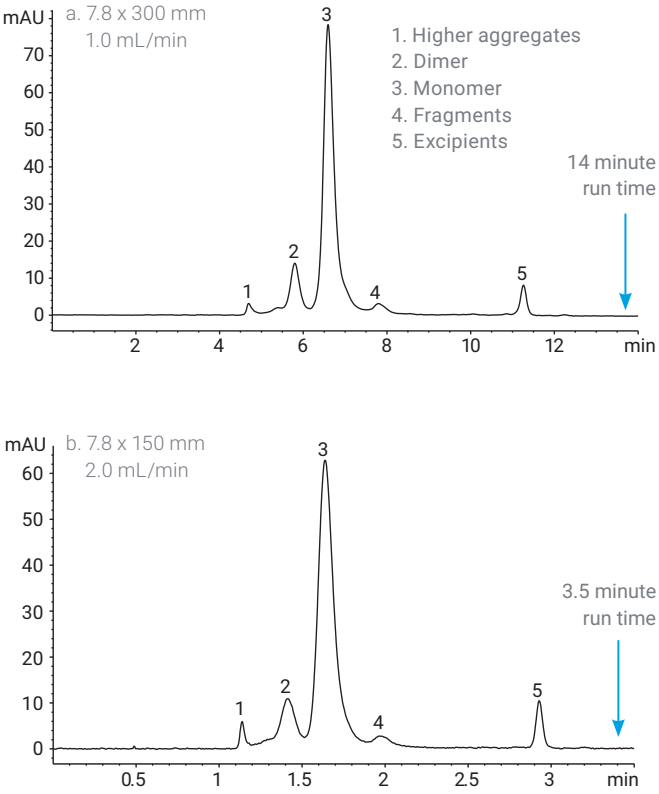

Figure 5. Comparison of Polyclonal IgG analysis using 300 mm vs. 150 mm AdvanceBio SEC columns to demonstrate time savings.

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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 that have a discrete molecular weight are best suited to silica-based stationary phases (Table 1).

Agilent Bio SEC columns for biomolecule separations, including protein aggregation, and Agilent GPC columns for natural polymer analysis, including polysaccharide molecular weight determination.It is important to remember that proteins contain numerous amino acids with differing side chain functionalities: acidic, basic, hydrophobic, and neutral/hydrophilic. 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.

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

Size Exclusion Chromatography (SEC)

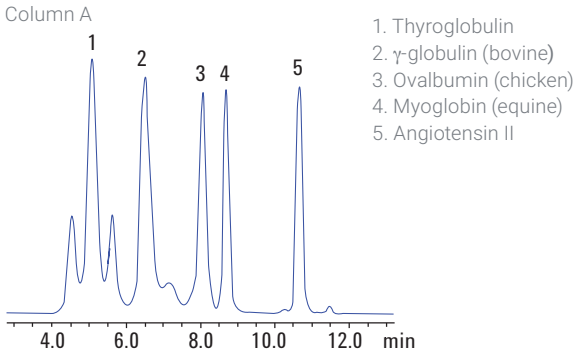
Table 1. Agilent offers a diverse variety of SEC columns to cover all of your size exclusion needs.

Application	Agilent Columns	Notes
Proteins		
SEC-UV/DAD, native SEC-MS, or LS analysis of mAbs, proteins, and peptides.	Agilent AdvanceBio SEC	The latest innovative technology that provides resolution to eliminate sample re-analyses and speed to reduce analysis time, so improving lab productivity. PEEK lined option for native SEC-MS.
Large biomolecules and samples with multiple weight components.	Agilent Bio SEC-5	More pore size options (100 Å, 150 Å, 300 Å, 500 Å, 1000 Å, and 2000 Å) 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	Legacy products that should be employed where protocols still require use of USP designation L35.

Pore size

Proteins are relatively small and compact compared to other biopolymers, and so 300 Å pore size is a good choice for an initial column selection. Figure 6 compares the resolution of a five-protein mix reference standard and a polyclonal IgG sample on columns with different pore sizes and clearly shows the effect of pore size on the resolution.

Agilent AdvanceBio SEC Protein Standards



Conditions

Parameter	Value
Column A:	AdvanceBio SEC 300 Å 4.6 x 300 mm, 2.7 µm (p/n PL1580-5301)
Column B:	AdvanceBio SEC 130 Å 4.6 x 300 mm, 2.7 µm (p/n PL1580-5350)
Sample:	Agilent AdvanceBio SEC Protein Standards (300 Å p/n 5190-9417 and 130 Å p/n 5190-9416)
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC System
Mobile phase:	150 mM phosphate buffer, pH 7.0
Flow rate:	0.35 mL/min
Detector:	UV, 220 nm

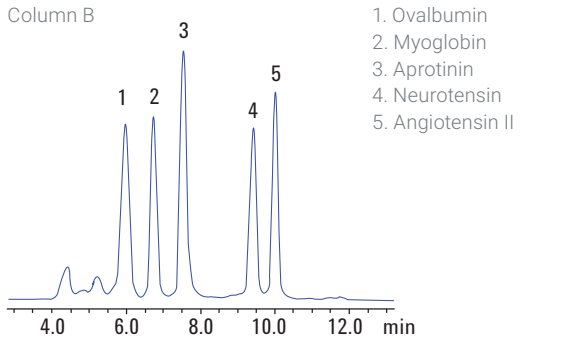
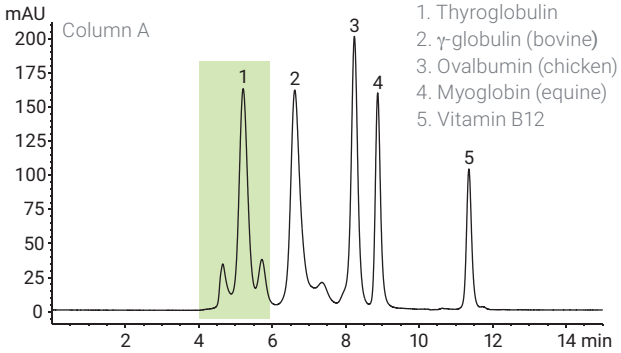


Figure 6: Comparison of pore sizes on the resolution of the Agilent AdvanceBio SEC 300 Å standard. The area highlighted in green shows the difference in resolution between the two pore sizes. The larger pore size is needed for analysis of larger proteins.

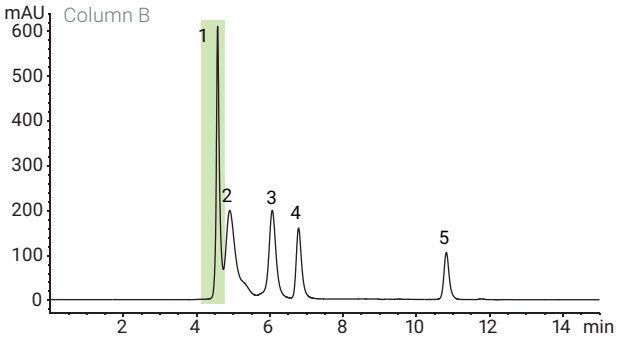
With 300 Å pores, the largest protein thyroglobulin and the IgG dimer are resolved, but as the pore size decreases, the largest proteins are excluded and there is no separation.

BioRad gel filtration standards mix



Conditions

Parameter	Value
Column A:	AdvanceBio SEC 300 Å 4.6 x 300 mm, 2.7 µm (p/n PL1580-5301)
Column B:	AdvanceBio SEC 130 Å 4.6 x 300 mm, 2.7 µm (p/n PL1580-5350)
Sample:	BioRad gel filtration standards mix
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC System
Mobile phase:	150 mM phosphate buffer, pH 7.0
Flow rate:	0.35 mL/min
Detector:	UV, 220 nm



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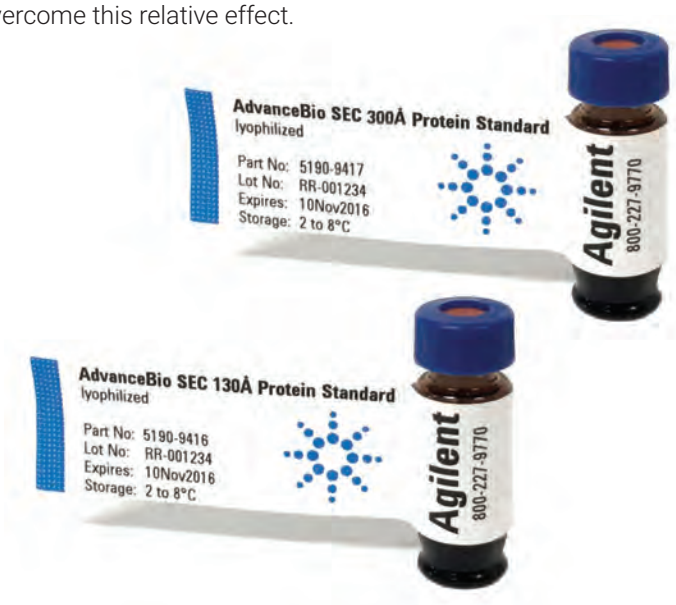
Evaluating SEC permeation ranges

With proteins, it is important to recognize that the SEC mechanism works by separating solutes depending 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 environment to which they are exposed, such as pH or ionic strength. The chains will form the shape that is most suited to them and so their 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 effect.



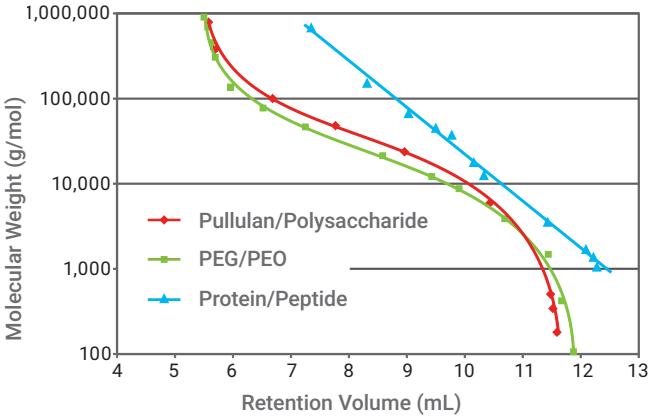


Figure 7: Comparison of calibration plots generated for three types of calibrant.

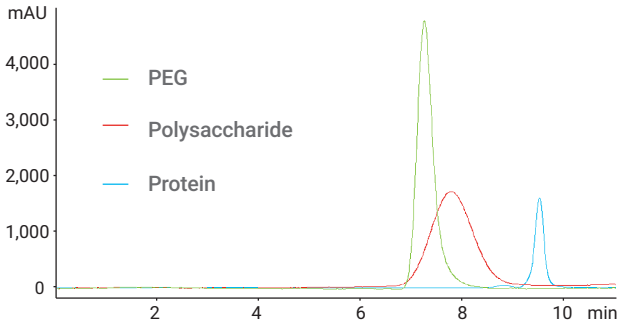


Figure 8: Overlay of chromatograms obtained for calibrants of similar molecular weight.

130 Å AdvanceBio SEC calibration standard (p/n5190-9416 130 Å AdvanceBio SEC calibration standard, 2 mL vial)

A protein mix consisting of 5 carefully selected proteins (Ovalbumin, Myoglobin, Aprotinin, Neurotensin, Angiotensin II – molecular weights ranging from 43 to 1.1 kDa) designed to calibrate Agilent's 130 Å AdvanceBio SEC columns. This standard can be used regularly to calibrate the column and ensure ideal system performance in various applications involving protein purification and analysis.

300 Å AdvanceBio SEC calibration standard (p/n5190-9417 300 Å AdvanceBio SEC calibration standard, 2 mL vial)

A protein mix consisting of 5 carefully selected proteins (Thyroglobulin, γ-Globulin, Ovalbumin, Myoglobin, Angiotensin II) designed to calibrate Agilent's 300 Å AdvanceBio SEC columns. This standard can be used regularly to calibrate the 200 Å and 300 Å AdvanceBio SEC columns and ensure ideal system performance in various applications involving protein purification and analysis.

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Particle size

Particle size is also an important consideration in column selection. Smaller particle sizes provide more efficient separation, and therefore higher resolution, but at the risk of degrading (shearing/deforming) larger proteins. Figure 9 shows a comparison between 3 µm and 5 µm particles in Agilent Bio SEC 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 column lifetime.

Comparison between 3 µm and 5 µm SEC particles

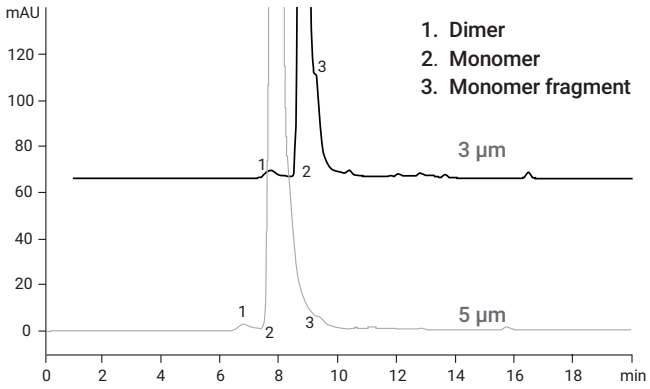

Figure 9: Comparison of Agilent Bio SEC-3 versus Agilent Bio SEC-5 particles. The 3 µm column gives better separation.

Column diameter

Column diameter can also be important depending on the amount of sample. 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, and so it is extremely useful for fractionation of complex samples or isolation of a sample component for further analysis. Larger diameter columns, such as 21.2 mm as found in the Agilent SEC-3 and SEC-5 product range, mean that lab prep separations can be performed using analytical HPLC systems.

Conditions, analysis of monoclonal antibody

Parameter	Value
Column A:	Bio SEC-3, 300 Å 7.8 x 300 mm, 3 µm (p/n5190-2511)
Column B:	Bio SEC-5, 300 Å 7.8 x 300 mm, 5 µm (p/n5190-2526)
Sample:	Humanized monoclonal antibody
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

Agilent AdvanceBio SEC columns 7.8 x 300 mm and 4.6 x 300 mm

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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 versus the conventional 300 mm–or flow rates can be increased or both. However, this could have a detrimental effect on resolution, because SEC relies on diffusion into and out of a pore to create differential path lengths through the column. Nonetheless, as shown in Figure 10, it is possible to obtain sufficient resolution to quantify an IgG dimer and monomer in under 4 minutes when using a 150 mm column at a flow rate of 2 mL/min.

Conditions

Parameter	Value
Column:	AdvanceBio SEC 300 Å, 7.8 x 150 mm, 2.7 µm (p/nPL1180-3301)
Sample:	IgG (2 mg/mL)
Eluent:	150 mM phosphate buffer, pH 7.0
Flow rate::	0.5, 1.0, 1.5 mL/min (52, 102, 152 bar)
Detector:	UV, 220 nm
Injection:	5 µL

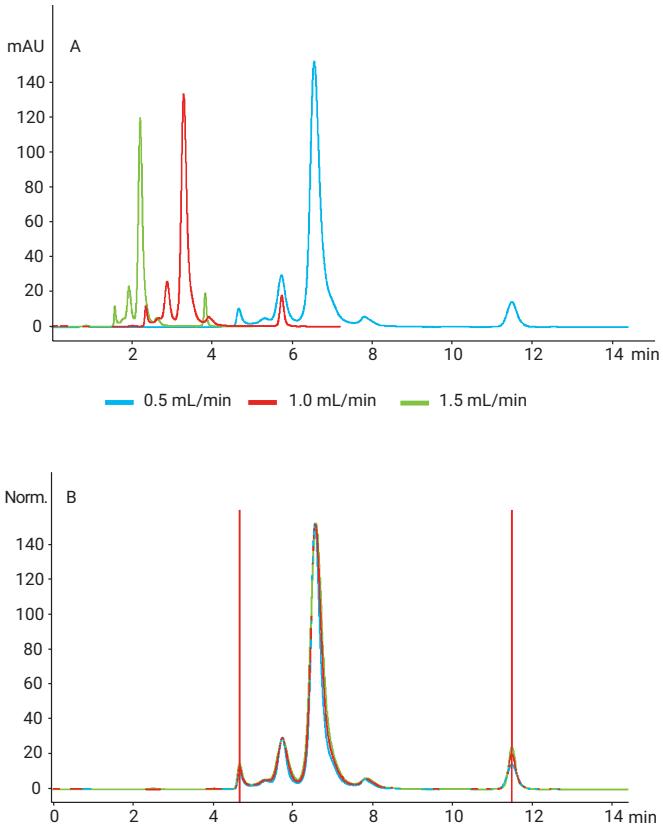

Figure 10: Increasing the flow rate reduces the analysis time from 12 to 4 minutes (A). When the retention times are normalized and overlayed (B) it is evident that the retention times are consistent and that there is minimal reduction in resolution.

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 appear when they should not, 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 Quaternary LC system
Poor retention/resolution of analytes	Insufficient pore size for molecule size	Check your pore size; see pages 6 and 12 for more information

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Mobile phase selection

Secondary interactions can cause difficulties

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.

Conditions

Parameter	Value
Column:	Agilent Bio SEC-3 300 Å 4.6 mm x 300 mm, 3 µm (p/n5190-2513)
Sample:	Protein (1 mg/mL 20 mM phosphate buffer, pH 7)
Instrument:	150 mM phosphate buffer, pH 7.0
Flow rate:	0.35 mL/min
Detector:	UV, 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

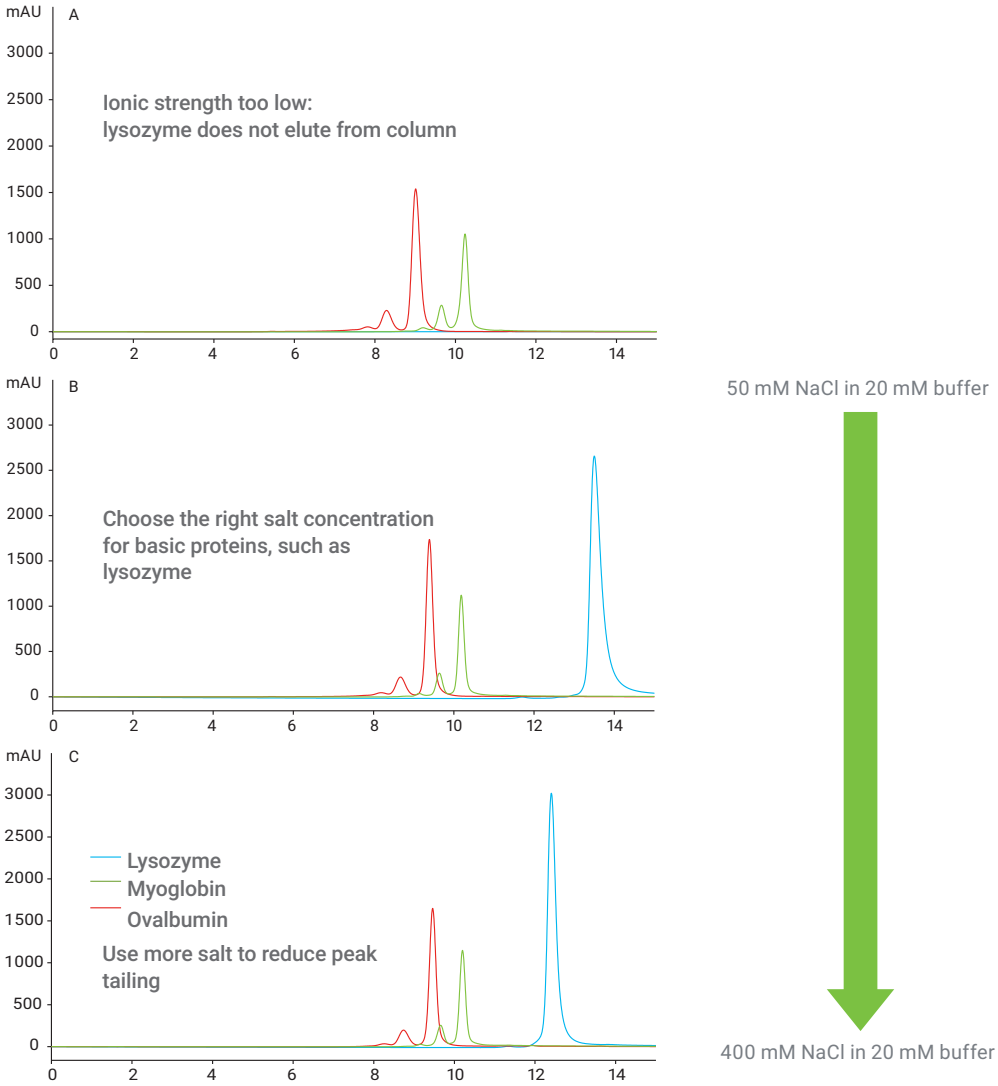

Figure 11: Effect of too much or too little ionic strength on achieving your desired separation.

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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 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.

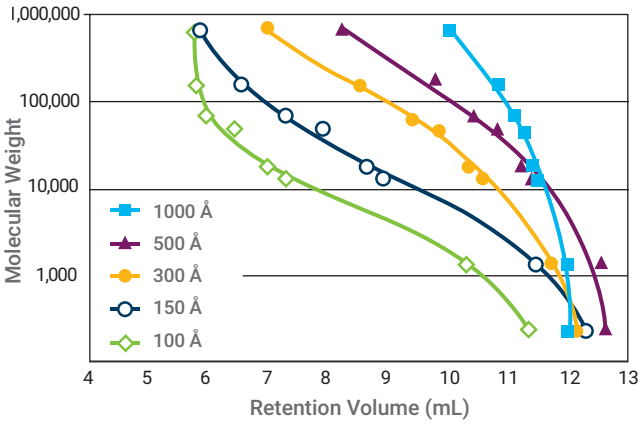

Figure 12: Calibration curves obtained by plotting retention time against molecular weight.

		Retention Volume				
Proteins	**MW**	**1000 Å**	**500 Å**	**300 Å**	**150 Å**	**100 Å**
Thyroglobulin	670,000	10.07	8.23	7.03	5.82	5.77
γ-globulin	158,000	10.88	9.80	8.57	6.55	5.79
BSA	67,000	11.13	10.44	9.44	7.29	6.00
Ovalbumin	45,000	11.28	10.83	9.89	7.90	6.40
Myoglobin	17,000	11.44	11.28	10.42	8.66	7.05
Ribonuclease A	12,700	11.52	11.41	10.58	8.93	7.32
Vitamin B12	1,350	12.00	12.59	11.78	11.49	10.30
Uracil	112	12.08	12.68	12.21	12.13	11.41

Conditions

Parameter	Value
Column:	Agilent Bio SEC-5 7.8 x 300 mm, 5 µm (p/n5190-2521)
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

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, it will be necessary to take further action. Centrifugation or filtration should be used to remove insoluble matter before 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.

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Advanced detection techniques

Further SEC considerations include choice of detector. UV or diode array (DAD) is commonly used for protein separations. Best results, i.e. 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 extra information is obtained without sacrificing chromatographic performance.

In special cases, mass spectrometry (MS) may be used with SEC, either in native mode (standard aqueous buffer mobile phase) or denaturing (organic solvent included in the mobile phase). In the case of MS, smaller i.d. columns are often preferable due to the low flow rates used for native mode MS. In some cases there is benefit to PEEK-lined columns to eliminate undesirable sample-metal interactions. AdvanceBio SEC 1.9 µm is available in 2.1 mm i.d. and PEEK-lined hardware for these circumstances.

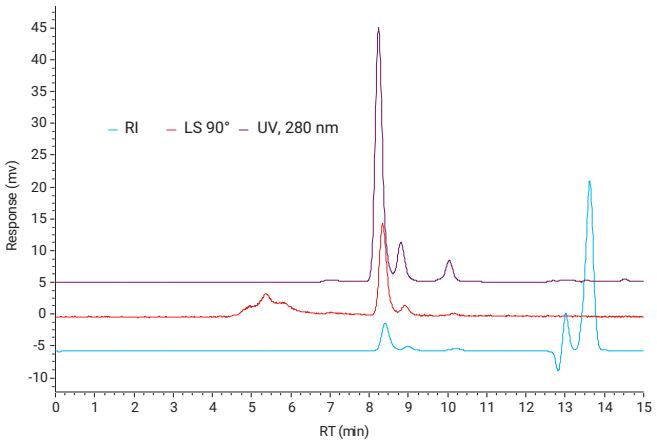

Figure 13: Results of using different detectors on a protein separation.

Conditions

Parameter	Value
Column:	Agilent AdvanceBio 300 Å, 7.8 x 300 mm, 2.7 µm
Sample:	Degraded monoclonal antibody
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC System with Agilent 1260 Infinity Multi-Detector GPC/SEC
Mobile phase:	150 mM sodium phosphate, pH 7.0
Flow rate:	0.8 mL/min
Temp.:	30 °C
Detector:	UV, 280 nm + RI + LS 90°
Injection:	5 µL

Conjugated proteins

Therapeutic proteins are subjected to aggregation and degradation during all stages of development, such as expression, refolding, downstream processing, formulation, sterilization, and storage. Although aggregates/degradents are present in extremely low concentrations, they may have a big impact on the quality of biologics, leading to activity loss, decreased solubility, and increased immunogenicity. Size exclusion chromatography is the standard method used to characterize protein aggregation and is also required for regulatory submission and approval.

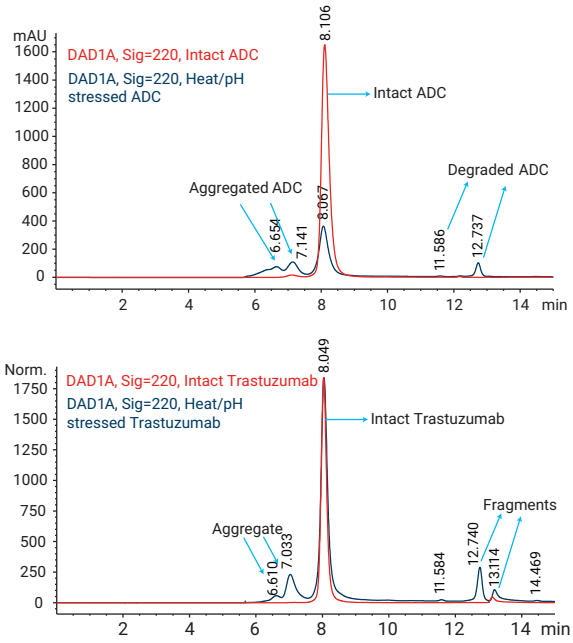

Figure 14: The same aqueous mobile phase was used for the mAb and the more hydrophobic ADC.

To improve the delivery, increase half life, and increase potency, proteins, including monoclonal antibodies, can be conjugated. Water-soluble polymers, such as polyethylene glycol, are conjugated with the protein to enhance pharmacological activities, increase their half life in the blood stream, and reduce immunogenicity. More recently, there has been interest in antibody drug conjugates, ADCs, where monoclonal antibodies are conjugated to a cytotoxic agent for targeted drug delivery and increased efficacy of treatment. After conjugation, the same aggregation studies are required because the change in sample characteristics can present a greater challenge to achieving an SEC separation. Columns with very low non specific binding, such as AdvanceBio SEC, are required for the analysis of both the antibody and the ADC using aqueous mobile phases. See Figure 14.

Conditions

Parameter	Value
Column:	AdvanceBio SEC 300 Å 7.8 x 300 mm, 2.7 µm
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC System
Mobile phase:	PBS, 50 mM sodium phosphate containing 150 mM sodium chloride, pH 7.4
TCC Temp.:	Ambient
Injection:	10 µL
Flow rate::	0.8 mL/min
Detector:	UV, 220 nm

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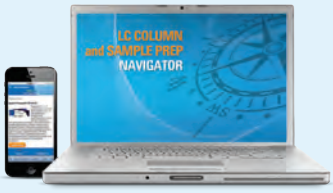

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Sample preparation

- 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
- 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 150 or 300 mm
- Normal flow rate is 1.0 mL/min on a 7.5 or 7.8 mm i.d. column, 0.35 mL/min on a 4.6 mm i.d. column, and 0.1 mL/min on a 2.1 mm i.d. 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 to avoid exclusion of sample components and maximize volume in the required separation region
- **Particle size**–use smaller particles for higher resolution (but higher backpressure)
- **Column length**–compromise between resolution and analysis time
- **Column i.d.**–use smaller columns for reduced solvent consumption and smaller injection volume

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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 to remove particulates 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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Application Note

Aggregate/Fragment Analysis

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Elevate Your mAb Aggregate Analysis

High-resolution SEC with the Agilent 1290 Infinity II Bio LC SystemBio LC System

Author

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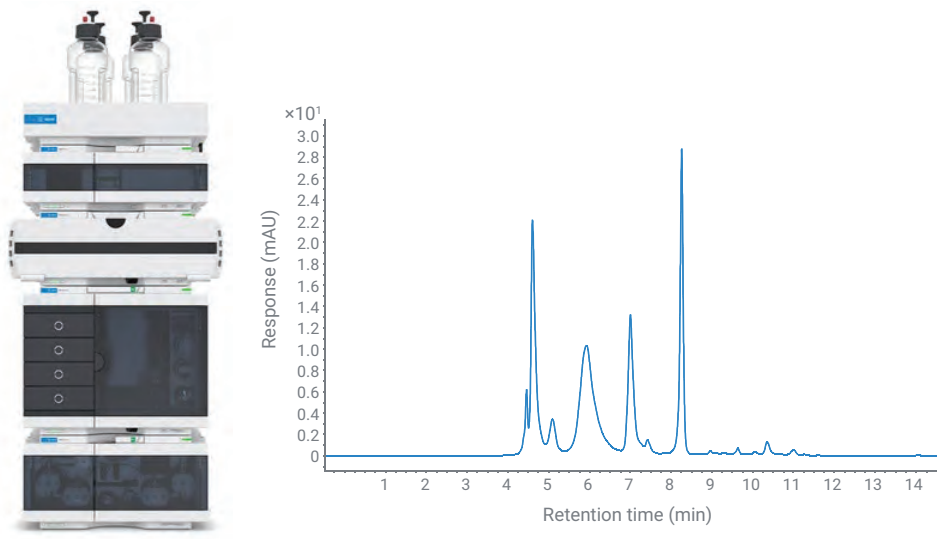
Abstract

This application note demonstrates the superior resolution in size exclusion chromatography (SEC) protein separation made possible by the Agilent 1290 Infinity II Bio LC System equipped with the Agilent AdvanceBio SEC column and ultralow dispersion capillaries. The biocompatible UHPLC system enables analysis using corrosive salty buffers and therefore saves maintenance expense. The resolution in SEC analysis was compared for capillaries of different inner diameter (0.17, 0.12, and 0.07 mm). A protein standard mixture and monoclonal antibodies (mAbs), including aggregates, were separated and compared for resolution. In addition, molecular weight was determined by the Agilent OpenLab GPC/SEC add-on software in one software solution, enabling a one-step workflow.



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Introduction

Modern biopharmaceuticals, such as mAbs, are highly heterogeneous compounds. Aggregation monitoring, one of the most important critical quality attributes (CQAs), is typically executed by SEC. With this technique, the identity of the compound can be determined by the calculation of the molecular weight after a standard column calibration. In addition, it confirms the purity by showing the presence of unwanted higher molecular weight compounds such as dimeric and higher aggregates. To achieve the necessary resolution, modern SEC columns with sub-2 µm particle material are recommended. To enable optimal performance, a combination of sub-2 µm columns and a UHPLC instrument with dead volumes as low as possible is preferred. Large dead volumes destroy the resolution obtained by these columns due to dispersion effects. In addition, the completely biocompatible 1290 Infinity II Bio LC perfectly copes with the high salt concentrations often found in SEC buffers, providing confident results at the lowest maintenance cost.

This application note demonstrates the use of modern sub-2 µm SEC columns on the 1290 Infinity II Bio LC and illustrates the benefit of using instruments with the lowest possible dead volumes. To demonstrate the effect of dead volume on the separation of proteins and aggregates, capillaries with different inner diameters were used. The well-characterized NISTmAb will be used to generate more aggregates by pH and thermal stress with subsequent separation of dimers, trimers, and higher aggregates.

Experimental

Instrument

- Agilent 1290 Infinity II Bio High-Speed Pump (G7132A)
- Agilent 1290 Infinity II Bio Multisampler (G7137A) including integrated Sample Thermostat (#101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with biocompatible Heat Exchanger
- Agilent 1290 Infinity II Variable Wavelength Detector (G7114B), equipped with a biocompatible micro flow cell, 3 mm, 2 µL

Additional parts

Agilent 1290 Infinity II Bio Ultra Low Dispersion Kit (G7132A#006)

Software

Agilent OpenLab Version 2.5 and GPC/SEC add-on software V. 1.2

LC Method

Parameter	Valve
Solvent	Phosphate-buffered saline (PBS), pH 7.4
Flow rate	0.35 mL/min
Isocratic separation	
Column temperature	30 °C
Sample temperature	4 °C
Needle wash	3 s water
Injection volume	5 µL
Detection (VWD)	280 nm, data rate 20 Hz

Column

Agilent AdvanceBio SEC, 200 Å, 4.6 × 300 mm, 1.9 µm (part number PL1580-5201)

Samples

- Protein mix for calibration (part number 5190-9417): thyroglobulin (670,000 Da), γ-globulin (150,000 Da), ovalbumin (45,000 Da), myoglobin (17,000 Da), angiotensin II (1,000 Da)
- Humanized monoclonal antibody (mAb) trastuzumab, marketed as Herceptin, was obtained from Roche (Basel, Switzerland). The trastuzumab was dissolved in 30 mM phosphate buffer, pH 6.8.
- Agilent NISTmAb, humanized IgG1κ mAb (part number 5191-5745)(Steinheim, Germany).

Protocol for pH/temperature-stressed NISTmAb

The mAb was diluted in the mobile phase to a final concentration of 2 mg/mL. pH stress was carried out as described elsewhere with slight modification:1 1 M HCl was slowly added dropwise to the sample solutions to change the pH from 6.0 to 1.0. Then, 1 M NaOH was added to adjust the pH to 10.0. Finally, 1 M HCl was added again to adjust the pH back to 6.0. There was approximately 1 minute waiting time between the pH shifts, with constant, slight stirring. The resulting solution was incubated at 60 °C for 60 minutes.

Solvents and chemicals

- **PBS:** One tablet dissolved in 200 mL of deionized water yields 0.01 M phosphate buffer, 0.0027 M potassium chloride, and 0.137 M sodium chloride, pH 7.4, at 25 °C.
- Chemicals were purchased from VWR, Germany.
- Fresh ultrapure water was obtained from a Milli-Q integral system equipped with LC-Pak polisher and a 0.22 µm membrane point-of-use cartridge (Millipak).

Results and Discussion

Modern columns for SEC separation of proteins comprise material with sub-2 µm particles for optimum resolution. However, this requires instruments that have optimized low dead volume, because especially capillaries of larger inner diameters can destroy the achieved resolution. The separation of a mixture of five proteins, including three dimers, with the 1290 Infinity II Bio LC is shown in Figure 1A. To minimize dead volume and dispersion effects, capillaries with an inner diameter of 0.07 mm were used for the separation. Even the early-eluting dimer of thyroglobulin (4.947 min) was partially separated. To set up a calibration for molecular weight determination, all the proteins in this mixture were used to generate the calibration curve (Figure 1B). The best curve fit was obtained for a fourth order.

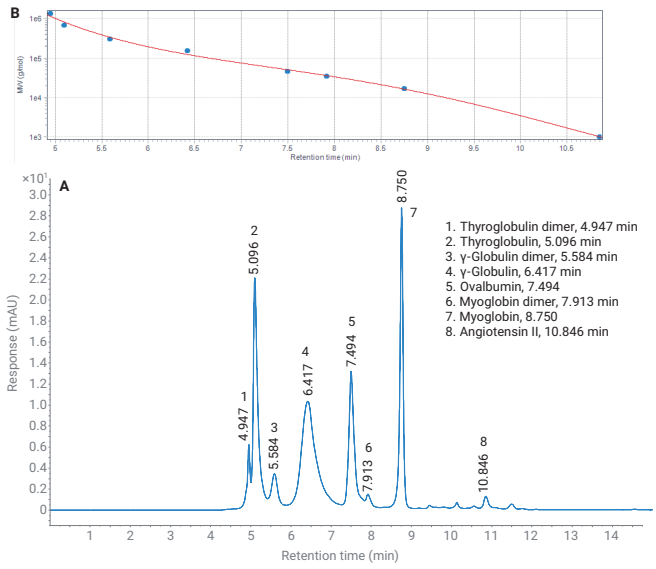
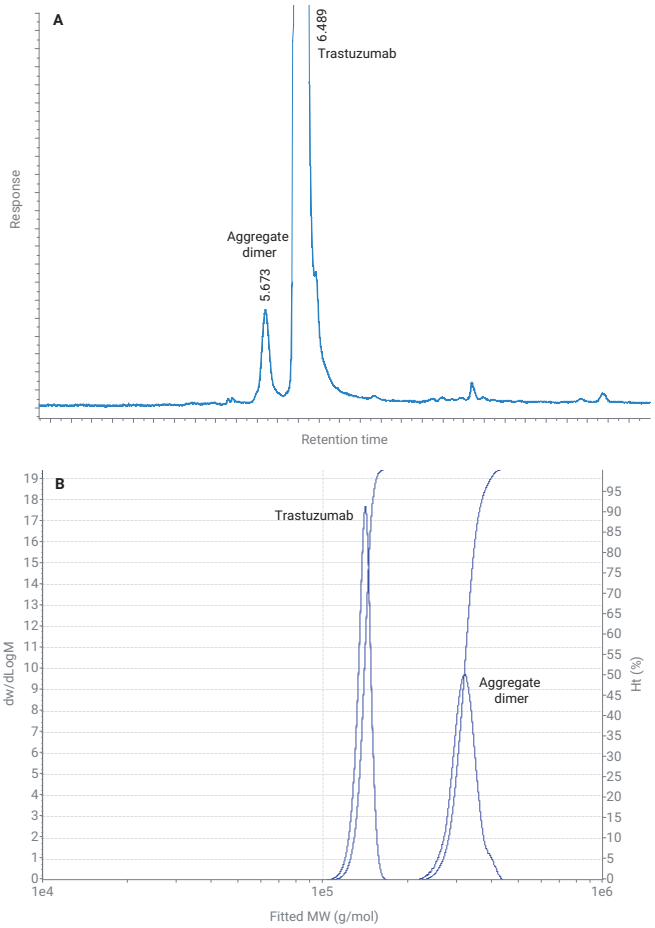


Figure 1. (A) Separation of a five-protein mixture including three dimers on the Agilent AdvanceBio SEC, 200 Å, 4.6 × 300 mm, 1.9 µm in combination with the Agilent 1290 Infinity II Bio Ultra Low Dispersion Kit comprising 0.07 mm capillaries. (B) Calibration curve of SEC for molecular weight determination with a protein mixture featuring some dimers.

The calibration was used to determine the molecular weight of the mAb trastuzumab and a comprised dimeric aggregate (Figure 2). The antibody elutes at 6.489 minutes and the corresponding dimeric aggregate elutes at 5.673 minutes (Figure 2A). The determined molecular weight at the peak maximum of trastuzumab and the dimer were Mp 141,566 Da and Mp 321,609 Da, respectively. The molecular weight distribution is shown in Figure 2B and the calculated molecular weights are outlined in the included table (2C).



C

Compound	RT (min)	Mp (g/mol)
Trastuzumab Aggregate	5.673	321609
Trastuzumab	6.489	141566
D4	0.072	0.071
D6	0.065	0.262
D8	0.066	0.281

Figure 2. Trastuzumab and dimeric aggregate, determination of molecular weights. (A) SEC separation of the monomer of trastuzumab from a dimeric aggregate. (B) The molecular weight of trastuzumab and the dimeric aggregate. (C) Table with molecular weights of trastuzumab and its aggregate. Mp: molecular weight at peak maximum.

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The influence of the inner diameter of the used capillaries could be shown in a comparison of capillaries with an inner diameter of 0.07 (ULD), 0.12 and 0.17 mm. To demonstrate the effect, the resolution of the second and third peak of the protein mixture (shown in Figure 1) was determined (Figure 3). The best values of resolution for peak 2 and peak 3 could be obtained by means of capillaries with 0.07 mm inner diameter (table in Figure 3). For the determination of the peak width at half-height, peak 2 of the protein mixture was used. From the measured values, it could be seen that the peak width increases when using capillaries of larger inner diameters.

The influence of the capillaries on the separation of trastuzumab and its aggregate is shown in Figure 4. Here, it can be seen that an additional lower molecular weight compound was hidden under the main peak, which is only separated as a slight shoulder with the 0.17 mm capillaries and is more clearly visible using the 0.07 mm capillaries.

	Resolution		Width (min)
Capillaries (µm)	Peak 2	Peak 3	Peak 2
0.07	0.94	1.98	0.110
0.12	0.86	1.88	0.120
0.17	0.71	1.73	0.142

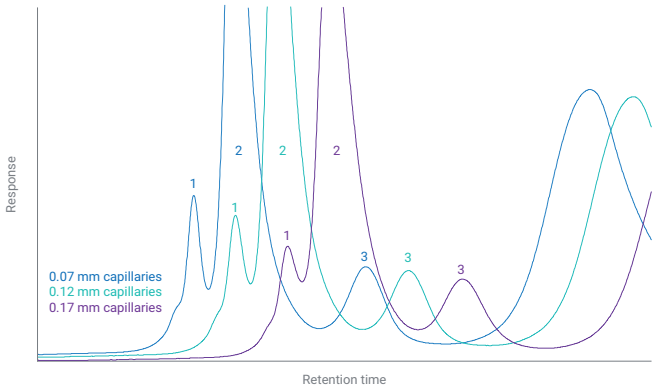

Figure 3. Comparison of capillaries with increasing inner diameters and their influence on resolution and peak width.

Capillaries (µm)	Resolution	Width (min) Herceptin	Width (min) Aggregate
0.07	3.34	0.096	0.189
0.12	3.05	0.107	0.205
0.17	2.83	0.121	0.216

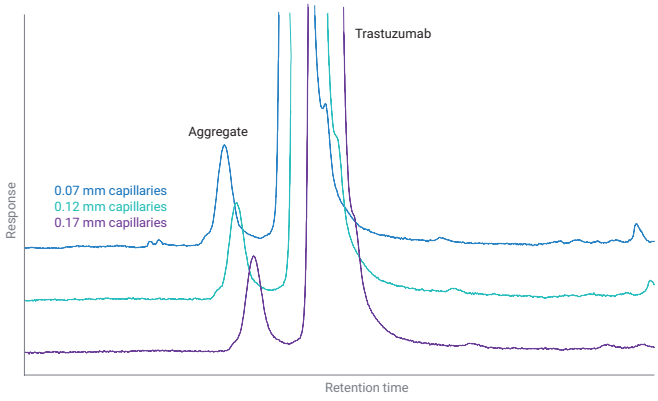

Figure 4. Resolution of trastuzumab from its dimeric aggregate and peak width depending on the inner diameter of the used capillaries.

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The RSD values of retention time and peak area are excellent for all capillaries (Table 1).

As another example, the well-characterized NISTmAb (humanized IgG1κ mAb) was used for separation from aggregates and determination of molecular weights (Figure 5).

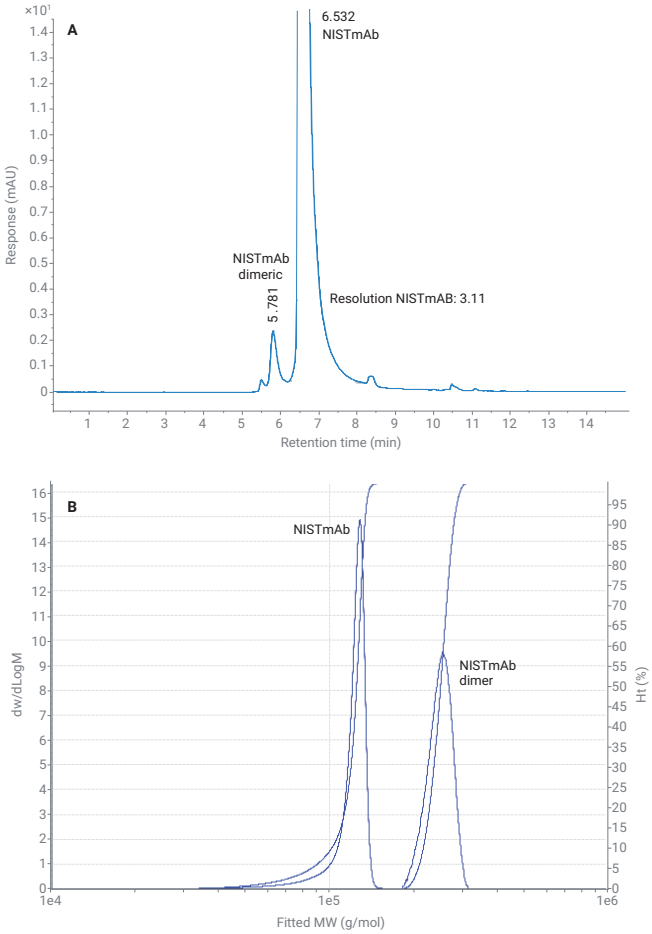

Figure 5. (A) Separation of NISTmAb from its main dimeric aggregate with resolution 3.11. (B) Molecular weights in the NISTmAb and its aggregate. (C) Table showing the values of major peak characterization from NISTmAb and its aggregate. (D) Tables showing the molecular mass of NISTmAb and its aggregate.

Table 1. Retention time and peak area RSDs of trastuzumab for all used capillaries. The increase in retention times is due to the increasing volumes of the different sets of capillaries.

	0.07 mm Capillaries		0.12 mm Capillaries		0.17 mm Capillaries	
	RT	Area	RT	Area	RT	Area
Average	6.464	1736.13	6.500	1727.05	6.554	1717.29
RSD (%)	0.02	0.10	0.01	0.28	0.01	0.25

C

RT (min)	Area	Height	Resol. USP	Tailing	Width 50%
5.781	23.80	2.08	7.59	1.940	1.940
6.532	3006.00	409.40	3.11	1.569	0.100

D

RT (min)	Mp (g/mol)
5.781	305626
6.532	144767

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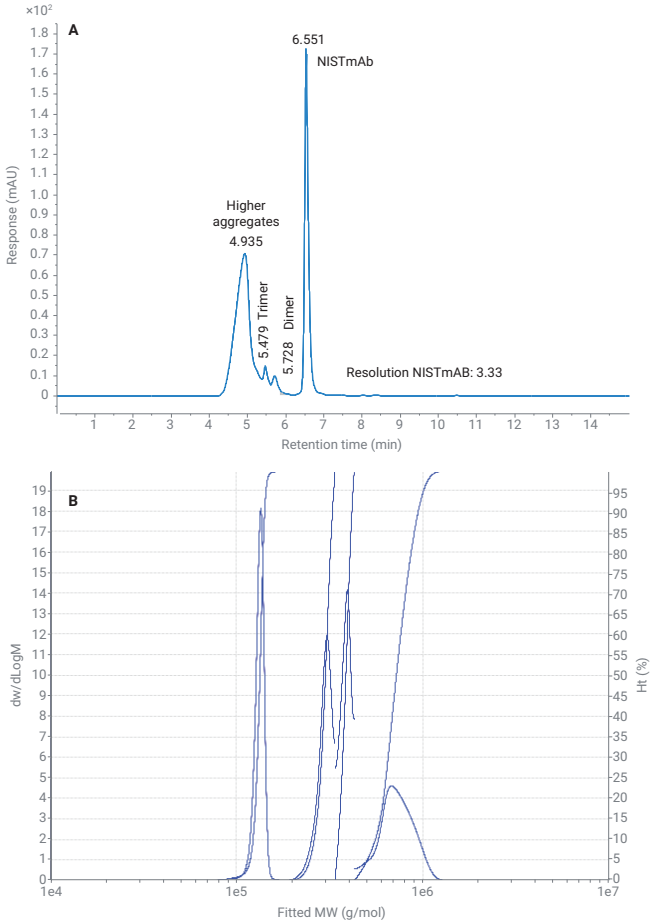

Figure 6. (A) Separation of aggregates from a pH-stressed NISTmAb. (B) Distribution of molecular weight of aggregates occurring under stress conditions from NISTmAb. (C) Table of values of major peak characterization of NISTmAb and its aggregates. (D) Tables showing the molecular mass of NISTmAb and its aggregates.

Under pH- and temperature-stress conditions (see Experimental section), this mAb can form higher aggregates (Figure 6). With the ultralow dispersion capillaries, the higher aggregates could be separated (Figure 6A). Their molecular weight distribution and values for peak characterization are outlined in Figure 6B and the associated tables.

C

RT (min)	Area	Height	Resol. USP	Tailing	Width 50%
4.913	1926.46	70.80	7.59	0.781	0.398
5.479	129.63	14.58	0.85	1.277	0.168
5.728	106.16	9.5	0.91	1.985	0.185
6.551	1208.07	172.21	3.33	1.116	0.110

D

RT (min)	Mp (g/mol)
4.913	706827
5.479	411615
5.728	321609
6.551	141918

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Conclusion

This application note demonstrates the capability of the 1290 Infinity II Bio LC together with the AdvanceBio SEC column to separate proteins and their aggregates with the highest resolution due to minimized system dead volume and ultralow dispersion capillaries. The 1290 Infinity II Bio LC is a completely biocompatible system capable of operating with highly salted buffers. This offers the lowest maintenance costs at the highest resolution performance.

References

1. Quantitation of mAb and ADC Aggregation Using SEC and an Aqueous Mobile Phase. *Agilent Technologies application note*, publication number 5991-6303EN, **2016**.

Published in the USA, October 22, 2020
5994-2709EN

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Application Note

Aggregate/Fragment Analysis

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High-Resolution, High-Throughput Size Exclusion Chromatography Analysis of Monoclonal Antibodies

Using an Agilent AdvanceBio SEC 200 Å 1.9 µm column

Author

Veronica Qin

Agilent Technologies, Inc.

Abstract

This Application Note demonstrates the use of the Agilent AdvanceBio SEC 200 Å 1.9 µm column for high-resolution and high-throughput size exclusion chromatography (SEC) analysis of a monoclonal antibody (mAb). The optimized sub-2 µm particle enables faster separations and uncompromised high resolution for accurate quantitation.

Introduction

Aggregates and fragments are critical quality attributes of biotherapeutic proteins that need to be well characterized. Size exclusion chromatography (SEC) is commonly used to analyze these size variants. There are cases where high-throughput SEC analysis is in high demand. Examples are in the early stage of drug development during clone selection, or process development, where large numbers of samples need to be analyzed daily. The AdvanceBio SEC 200 Å 1.9 µm column, with its unique, durable sub-2 µm particles, offers fast analysis with high resolution. These features significantly improve sample throughput, while delivering robust and accurate results.

Experimental

Materials

SILu Lite SigmaMAb universal antibody standard was purchased from MilliporeSigma and reconstituted with water to 1 mg/mL. Monobasic and dibasic sodium hydrogen phosphate and sodium chloride were purchased from MilliporeSigma. All chemicals used were ≥99.5 % pure. Water was purified from a Milli-Q A10 water purification system (Millipore). Solutions were prepared fresh daily, and filtered through a 0.22 µm membrane filter prior to use.

Instrumentation

An Agilent 1260 Infinity LC with the following configuration was used:

- Agilent 1260 Infinity II bio-inert quaternary 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 variable wavelength detector (G7114A)

Columns

- Agilent AdvanceBio SEC 200 Å 1.9 µm, 4.6 × 300 mm (p/n PL1580-5201)
- Agilent AdvanceBio SEC 200 Å 1.9 µm, 4.6 × 150 mm (p/n PL1580-3201)bio-inert flow cell (option #028)

Software

Agilent OpenLab 2.2 CDS.

Results and discussion

Materials

Figure 1 shows SEC chromatograms of mAb with aggregates and fragments using 300 mm columns with flow rates at 0.35, 0.4, and 0.5 mL/min. Excellent resolution of both dimer/monomer and monomer/fragment1 was achieved even at 0.5 mL/min (Table 1) by saving 28 % of run time versus 0.35 mL/min flow rate without compromising resolution.

Instrument conditions

Parameter	HPLC conditions
Column temperature	25 °C
Mobile phase	50 mM sodium phosphate, 200 mM NaCl, pH 7.0
Flow rate	0.3 to 0.7 mL/min
Injection volume	1 µL
Direction	UV at 220 nm

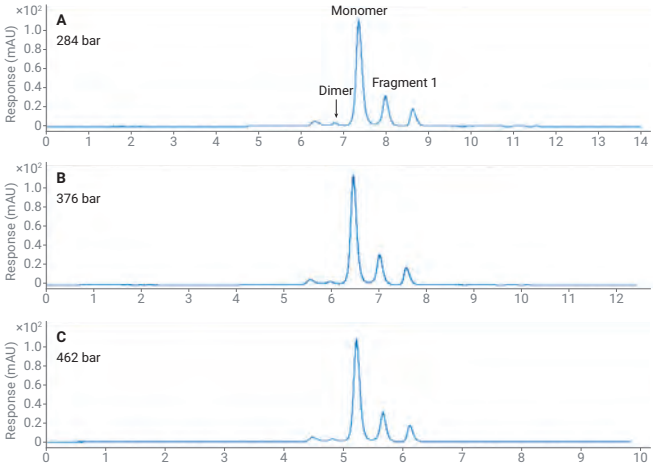

Figure 1. Size exclusion chromatograms of SigmaMAb (mixed with its F(ab')2 and Fc fragments) using 4.6 × 300 mm SEC columns running with 50 mM sodium phosphate, 200 mM NaCl, pH 7.0 at A) 0.35 mL/min; B) 0.4 mL/min; C) 0.5 mL/min.

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Aggregates and fragments are critical quality attributes of biotherapeutic proteins that need to be well characterized. Size exclusion chromatography (SEC) is commonly used to analyze these size variants. There are cases where high-throughput SEC analysis is in high demand. Examples are in the early stage of drug development during clone selection, or process development, where large numbers of samples need to be analyzed daily. The AdvanceBio SEC 200 Å 1.9 µm column, with its unique, durable sub-2 µm particles, offers fast analysis with high resolution. These features significantly improve sample throughput, while delivering robust and accurate results.

Table 1. Peak tailing factor and resolution under different flow rates.

Flow Rate (mL/min)	Tailing Factor (Monomer)	Resolution (Dimer/Monomer)	Resolution (Monomer/Fragment 1)
0.35	1.18	1.98	2.37
0.4	1.16	1.96	2.36
0.5	1.14	1.91	2.29

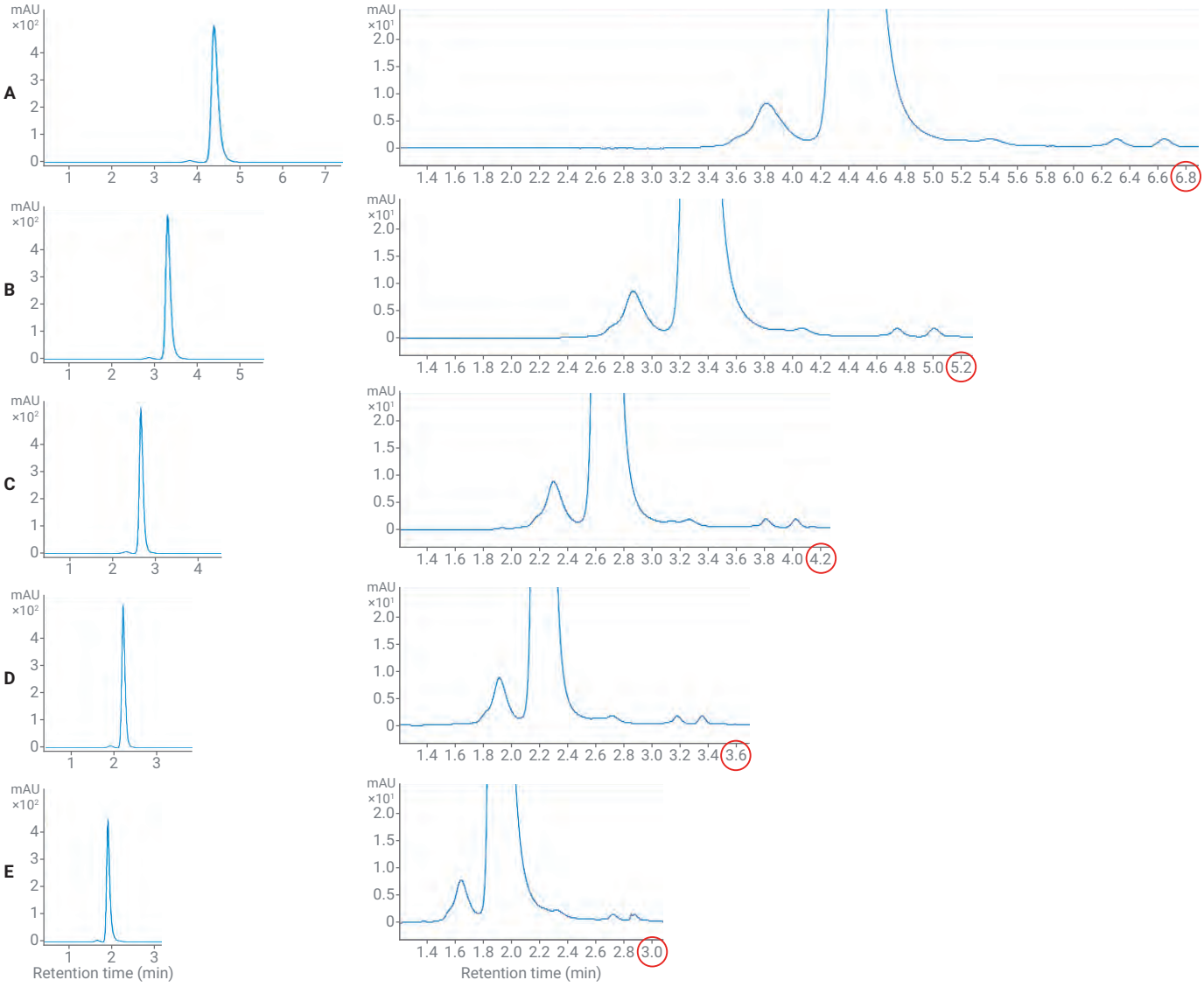

Figure 1. Size exclusion chromatograms of SigmaMAb using 4.6 × 150 mm SEC columns running with 50 mM sodium phosphate, 200 mM NaCl, pH 7.0 at A) 0.3 mL/min; B) 0.4 mL/min; C) 0.5 mL/min; D) 0.6 mL/min; E) 0.7 mL/min.

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The unique particles in this column enable excellent stability at much higher flow rates with high resolution of dimer/monomer for accurate quantitation of the dimer peak area (Table 2).

Table 2 calculates the effect of flow rate on sample throughput. When increasing the flow rate from 0.3 to 0.7 mL/min, 480 samples can be analyzed per day, which is a 2.3-fold improvement in throughput. Compared to running the 300 mm column at 0.3 mL/min, which can only analyze 105 samples per day, the throughput increases 4.6-fold..

Conclusion

This study demonstrates the ability of the AdvanceBio SEC 200 Å 1.9 µm column to be used for fast analysis of mAb aggregates. The durable particles enable running at a higher flow rate without loss of high resolution. By reducing column length from 300 to 150 mm, and by increasing flow rate from 0.3 to 0.7 mL/min, we can enhance sample throughput 4.6-fold.

Table 2. Effect of flow rate on resolution, monomer area percentage, and sample throughput.

RT	Run time (min)	Backpressure (bar)	Resolution	Dimer Area %	Samples per hour	Samples per day 24-hours
0.3	6.8	164	1.81	2.33	8-9	211
0.4	5.2	218	1.79	2.35	11-12	276
0.5	4.2	272	1.78	2.35	14	342
0.6	3.6	324	1.77	2.39	16-17	400
0.73	3.0	380	1.58	2.30	20	480

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Application Note

Aggregate/Fragment Analysis

Agilent

Trusted Answers

Sensitive Native Mass Spectrometry of Macromolecules Using Standard Flow LC/MS

Authors

David L. Wong

Agilent Technologies, Inc.

Abstract

Native mass spectrometry can be used for a variety of protein-based applications, such as protein-protein interaction, protein-ligand binding, protein complex structures, protein folding and antibody-drug conjugates. Most of the native MS analyses are using a nano-electrospray approach which faces significant challenges.

This application note describes a robust and sensitive LC/MS method using standard LC flow for the analysis of native protein analysis. The workflow comprised the Agilent 1290 Infinity II LC, the AdvanceBio SEC column, the 6545XT AdvanceBio LC/Q-TOF, and MassHunter BioConfirm software.

Introduction

Native mass spectrometry (MS) has emerged as a widely used technique for the characterization of intact proteins and noncovalent protein complexes. Various sizes of protein complex structures (protein-ligand binding or protein-protein interaction) ranging from a few kDa to more than 1 MDa have successfully been analyzed and studied by this technique despite its tremendous analytical challenges.1-4 Without organic solvent and acid to enhance sample desolvation and ionization, native MS analysis of protein samples at neutral pH conditions tends to have fewer charges per molecule and much lower abundance MS signals at higher m/z ranges. In the past decade, the nano-electrospray ionization (nESI) approach has become a crucial method used in native protein analysis. The nESI forms fine charged droplets, which can dramatically increase the sample desolvation and ionization efficiency while preserving the noncovalent protein-protein complexes. However, it has commonly been observed that the neutral aqueous protein samples tend to aggregate easily under the unstable nanoflow rate condition and cause the nanospray emitter to clog. Also, well trained or experienced researchers are needed to produce good-quality MS data using the nESI technique.

In this study, we demonstrate a highly sensitive analytical flow LC/MS methodology for the analysis of native proteins and protein complexes. This workflow uses the AdvanceBio size exclusion chromatography (SEC) column for online sample separation. The 6545XT AdvanceBio LC/Q-TOF, featuring large molecule SWARM autotune and 30,000 m/z extended mass range, was used for rapid and reproducible native protein analysis (Figure 1).

Experimental

Materials and methods

Monoclonal antibody standard, RM 8671, was purchased from the National Institute of Standards and Technology (NIST), often referred to as NIST-mAb. The formulated Herceptin (trastuzumab) was obtained from Genentech (South San Francisco, California, USA). The formulated trastuzumab emtansine (TDM1, ADC) was also from Genentech. All other protein samples and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Sample preparation

To perform native MS analysis, it is crucial to preserve the protein samples at neutral pH and volatile aqueous solutions, such as ammonium acetate or ammonium formate. Therefore, sample desalting and buffer exchange are usually needed prior to the MS analysis. Briefly, protein stock solutions (1 to 10 mg/mL) were desalted and solvent exchanged into 100 mM ammonium acetate using Bio-Rad Bio-Spin P-6 (6,000 MW limit) or P-30 (40,000 MW limit) cartridges. The cartridge was first fully equilibrated with 100 mM ammonium acetate buffer. Protein sample was then pipetted to the top of the column and centrifuged for 5 min at 1000 × g. The protein was then buffer exchanged into the 100 mM ammonium acetate and was ready for MS analysis. This desalting protocol caused minimal sample loss and much less structural alteration of the protein molecule.

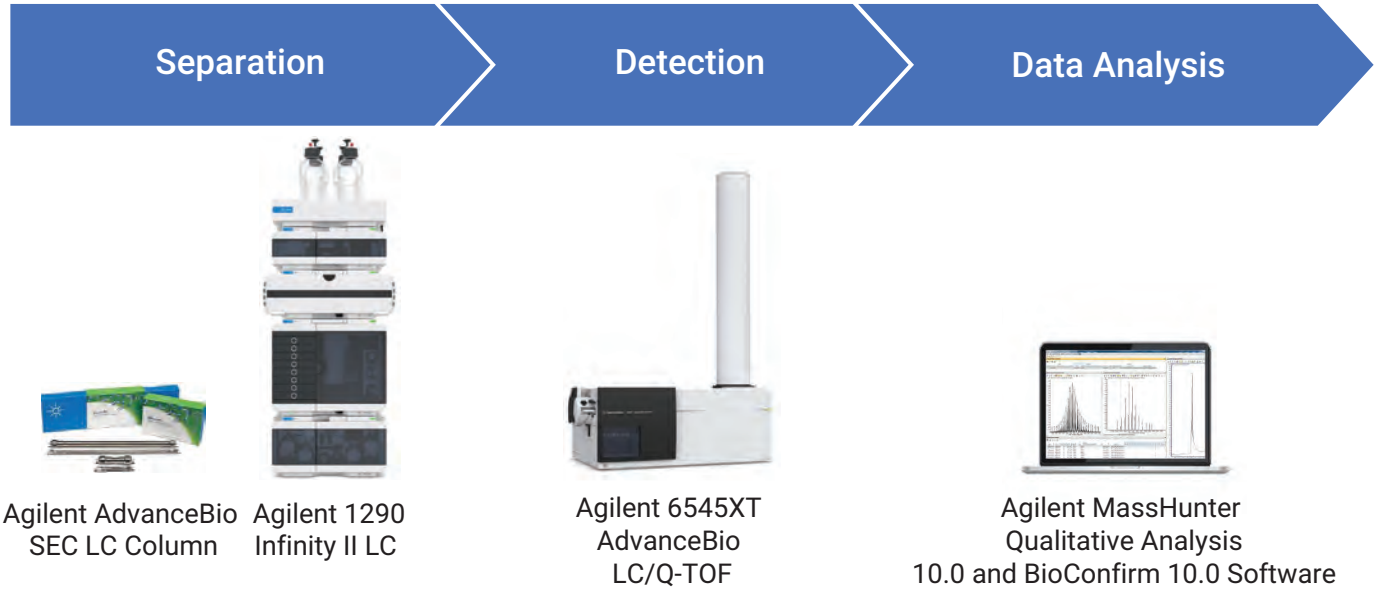

Figure 1. Analytical components of the native protein analysis workflow.

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Instrumentation

Agilent 1290 Infinity II LC including:

- Agilent 1290 Infinity II high-speed pump (G7120A)
- Agilent 1290 Infinity II multisampler (G7167B)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 6545XT AdvanceBio LC/Q-TOF)

LC/MS analysis

LC/MS analyses were conducted on a 1290 Infinity II LC coupled with a 6545XT AdvanceBio LC/Q-TOF system equipped with an Agilent Jet Stream source. Agilent MassHunter Acquisition (B.09.00) workstation software with the large molecule SWARM autotune feature was used.

LC separation was obtained with an Agilent AdvanceBio SEC guard column (4.6 × 30 mm, 200 Å, 1.9 µm).

Tables 1 and 2 list the LC/MS parameters used.

Data processing

All MS data of the native intact mAbs or protein complexes were processed using Agilent MassHunter Qualitative Analysis 10.0 and BioConfirm 10.0 software.

Results and discussion

Method optimization for native protein and protein complex analysis

To overcome the challenges of native protein analysis, some key method developments and optimizations were made:

- The use of offline desalting cartridges (Bio-Rad Bio-Spin P-30) for sample preparation (desalting and buffer exchange) prior to the MS analysis and online SEC column further separated the target protein from background salts, which led to higher MS sensitivity and improved MS data quality.
- The use of a conventional flow rate (0.2 mL/min) of 100 mM ammonium acetate buffer not only eliminated the sample aggregation but also improved LC/MS analytical reproducibility for well-preserved native protein samples.
- The 6545XT AdvanceBio LC/Q-TOF system was equipped with large molecule SWARM autotune for optimizing macromolecular ions transmission, and the extended mass range of up to m/z 30,000 for the native protein complex analysis with high sensitivity.

Table 1. Liquid chromatography parameters.

	Agilent 1290 Infinity II LC
Column	AdvanceBio SEC (200 Å, 4.6 × 30 mm, 1.9 µm) (p/n: PL1580-1201)
Thermostat	4 °C
Solvent (A)	100 mM NH4OAc (pH 7)
Isocratic Elution	0–5 min, 100% A
Column temperature	Room temperature
Flow rate	0.2 mL/min
Injection volume	1–5 µL

Table 2. Native MS data acquisition parameters.

	Agilent 1290 Infinity II LC		
Sample Type	Myoglobin	Intact mAbs	Intact Macroprotein Complexes
Source	Agilent Jet Stream	Agilent Jet Stream	Agilent Jet Stream
Dry Gas Temperature	150 °C	365 °C	150 °C
Dry Gas Flow	10 L/min	12 L/min	10 L/min
Nebulizer	30 psig	35 psig	30 psig
Sheath Gas Temperature	150 °C	300 °C	150 °C
Sheath Gas Flow	10 L/min	12 L/min	10 L/min
VCap	5000 V	5500 V	5000 V
Nozzle Voltage	2000 V	2000 V	2000 V
Fragmentor	250 V	300 V	250 V
Skimmer	100 V	220 V	100 V
Quad AMU	m/z 500	m/z 1000	m/z 3000
Mass Range	m/z 300–7000n	m/z 3000–10000n	m/z 5000–25000n
Acquisition Rate	1.0 spectrum/s	1.0 spectrum/s	1.0 spectrum/s
Acquisition Mode	Positive, extended (m/z 10,000 mass range	Positive, extended (m/z 10,000 mass range	Positive, extended (m/z 25,000 mass range

Native MS analysis of intact myoglobin (with heme)

Native MS analysis of noncovalent interactions of myoglobin has been well-studied.4 In myoglobin, heme is noncovalently attached to the globin through hydrogen bonds and hydrophobic interactions. When the heme is attached to the globin, the protein is referred to as holomyoglobin (the native conformation). Monitoring of the charge state distributions of myoglobin ions in mass spectra of ESI-MS has been used in protein folding/unfolding studies.4 The apomyoglobin (with no heme) with high charge states indicated the disruption of the native heme-protein interaction, which led to a considerable degree of protein unfolding. As shown in Figure 2A, myoglobin was denatured in the organic and acid solvent, and under harsh MS source conditions.

The charge envelope of the denatured myoglobin ranged from m/z 1,000 (17+) to 3,500 (5+) while the most intense charged ion was 12+.Most of the native holomyoglobin was denatured into apomyoglobin and heme (inset in Figure 2A). Our optimized native MS analysis of myoglobin clearly demonstrated that the native conformation of myoglobin was retained (Figure 2B). Only trace amounts of apomyoglobin and heme could be detected. The charge envelope of holomyoglobin was from 9+ to 5+ and the charge state of 8+ was the most abundant ion. The overall MS signal intensities of the native MS ions were about 1/10 of those in the denatured MS spectrum.

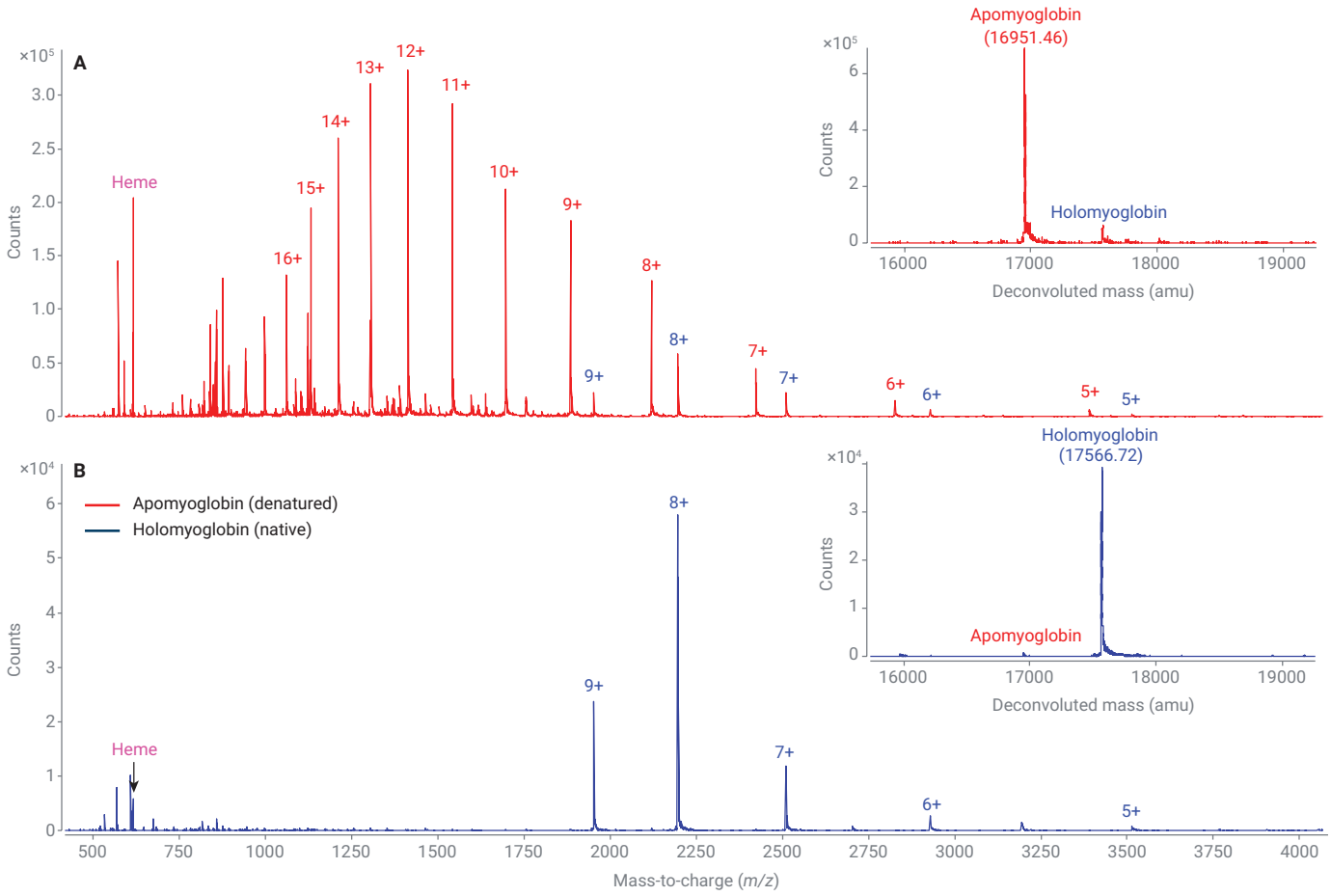

Figure 2. LC/MS analysis of intact myoglobin sample.
A) Myoglobin sample was analyzed under denatured LC/MS conditions (previous studies).
The heme group was dissociated from the protein complex and the majority of the protein was apomyoglobin (inset figure).
B) Native MS analysis of myoglobin. The holomyoglobin (with heme) structure was preserved and only trace amount of heme was detected.

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The native MS analysis results confirmed that ionic strength of the SEC column mobile phase also played a key role in maintaining the protein native conformation.2 Figure 3 demonstrates the native MS analysis of alcohol dehydrogenase (ADH, tetramer) under two mobile phase conditions. Even though both mobile phases were at neutral pH, protein dissociation products (dimer) were observed when ammonium formate was substituted for ammonium acetate in the mobile phase (Figure 3A and 3B). Also, the charge state envelope of the intact native ADH in the 50 mM ammonium formate was shifted to a lower m/z range compared to that in the 100 mM ammonium acetate (Figure 3C).

The results indicate that use of ammonium formate in the mobile phase increases the number and extent of multiply charged ions (max at 26+ compared to 23+ using ammonium acetate, Figure 3), although the ADH species may still be considered an intact protein tetrameric complex. Therefore, we believe that the 100 mM ammonium acetate solution offered better structural protection to protein complexes during the native MS analysis.

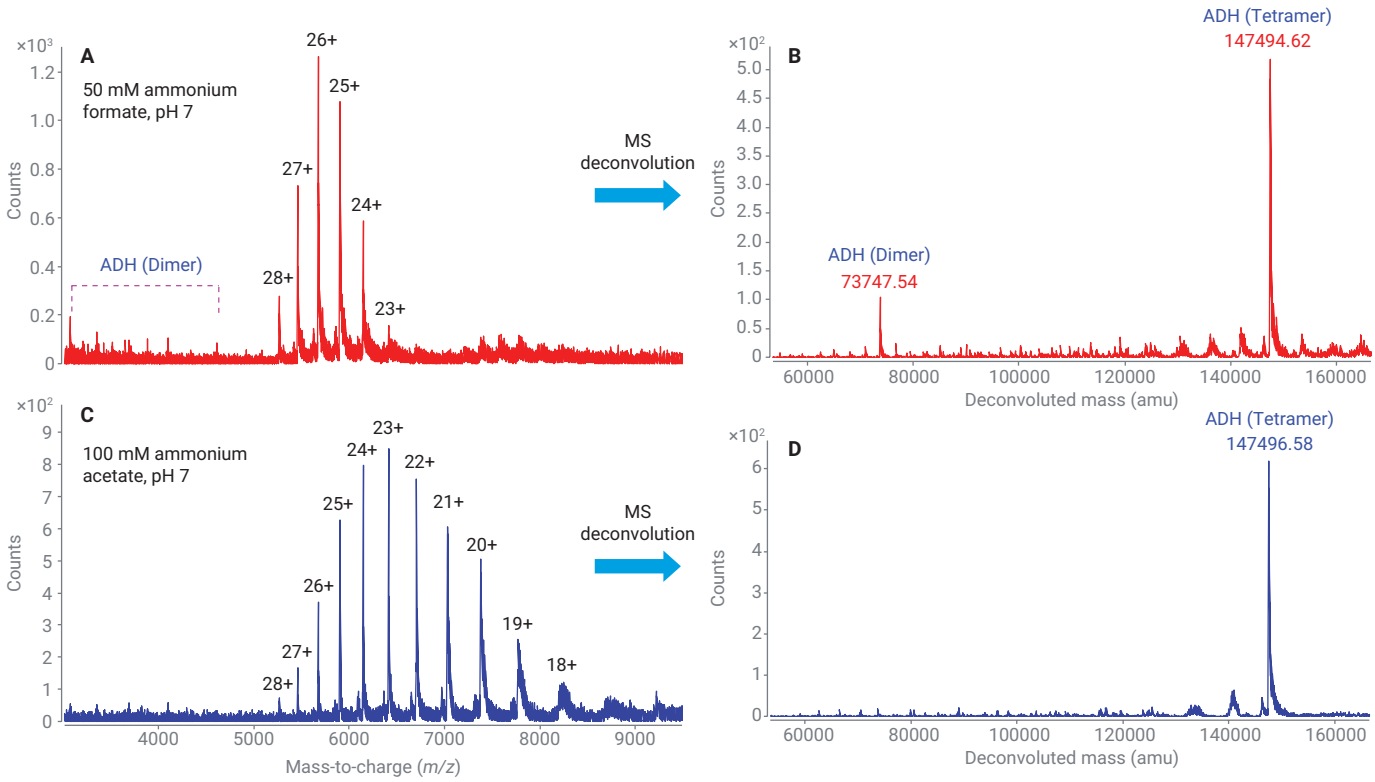

Figure 3. Native alcohol dehydrogenase (ADH, tetramer) analysis under various solvent conditions.
A) Native ADH in 50 mM ammonium formate, pH 7. C) Native ADH in 100 mM ammonium acetate, pH 7. B) and D) deconvoluted spectrum of both samples.

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Native MS aanalysis of intact mAbs

Monoclonal antibodies (mAbs) and their derivative products have quickly become an important class of biopharmaceutical molecules with a wide range of therapeutic applications. Native MS analysis of mAbs can provide valuable information, such as: protein folding, mAb aggregation (mAb dimer or trimer), antibody drug conjugates (ADCs), bispecific mAbs, etc.

In this study, we applied the online SEC method for rapid and robust native mAbs MS analysis. Approximately 0.5 to 1.0 µg of mAb was injected onto an AdvanceBio SEC guard column using a 5 min isocratic flow at 0.2 mL/min of 100 mM ammonium acetate solvent. The Q-TOF source conditions were optimized for excellent quality of native MS spectra over the mass range from m/z 5,000 to 10,000.

Figure 4 demonstrates the LC/MS analysis of intact NIST mAb standard under the denaturing MS conditions (Figure 4A and 4B) as well as the native MS conditions (Figure 4C and 4D). In both conditions, all major glycoforms of the NIST mAb were well resolved (Figure 4A and 4C, inset). The charge state distribution of denatured NIST mAb spanned the mass range of m/z 2,000 to 5,000 (30+ to 75+), while the native NIST mAb had a charge envelope in the range of m/z 5,000 to 10,000 (15+ to 30+). As shown in the MS deconvoluted spectra (Figure 4B and 4D), low ppm in mass errors were obtained for all major glycoforms. We also achieved very good agreement with the data for the intact NIST mAb analysis under both MS conditions.

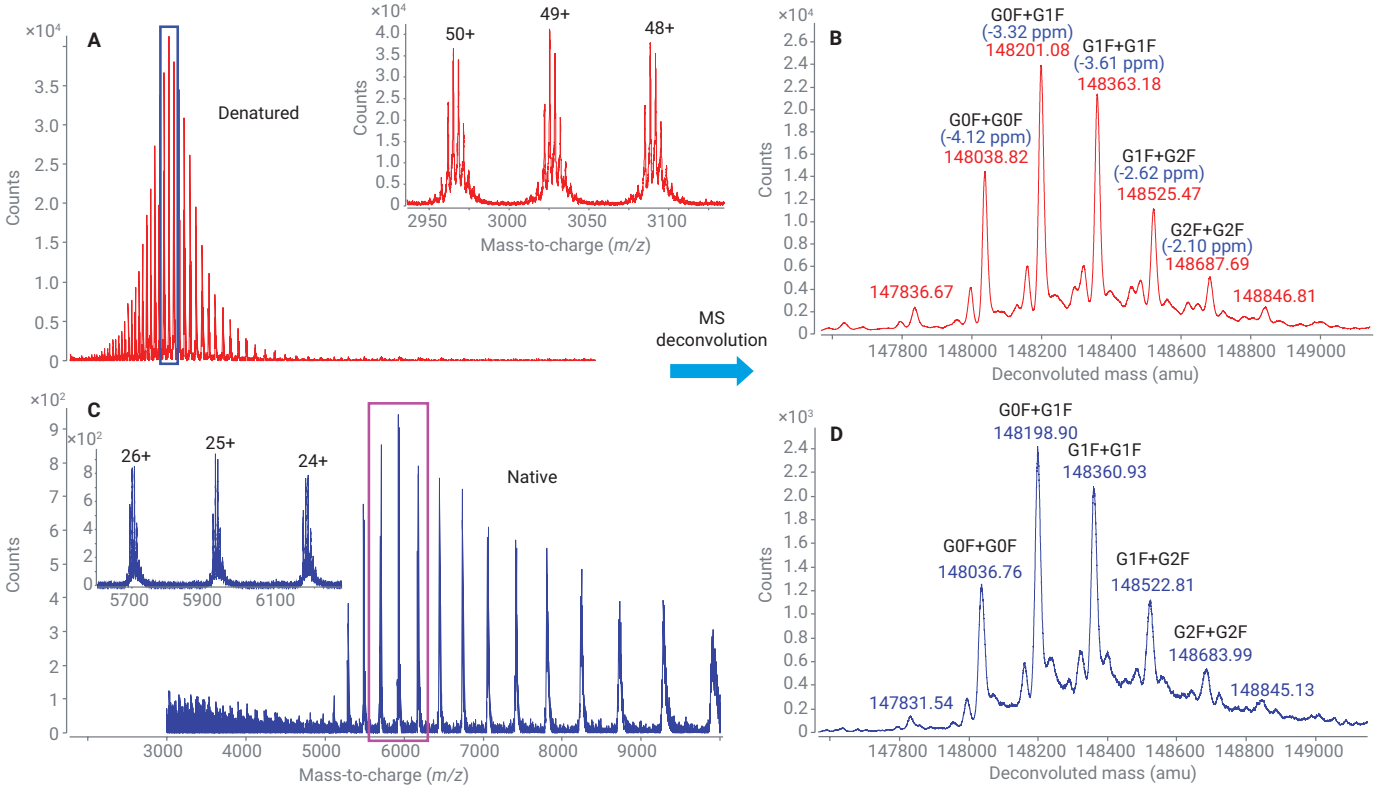

Figure 4. LC/MS analysis of NISTmAb under: A) denaturing MS conditions (acetonitrile and 0.1% formic acid) (previous work, Ref. 5) and C) native MS conditions (in 100 mM ammonium acetate, pH 7). The deconvoluted MS spectra of both samples are shown in B) and D), respectively.

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Similarly, native MS analysis of a biotherapeutic drug (trastuzumab, brand name: Herceptin) and its ADC (trastuzumab emtansine, T-DM1) was performed and compared. Figure 5A illustrates the native mass spectrum of intact Herceptin, showing a nicely distributed charge envelope from m/z 5,000 to 10,000 with charge states between 15+ and 28+. The most prominent charge state was at 24+ which indicated the intact Herceptin was in its native/folded conformation. High mass accuracies for the major glycoforms were achieved as shown in the inset deconvoluted spectrum.

Native MS analysis enables probing of protein molecules while preserving their native structural conformation. As this method minimizes the interferences from organic solvent and acid in the mobile phase, it is an ideal analytical tool for noncovalent protein complexes or acid labile protein conjugates, such as some ADCs. Figure 5 shows the native raw and deconvoluted (inset) MS spectrum of T-DM1. The average DAR value calculated using the BioConfirm DAR Calculator was 3.5 (Figure 5B, inset), which is consistent with the DAR values of the intact ADC reported by Genentech (the manufacturer).

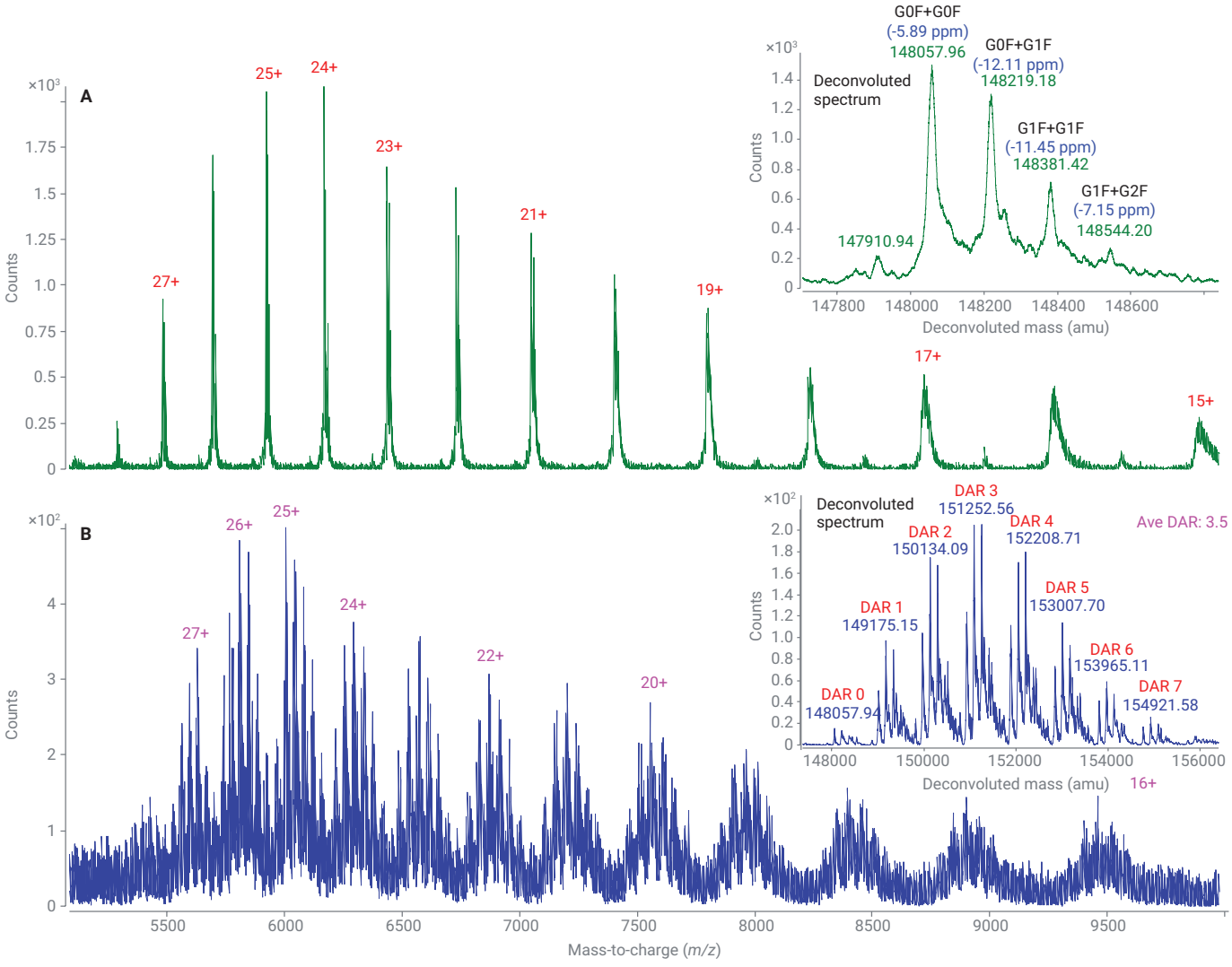

Figure 5. Native LC/MS analysis of mAb and its antibody drug conjugate (ADC): A) Herceptin and B) T-DM1. The deconvoluted MS spectra of both samples are shown in the inset figures.

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Native MS analysis of intact protein complexes

The optimized native MS analysis method was also evaluated by three protein complex samples. They were: tetrameric pyruvate kinase (PK, 232 kDa), hexameric glutamate dehydrogenase (GDH, 335 kDa), and tetrameric β-galactosidase (466 kDa). The 6545XT AdvanceBio LC/Q-TOF system offers large molecule SWARM autotune for optimizing macromolecular ions transmission in the extended mass range up to m/z 30,000. It is an ideal LC/MS system for native protein complex analysis. Figure 6A shows the native mass spectrum of the tetrameric pyruvate kinase. Two major charge envelopes ranging from m/z 6,000 to 10,000 with charge state of 24+ to 37+ were detected.

The deconvoluted spectrum revealed that there were two multi-proteoform complexes of PK tetramers in the sample: full-length pyruvate kinase and truncated PK tetramer (three intact subunits plus one PK proteoform with N-terminal cleavage).[3]

The 6545XT system also demonstrated excellent detection sensitivity for protein complexes at higher m/z (>m/z 10,000). Figure 6B and 6C show the native MS spectrum of GDH and β-galactosidase. Both of their protein charge envelopes were greater than m/z 8,000, whereas the most abundant ions were at m/z 9,566 (35+) for GDH and m/z 10,832 (43+) for β-galactosidase, respectively. The molecular mass of the intact hexameric GDH was determined to be 334,754 and 465,788 Da for the tetrameric β-galactosidase with 1 to 10 µg sample injections.

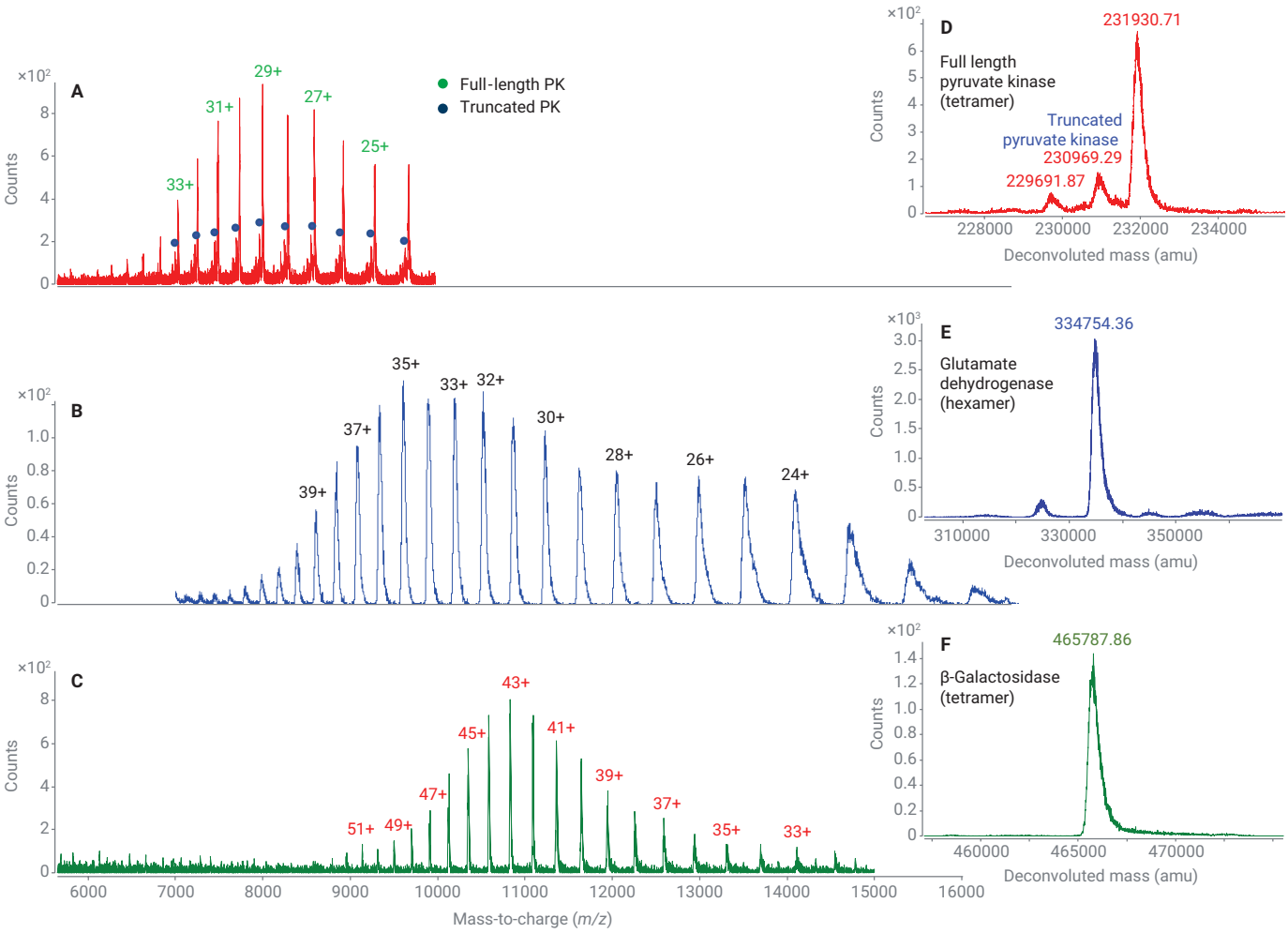

Figure 6. Native LC/MS analysis of various intact protein complexes. A) Pyruvate kinase (PK, tetramer, 232 kDa), B) glutamate dehydrogenase (GDH, hexamer, 335 kDa) and C) β-galactosidase (tetramer, 466 kDa). The deconvoluted spectra are shown in D) to F), respectively. The raw MS spectrum in Figure 6B was smoothed using the mMass open-source MS software tool

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Conclusion

We have developed a highly sensitive and robust LC/MS workflow methodology for native protein analysis. This optimized workflow utilizes the 1290 Infinity II LC with the AdvanceBio SEC column, the 6545XT AdvanceBio LC/Q-TOF with extended mass range up to m/z 30,000, and MassHunter BioConfirm software. The following benefits have been demonstrated by this native MS analysis method:

- Use of an online SEC column at typical analytical LC flow rates eliminates the challenging issues (protein aggregation and instable spraying flow) associated with nanoESI analysis.
- The optimized native MS conditions provide high confidence in ADC characterization, with accurate determined DAR values.
- The large molecule SWARM autotune feature, along with the extended mass range of the 6545XT AdvanceBio LC/Q-TOF, enables the sensitive detection and characterization of native intact macroprotein complexes.

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Application Note

Aggregate/Fragment Analysis

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Analysis of Camelid Single-Domain Antibodies Using Agilent AdvanceBio SEC 120 Å 1.9 µm and AdvanceBio HIC Columns

Authors

Te-Wei Chu and Greg Staples

Agilent Technologies, Inc.

Abstract

This application note describes the use of size exclusion chromatography (SEC) and hydrophobic interaction chromatography (HIC) for the analysis of camelid single-domain antibodies (nanobodies). Nanobodies are a growing class of single-domain antibody fragments used for therapeutic purposes. The Agilent AdvanceBio SEC 120 Å 1.9 µm column provides a unique advantage over other SEC columns for high-resolution separation of nanobody aggregates and fragments. The Agilent AdvanceBio HIC column enables analysis of nanobody post-translational modifications (PTMs) such as glutamine/pyroglutamate conversion. An SEC-based native LC/MS method is also demonstrated for greater understanding of PTM and impurity characterization.

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Introduction

Despite the success of biotherapeutics such as monoclonal antibodies (mAbs), many significant drawbacks still exist for this class of drugs. For example, IgG mAbs, the most widely used biologic drugs, have a complex structure and rather large size (150 kDa). The large size hampers their efficient in vivo delivery to diseased cells such as those found in tumors. Alternatively, single-domain antibodies, also known as nanobodies, provide tremendous opportunity in terms of reaching their intended targets.[1]

Nanobodies are small (~15 kDa), natural single-domain proteins derived from the camelid heavy chain antibody (Figure 1). They are recombinantly produced antigen-binding V_{HH} fragments with binding affinity equivalent to that of conventional IgG mAbs. Due to their small size, nanobodies can bind to antigen motifs that are frequently inaccessible to conventional mAbs, providing access to presently "undruggable" targets. In addition, the relatively simple protein conformation offers many advantages to drug developers such as ease of manufacturing and different administrative routes.[1] These promising features make nanobodies and V_{HH} fragment-derived biologics the rising stars in the biopharma research and development pipeline.[2]

This application note presents thorough characterization of two V_{HH} fragments (anti-PD1 and anti-PDL1 single-domain antibodies) using SEC and HIC, both with UV detection. Furthermore, we demonstrate SEC-based native LC/MS analysis of the two samples. The Agilent AdvanceBio SEC 120 Å 1.9 µm column is designed for aqueous SEC separation of small biomolecules such as proteins in the molecular weight range of 1 to 80 kDa. The column is perfectly suitable for nanobody analysis. The proprietary sub-2 µm hydrophilic polymer-coated silica packing technology enables higher resolving separation of aggregates and fragments compared to SEC columns from other vendors. HIC analysis allows reserved-phase-like separation of protein variants in a native condition. The Agilent AdvanceBio HIC column exhibits optimal hydrophobicity and selectivity for nanobody PTM characterization. Using a generic HIC method (without organic solvent modifier), a common PTM, glutamine/pyroglutamate conversion is revealed. The combination of these approaches offers a complete solution for in-depth analysis of nanobody purity and critical quality attribute assessment.

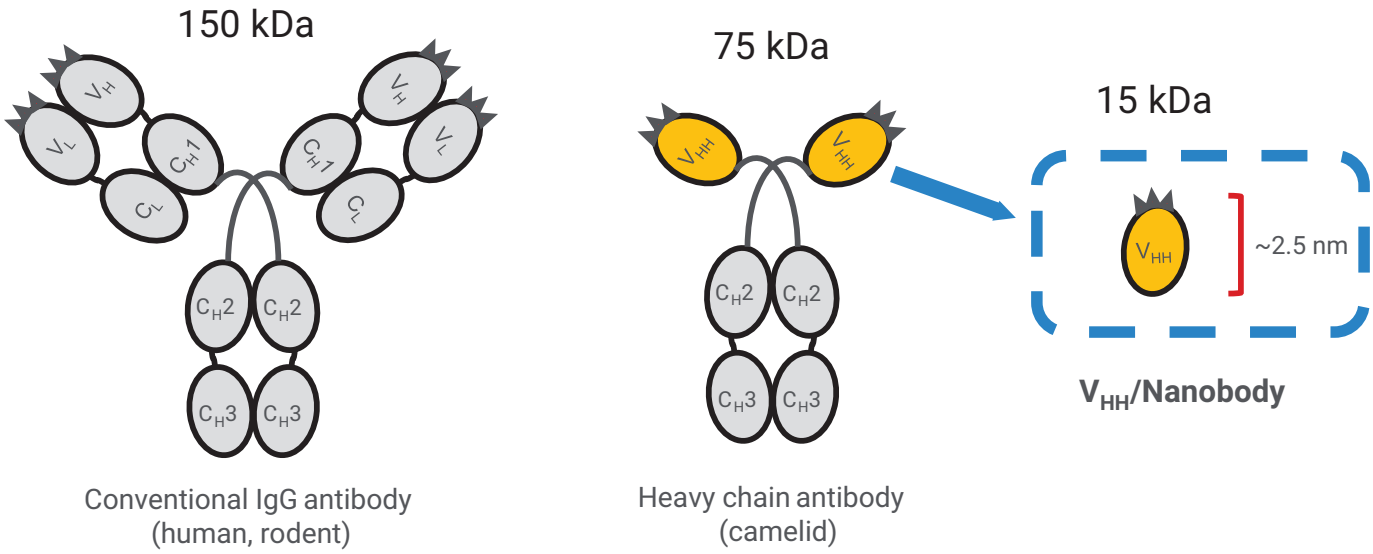

Figure 6. Schematic of a V_{HH} single-domain antibody (nanobody) compared to a conventional mAb.

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Experimental

Samples and chemicals

- Llama anti-PD1 single-domain antibody [F12A8]; purchased from ProSci Inc (Poway, CA)
- Llama anti-PDL1 single-domain antibody [F2G2]; purchased from ProSci Inc (Poway, CA)
- Glutaminyl-peptide cyclotransferase; purchased from R&D Systems (Minneapolis, MN)
- Human anti-IL8 IgG monoclonal antibody; produced in house from CHO cells

All chemicals and solvents used were HPLC grade or higher. Sodium phosphate monobasic and dibasic, sodium chloride, and ammonium sulfate were from Sigma-Aldrich. Water was purified using a Milli-Q Integral system equipped with LC-Pak Polisher and a 0.22-µm membrane point-of-use cartridge (Millipore).

Columns

- Agilent AdvanceBio SEC 1.9 µm 120 Å, 4.6 × 300 mm (p/n PL1580-5250)
- Agilent AdvanceBio SEC 1.9 µm 120 Å, 4.6 × 150 mm (p/n PL1580-3250)
- Agilent AdvanceBio HIC, 4.6 × 100 mm (p/n 685975-908)

Instrumentation

For HPLC experiments, an Agilent 1260 Infinity II Bio-inert LC system was used 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 Variable Wavelength Detector (G7114A)

For LC/MS experiments, an Agilent 6224 accurate-mass time-of-flight (TOF) LC/MS and 1290 Infinity II LC were used comprising::

- Agilent 1290 Infinity II High Speed Pump (G7120A)
- Agilent 1290 Infinity II Multisampler (G7167B)
- Agilent 1290 Infinity II Thermostatted Column Compartment (G7116B)
- Dual ESI Agilent 6224 accurate-mass time-of-flight (TOF) LC/MS

Results and discussion

SEC is a gold standard technique for characterizing aggregation of biologics. The chromatographic separation mechanism is unique in that analytes are not retained by the stationary phase; instead, they are separated based on accessibility to available particle pore volume. Therefore, careful selection of column pore size based on the protein analyte's size in solution (hydrodynamic radius) is important. Nanobodies are small proteins with molecular weight of approximately 15 kDa, translating to a hydrodynamic radius of approximately 25 Å. SEC columns packed with narrow pore (120 to 130 Å) particles offer linear separation in the range suitable for such an application.[3,4] Figure 2 shows an SEC separation of anti-PD1 single-domain antibody (sdAb) using a standard method with sodium phosphate pH 7 as the mobile phase (to maintain the native state of the protein). Excellent separation of sdAb monomer from impurities, i.e., high-molecular weight (HMW) and low molecular weight (LMW) species, can be seen with the AdvanceBio SEC 1.9 µm 120 Å column.

The resolution of HMW and LMW species as well as the peak width and tailing factor of the monomer peak are significantly better compared to columns packed with larger (2.7 µm) particles. Compared to other vendors' offering of sub-2 µm SEC technology, the AdvanceBio SEC 1.9 µm 120 Å column exhibits best-in-class performance. This is due to careful design of particle surface bonding coverage to eliminate undesirable secondary interactions.[3,4]

Method conditions

Parameter	HPLC Conditions (SEC Analysis)
Column	Agilent 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	0.35 mL/min
Column temperature	25 °C
Injection volume	5 µL, 1 mg/mL
Total run time	15 min
Detection	UV at 214 nm

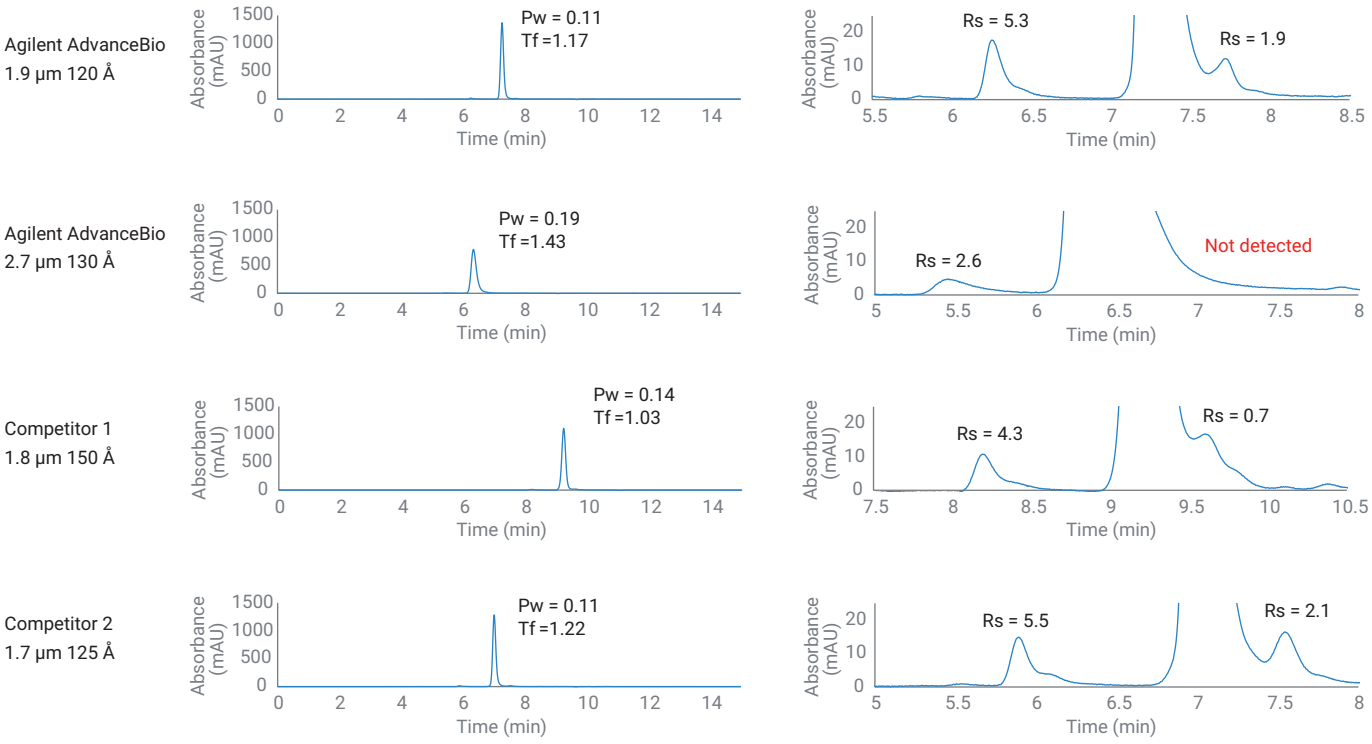

Figure 2. SEC of anti-PD1 single-domain antibody. Right panels show the magnified baseline of the same run on the left. Resolution of the HMW aggregate peak (eluted earlier) or the LMW fragment peak (eluted later) compared to the monomer peak are shown.

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SEC is a relatively straightforward chromatographic method where the column is run in isocratic mode. Method development and optimization involve the selection of mobile phase parameters (pH, salt concentration, etc.) to minimize potential secondary interactions such as ionic or hydrophobic interaction. Figure 3 shows SEC salt plot studies for method optimization of anti-PD1 and anti-PDL1 sdAb analysis. A shorter column of 15 cm length was selected for this experiment to achieve higher throughput and speed up the method optimization process. Results showed that anti-PD1 sdAb had minimal or no secondary interaction with the column at the range of mobile phase NaCl concentration used. This is evident from the similar chromatographic peak shapes and retention times at each mobile phase condition. In contrast, anti-PDL1 sdAb had slightly deteriorated peak shape with delayed retention time at higher salt concentration mobile phase, indicative of potential hydrophobic interaction between analytes and the column. Interestingly, at 250 mM NaCl and above, a secondary (shoulder) peak became evident.

Method conditions

Parameter	HPLC Conditions (SEC Salt Plot Study)
Column	Agilent AdvanceBio SEC 1.9 µm 120 Å, 4.6 × 150 mm (p/n PL1580-3250)
Mobile phase	20 mM sodium phosphate, pH 7.0 with concentration of sodium chloride indicated in Figure 3
Flow rate	0.35 mL/min
Column temperature	25 °C
Injection volume	2 µL, 1 mg/mL
Total run time	7 min
Detection	UV at 214 nm

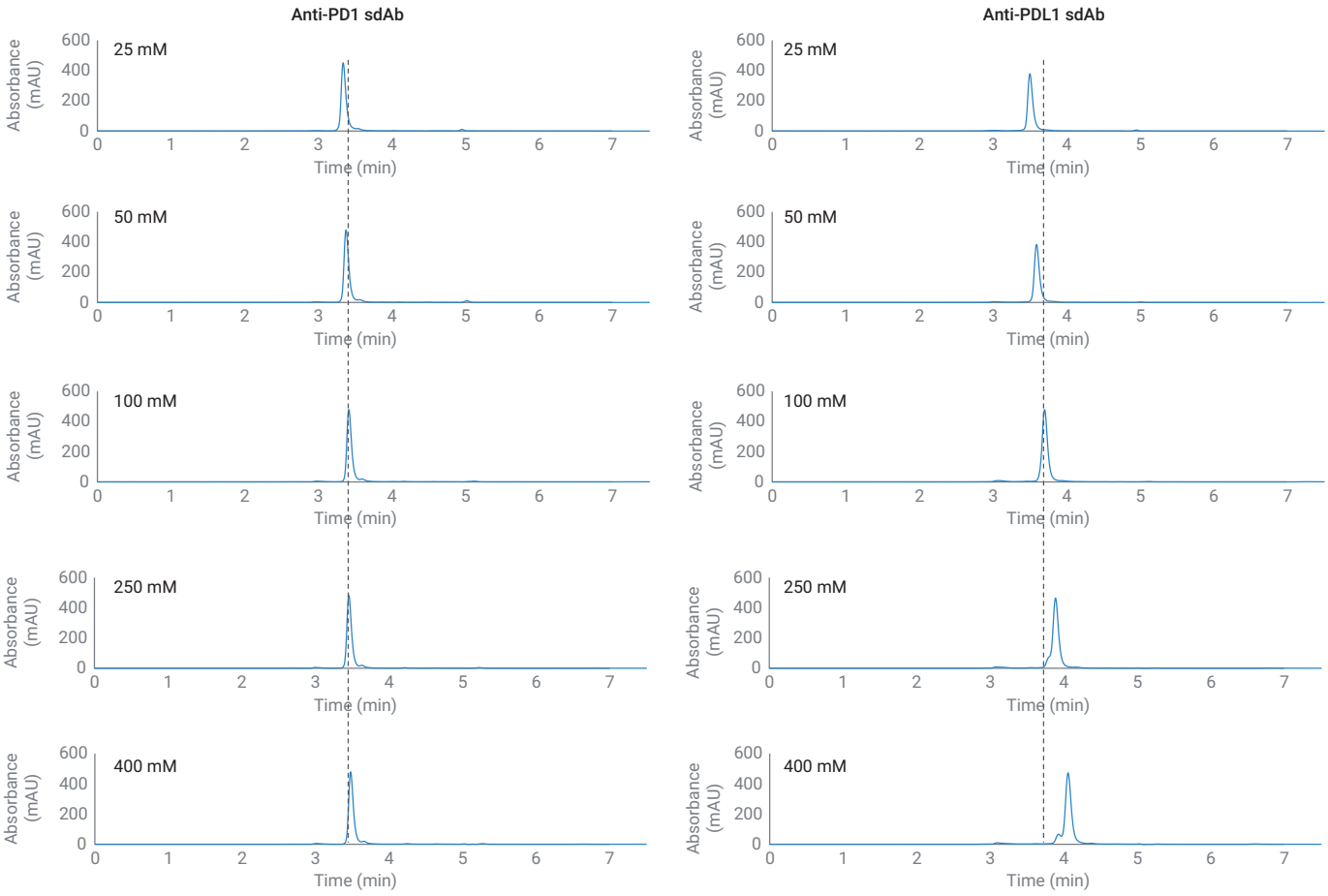

Figure 3. Salt plot studies of anti-PD1 (left) and anti-PDL1 (right) single-domain antibodies using the Agilent AdvanceBio SEC 1.9 µm 120 Å column.

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This phenomenon is commonly encountered with challenging proteins that contain highly hydrophobic motifs or which have extreme isoelectric points. As nanobodies contain only the variable domain of an antibody, the amino acid sequence varies significantly when comparing one nanobody to another, potentially causing large differences in protein physicochemical characteristics. The method demonstrated here is useful for fast screening of SEC mobile phase conditions to determine the optimum conditions for analyzing different samples. The data inform the use of lower salt concentration (50 mM or below) in the mobile phase for anti-PDL1 sdAb.

To further characterize the two nanobodies, native SEC-LC/MS experiments were conducted (Figure 4). Results showed that the AdvanceBio SEC 1.9 µm 120 Å column was suitable for SEC-MS, where low concentrations of volatile aqueous buffer (i.e., 50 mM ammonium acetate) are used. Excellent ion chromatograms can be seen for both sdAb samples, together with high-resolution mass spectra. The deconvoluted MS results showed accurate molecular weight measurement for both samples: anti-PD1 sdAb (16,528 Da) and anti-PDL1 sdAb (16,895 Da).

Method conditions

Parameter	Native SEC-LC/MS Conditions
Column	Agilent AdvanceBio SEC 1.9 µm 120 Å, 4.6 × 300 mm (p/n PL1580-5250)
Mobile phase	50 mM ammonium acetate, pH 7.0
Flow rate	0.35 mL/min
Column temperature	25 °C
Injection volume	20 µL, 1 mg/mL
Total run time	15 min
MS Detection	Min range: 300 m/z Max range: 7,000 m/z Ion polarity: Positive
MS Source Parameters	5Gas temperature: 325 °C Gas flow: 5 L/min Nebulizer: 30 psi Vcap: 5,500 V Fragmentor: 250 V Skimmer: 65 V Octopole RF peak: 750 V

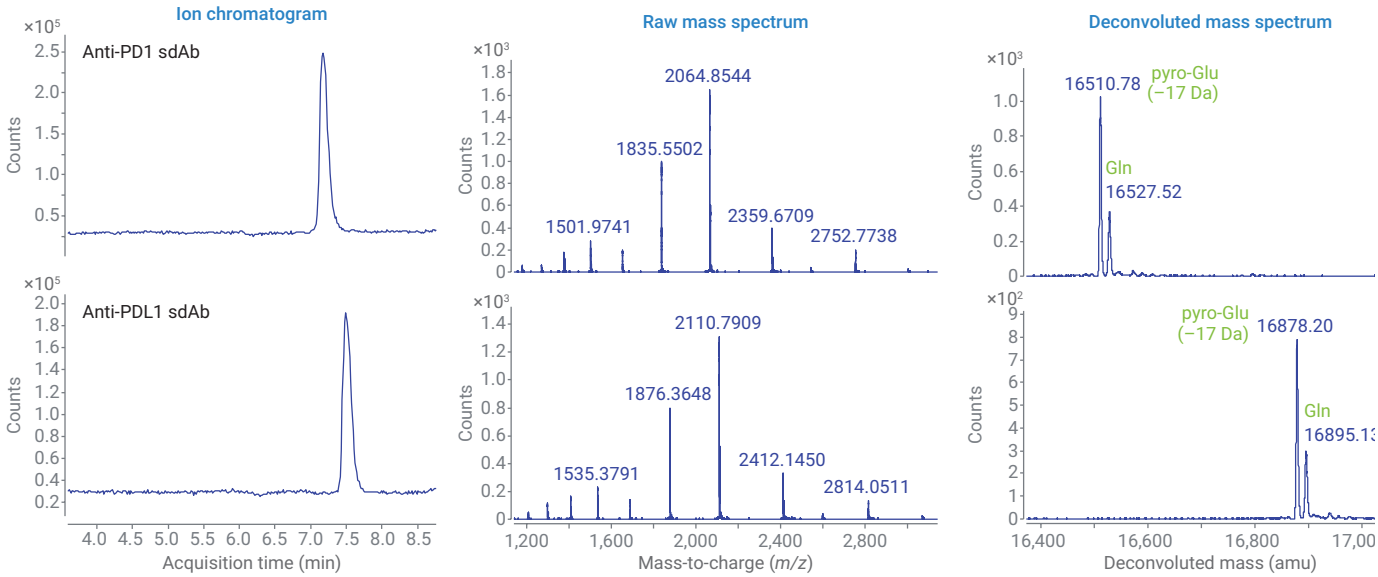

Figure 4. Native SEC-LC/MS analysis of anti-PD1 (top) and anti-PDL1 (bottom) single-domain antibodies.

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Interestingly, MS results revealed a large amount of a –17 Da modification on both samples. This is a typical mass shift associated with cyclization of N-terminal glutamine (Gln) to form pyro-glutamic acid (pyro-Glu).[5] The Gln/pyro-Glu conversion commonly occurs at the heavy chain variable domain of the antibody; thus, it is not surprising to observe this PTM in nanobodies (V_{HH} fragment). Both sdAb samples analyzed here were produced in bacteria (E. coli). It has been well documented that production in prokaryotic systems may result in proteins being recovered as inclusion bodies, thus leading to unusual PTMs.[5] Because the N-terminal Gln residues of V_{HH} are near the complementarity-determining region (CDR), pyro-Glu formation can potentially have significant impact on target binding.[1] Therefore, careful characterization and documentation of this PTM is typically required.[5]

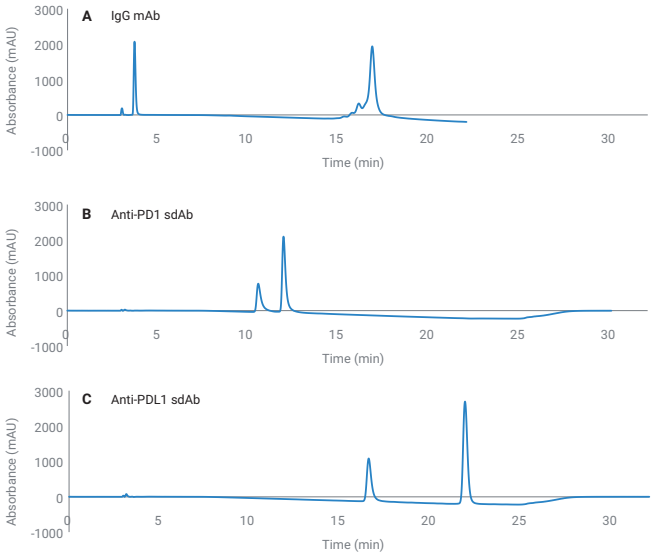

Figure 5. Hydrophobic interaction chromatograms of (A) an IgG mAb, (B) anti-PD1 single-domain antibody, and (C) anti-PDL1 single-domain antibody.

Method conditions

Parameter	HPLC Conditions (HIC analysis)
Column	Agilent AdvanceBio HIC, 4.6 × 100 mm (p/n 685975-908)
Mobile phase	A) 2 M ammonium sulfate in 50 mM sodium phosphate, pH 7.0 B) 50 mM sodium phosphate, pH 7
Gradient	0 min: 50% B 2 min: 50% B 17 min: 100% B 20 min: 100% B 22 min: 50% B 32 min: 50% B
Flow rate	0.4 mL/min
Column temperature	30 °C
Injection volume	5 µL, 0.8 mg/mL (mAb) 5 µL, 1.0 mg/mL (sdAb)
Detection	UV at 214 nm

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To characterize protein Gln/pyro-Glu conversion, chromatographic methods such as ion-exchange chromatography and HIC can be used. Here, HIC was chosen because, in addition to PTM analysis, it also provided an assessment of hydrophobicity for the two sdAb samples. HIC uses a salting-out mechanism to separate intact, native proteins based on hydrophobicity under near physiological conditions. Figure 5 shows the HIC separation of anti-PD1 and anti-PDL1 sdAbs and an IgG mAb using a generic ammonium sulfate gradient method. For both Nbs, two chromatographic peaks were well resolved, indicating that both samples contained two species of different hydrophobicity. This result was consistent with the LC/MS data showing the Gln/pyro-Glu conversion. In addition, HIC data suggested that the anti-PDL1 sdAb was very hydrophobic. The anti-PDL1 sdAb retention time was much longer than anti-PD1 sdAb and the IgG mAb (with a molecular weight that is 10 times larger). These results help explain the observations from the SEC salt plot study (Figure 3), suggesting major physicochemical property differences between the two single-domain antibodies.

To further confirm whether the two peaks separated using HIC were indeed sdAb species that contained Gln or pyro-Glu, a biochemical approach was developed using glutaminyl-peptide cyclotransferase (QPCT) (Figure 6). The enzyme is known to catalyze the conversion of N-terminal glutaminyl residues of proteins to pyroglutamyl groups.[6] Indeed, treatment of both sdAb samples with QPCT resulted in chromatograms containing only the pyro-Glu peak. The experiments presented here using the AdvanceBio HIC column demonstrated excellent selectivity for nanobody PTM analysis.

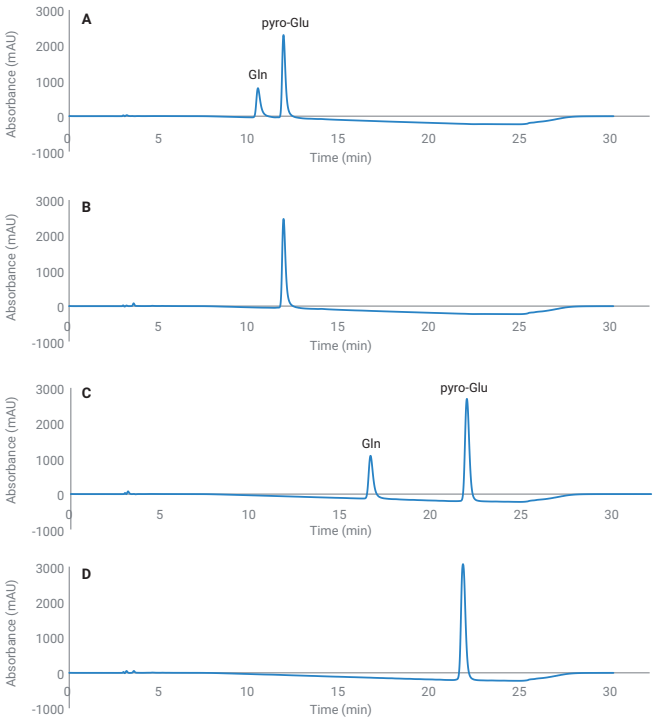

Figure 6. HIC analysis of (A) anti-PD1 sdAb, (B) anti-PD1 sdAb + QPCT, (C) anti-PDL1 sdAb, and (D) anti-PDL1 sdAb + QPCT. For enzyme treatment, 20 µL of sdAb (1 mg/mL) was incubated with 4 µL QPCT at 37 °C for 16 to 18 hours.

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Conclusion

This Nanobodies are revolutionary, new biotherapeutic modalities that offer many advantages over conventional mAb therapy. For research and development of this novel class of biologic, it is of utmost importance to characterize and document quality attributes that can be formed or changed during the process of production and storage. These attributes have been shown to impact drug potency, pharmacokinetics, immunogenicity, and safety. Reliable and robust analytical tools and methods are needed. This application note presents SEC, HIC, and SEC-LC/MS techniques that can successfully be applied to nanobody characterization. Important critical quality attributes (CQAs) such as HMW aggregates and LMW fragments GluGlncan be characterized using SEC in a high-resolution and high-throughput manner. Subtle changes on the molecules such as post-translational Gln/pyro-Glu conversion can be detected using HIC and SEC-MS approaches. The methods described here offer guidance for careful analysis of nanobodies in native, non-denaturing modes.

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Application Note

Aggregate/Fragment Analysis

Agilent

Trusted Answers

High Resolution Size Exclusion Chromatography Analysis of Small Therapeutic Proteins

Authors

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Agilent Technologies, Inc.

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

Parameter	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
Total run time	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

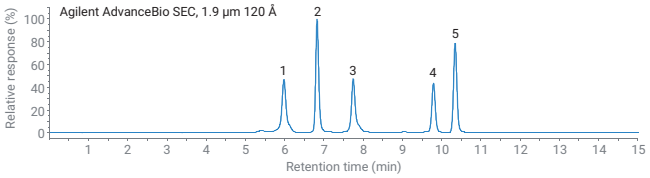

Figure 1. Size-exclusion chromatogram of low molecular weight protein and peptide mix at 0.35 mL/min.

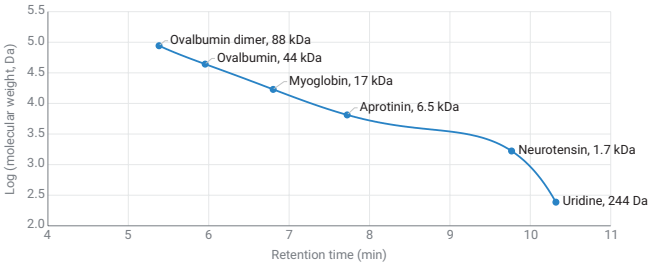

Figure 2. AdvanceBio SEC 1.9 µm 120 Å calibration curve of low molecular weight protein and peptide standards.

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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 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)

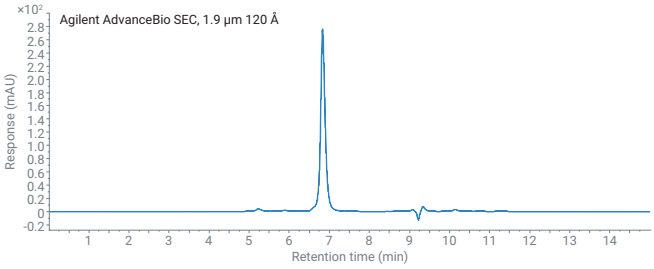

Figure 3. Size-exclusion chromatogram of hG-CSF on an Agilent AdvanceBio SEC 1.9 µm 120 Å 4.6 × 300 mm column at 0.35 mL/min.

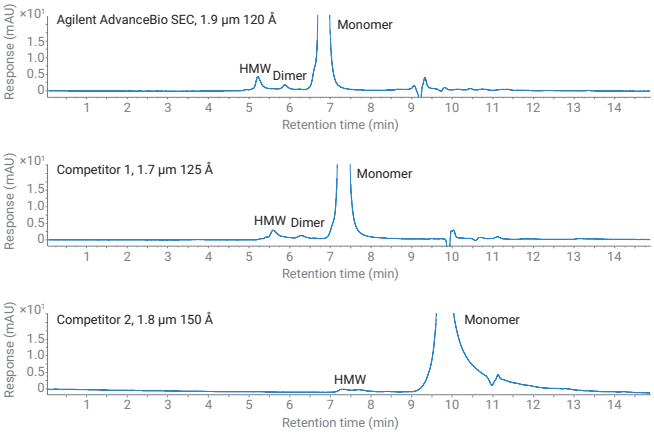

Figure 4. Close up of size-exclusion chromatograms of hG-CSF at 0.35 mL/min.

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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

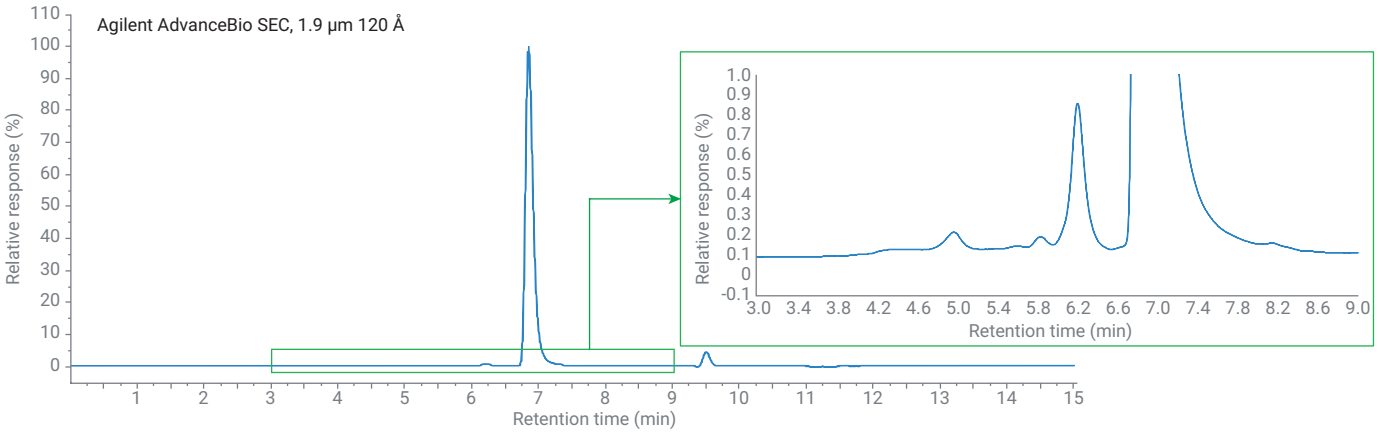

Figure 5. Size-exclusion chromatogram of somatropin (rhGH).

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

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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.

Method conditions

Parameter	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

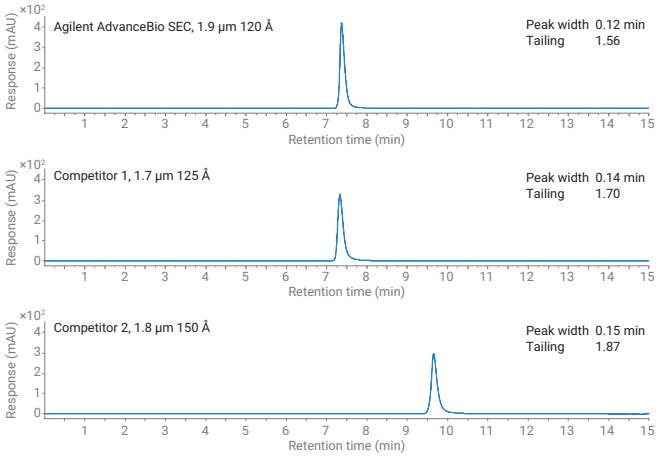

Figure 6. Size-exclusion chromatograms of interferon α-2b.

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.

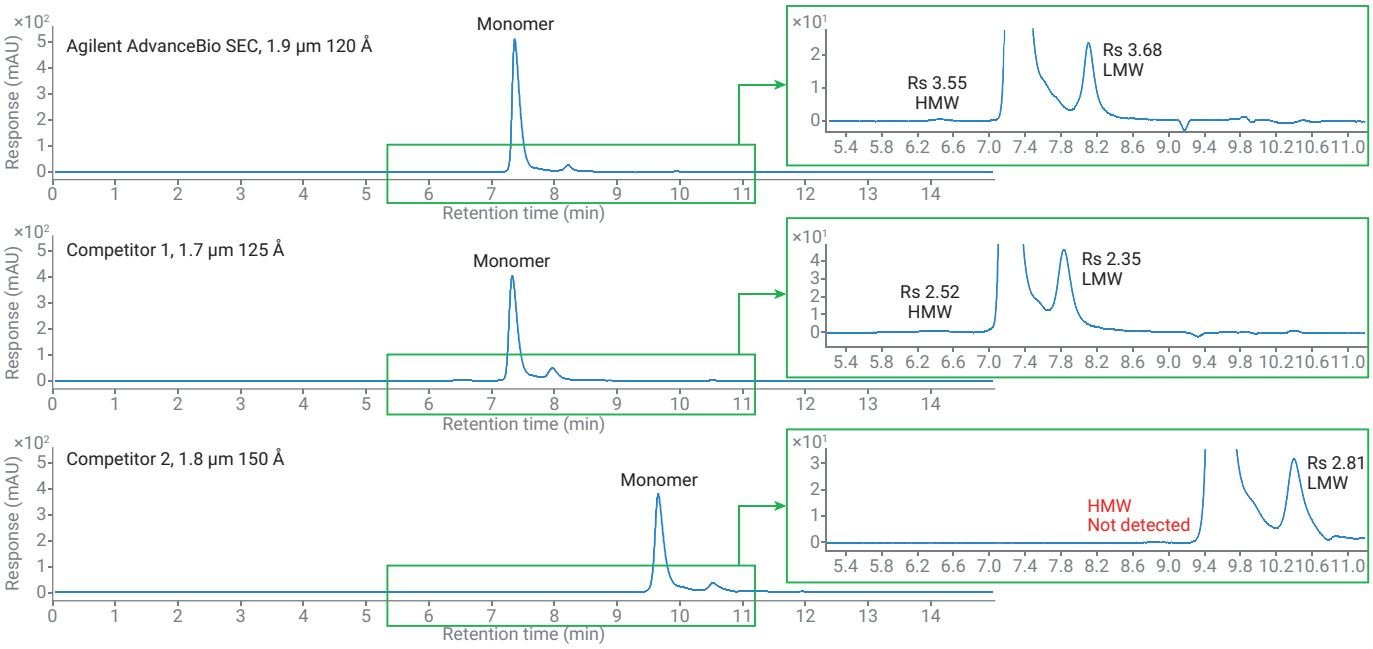

Figure 7. Size-exclusion chromatograms of interferon α-2b.

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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.

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Application Note

Aggregate/Fragment Analysis

Agilent

Trusted Answers

Analysis of Covalent High Molecular Weight Insulin

Improvements in speed and resolution with high performance size exclusion chromatography

Author

Sandeep Kondaveeti

Agilent Technologies, Inc.

Abstract

The analysis of insulin was done with an HPLC size exclusion chromatography (SEC method using an Agilent AdvanceBio SEC 120 Å pore size, sub-2 µm hydrophilic polymer coated silica packing material. The results of the analysis were compared to traditional methods and competitor columns for performance and efficiency. Resolution of insulin and high molecular weight (HMW) proteins were significantly improved over results from traditional methods with the AdvanceBio column. Chromatographic run times were shorter, and high-throughput insulin sample analysis became a reality.

Introduction

Insulin is a small polypeptide hormone that controls blood glucose homeostasis and is widely used in diabetes treatment. Genetic engineering techniques have enabled biopharma companies to develop diverse, long-acting insulin analogs. It has long been known that, when subjected to acidic conditions and high temperature, insulin monomers form amyloid-like fibrils.[1] For the insulin analogue manufacturer, this is especially problematic, since insulin analogs can have a higher propensity for aggregation than native insulin.[2] One of the critical quality control attributes for injectable insulin is the control of insulin fibrillation, commonly known as high molecular weight (HMW) proteins. The current US (USP) and European (EP) pharmacopoeia monograph methods for HMW aggregates determination are based on HPLC size exclusion chromatography (SEC).[3,4] According to the EP method, the use of a "hydrophilic silica gel for chromatography R (5 to 10 µm) with a pore size of 12 to 12.5 nm, of a grade suitable for the separation of insulin monomer from covalent dimer and polymers" with a length of 30 cm and a minimum internal diameter of 7.5 mm are prescribed. However, the method requires a lengthy 35 minute run time that is cost prohibitive for any laboratory performing high throughput sample analysis. The application presented here describes a SEC method developed using an Agilent AdvanceBio SEC 120 Å pore size, sub-2 µm hydrophilic polymer coated silica packing material. Some of the advantages of this method include faster run times and higher resolving separations of insulin and covalent insulin HMW compared to traditional pharmacopoeia methods.

Method conditions

Parameter	HPLC Conditions
Column	Agilent AdvanceBio SEC 1.9 µm 120 Å, 4.6 × 300 mm (p/n PL1580-5250)
Mobile phase	Arginine (1.0 g/L)/acetic acid/acetonitrile (65/15/20 v/v/v)
Flow rate	0.30 mL/min
Column temperature	25 °C
Injection volume	2 µL
Samples	Human insulin control, Heat-stressed insulin (60 °C for six hours)
Total run time	15 min

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)..

Sample preparation

To perform native MS analysis, it is crucial to preserve the protein samples at neutral pH and volatile aqueous solutions, such as ammonium acetate or ammonium formate. Therefore, sample desalting and buffer exchange are usually needed prior to the MS analysis. Briefly, protein stock solutions (1 to 10 mg/mL) were desalted and solvent exchanged into 100 mM ammonium acetate using Bio-Rad Bio-Spin P-6 (6,000 MW limit) or P-30 (40,000 MW limit) cartridges. The cartridge was first fully equilibrated with 100 mM ammonium acetate buffer. Protein sample was then pipetted to the top of the column and centrifuged for 5 min at 1000 × g. The protein was then buffer exchanged into the 100 mM ammonium acetate and was ready for MS analysis. This desalting protocol caused minimal sample loss and much less structural alteration of the protein molecule.

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 Variable Wavelength Detector (G7114A)

Software

Agilent OpenLab 2.2 CDS.

Sample preparation

The control human insulin (Sigma, I2643) and heat-stressed insulin samples were prepared as per the Ph. Eur.

Sample was reconstituted and diluted to 4.0 mg/mL in 0.01 N hydrochloric acid solution, then further diluted to 2 mg/mL as the final concentration.

Results and discussion

The focus of this application is a performance evaluation of the AdvanceBio SEC 120 Å, 1.9 µm, 4.6 × 300 mm column under the conditions provided by the USP and EP monographs for the analysis of HMW species in insulin samples. The acidic mobile phase prescribed by both of these pharmacopeial methods is composed of 0.65 g/L L-arginine, 15% acetic acid, and 20% (v/v) acetonitrile. This mobile phase provides an assessment of the levels of covalent HMW present in these preparations while disrupting noncovalent insulin self-association and column interactions. The featured AdvanceBio SEC 1.9 µm columns are designed with hydrophilic polymer coating to minimize undesired secondary effects between analyte and surface particle chemistry. The columns provide superior resolution and accurate HMW protein quantification.

Figure 1 shows the chromatogram for system suitability with Ph. Eur. insulin control standard. Monomer and HWM species for the 1.9 µm column have a resolution of 4.03, which far exceeds the resolution of the monograph system suitability requirement of ≥2.0. Peak areas (Table1) for HMW proteins in the insulin control sample are within the <1% suitability requirement. Note the total analysis time required for this method is approximately 15 minutes using AdvanceBio SEC 120 Å, 1.9 µm, 4.6 × 300 mm column compared to reported 35 minutes in Ph. Eur. monograph with traditional hydrophilic silica column using higher particle size.

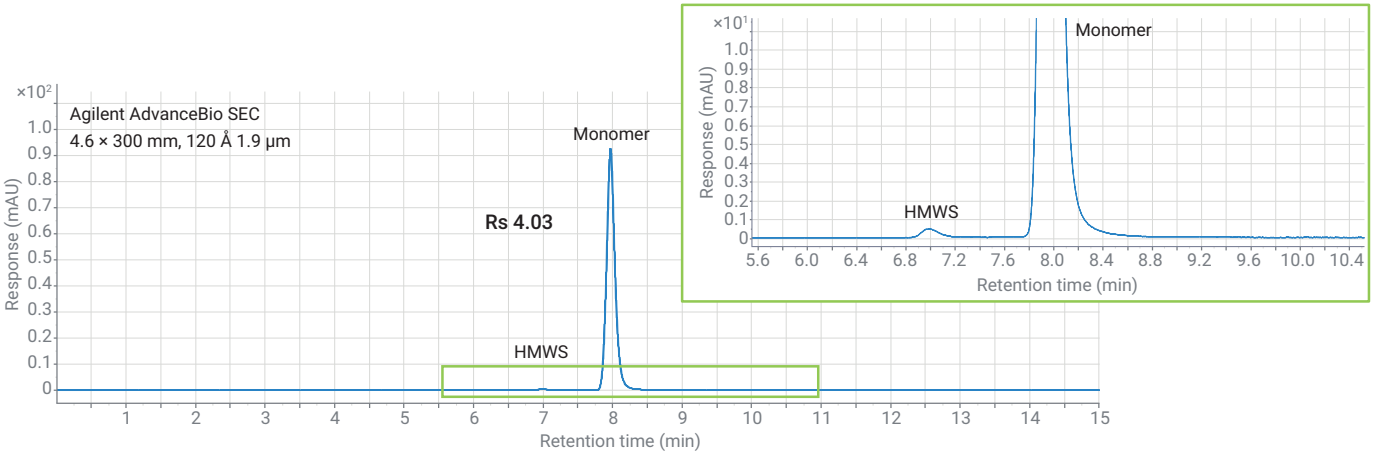

Figure 1. Size-exclusion chromatograms of human insulin control with monomer and HMW species.

Table 1. Results summary for SEC analysis of human insulin control.

Peak ID	RT (min)	% Area	Resol. USP	Tailing	Width 50%
Insulin HWM	6.99	0.67		1.48	0.15
Insulin Monomer	7.96	99.33	4.03	1.04	0.15

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The insulin SEC HMW determination method in the EP monograph prescribes an SEC particle size of 5 to 10 µm while the USP monograph does not specify a particle size limit. As part of this study, a comparison was performed among AdvanceBio SEC 120 Å 1.9 µm column and other SEC column vendors with sub-2-µm particles and equivalent 300 mm length with 4.6 mm id (Figure 2). The 1.9 µm Agilent column demonstrates a significant increase (>50%) in resolution as compared to the competitor columns. The improved resolution is also apparent in the insulin monomer peak tail, in which lower molecular weight fragment peaks are better resolved with Agilent SEC compared to the vendor 2 SEC column. Significant peak tailing observed in the vendor 2 SEC column might be due to undesired secondary interactions. It is important to note that different elution times of insulin peaks are due to pore size differences between these columns.

The percent peak areas of HMW species in the heat stressed insulin sample exceeds 1% for all the columns, indicating that sample would not pass the suitability test limits. However, higher % aggregates were resolved using Agilent SEC 120 Å, 1.9 µm column compared to other vendor columns.

Data are summarized in Table 2. Improvements are seen in efficiency for monomer and covalent dimer peaks in the AdvanceBio SEC 120 Å, 1.9 µm, 4.6 × 300 mm column method, again lending to the increased resolution and reduced run times with the updated method. According to USP (USP37-NF32S1) and EP guidelines, a 50% reduction in particle size and a 25% change in the column inner diameter for isocratic methods are the maximum allowable adjustments. Based on these requirements, featured SEC methods for insulin analysis with sub-2 µm particle size and 4.6 mm id would require further method validation and optimization to incorporate modern particle technology into established methods.

Table 2. Results summary of competitor SEC analysis for stressed insulin sample.

	AdvanceBio SEC 1.9 µm 120 Å			Vendor 1 SEC 125 Å 1.7 µm			Vendor 2 SEC 150 Å 1.8 µm		
Peak ID	%Area	Peak Tailing	Peak Width 50%	%Area	Peak Tailing	Peak Width 50%	%Area	Peak Tailing	Peak Width 50%
HMWS	1.93			1.57			1.10	2.01	
Insulin Monomer	97.66	1.00	0.13	97.85	1.10	0.13	98.80	1.37	0.14
LMWS	0.41			0.58			0.20	97.99	

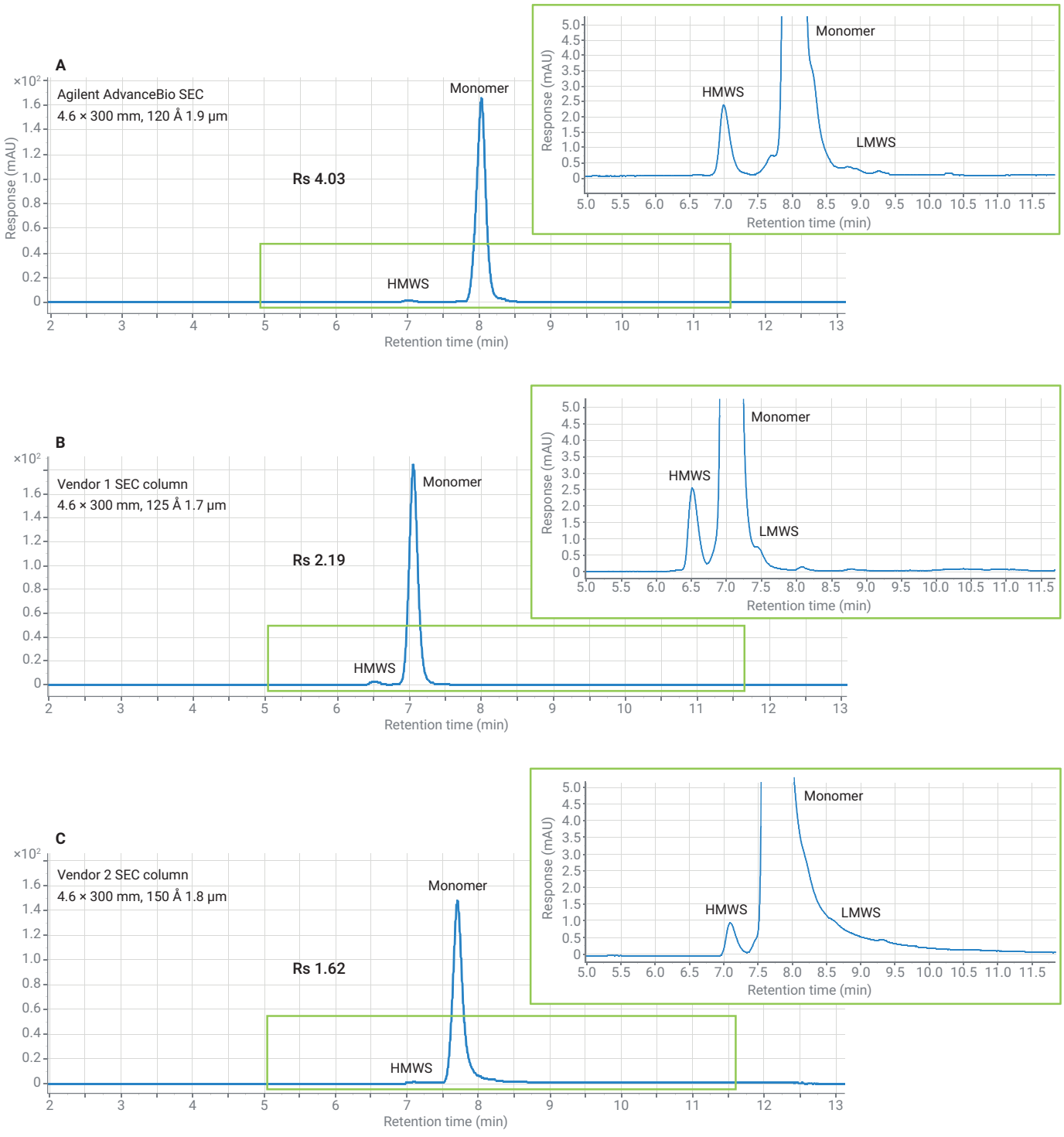

Figure 2. Competitor SEC analysis of stressed insulin with aggregates and low molecular weight fragments.

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Conclusion

Size exclusion chromatography is the USP and EP standard method for the analysis of covalent HMW insulin in therapeutic preparations. The chromatographic profiles demonstrating the performance of this method using SEC columns of different competitors have been presented. Based on these results the use of Agilent BioAdvance SEC 120 Å pore size, AdvanceBio SEC 1.9 µm column, and Agilent Infinity II Bio-inert liquid chromatography instrumentation for this SEC-based analysis provides significant improvements in resolution compared to traditional SEC-HPLC methods while reducing analysis time and mobile phase use.

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2. Librizzi; Fabio; Rischel. The Kinetic Behavior of Insulin Fibrillation is Determined by Heterogeneous Nucleation Pathways. *Protein Science* **2005**, *12*, 3129–3134.
3. Insulin Monograph, *USP Pharmacopeial Forum*, *31(5)*, 1375.
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5994-1566EN

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Application Note

Aggregate/Fragment Analysis

Agilent

Trusted Answers

Calibrating your Agilent AdvanceBio SEC Columns

Technical Overview

Authors

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Agilent Technologies, Inc

Introduction

Agilent AdvanceBio SEC columns are specifically 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 proteins, polysaccharides, or aqueous soluble polymers.

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. For polysaccharide analysis, pullulan polysaccharide molecular weight standards should be used, and for synthetic polyethylene glycol or polyethylene oxide, PEG/PEO standards are suitable.

SEC is a relatively straightforward technique. It 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. This provides two key aspects for the successful use of SEC:

- There should be no secondary interactions between the analyte and the stationary phase
- The pore size should be chosen to match the size of molecules being analyzed

Proteins are complex molecules containing numerous side chain functionalities: acid, 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. 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 [1]. This is a particular issue for molecules such as monoclonal antibodies, where the presence of aggregated proteins can lead to adverse effects if administered. SEC provides the ideal tool for quantifying and monitoring protein aggregation. In comparison, polysaccharides and synthetic polymers such as polyethylene oxide do not possess a wide range of functional groups, but are often made up of a series of closely related oligomers. In this case, SEC can provide the analysis of molecular weight, molecular weight distribution, and branching information [2].

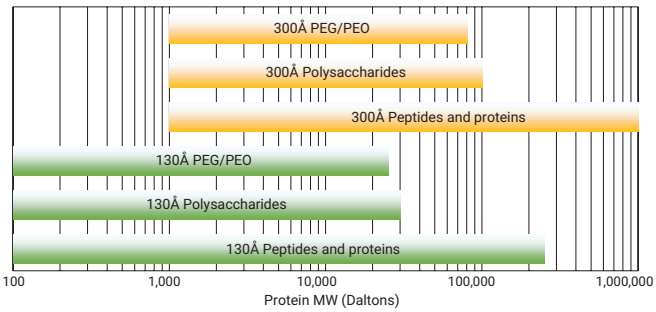

Figure 1. Resolving ranges for Agilent AdvanceBio SEC columns.

Calibrating your Agilent AdvanceBio SEC column

Choose the eluent for analysis. The samples need to be soluble in the mobile phase, and there should be no secondary interactions that lead to molecules eluting earlier or later than expected. For proteins, we recommend 150 mM sodium phosphate, pH 7.0 as this eliminates sodium chloride, which may lead to corrosion, from your HPLC system. However, other mobile phases including phosphate buffered saline (PBS), or high ionic strength mobile phases such as 0.1 M sodium phosphate/0.1 M sodium sulphate may still be used (see Pages 10–11).

Dissolve the standards in the mobile phase. Ensure that the solution is clear and tha there is no insoluble material present. If necessary, filter the sample. Consider changing the mobile phase to ensure that the samples fully dissolve.

Record the chromatogram for each of the standards, and plot the retention time (x-axis) versus the molecular weight (y-axis) to create the calibration curve. Notice that the y-axis is plotted on a logarithmic scale.

Conditions

Parameter	Value
Column:	Agilent AdvanceBio SEC 130 Å, 2.7 µm, 7.8 × 300 mm (p/nPL1180-5350) Agilent AdvanceBio SEC 300 Å, 2.7 µm, 7.8 × 300 mm (p/nPL1180-5301)
Samples:	Agilent polyethylene glycol calibration kit, PEG-10, 10 × 0.2 g (p/nPL2070-0100) Agilent polyethylene oxide calibration kit, PEO-10, 10 × 0.2 g (p/nPL2080-0101) AdvanceBio SEC 130 Å Protein Standard, lyophilized, 1.5 mL (p/n5190-9416) AdvanceBio SEC 300 Å Protein Standard, lyophilized, 1.5 mL (p/n5190-9417) Protein standards (Sigma-Aldrich) Samples prepare at 0.5 – 1.0 mg/mL in mobile phase
Eluent A:	150 mM Sodium phosphate buffer, pH 7.0, PBS, pH 7.4 (10 mM phosphate, 140 mM NaCl) or 100 mM Sodium phosphate + 100 mM sodium sulfate, pH 7.0
Flow rate:	1.0 mL/min
Detector:	RI for polysaccharides and PEG / PEO standards UV, 220 nm for peptides and proteins
System:	Agilent 1260 Infinity Bio-inert LC (with additional refractive index detector)

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Peptide and Protein Calibration

Proteins and peptides are generally very compact molecules containing multiple hydrogen bonds, electrostatic interactions, or covalent bonds such as disulphide bridges. Choosing a range of standards covering the operating range of the column for calibration is preferred. It is also possible to use prepared standard mixtures, as peaks are likely to be clearly defined and well resolved (Figures 3A and 3B).

The presence of a diverse range of side chain functionality from different amino acids may mean undesirable secondary interactions can occur, and so different mobile phase compositions may need to be tested.

The peaks shown are generally very sharp and well defined since they represent a single molecular species. It is quite common for proteins to contain aggregates, and these too are typically well resolved – evident as an earlier eluting peak or series of peaks.

		Retention time (min)	
Protein/Peptide	**MW**	**130 Å**	**300 Å**
Thyroglobulin	670,000	4.60	5.14
γ-Globulin	150,000	4.90	6.53
BSA	66,000	5.53	7.57
Ovalbumin	44,300	6.04	8.13
Myoglobin	17,600	6.77	8.79
Cytochrome C	12,327	6.95	8.92
Aprotinin	6,511	7.56	9.38
Neurotensin	1,672	9.42	10.54
Angiotensin-II	1,040	9.94	10.82

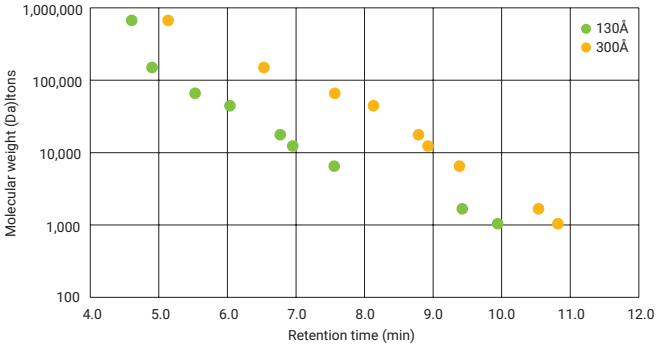

Figure 2. Peptide and protein calibration on Agilent AdvanceBio SEC columns.

Representative chromatograms for protein and peptide standards

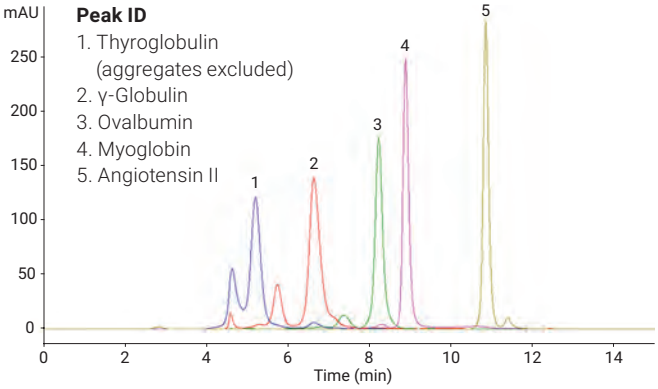

Figure 3A. Individual peptide and proteins contained in Agilent AdvanceBio SEC 300 Å Protein Standard (p/n5190–9417) on an Agilent AdvanceBio SEC 300 Å column.

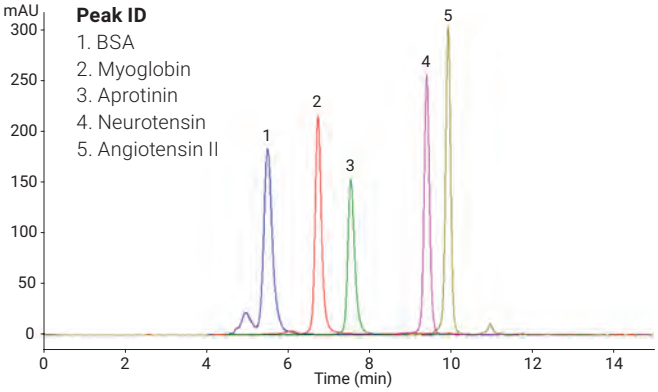

Figure 3B. Individual peptides and proteins contained in Agilent AdvanceBio SEC 130 Å Protein Standard (p/n5190–9416) on an Agilent AdvanceBio SEC 130 Å column.

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Polysaccharide Calibration

Calibration with polysaccharide standards should be used when your analyte is also a polysaccharide, for example starch or cellulose. There are many types of polysaccharide, including linear, branched, or sulfated molecules. Unlike peptides or proteins, they comprise of a range of chain lengths and can extend to very large sizes. Therefore, it is important to ensure that the column you are using is capable of resolving the molecular weight range of polysaccharide you are investigating.

The mass difference of 162 for each additional hexose means that separating individual oligomers is very difficult unless the sample is sufficiently small (Figure 5C), achieved using two columns in series for additional resolution.

The combination of two columns run in series can increase resolution to the extent that individual oligomers are becoming visible in the 5,800 Da polysaccharide sample.

This sample contains oligomers of approximately 30–40 units, with a mass difference of 162 Da each.

		Retention time (min)	
Polysaccharide	**MW**	**130 Å**	**300 Å**
788K	788,000	4.48	4.74
380K	380,000	4.58	4.84
100K	100,000	4.73	5.33
48K	48,000	4.89	6.19
23.7K	23,700	5.39	7.42
5.8K	5,800	7.16	9.22
Maltotriose	504	9.46	10.72
Maltose	342	9.73	10.82
Glucose	180	10.01	11.09

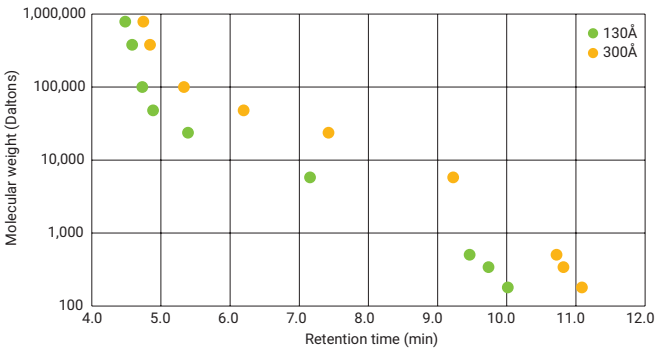

Figure 4. Polysaccharide calibration on Agilent AdvanceBio SEC columns.

Representative chromatograms for polysaccharide standards

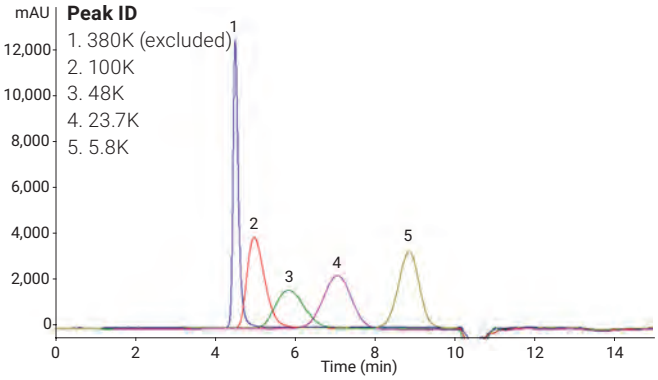

Figure 5A. Polysaccharide standards on an Agilent AdvanceBio SEC 300 Å column.

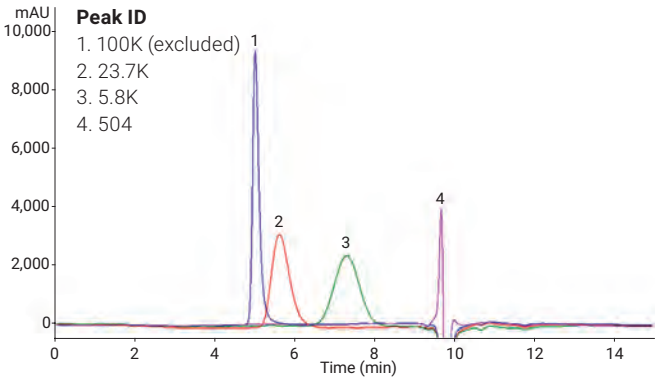

Figure 5B. Polysaccharide standards on an Agilent AdvanceBio SEC 130 Å column.

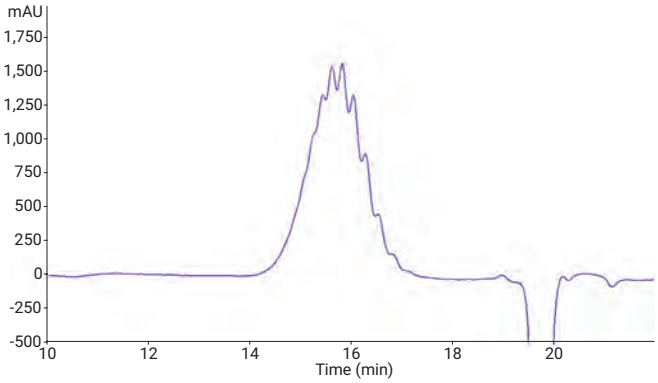

Figure 5C. Polysaccharide 5.8K run on Agilent AdvanceBio SEC columns 300 Å + 130 Å in series.

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PEG/PEO Calibration

Polyethylene glycol (PEG) and polyethylene oxide (PEO) are synthetic polyethers, available in a range of sizes covering a diverse molecular weight range. Similar to polysaccharides, they comprise of a range of chain lengths, however the mass difference is just 44 Da.

		Retention time (min)	
PEG/PEO	MW	130 Å	300 Å
905K	905,000	4.41	4.73
692K	692,000	4.41	4.73
498.6K	498,600	4.58	4.83
305.5K	305,500	4.55	4.85
135.8K	135,800	4.65	4.93
77.35K	77,350	4.71	5.20
46.47K	46,470	4.77	5.67
21.3K	21,300	5.11	6.81
12.14K	12,140	5.65	7.74
8.73K	8,730	6.10	8.28
3.87K	3,870	7.26	9.32
1.48K	1,480	8.59	10.26
420	420	9.79	11.03
106	106	10.64	11.44

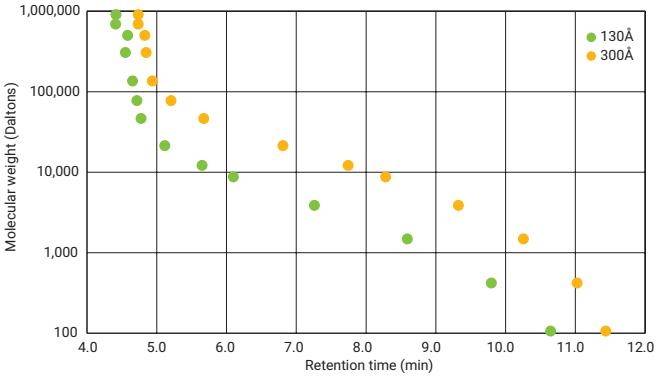

Figure 6. PEG/PEO Calibration on Agilent AdvanceBio SEC columns.

Representative chromatograms for PEG/PEO standards

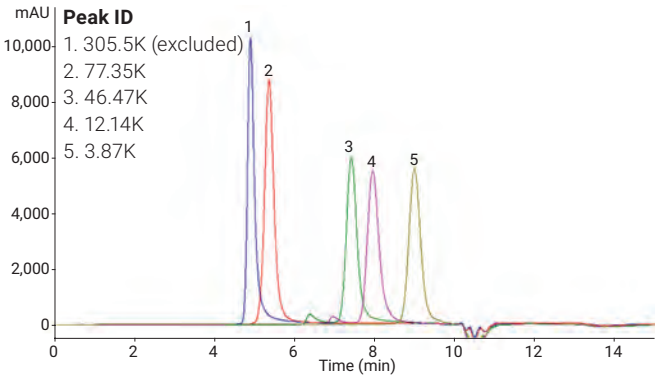

Figure 7A. PEG/PEO standards on an Agilent AdvanceBio SEC 300 Å column.

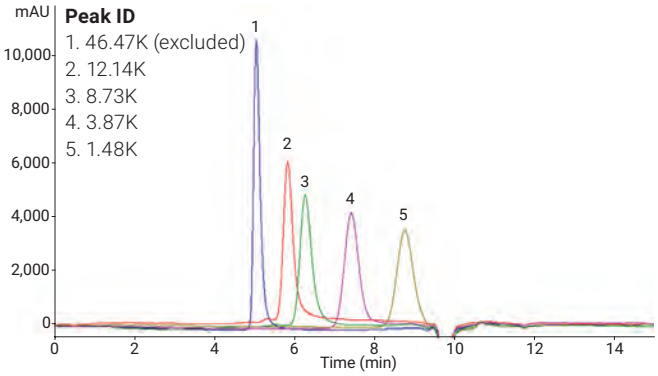

Figure 7B. PEG/PEO standards on an Agilent AdvanceBio SEC 130 Å column.

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Effect of Mobile Phase Composition

The impact of mobile phase on the size of the molecule in solution, or in overcoming secondary interactions needs to be taken into consideration, as this will influence the retention time and alter the shape of the calibration curve. This is clearly visible in the comparison of three different mobile phases: 150 mM sodium phosphate, pH 7.0; PBS, pH 7.4 (approximately 10 mM sodium phosphate, 140 mM NaCl); 0.1 M sodium phosphate + 0.1 M sodium sulfate, pH 7.0 (Figure 8).

For molecules that do not present secondary interactions, such as polysaccharides, the effect of mobile phase composition is minimal (Figure 9).

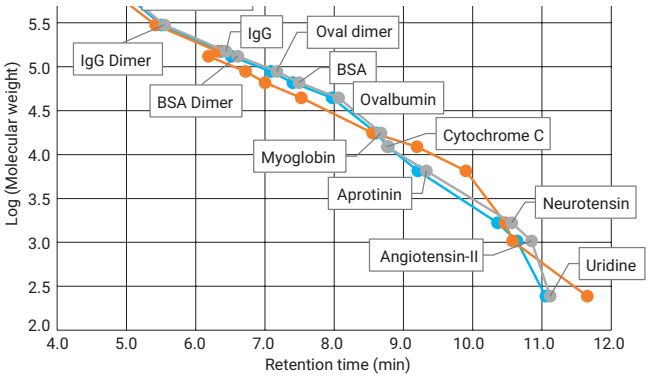

Figure 8. Effect of mobile phase composition on column calibration with protein standards.

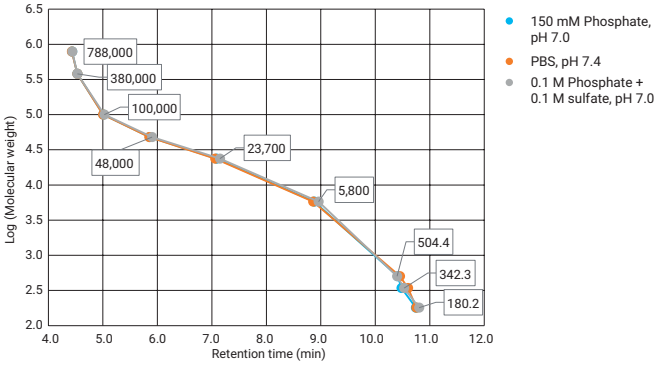

Figure 9. Effect of mobile phase composition on column calibration with polysaccharide standards.

When choosing a mobile phase for peptide or protein analysis by SEC, it is important to determine the effect that differences in pH or ionic strength may have on the sample of interest. Figures 10A, 10B, and 10C show a noticeable shift in the retention time of BSA under different mobile phase conditions. Resolution factors between dimer and monomer peaks may also be affected, so method optimization and method robustness should be fully explored.

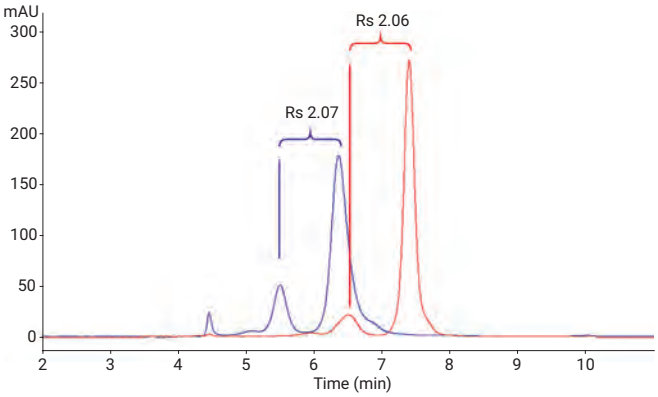

Figure 10A. γ-Globulin and BSA on an Agilent AdvanceBio SEC 300 Å column using 150 mM sodium phosphate, pH 7.0.

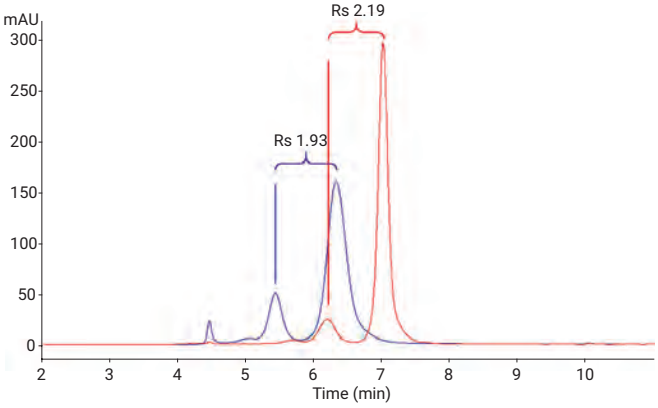

Figure 10B. γ-Globulin and BSA on an Agilent AdvanceBio SEC 300 Å column using PBS, pH 7.4.

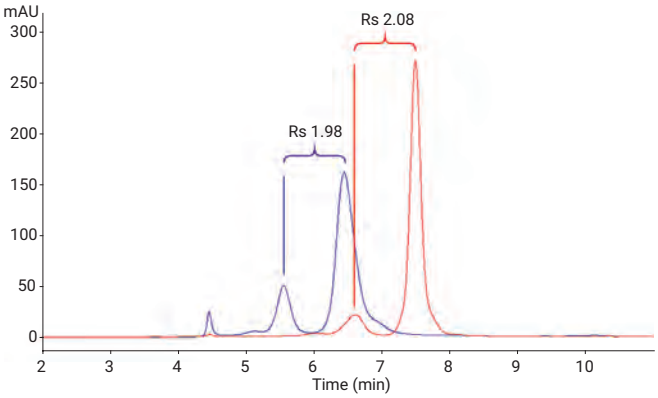

Figure 10C. γ-Globulin and BSA on an Agilent AdvanceBio SEC 300 Å column using 100 mM sodium phosphate + 100 mM sodium sulfate, pH 7.0.

Conclusion

Calibrating your Agilent AdvanceBio SEC size exclusion column with appropriate standards ensures you understand the correct working range, and can 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 a period of time, allowing early detection of potential problems. In turn, corrective action can be taken, ultimately reducing system downtime and improving productivity.

Agilent offers a range of column dimensions and different pore sizes suitable for differently sized molecules. Together with the extensive range of Agilent LC instrumentation, consumables, and technical support, we can provide the ultimate workflow solution for your needs.

Ordering Information

Part Number	Description
PL1180-5301	Agilent AdvanceBio SEC 300 Å, 2.7 µm, 7.8 × 300 mm
PL1180-3301	Agilent AdvanceBio SEC 300 Å, 2.7 µm, 7.8 × 150 mm
PL1180-1301	Agilent AdvanceBio SEC 300 Å, 2.7 µm, 7.8 × 50 mm guard
PL1580-5301	Agilent AdvanceBio SEC 300 Å, 2.7 µm, 4.6 × 300 mm
PL1580-3301	Agilent AdvanceBio SEC 300 Å, 2.7 µm, 4.6 × 150 mm
PL1580-1301	Agilent AdvanceBio SEC 300 Å, 2.7 µm, 4.6 × 50 mm guard
5190-9417	Agilent AdvanceBio SEC 300 Å Protein Standard, lyophilized, 1.5 mL
PL1180-5350	Agilent AdvanceBio SEC 130 Å, 2.7 µm, 7.8 × 300 mm
PL1180-3350	Agilent AdvanceBio SEC 130 Å, 2.7 µm, 7.8 × 150 mm
PL1180-1350	Agilent AdvanceBio SEC 130 Å, 2.7 µm, 7.8 × 50 mm guard
PL1580-5350	Agilent AdvanceBio SEC 130 Å, 2.7 µm, 4.6 × 300 mm
PL1580-3350	Agilent AdvanceBio SEC 130 Å, 2.7 µm, 4.6 × 150 mm
PL1580-1350	Agilent AdvanceBio SEC 130 Å, 2.7 µm, 4.6 × 50 mm guard
5190-9416	Agilent AdvanceBio SEC 130 Å Protein Standard, lyophilized, 1.5 mL

See Also

1. Size exclusion chromatography for Biomolecule analysis: A "How-To" Guide; 5991-3651EN.

2. GPC/SEC standards: Product guide; 5990-7996EN.

3. Calibrating GPC columns: A Guide to Best Practice; 5991-2720EN.

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Printed in the Printed in the USA, January 9, 2017
5991-7799EN

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Application Note

Aggregate/Fragment Analysis

Agilent

Trusted Answers

Size Exclusion Chromatography of Biosimilar and Innovator Insulin

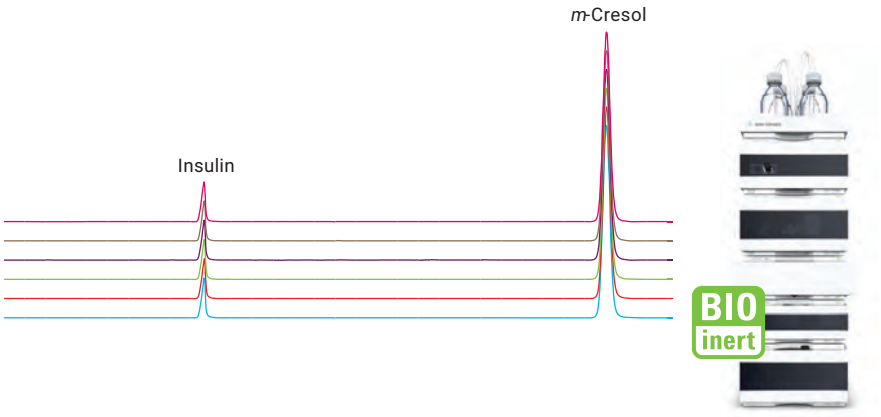
Using the Agilent AdvanceBio SEC column

Authors

M. Sundaram Palaniswamy and Andrew Coffey
Agilent Technologies, Inc.

Abstract

Insulin is a small polypeptide hormone that controls blood glucose homeostasis. Genetic engineering techniques have enabled biopharma companies to develop diverse, long-acting insulin analogs. There is no pharmacopeia method available for the analysis of insulin analogs. An SEC method identifying innovator and biosimilar insulin analog, following a draft EP method, was developed using an Agilent AdvanceBio SEC 130 Å, 7.8 × 300 mm, 2.7 µm column. The effectiveness of this method, for routine analysis, was confirmed using a system suitability test, and retention time (RT) and area precision studies using innovator insulin as a reference material. This Application Note also presents the application of this column for detecting impurities with molecular masses greater than that of insulin for quantitation studies.



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Introduction

Novel insulin analogs are alternatives to human insulin products. Clinical trials have demonstrated equal or superior efficacy outcomes when these analogs are compared with human insulin. Insulin analogs are currently the long-acting basal human insulin on the market. Insulin analog was approved for use by the US Food and Drug Administration (USFDA) in April 2000. Unlike small molecules, biotherapeutics are created using biological processes. Each manufacturer uses an in-house developed process for the production of drug substance and drug product. These production methods can result in impurities derived from the drug substance, such as aggregates and degradation products. Due to the increased demand for antidiabetic drugs, it is a crucial yet challenging task to produce drugs free from impurities, and provide safe medicine free from side effects. In the biopharma industry, LC with UV detection is a versatile tool for lot release and characterization studies[1]. Size exclusion chromatography (SEC) is the method of choice for purity analysis, and for detecting aggregates of drug product. This Application Note describes a SEC-UV approach to determine the molecular similarity between insulin biosimilar and its innovator reference, following system suitability and method precision analysis[2]. These tests ensure that the method can generate results of acceptable accuracy and precision. The criteria selected is based on critical chromatographic parameters and their variation within acceptable limits, which are defined during the method evaluation experiments. An excellent correlation coefficient was observed for the linearity curve of insulin in the range of 10.6 to 3,400 µg/mL, indicating that the method is quantitative. Use of the Agilent AdvanceBio SEC column to monitor and separate impurities with molecular masses greater than the drug product, as determined by forced-stress studies, is also shown.

Table 1. Chromatographic parameters used for SEC HPLC.

Parameters	Conditions
Mobile phase	200 mL of anhydrous acetic acid, 300 mL of acetonitrile, and 400 mL of water, adjusted to pH 3.0 with concentrated ammonia, and diluted to 1,000.0 mL with water.
TCC temperature	Ambient
Isocratic run	Mobile phase A
Injection volume	10 µL
Flow rate	0.5 mL/min
UV detection	276 nm

Materials and Methods

Instruments

- A completely biocompatible Agilent 1260 Infinity Bio-inert Quaternary LC System with a maximum pressure of 600 bar was used, consisting of:
- 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 containing bio-inert click-in heating elements (G1316C, option 19)
- Agilent 1260 Infinity DAD VL (G1315D with Bio-inert standard 10-mm flow cell)
- Agilent AdvanceBio SEC, 130 Å, 7.8 × 300 mm, 2.7 µm (p/nPL1180-5350)
- Software
- Agilent ChemStation B.04.03 (or higher)
- Size exclusion chromatography parameters
- Table 1 shows the chromatographic parameters for size exclusion chromatography using an Agilent 1260 Infinity Bio-inert LC System.

Reagents, samples, and materials

Commercial innovator and biosimilar insulin were purchased from a local pharmacy, and stored according to manufacturer's instruction. Acetic acid and ammonia were purchased from Sigma-Aldrich. All chemicals and solvents used were HPLC grade, and highly purified water from a Milli-Q water purification system (Millipore Elix 10 model, USA) was used.

Procedure

A 10 µL volume of mobile phase was injected as a blank, followed by individual linearity levels in triplicate. Area and retention time (RT) of each level were used to calculate standard deviation (SD) and relative standard deviation (RSD %) values. Limits of detection (LODs) and limits of quantitation (LOQs) were established from the lower linearity level injections. The average area of each linearity level was plotted against the concentration of insulin to determine the calibration curve for the monomers.

Linearity and range

The calibration curve was constructed with nine standard concentrations of innovator insulin in the range 10.6 to 3,400 µg/mL.

LOQ and LOD

The insulin concentration that provides a signal-to-noise ratio (S/N) of > 3 was considered as the LOD, and S/N > 10 was considered as the LOQ.

Preparation of insulin aggregates

Aggregates of insulin were prepared following temperature stress. Briefly, about 3.4 mg/mL of the drug product was incubated at 60 °C for 6 hours in a polypropylene tube. Samples were cooled to room temperature, and immediately analyzed.

System suitability

As per the draft monograph, the following are the system suitability requirements:

- Symmetry factor: Maximum 2.0 for the peak due to insulin analog
- Peak-to-valley ratio: Minimum 2
- Total of all impurities with a retention time less than that of insulin analog: Not more than 0.3 % of the total area of the peaks, disregarding any peak with a RT greater than that of the insulin peak

Results and Discussion.

Separation and detection

The biosimilar insulin was compared using the innovator as the reference standard. The optimized SEC HPLC separation of intact biosimilar and innovator insulin on the AdvanceBio SEC 130 Å, 7.8 × 300 mm, 2.7 µm column achieved excellent separation. Homogenous profiles without any indication of aggregation were demonstrated within a total run time of 55 minutes. A peak due to the preservative m-cresol was also observed, eluting at approximately 49 minutes (Figure 1).

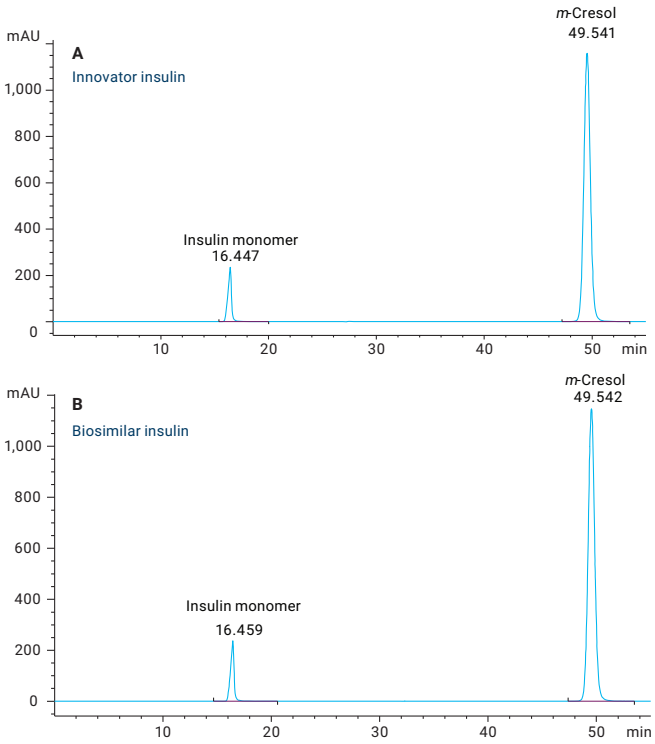

Figure1. SEC HPLC profile of insulin innovator and biosimilar on an Agilent AdvanceBio SEC, 130 Å, 7.8 × 300 mm, 2.7 µm column.

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Precision of retention time and area

Figure 2 shows the overlays of six replicates of innovator and biosimilar insulin, demonstrating excellent separation reproducibility. Table 2 lists the average RTs and peak area RSDs for the insulin monomer from six replicates. The RT and peak area RSDs for the insulin monomer were within the acceptable limit of ±3 % and ±5 %, respectively, demonstrating the excellent reproducibility and precision of this method.

System suitability

Table 3 tabulates the acceptance criteria for this system suitability study for insulin analog, and Table 4 presents the summary of the system suitability results.

These results of the system suitability test for insulin innovator and biosimilar demonstrate that the method performed using an Agilent Bio-inert LC and an AdvanceBio SEC column meets the stringent performance requirements for insulin QA/QC analysis.

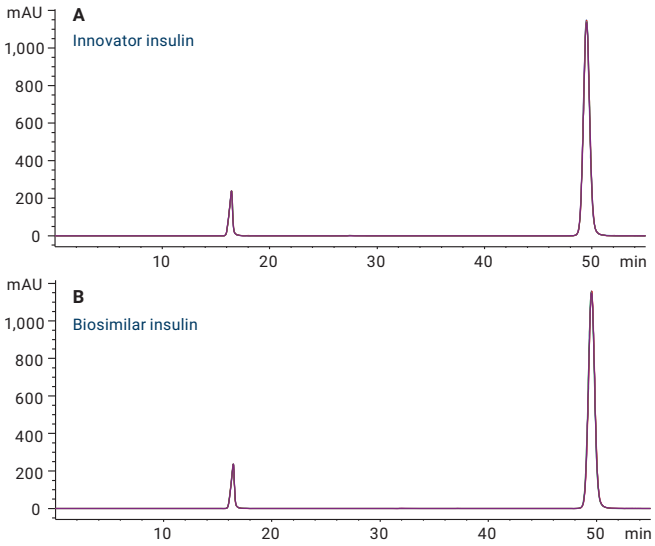

Figure2. Overlay of six replicates of innovator and biosimilar insulin separated on an Agilent AdvanceBio SEC, 130 Å, 7.8 × 300 mm, 2.7 µm column.

Table 2. RT and peak area precision (n = 6).

	RT		Peak area	
Sample	Mean (min)	RSD	Mean (mAU/min)	RSD
Innovator insulin	16.450	0.057	5,544.91	0.285
Biosimilar insulin	16.460	0.044	5,459.55	0.662

Table 3. Acceptance criteria.

Parameter	Limit
Symmetry factor	Maximum 2.0 for the peak due to insulin analog
Peak-to-valley ratio	Minimum 2
Total of all impurities with an RT less than that of insulin analog	Not more than 0.3 % of the total area of the peaks

Table 4. Summary of system suitability test results.

	Results on an Agilent AdvanceBio SEC, 130 Å, 7.8 × 300 mm, 2.7 µm column			
Sample	Symmetry factor	Peak-to-valley ratio	Total of all impurities with an RT less than that of insulin analog	Passed (Yes/No)
Innovator insulin	1.71	–	0.167	Yes
Biosimilar insulin	1.72	–	0	Yes

Table 5. LOD, LOQ, and S/N results (n = 3) for insulin innovator.

Concentration (µg/mL)	S/N	Average area
10.6 (LOD)	11.9	12.8
31.8 (LOQ)	34.7	37.4

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LOD and LOQ

The LOD and LOQ were tested for insulin innovator, and were found to be 11.3 µg/mL and 28 µg/mL, respectively, indicating that the method is sensitive. Table 5 shows the observed LOD and LOQ values of insulin innovator.

Linearity

Linearity curves for insulin innovator were constructed from the LOD level to the label claim (3.4 mg/mL) in the study, using the area response and concentration of insulin. Figure 3 shows the linearity curve for insulin in the concentration range 10.6 to 3,400 µg. The R2 value observed was more than 0.99, suggesting excellent dose-dependent correlation between the peak area and the concentration of insulin.

Aggregation/degradation analysis and quantification

The impurity profile of biotherapeutics is of increasing importance in drug safety. Although aggregates are present in extremely low concentrations, they may have a big impact on the quality of the product. The AdvanceBio SEC column is designed to have minimum interaction with biomolecules, enabling distinct baseline separation of insulin aggregates. These insulin aggregates elute from the AdvanceBio SEC column at 11.181 and 13.884 minutes, respectively, as shown in Figure 4.

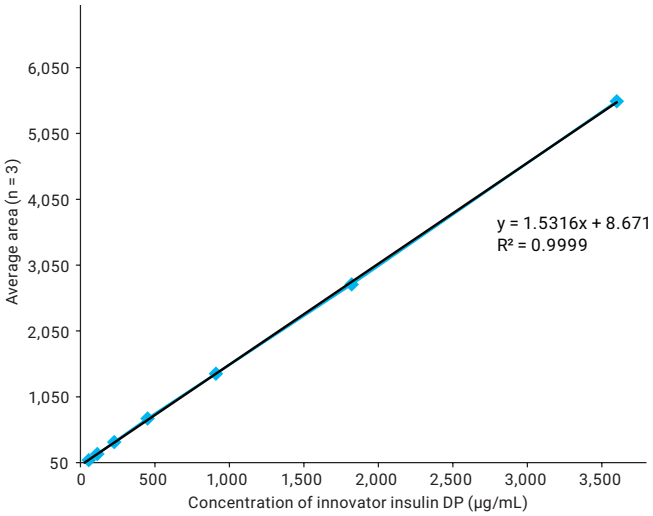

Figure 3. Linearity curve with standard concentrations of insulin ranging from 10.6 to 3,400 µg/mL, showing excellent coefficient value.

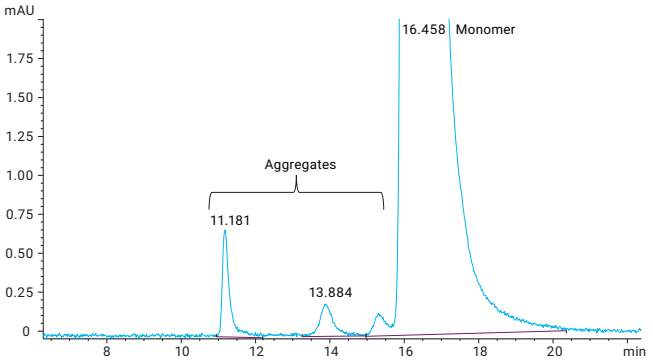

Figure 4. An Agilent AdvanceBio SEC profile of heat stressed insulin showing baseline separation of insulin aggregates.

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Economic value and lifetime studies

A laboratory head or group leader may primarily consider cost, particularly when comparing the cost of the AdvanceBio SEC column and other column types. In SEC separation, apart from the cost of the operator and instrument, the most expensive component is the cost of the column itself. If the columns do not last long enough, or there are column-to-column reproducibility issues, multiple columns may need to be screened. Ensuring batch-to-batch reproducibility through control of the entire production process is essential. Figure 5 shows the separation of AdvanceBio 130 Å protein markers on four separate batches of the AdvanceBio SEC 130 Å media, ensuring thorough control of the entire production process.

One of our objectives is to ensure extended column lifetime throughout our customers' development processes. This extended column lifetime provides extra benefits, as the downtime is greatly reduced. Figure 6 shows six overlaid chromatograms of the 250 injections of 3 mg/mL insulin drug substance taken at an interval of 50 runs. Table 6 shows the RT, area, tailing factors, and theoretical plates from the selected runs.

The results clearly demonstrate that there is virtually no change in RT, area, as well as tailing factor over the course of 250 injections. The theoretical plates, a measure of the efficiency of the column, also do not vary significantly.

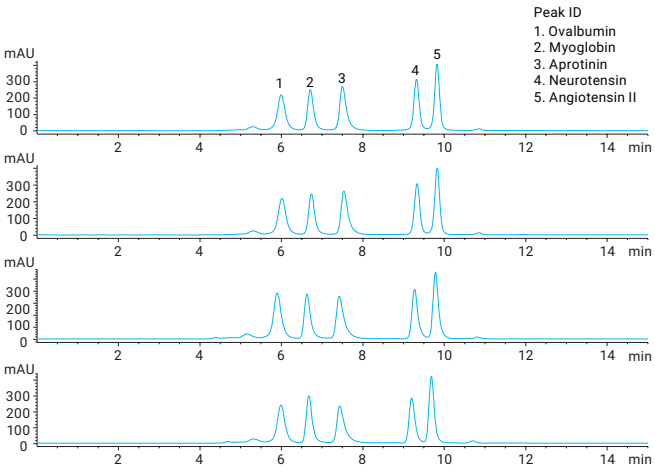

Figure 5. Separation of Agilent AdvanceBio 130 Å protein standards on four separate batches of an Agilent AdvanceBio SEC 130 Å, 7.8 × 300 mm, 2.7 µm media.

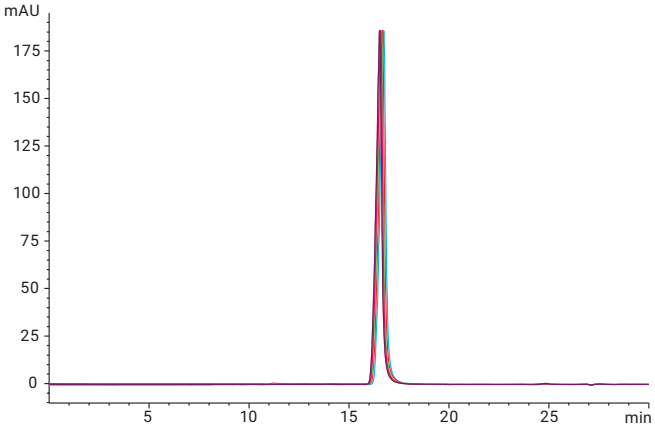

Figure 6. Overlay of six chromatograms for the 250 injection at an interval of 50 runs.

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Conclusion

Size exclusion chromatography is the workhorse for detecting and monitoring aggregates and monomers for biopharmaceuticals. This Application Note demonstrates the suitability of an Agilent AdvanceBio SEC 130 Å column as an excellent choice to study insulin analogs. We used the draft pharmacopeia method to develop a simple UV-based approach to define the molecular similarity between biosimilar and innovator insulin drug product using an AdvanceBio SEC 130 Å, 7.8 × 300 mm, 2.7 µm column. RT and area precision of the method were excellent, and met the system suitability requirements. A linear relationship between the peak area and eight standard concentrations of the insulin drug product was observed, with an outstanding coefficient of linearity value. The observed LOD and LOQ was found to be 10.6 and 31.8 µg/mL, respectively, indicating the sensitivity of the method. The AdvanceBio SEC column was able to separate and monitor aggregates analyzed by forced stress study. We have also shown the greater economic benefits of using an AdvanceBio SEC column, some of which are reducing lot-to-lot manufacturing variations, and prolonged column lifetime with reproducible and robust outcomes. This simple and reproducible method, coupled with a bio-inert and corrosion-resistant instrument is considered to be reliable and suitable for routine quality checks of insulin throughout the development process.

Table 6. Observed RT, area, tailing factor, and theoretical plates for 250 injections of insulin drug substance.

Injection no.	RT (min)	Area	Tailing factor	Theoretical plates
1	16.657	3944	0.899	16,001
50	16.671	3966	0.890	15,849
100	16. 681	3968	0.898	15,982
150	16.622	3942	0.893	15,942
200	16.634	3953	0.895	15,919
250	16.634	3963	0.890	15,944

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Application Note

Aggregate/Fragment Analysis

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A Comprehensive Workflow to Optimize and Execute Protein Aggregate Studies

Combining Size Exclusion Chromatography with Method Development and Light Scattering

Authors

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Abstract

This Application Note illustrates a comprehensive aggregate analysis workflow to:

- Optimize mobile phase conditions for high-performance size exclusion chromatography (SEC) of monoclonal antibodies
- Characterize aggregation profiles that include monomers, dimers, and higher-order aggregates

We used Agilent Buffer Advisor software to automate complex SEC optimization experiments that use the full capabilities of the bio-inert quaternary pump of the Agilent 1260 Infinity II Bio-inert LC system to mix a wide range of buffer compositions, automatically, in real time during a series of fast LC runs. The Agilent 1260 Infinity Bio-MDS multidetector suite provided dynamic light scattering detection capability to reveal higher-order protein aggregates, determine absolute molecular weights, and augment quantitative measurements made by a UV detection system.

Introduction

Some monoclonal antibodies (mAbs) and proteins are prone to aggregating spontaneously in solution[1,2]. For many biopharmaceutical applications, the extent of such aggregation must be characterized and quantified precisely under a variety of conditions. Size exclusion chromatography (SEC) is a powerful technique to characterize and quantify protein aggregation, but accurate measurements require excellent chromatography under conditions that accommodate natural protein conformation. To improve chromatographic peak shape for a particular protein, and thus improve resolution, it is often necessary to evaluate a variety of different mobile phase conditions.

The utility of optimizing buffer conditions is sometimes overlooked with SEC techniques. Historically, buffer conditions were optimized to overcome undesirable nonspecific interactions with stationary phase materials, but optimizing for column deficiencies can introduce a risk of disrupting the very aggregation that the technique seeks to measure. However, the inert surface coating of Agilent AdvanceBio SEC columns helps to reduce secondary interactions across a wide range of buffer conditions, and provides greater flexibility to optimize buffer chemistry for protein conformation and chromatographic resolution. The sheer complexity of parameter-scouting experiments has been another impediment to routinely optimizing SEC buffer conditions for aggregate analysis. Optimization experiments required the design of complex tables of mobile phase possibilities, and the tedious manual creation of many different solutions to evaluate the matrix of salts, buffers, and pH variations experimentally. However, modern tools have greatly streamlined workflows for optimizing SEC conditions, characterizing and quantifying aggregates, and deploying optimized techniques in daily use.

This Application Note demonstrates the utility of a full workflow solution for aggregation studies to:

- Automatically mix a specified list of LC buffers from four simple stock solutions and to adjust pH and buffer concentrations in real time using Agilent Buffer Advisor software and a high-resolution bio-inert quaternary LC pump
- Measure higher-order protein aggregates with dynamic light-scattering detection, complementing UV detection to extend high-sensitivity across a broad mass range
- Characterize absolute molecular weight and hydrodynamic radius of aggregated and monomeric proteins by light-scattering detection

For the present analyses, we used an Agilent AdvanceBio SEC 150 mm column to provide fast separations for rapid screening. It is important to note that AdvanceBio SEC columns come in longer (and shorter) variations, so the methods can easily be adapted to the 300 mm format, or even multiplexed columns, where additional resolution may be required. Monoclonal antibody samples in this study include commercially available rituximab and a commercially available rituximab biosimilar.

Materials and Methods

Reagents, samples, and materials

Monobasic and dibasic sodium hydrogen phosphate and sodium chloride were purchased from VWR. All the chemicals and solvents used were ≥ 99.7 % pure. >18 MΩ Water was used from a Milli-Q A10 water purification system (Millipore, USA). Solutions were prepared fresh daily and filtered through 0.22 µm membrane filter prior to use.

Instrument

Agilent 1260 Infinity II Bio-inert LC System for aggregate analysis, comprising:

- Agilent 1260 Infinity II Bio-inert Quaternary Pump (G5654A)
- Agilent 1260 Infinity II Bio-inert Multisampler with sample cooler and multi-wash (G5668A)
- Agilent 1260 Infinity II Multicolumn Thermostat with bio-inert heat exchangers (G7116A)
- Agilent 1260 Infinity II Diode Array Detector WR with bio-inert standard flow cell (G7115A)
- Agilent 1260 Infinity Bio-SEC Multi Detector System (G7805AA)
- Agilent AdvanceBio SEC 300 Å, 7.8 × 150 mm, 2.7 µm column (p/n PL1180-3301) or
- Agilent AdvanceBio SEC, 2.7 µm 300 Å 7.8 × 300 mm column (p/n PL1180-5301)

Instrument conditions

Parameter	Value
Mobile phase	See Table 1
Flow rate	0.8 mL/min
Temperature	25 °C
Injection	1–25 µL (dependent on sample concentration)
Detection	220 nm, 280 nm, LS 90°, and DLS
Samples	Rituximab innovator, rituximab biosimilar, and BSA

Results and Discussion

To identify optimal mobile-phase compositions for each analyte mixture, we evaluated three different mobile phases across four different pH levels, representing a matrix of 12 experimental conditions. Buffer compositions were:

- 150 mM Sodium phosphate
- 10 mM Sodium phosphate + 140 mM NaCl (emulating PBS at different pH values)
- 100 mM Sodium phosphate + 150 mM NaCl

Each mobile phase was tested at pH 6.2, 6.6, 7.0, and 7.4.

To execute each experiment, we specified buffer composition and pH for each treatment in Agilent Buffer Advisor. The software automatically calculated the appropriate mixtures of stock solutions A–D to achieve the desired mobile phase specifications in real time during the LC parameter-scouting run. Table 1 summarizes the 12 experimental conditions.

These conditions were used to analyze a commercial sample of rituximab, a sample of rituximab biosimilar, and a commercial BSA protein standard mixture (10 mg/mL solution for instrument calibration purposes).

By using an Agilent AdvanceBio SEC 300 Å 150 × 7.8 mm column, we were able to perform screening experiments in less than 10 minutes per sample.

Table 1. Experimental conditions and corresponding mobile phase compositions.

	User-specified parameters				Software-calculated parameters			
Experiment	pH	Buffer (mM)	NaCl (mM)	Total conc. (mM)	% A	% B	% C	% D
1	6.2	150	0	150	25.0	0.0	57.0	18.0
2	6.6	150	0	150	25.0	0.0	42.3	32.7
3	7.0	150	0	150	25.0	0.0	26.3	48.7
4	7.4	150	0	150	25.0	0.0	13.8	61.2
5	7.4	10	140	150	67.0	28.0	0.9	4.1
6	7.0	10	140	150	67.0	28.0	1.8	3.2
7	6.6	10	140	150	67.0	28.0	2.9	2.1
8	6.2	10	140	150	67.0	28.0	3.9	1.1
9	6.2	100	150	250	20.0	30.0	36.3	13.7
10	6.6	100	150	250	20.0	30.0	26.1	23.9
11	7.0	100	150	250	20.0	30.0	15.6	34.4
12	7.4	100	150	250	20.0	30.0	7.8	42.2

A = Water
B = 500 mM NaCl
C = 200 mM NaH_2PO_4
D = 200 mM Na_2HPO_4

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An initial review of the chromatographic data revealed that the mobile phase conditions impacted the peak shapes of the rituximab innovator and rituximab biosimilar during the experiment, as shown in Figure 1 and Figure 2. The two versions of the molecule appear similar in profile, and demonstrate the same unusual behavior with the mobile phase composition of 10 mM sodium phosphate with 140 mM NaCl. This mobile phase composition produced a noticeable increase in peak tailing with a reduction in peak height. This type of behavior may also be seen with other proteins, illustrating the need to carefully evaluate the effect of mobile phase composition for both method development and method robustness.

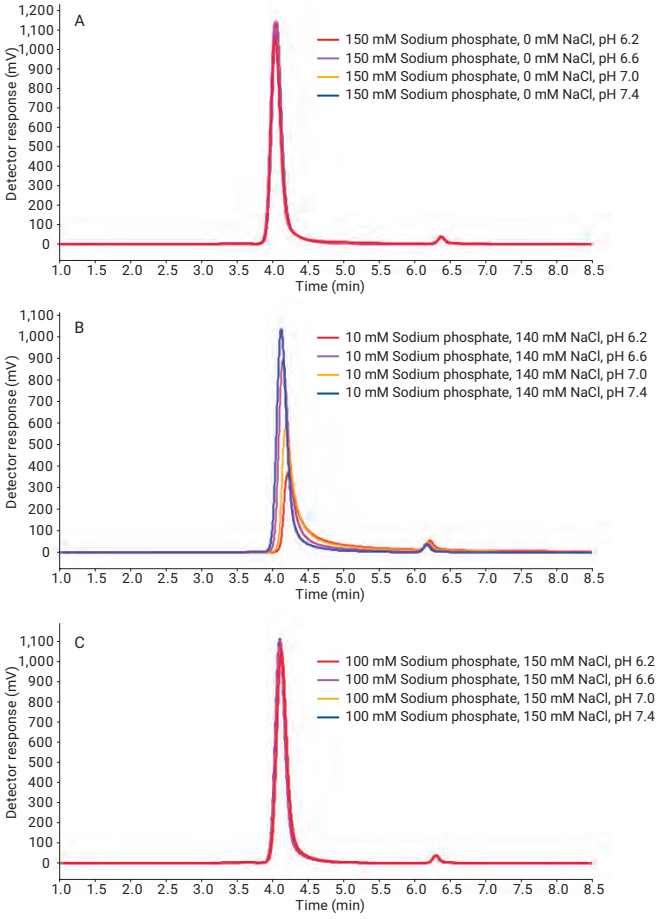

Figure 1. Rituximab innovator (1 µL injection) run consecutively at 12 different mobile phase conditions (Experiments 1–12: see Table 1).

For quantification of aggregate content, it is necessary to use the UV detector. Integration of monomer peak and aggregate peak (where detected) enables the peak area aggregate percentage to be determined. Results are reported in Figure 3A for rituximab innovator and Figure 3B for rituximab biosimilar. The most consistent results were obtained at pH 7.0 using 150 mM sodium phosphate or 100 mM sodium phosphate with 150 mM NaCl (Experiments 3 and 11).

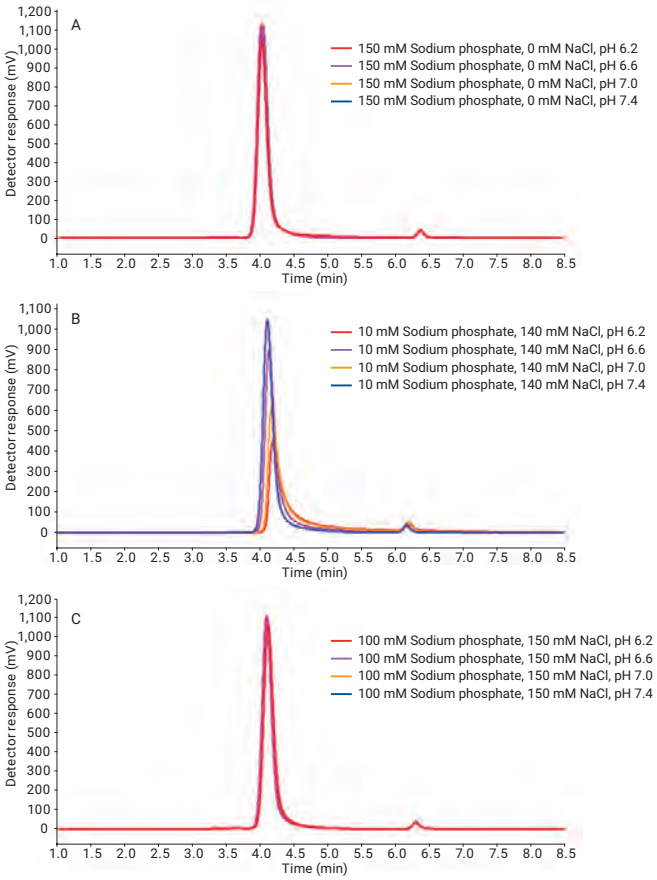

Figure 2. Rituximab biosimilar (1 µL injection) run consecutively at 12 different mobile phase conditions (Experiments 1–12: see Table 1).

Figure 4, showing chromatograms obtained at pH 7.0 in the three different buffer compositions, clearly illustrates that aggregates (highlighted with an arrow) were not detected when 10 mM sodium phosphate with 140 mM NaCl was used as mobile phase for the analysis of this molecule.

Adding a light scattering detector to the aggregate analysis workflow is optional, but it reveals more useful information about protein aggregates. Following a simple instrument calibration with a single injection of a well-defined molecule, in this case BSA, the inter-detector delay and instrument constants can be determined rapidly. Agilent Bio-SEC software can be used to report molecular weight information for individual peaks from any other chromatogram obtained under the same column and flow rate conditions.

Figure 5 shows the light scattering analysis results of the rituximab innovator versus rituximab biosimilar run under the high salt conditions of Experiment 11. Focusing analysis on the monomer peak only, the light scattering analysis reported molecular weights close to the accurate mass values seen in a previous Application Note[1]. The biosimilar molecule is expected to have a slightly higher mass due to the presence of C-terminal lysine variants not evident in the originator molecule.

The formation of larger aggregates and subvisible particles is a particular concern in biopharmaceutical applications. The sensitivity of light-scattering detection toward highly aggregated samples complements the concentration data obtained from UV detection.

Despite the very similar results for monomers and dimers analyzed by UV detection, the light scattering detector proved more responsive to higher order aggregates, and revealed some differences in the more extensive aggregation of the rituximab innovator and biosimilar under certain mobile phase conditions as shown in Figure 6.

The addition of DLS capability further enhances the level of information that can be gained, providing hydrodynamic radius measurements to be made (Figure 7).

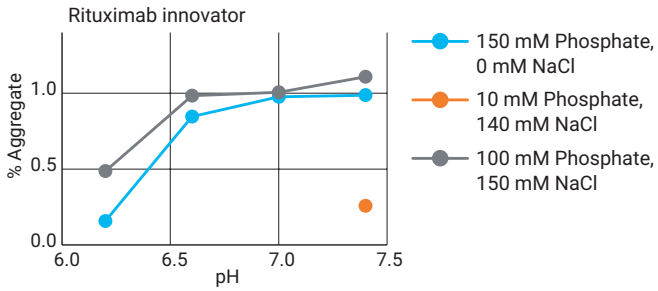

Figure 3A. Peak area percentage of aggregate content of rituximab innovator (Experiments 1–12: see Table 1). Points are shown for experiments where aggregates were detected.

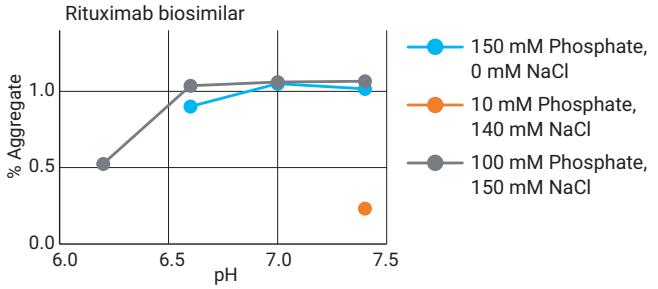

Figure 3B. Peak area percentage of aggregate content of rituximab biosimilar (Experiments 1–12: see Table 1). Points are shown for experiments where aggregates were detected.

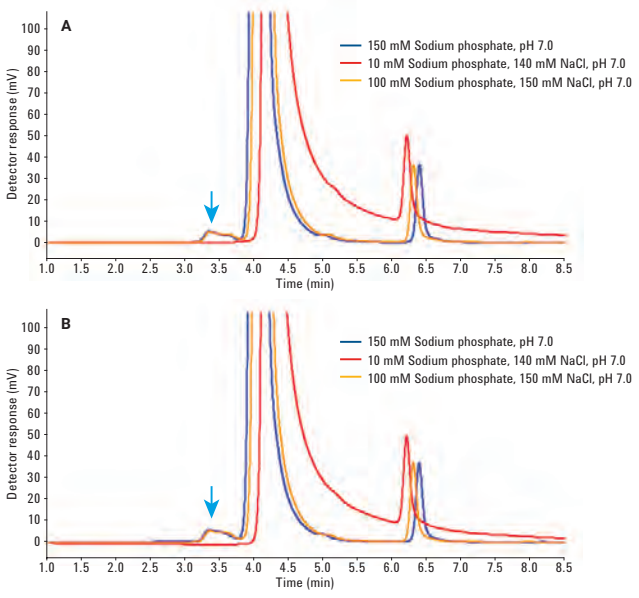

Figure 4. Baseline zoom of UV 220 nm signals of rituximab innovator (A) and rituximab biosimilar (B) run with different buffer salt concentrations at the optimized pH 7.0 (Experiments 3, 6, and 11 in Table 1).

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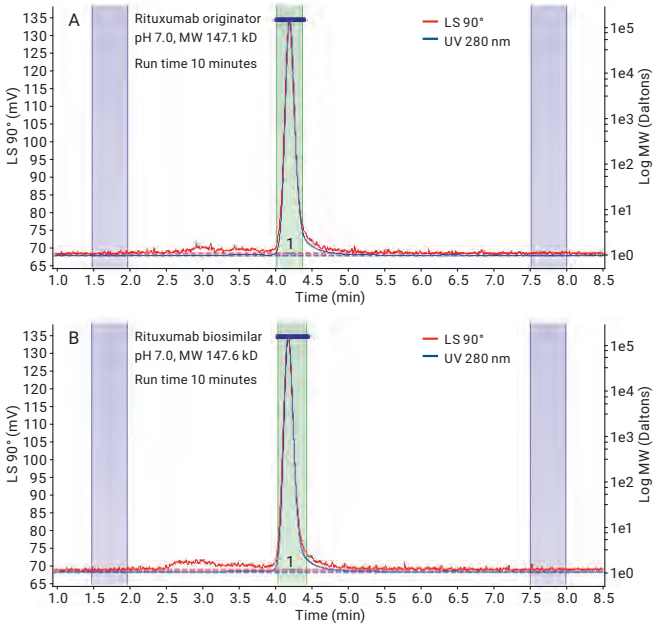

Figure 5. Comparison of LS analysis rituximab innovator (A) and rituximab biosimilar (B) run at 100 mM sodium phosphate with 150 mM NaCl, pH 7.0 (Experiment 11 in Table 1).

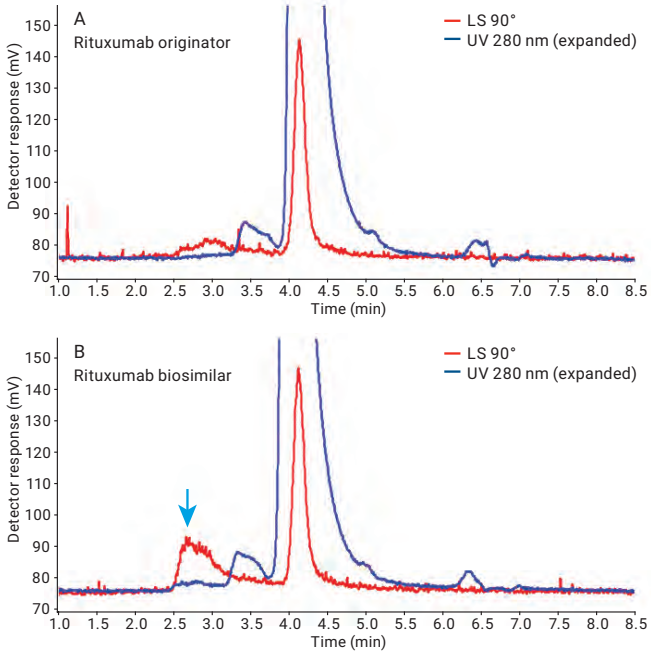

Figure 6. Comparison of LS analysis rituximab innovator (A) and rituximab biosimilar (B) run at 150 mM sodium phosphate, pH 7.0 (Experiment 3 in Table 1).

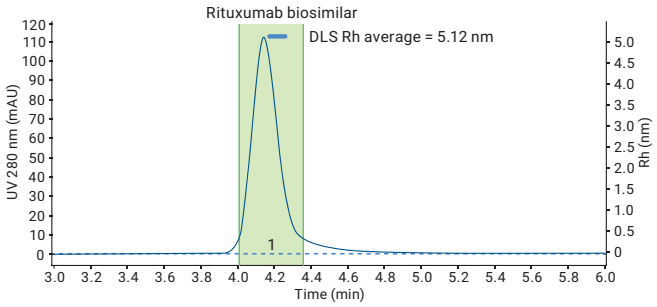

Figure 7. DLS analysis of rituximab biosimilar showing hydrodynamic radius results from LS detector.

Conclusion

The Agilent 1260 Infinity II Bio-inert LC System containing a fully bio-inert flow path, with Agilent Buffer Advisor software, provides a simple way of performing method optimization for size exclusion chromatography for protein aggregate quantitation. Faster separations are possible using a shorter Agilent AdvanceBio SEC 300 Å 150 mm column, which greatly increases throughput and reduces the time required for screening a wide range of analysis conditions. To gain more resolution and higher accuracy, use a longer 300 mm column.

The AdvanceBio SEC column shows additional benefits such as low nonspecific binding. The Bio-MDS Multidetector Suite with Bio-SEC software can be used to reveal low levels of high molecular weight aggregates that are difficult to detect by any other means. Ultimately, the Bio-MDS can be used to determine protein molecular weight, or determine hydrodynamic radius information in conjunction with DLS detection. This suite of technologies comprises a comprehensive workflow solution to optimize SEC conditions rapidly, quantify aggregates accurately across their entire molecular weight range, and characterize the aggregation dynamics of monoclonal antibodies in relevant buffer conditions.

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Application Note

Aggregate/Fragment Analysis

Agilent

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Size Exclusion Chromatography in the Presence of an Anionic Surfactant

Intact Protein Profiling

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Abstract

Sodium dodecyl sulfate (SDS, or SLS) is a well known anionic detergent, frequently used to denature proteins. It is commonly used in polyacrylamide gel electrophoresis (SDS-PAGE), where a remarkably consistent level of binding across a wide range of proteins imparts a reliable charge-to-mass ratio. This allows separation of denatured proteins based on relative size due to their relative ion mobility. Conversely, size exclusion chromatography (SEC) for size-based separation of proteins is normally performed under nondenaturing conditions using predominantly aqueous buffers as mobile phase.

This Application Note used SEC with light scattering detection to investigate the impact of varying SDS concentration on the protein molecule. This was achieved by studying the changes in RT, apparent molecular weight, and hydrodynamic radius.

Introduction

To denature proteins, sodium dodecyl sulfate (SDS) is used above its critical micelle concentration (CMC). The CMC of SDS in pure water is 8.2 mM (approximately 0.2 % w/v). However, the presence of buffer salts or changes in pH and temperature may reduce the CMC significantly. CMC values of around 1.0 mM (approximately 0.04 % w/v) are observed in phosphate buffered saline (PBS), for example. A micelle forms when approximately 62 SDS molecules coalesce into a spherical shape with a hydrophobic core surrounded by an anionic surface, as depicted in Figure 1. Typically, SDS is used at significantly higher concentrations of 2 % w/v for sample preparation for SDS-PAGE, but also with a reducing agent to cleave disulfide bonds within the protein molecule. The resulting saturated SDS-protein complex has, on average, 1.4 g SDS per gram of protein [1]. Such denatured proteins are considered to adopt a rigid cylindrical shape resulting in the observation that ion mobility in gel electrophoresis is proportional to molecular weight. By omitting the reduction of disulfide bonds, it has been observed that the ratio of SDS to protein decreases significantly and the time to reach equilibrium may be doubled [2].

In contrast, adding SDS (approximately 10:1 mol ratio) has been found to arrest heat denaturation of BSA solution, which otherwise leads to creation of high molecular weight oligomers [3], or reduces the level of noncovalent aggregation. Historically, it was recommended to use 0.1 % w/v SDS in the mobile phase to reduce retention time (RT), and improve peak shape in protein size exclusion chromatography (SEC) [4].

The mechanism by which SDS denatures a protein is not fully understood. Since both the detergent molecule and the protein possess complementary ionic and hydrophobic regions, it is clear that a combination of mechanisms may be involved. Thermodynamic studies have suggested that interactions are not ionic alone. It is conceivable that SDS first begins to bind through an ionic interaction with positively charged amino acids side chains (Lys and Arg) on the surface of the protein. Increasing SDS concentration leads to higher levels of incorporation, and it is postulated that ultimately, the flexible protein chain is decorated with SDS micelles rather than the rigid cylinder model originally proposed [5].

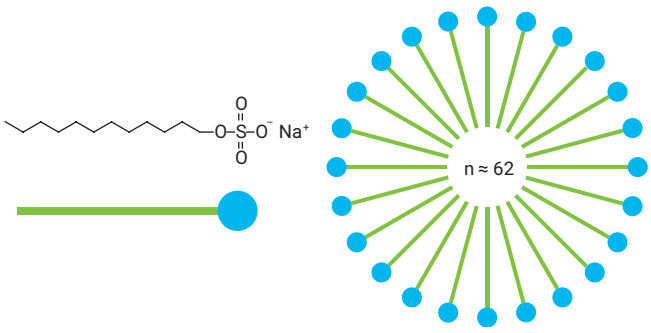

Figure 1. Cartoon depicting SDS micelle formed at concentrations above CMC (aggregation number ~ 62).

Bovine serum albumin (BSA) is a well studied protein due to its capability of acting as a carrier of smaller molecules between tissues and cells. The structure and size (hydrodynamic radius) of the BSA monomer has been determined using multiple analytical techniques. Its primary structure is 583 amino acids with a molecular weight of 66,463 Da, Figure 2. Commercial samples frequently contain varying amounts of dimer and higher-order aggregates, and different isolation techniques may result in varying amounts of these oligomers. Since BSA contains 35 cysteine residues, there are 17 disulfide bridges and one free thiol group. It is believed that BSA oligomers are predominantly covalently linked through disulfide bonds involving the extra thiol group. Therefore, in the absence of reducing agents, denaturation with SDS will not result in such covalently linked dimers and higher aggregates reverting to a monomeric form.

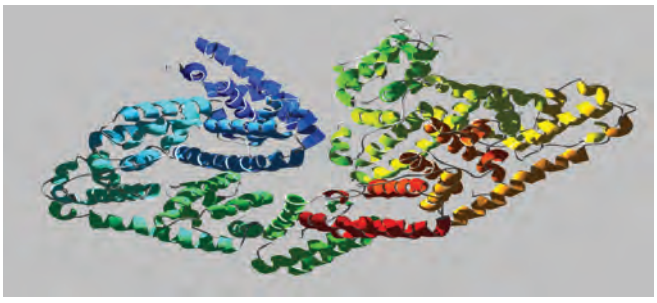

Figure 2. Native heart-shaped conformation of bovine serum albumin.

To explore the effect of SDS denaturation on the characteristics of BSA, a series of experiments were performed. Using SEC of the nonreduced BSA protein, the amount of dimer and higher-order aggregates could be determined. The addition of a light scattering detector with dynamic light scattering (DLS) capability allowed the measurement of both molecular weight and hydrodynamic radius (Rh). The eluent chosen for this series of experiments was phosphate buffered saline (PBS), containing 10 mM phosphate buffer, and 140 mM NaCl, pH 7.4 (Eluent A). A second mobile phase was prepared in an identical manner, but with the addition of 2 % w/v SDS (Eluent B). The HPLC instrument was then run with increasing levels of SDS incorporated in the mobile phase by varying the proportions of Eluent A and Eluent B.

Throughout the experiment, the same BSA sample was used, prepared at a concentration of 10 mg/mL in PBS alone.

The experiment was designed to determine:

- If SDS can denature BSA oligomers
- What effect is observed on the molecular weight measured using inline light scattering detection
- What change in hydrodynamic radius is observed

Conditions

Parameter	Value
Column:	Agilent AdvanceBio SEC 300 Å, 2.7 µm, 7.8 × 300 mm (p/n PL1180-5301)
Samples:	BSA (Sigma-Aldrich)
	Sample prepared 10 mg/mL in Eluent A
Eluent A:	PBS, pH 7.4 (10 mM phosphate, 140 mM NaCl)
Eluent B:	PBS, pH 7.4 (10 mM phosphate, 140 mM NaCl) + 2.0 % w/v SDS
Gradient:	Isocratic elution at 0 % B; 10 %B; 20 % B; 30 % B; 40 % B; 50 % B; 60 % B; 70 % B
Flow rate:	0.8 mL/min
Detector:	UV, 280 nm; LS 15°, LS 90°
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC with Agilent 1260 Infinity Bio-SEC multidetector system.

Results and Discussion

Following two blank injections to allow for the column to become conditioned in the mobile phase, duplicate injections of BSA 10 mg/mL were made.

Figure 3 shows overlaid chromatograms (UV 280 nm signal) of four individual experiments at different mobile phase compositions. Table 1 contains RT information relating to the entire experimental series.

From Table 1, it is apparent that there is little change in total peak area for different runs. This indicates that increasing SDS concentration does not result in the absorption of the protein, or loss of protein through induced aggregation. RTs become shorter but stabilize from 0.6 to 1.4 % SDS (w/v), as seen in Figure 4. This shortening of RT also results in lower resolution between the peaks, so it proved difficult to accurately determine how much dimer and higher-order aggregates became denatured. It is clear that these multimer peaks are still abundant and must, therefore, be predominantly covalently linked.

Table 1. RT and total peak area.

	RT (min)			
% SDS In mobile phase	Higher-order aggregates	Dimer	Monomer	Total peak area
0.0 %	6.00	6.46	7.29	3,632
0.2 %	5.22	5.59	6.45	3,633
0.4 %	4.96	5.23	5.96	3,668
0.6 %	4.89	5.15	5.83	3,588
0.8 %	4.89	5.13	5.80	3,571
1.0 %	4.89	5.11	5.77	3,572
1.2 %	4.88	5.11	5.76	3,536
1.4 %	4.90	5.10	5.75	3,566

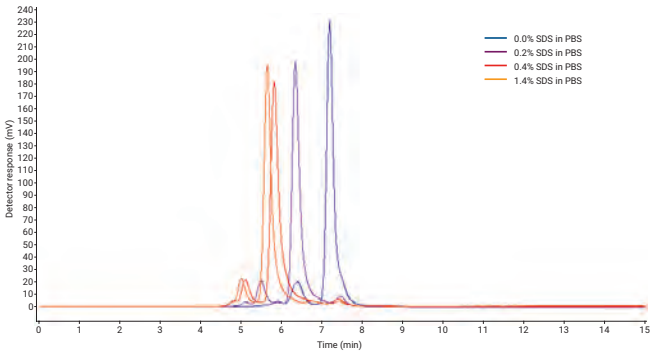

Figure 3. SEC chromatograms of BSA with increasing SDS concentration in mobile phase.

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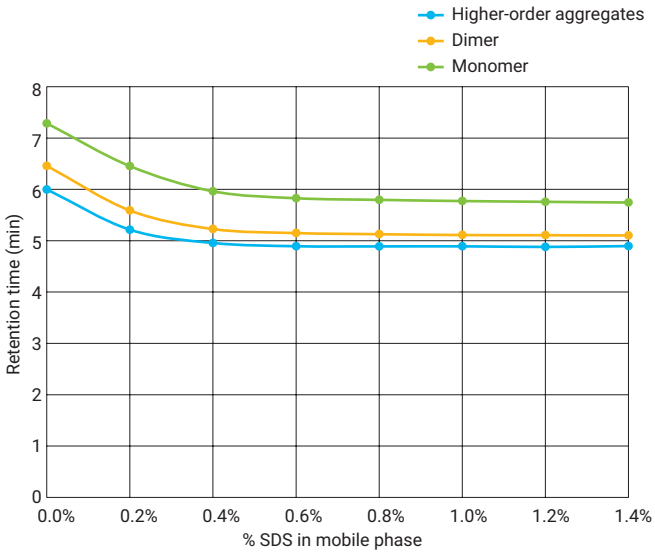

Figure 4. Effect of % SDS in mobile phase on RT.

In SEC, shorter RT is indicative of an increase in the size of a molecule in solution, but does not necessarily infer an increase in molecular weight. To ascertain the molecular weight of the three major peaks of the chromatogram, the BioSEC software was used to calculate the molecular weight using the relationship described in the following formulae [6]:

Where:

(*LS*) = Light scattering detector signal

K_{LS} = LS detector constant

dn/dc = Specific refractive index increment*

(*UV*) = UV detector signal (280 nm)

ε = Extinction coefficient

* Using the approximation of *dn/dc* ≈ 0.186 mL/g for nonglycosylated proteins

Figure 5 shows the regions of each peak chosen to perform the light scattering molecular weight determination to try to minimize interference due to anticipated peak overlap. Table 2 shows the results for the molecular weight measurements. This table includes columns where the relative number of monomer units has been calculated. It is important to recognize that the molecular weight of the higher-order aggregates peak is nearly always three times larger than the molecular weight of the monomer peak, indicating it is a trimer. The molecular weight of the dimer peak is two times larger than the molecular weight of the monomer peak, as expected. In addition, the molecular weight of each of the peaks increases with increasing SDS concentration until a plateau is reached at 0.6% w/v SDS, in agreement with the stabilization of RTs at this point.

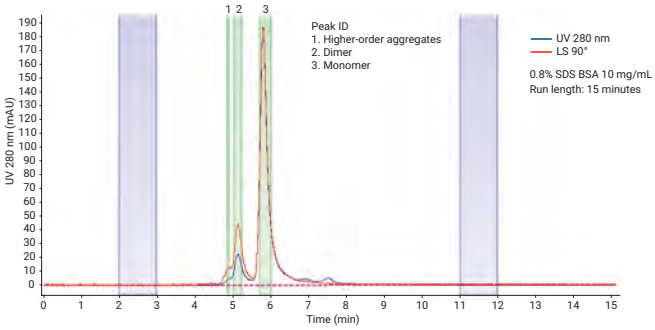

Figure 5. Molecular weight calculation regions for (1) higher-order aggregates, (2) dimer, and (3) monomer peaks of BSA.

Table 2. Molecular weight analysis results from light scattering detection.

	Mass gain			SDS:BSA (g/g)		
SDS w/v%	Higher-order aggregates	Dimer	Monomer	Higher-order aggregates	Dimer	Monomer
0.0	202,197	136,383	67,838	3.0	2.0	1.0
0.2	242,541	194,876	107,905	2.2	1.8	1.0
0.4	560,154	359,239	203,737	2.7	1.8	1.0
0.6	667,318	423,418	212,596	3.1	2.0	1.0
0.8	615,726	404,898	207,028	3.0	2.0	1.0
1.0	624,648	404,634	205,868	3.0	2.0	1.0
1.2	617,385	405,618	206,376	3.0	2.0	1.0
1.4	612,031	406,699	206,614	3.0	2.0	1.0

Table 3. Mass gain for BSA with increasing surfactant concentration, showing steady state at concentrations >0.6%.

	Mass gain			SDS:BSA (g/g)		
SDS w/v%	Higher-order aggregates	Dimer	Monomer	Higher-order aggregates	Dimer	Monomer
0.0	0	0	0	0.0	0.0	0.0
0.2	40,344	58,493	40,067	0.2	0.4	0.6
0.4	357,957	222,856	135,899	1.8	1.6	2.0
0.6	465,121	287,035	144,758	2.3	2.1	2.1
0.8	413,529	268,515	139,190	2.0	2.0	2.1
1.0	422,451	268,251	138,030	2.1	2.0	2.0
1.2	415,188	269,235	138,538	2.1	2.0	2.0
1.4	409,834	270,316	138,776	2.0	2.0	2.0

Since the observed molecular weight increases in line with the concentration of SDS present in the mobile phase, it may be inferred that each molecular species is actually gaining mass through accumulation of associated SDS. However, the observed mass gain is considerable and higher than expected, particularly since this BSA sample has not been reduced (Table 3). The steady state mass gain under the analysis conditions is 2 g SDS per 1 g protein, suggesting that SDS micelles may be accumulating along the protein molecule regardless of the number of oligomers it contains.

Closer inspection of the concentration-dependant UV trace of the chromatograms obtained at 0.0 % SDS and 1.4 % SDS shows further differences. There is clearly a reduction in resolution for the higher molecular weight species, however some smaller peaks eluting after the monomer peak have become evident in the 1.4 % SDS mobile phase composition (Figure 6). It was not possible to identify these lower molecular weight species.

Figure 7A shows the signals from the LS 90° detector for BSA at 0.0 % SDS and 1.4 % SDS concentrations. Since the light scattering detector is mass sensitive rather than concentration-dependant, it is not surprising that the signal obtained under the higher surfactant concentration conditions is much larger than the signal under native conditions.

In addition to the increased response seen in Figure 7A at the highest SDS concentration, there is also a vertical offset. The reason for this becomes apparent when the DLS data are analyzed to determine the Rh. Not only do we see an increase in the Rh of the monomer peak (from around 3.8 to 5.7 nm, close to literature values) [7,8,9], there is a continual background of particles detected of approximately 2.4–2.8 nm in size, almost certainly due to SDS micelles in solution.

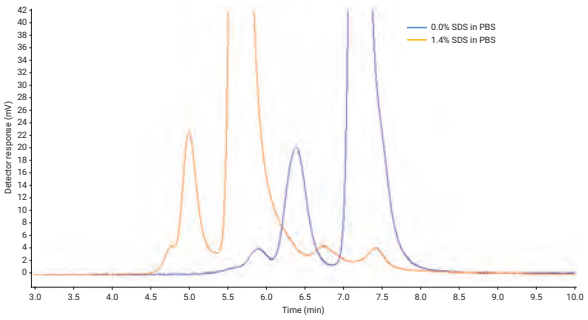

Figure 6. Close up of the UV 280 nm signal of BSA in PBS mobile phase containing 0.0 % SDS and 1.4 % SDS.

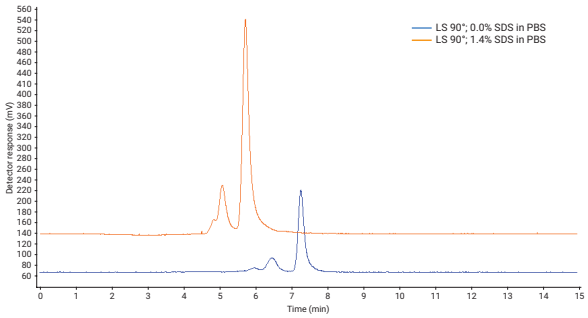

Figure 7A. Overview of the LS 90° signal of BSA in PBS mobile phase containing 0.0 % SDS and 1.4 % SDS.

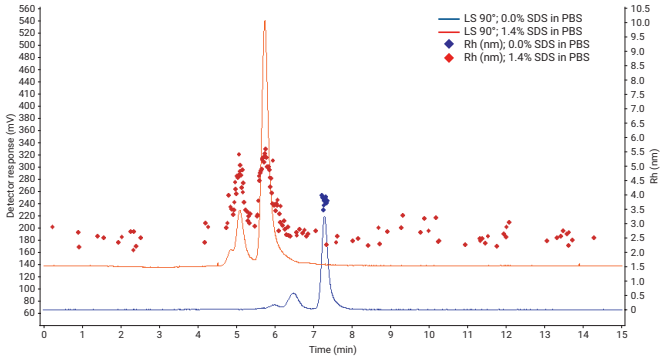

Figure 7B. Overview of the LS 90° signal of BSA in PBS mobile phase containing 0.0 % SDS and 1.4 % SDS with hydrodynamic radius (Rh) data added.

Conclusions

At the outset, this experiment was designed to determine if SDS can denature BSA oligomers using SEC with light scattering detection. It was found that accurate quantification of oligomer content was not possible through loss in resolution as the individual peaks corresponding to monomer, dimer, and higher-order aggregates eluted closer together. However, it is apparent the three individual species remain intact and become saturated with SDS to the same extent.

Simply denaturing the protein, making the molecule unfold, would be expected to give a larger structure in solution, leading to a shorter RT in SEC. However, as the SDS concentration was increased from 0.0 to 0.4 % w/v, the molecular weight of the monomer, dimer, and higher-order aggregates increased proportionally. At higher SDS concentrations (0.6–1.4 % w/v) the RTs (and, therefore, size in solution) and the molecular weight determined by light scattering stabilized as the molecular species were saturated by SDS.

Using DLS capability to determine hydrodynamic radius shows an increase in size comparable to literature values for a reduced BSA sample. The size increase does not reflect a change in conformation of the BSA sample run under nonreducing conditions, but replicates the observed increase in molecular weight as the molecule complexes with and becomes saturated by SDS micelles.

It is clear that, although the Agilent AdvanceBio SEC 300 Å column is able to tolerate SDS in the mobile phase, as seen by the good peak shape and reproducible recovery, the SEC separation is badly affected by the dramatic increases in size and molecular weight of the protein species being analyzed. The use of surfactants in the mobile phase for SEC should be avoided wherever possible.

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Bio SEC-3

Part Number	Title
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Additional Information

A number of standards are available to assist with method development, molecular weight estimates, and system QC testing.

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