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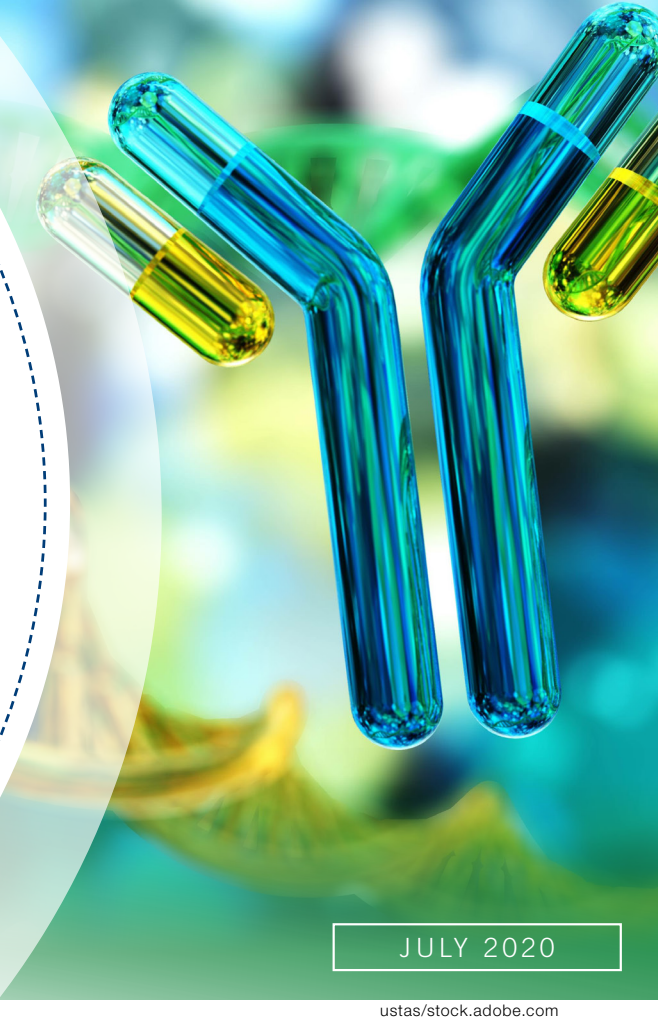
New Techniques and Workflows for Biopharmaceutical Analysis



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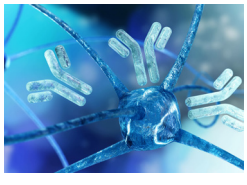
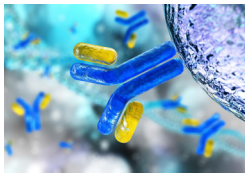


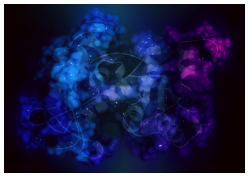
Workflows for Released N-Glycan Analysis and Sialic Acid Quantitation of Biotherapeutics

Aled Jones



Native and Denaturing Mode Size Exclusion Analysis of Biotherapeutics

Andrew Coffey and Veronica Qin



High-Resolution Size Exclusion Chromatography Analysis of Small Therapeutic Proteins

Sandeep Kondaveeti, Te-Wei Chu, and Andrew Coffey

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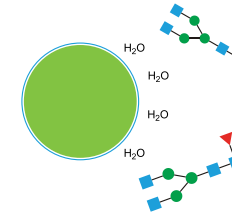

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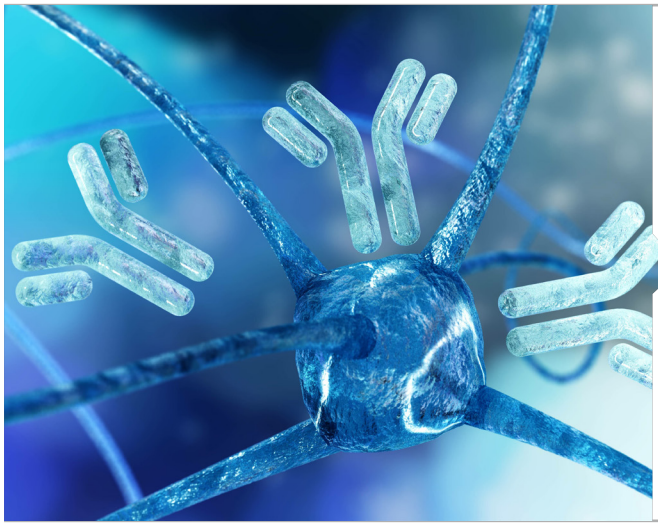

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Workflows for Released N-Glycan Analysis and Sialic Acid Quantitation of Biotherapeutics

Aled Jones

Rapid N-glycan sample preparation workflows for biotherapeutics.

Introduction

Approximately one-fifth of all proteins are glycosylated and the N-glycan moieties attached to biotherapeutic glycoproteins can affect immunogenicity, pharmacokinetics, and pharmacodynamics, making glycosylation a critical quality attribute (CQA). Therefore, the characterization of N-glycans is an essential part of the biotherapeutic glycoprotein development process. This summary discusses rapid N-glycan sample preparation workflows for biotherapeutics, the release and labeling of N-glycans, the separation of labeled N-glycans using liquid chromatography (LC) and capillary electrophoresis (CE), and glycan dyes that provide increased fluorescence and mass spectrometry (MS) detector response when using LC instruments. In addition, a plate-based method for the quantitation of total sialic acid content of glycosylated molecules is presented.

Importance of N-Glycan Analysis in Biotherapeutic Development

Although glycomics does not get, the same attention as genomics and proteomics, glycosylation can affect protein folding, stability, function and diversity based on differences in glycosylation sites, glycan composition, glycan length, and overall structure. More than 60% of all marketed biotherapeutics are glycosylated: in 2010, out of the 10 top-selling pharmaceutical products, only one was a glycosylated molecule. However, as of 2018, that number increased to seven. The two main types of glycosylation are the addition of O-linked glycans and N-linked glycans, but N-linked glycosylation,

where glycans are attached to the amide nitrogen of asparagine residues within a defined consensus sequon is the glycosylation type most seen in biotherapeutics.

Biotherapeutics are significantly more complex than small molecules. For example, aspirin is a standard small molecule drug that has a molecular weight of 180 Daltons with 21 atoms. A typical biopharmaceutical based on immunoglobulin G (IgG) has a molecular weight of 150,000 Daltons, around 20,000 atoms, and may have a variety of posttranslational modifications (PTMs) including glycosylation, all of which have to be monitored during production. The glycan profile of biosimilars (i.e., drugs that are high similar to an original innovator or reference biopharmaceutical) also must be assessed during production.

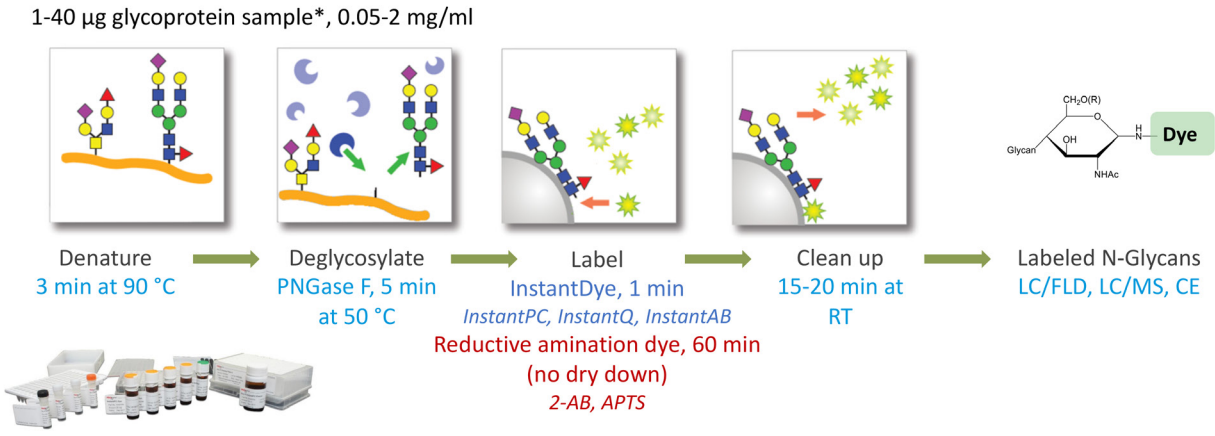
Effect of N-Glycan Structure on Biotherapeutics

Glycosylation is one of the PTMs that are monitored during biotherapeutic development. Monoclonal antibodies (mAbs), based on recombinantly expressed IgG, are the largest class of N-glycosylated biotherapeuticsand IgG has a single N-glycosylation site at asparagine 297, within the Fc region. There may also be additional N-glycan sites in Fc fusion biotherapeutics, if the fusion partner contains them, or in other classes of biotherapeutics such as blood products.

The effect of N-glycan structures present on a biotherapeutic vary depending on the molecule and the target. Sialic acid at the non-reducing terminal of the glycan can reduce the rate of clearance, increasing the amount of time that the therapeutic stays in the body. Terminal sialic acid may also be anti-inflammatory, as is the case for alpha-(2,6)-linked sialic acid on intravenous immunoglobulin (IVIG). Galactosylation can increase complement-dependent cytotoxicity (CDC) activity and core fucose reduces ADCC activity important in anti-cancer therapeutics. Some companies are focused on various methods to reduce the amount of core fucose in therapeutics in order to maximize the antibody-dependent cell-mediated cytotoxicity (ADCC) activity of a molecule. The presence of high mannose N-glycans can increase clearance via the mannose receptor, so this is a structure class that is frequently assessed when developing biotherapeutics. High mannose N-glycans, such as Man5, are immature N-glycan structures that may be indicative of a suboptimal cell culture process where, for example, cells are in a state of stress from being driven toward a higher titer, a frequent aim during production of therapeutics. Non-human glycan structures such as the alpha-Gal epitope

Workflows for Released N-Glycan Analysis and Sialic Acid Quantitation of Biotherapeutics

Figure 1: Schematic of the Gly-X N-glycan sample preparation platform.



and N-glycolylneuraminic acid (NGNA or NeuGc) are also monitored.

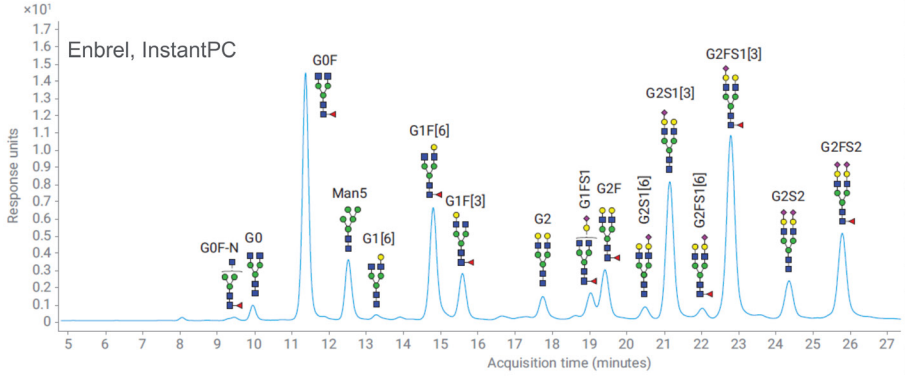
N-Glycan Analysis by Hydrophilic Interaction Liquid Chromatography (HILIC)

Knowing which glycan structures are present is part of understanding the activity of a biotherapeutic, particularly if glycosylation is known to be a CQA, and several analysis options are available for their characterization. Intact glycoprotein analysis with high resolution mass spectrometry after reduction or separation of the protein into domains can provide a good general idea of the glycan species present. Digestion of glycoproteins into peptides and glycopeptides followed by MS coupled with a database is useful for giving site-specific glycosylation and site occupancy information. Another option is to study "released" glycans, or glycans cleaved from the glycoprotein with enzymes like PNGase F, which are then labeled to enable fluorescence detection after HILIC or CE separations. Glycans may also be fragmented into individual monosaccharide species and quantified for sialic acid content.

Sample preparation of released N-glycans prior to analysis has traditionally been very time consuming, often taking several days. However, modern technology, using faster deglycosylation and reductive amination dyes has reduced sample preparation time to less than an hour or two, as shown in **Figure 1.** As seen in **Figure 1**, a sample is first denatured with a

Figure 2: HILIC/FLD separation of InstantPC N-glycans from etanercept (Enbrel).

- Etanercept: Fc fusion protein
- Tumor necrosis factor receptor (TNFR) fused to Fc of IgG1
- 3 N-glycan sites: 1 in Fc, 2 in receptor
- N = 4



	Average Rel % Area	Standard Deviation	%CV
G0	1.10	0.02	2.09
G0F	19.36	0.16	0.84
Man5	5.08	0.03	0.52
G1[6]	0.48	0.00	0.00
G1F[6]	10.48	0.04	0.39
G1F[3]	3.97	0.01	0.25
G2	2.08	0.01	0.55
G1FS1	1.84	0.05	2.49
G2F	4.26	0.09	1.99
G2S1[6]	1.18	0.01	0.49
G2S1[3]	13.91	0.04	0.31
G2FS1[6]	0.89	0.00	0.00
G2FS1[3]	20.54	0.08	0.37
G2S2	4.26	0.01	0.14
G2FS2	10.54	0.08	0.78

reagent which opens the protein for deglycosylation by PNGase F. The glycans are then labeled with a choice of fluorophore depending on the separation technique and cleaned up using a vacuum-driven filter plate. The labeled glycans are then ready for separation.

Several labeling choices are available that include either instant labeling or reductive amination. Instant dyes label the glycosylamine-form of the glycan in a very fast process. InstantPC (IPC) is a commercial label with a procaine core that facilitates fluorescence detection and a tertiary amine that results in a strong MS signal in the positive mode. It also has an NHS-carbamate linker that allows conjugation of the glyscosylamine form of N-glycans after they have been released from the protein by PNGase F. The IPC label is most commonly used for HILIC separations.

The most popular type of LC separation of N-glycans is HILIC paired with fluorescence detection (FLD). HILIC mobile-phase solvent gradients and methods can be adjusted depending on the molecule and the complexity of the glycan profile in the sample. An example of a HILIC/FLD separation of IPC-labeled N-glycans from etanercept (Enbrel) is presented in **Figure 2**, showing the structure of the N-glycan species.

MS hyphenation may also be used to assign masses to glycan peaks within a separation. An example of a HILIC separation with both FLD and positive-ion MS detection of the IPC-labeled N-glycans from Enbrel is presented in

Figure 3: HILIC separation with FLD and positive-ion MS detection of InstantPC N-glycans from etanercept (Enbrel).

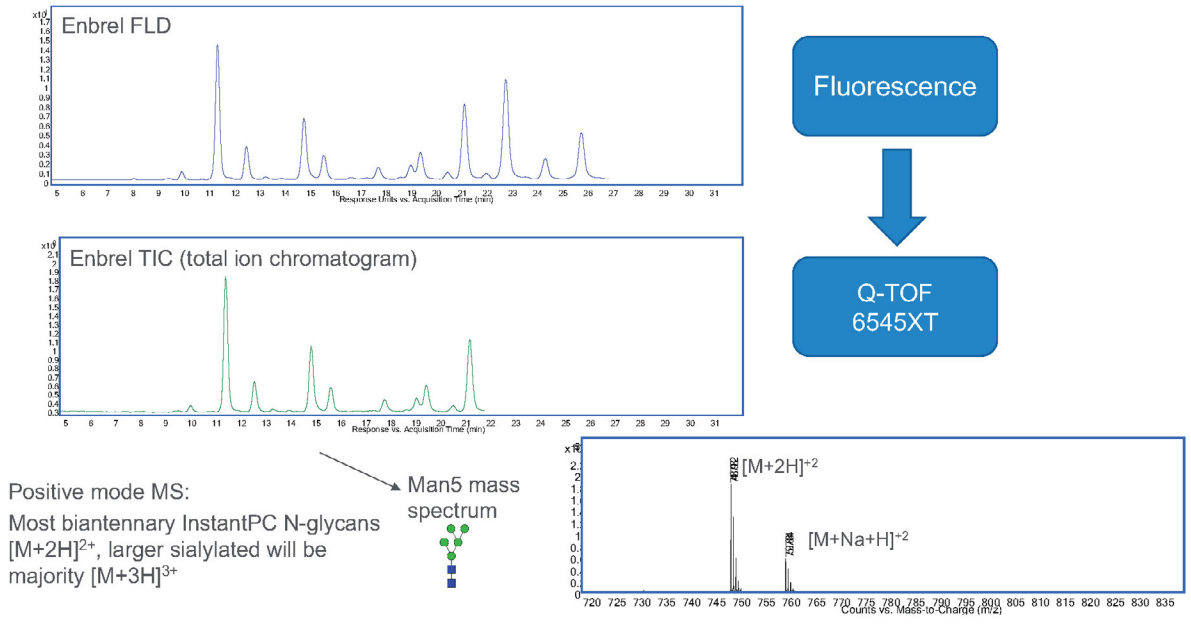


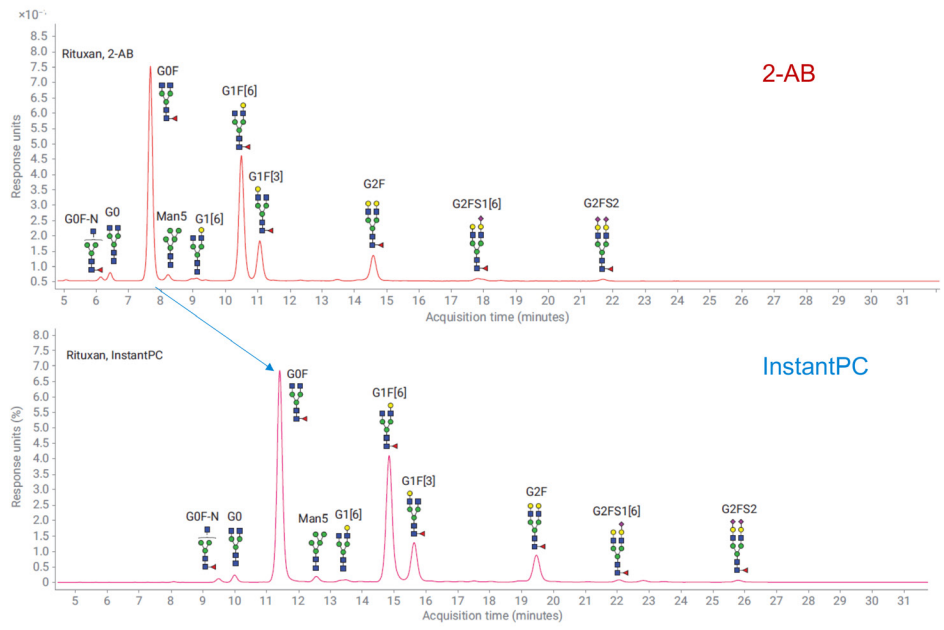
Figure 3. The FLD response is mirrored by the MS total ion chromatogram (TIC) with peak identification accomplished by extracting a mass spectrum at any point in the TIC. A range of qualitative N-glycan standards labeled with IPC are available to assess HILIC/FLD/MS retention times and masses.

Other labeling options like 2-AB take advantage of reductive amination. Since N-glycan labeling with 2-AB has been well-established in many laboratories it may also be used to compare with historic data. Labeling with 2-AB takes a little longer than IPC, and makes the N-glycan less polar and amendable to HILIC, but also introduces a negative charge. It does, however, result in a lower FLD and MS signal compared to IPC-labeled N-glycans. The elution order of 2-AB N-glycans is similar to that of IPC-labeled N-glycans, but the HILIC retention time is shorter, as presented in a separation of rituximab (Rituxan) N-glycans in **Figure 4**.

N-Glycan Analysis by CE

CE is another commonly used separation technique for the analysis of N-glycans using 8-aminopyrene-1,3,6-trisulfonic acid (APTS) labeling. APTS is a labeling option in the simple and rapid sample preparation technique described previously. After a cleanup step to remove free APTS that can

Figure 4: HILIC separations of rituximab: Gly-X 2-AB versus InstantPC.



N=4

	Average Rel % Area	Standard Deviation	%CV
G0F-N	0.78	0.09	11.94
G0	1.64	0.05	3.12
G0F	44.89	0.39	0.87
Man5	1.54	0.14	8.83
G1F[6]	31.39	0.09	0.27
G1F[3]	10.40	0.14	1.34
G2F	7.52	0.16	2.10
G2FS1	1.17	0.03	2.13
G2FS2	0.67	0.02	3.58

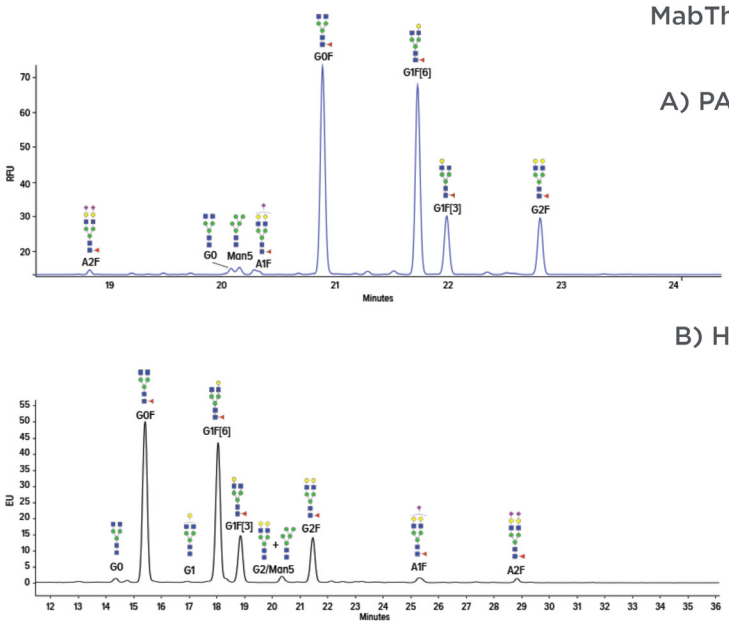
	Average Rel % Area	Standard Deviation	%CV
G0F-N	0.75	0.01	1.55
G0	1.47	0.02	1.18
G0F	46.82	0.07	0.15
Man5	1.21	0.01	0.83
G1[6]	0.75	0.02	2.67
G1F[6]	31.21	0.11	0.35
G1F[3]	9.27	0.05	0.54
G2F	7.04	0.04	0.51
G2FS1[6]	0.67	0.02	2.29
G2FS1[3]	0.37	0.06	15.98
G2FS2	0.45	0.03	6.67

interfere with detection early on in the analytical window, the APTS-labeled glycan samples can be separated by CE, as shown in **Figure 5** (top), or HILIC (bottom). While it provides useful information on its own, CE is also often used as an orthogonal method for HILIC since selectivity (elution order) is different. As demonstrated in Figure 5, it is particularly advantageous when there are co-elutions obtained with either technique. Sialylated glycans move faster through the capillary due to the extra charge, so they elute first in the CE separation followed by the neutral glycans. Similar to IPC, a range of APTS labeled N-glycan standards and sialylated bi-, tri- and tetra-antennary libraries are available to aid in identification and quantitation.

Sialic Acid Quantitation

Sialic acid serves a critical role in mediating the effectiveness of biotherapeutics. The presence or absence of sialic acid on the non-reducing terminal of N- or O-glycans can affect the pharmacokinetics of the protein as well as its immunogenicity. Therefore, it is essential that the sialic acid content of biotherapeutics is monitored. Total sialic acid quantitation can be performed using a plate-based colorimetric assay and a plate reader.

Figure 5: APTS N-glycan standards and libraries separated by CE (top) and HILIC (bottom).



Development of a Rapid APTS Sample Preparation Workflow for N-Glycan Release and Labeling
5994-0994EN

Total sialic acid quantitation is a sensitive, high-throughput approach based on a coupled enzyme reaction. Sialic acid residues are released using sialidase A, and a standard curve of N-acetylneuraminic acid (NANA or Neu5Ac) is prepared alongside samples. Sialic acids and are then converted enzymatically to hydrogen peroxide, which reacts with a dye stoichiometrically to form a fluorescent reporter dye complex, that may be read using a plate reader with fluorescence or absorbance detection. The plate-based approach allows enzymatic release of sialic acid, conversion, with detection and quantitation performed in a single well for fast and simple processing, and with a broad range of detection: 40–1,000 pmol with fluorescence, and 500–4,000 pmol when using absorbance. However, it does not provide information on the relative proportion of sialic acid species present. **Figure 6** shows total sialic acid quantitation data obtained with a number of biotherapeutic glycoproteins (MabThera, Enbrel, Zaltrap and EPO alfa) as well as a control protein, fetuin. Intra-assay %CVs are low, demonstrating the intra-assay reproducibility.

Conclusion

There are many workflows options available for N-glycan analysis of biotherapeutic glycoproteins. Simple, fast and automatable sample preparation for N-glycan analysis can

Figure 6: Total sialic acid quantitation data demonstrating intra-assay repeatability.

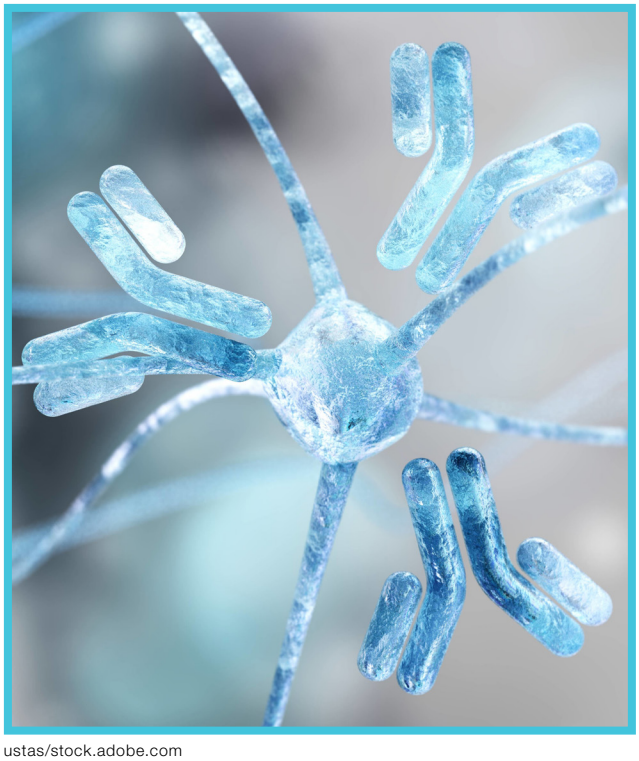
- Single operator, 3 replicates per sample
- SA - sialic acid

	Fetuin	MabThera	Enbrel	Zaltrap	Orencia	EPO alfa
Starting concentration (mg/mL)	0.25	10	0.25	0.5	0.5	1
MW (kDa)	48	145	150	115	92	30.4
nmol SA/10 µL sample	0.58	0.08	0.55	0.45	0.59	0.16
nmol SA/mg protein	232.53	0.79	220.93	90.27	117.27	15.50
mol SA/mol protein	11.16	0.11	33.14	10.38	10.79	0.47
%CV (n=3)	0.55%	2.65%	1.81%	0.46%	0.60%	3.59%
SD (mol SA/mol protein)	0.06	0.00	0.60	0.05	0.06	0.02

be accomplished using a choice of labeling techniques, including instant labeling by IPC for LC/FL/MS analysis, reductive amination by 2-AB for LC/FL/MS analysis, and APTS for open platform CE analysis. Glycoproteins can be further characterized for their total sialic acid content in a plate-based assay.

The characterization of N-glycans for their CQAs is an essential part of the biotherapeutic glycoprotein development process and the variety of chemistries, the availability of standards, multiple separation and detection technologies and the option of MS for the assignment of glycan structures to peaks by their predicted mass is essential to that success.

Aled Jones, PhD, *is the marketing manager for bioconsumables at Agilent.*



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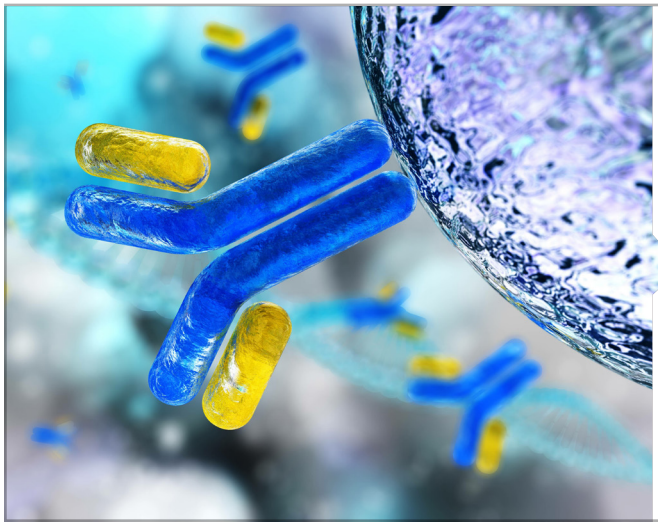

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Native and Denaturing Mode Size Exclusion Analysis of Biotherapeutics

Andrew Coffey and Veronica Qin

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Useful tips and examples.

Introduction

High-performance liquid chromatography (HPLC) and mass spectrometry (MS) are becoming fundamental methods for research and quality control of biotherapeutics. Detailed analytical characterization of these biopharmaceutical proteins to assess the aggregation levels and other potential variations is critical for ensuring product quality and safety. Size-exclusion chromatography (SEC) is a popular analytical method used to quantify the aggregation and purity of proteins. Agilent's SEC column portfolio, including the AdvanceBio SEC columns with sub-2 µm particle technology, delivers high-quality analysis of biotherapeutics for a variety of sample modalities. Selection of the appropriate column, particle size and pore size significantly impacts data quality in both denaturing and native mode biotherapeutic analysis.

Size-Exclusion Chromatography of Biotherapeutics

Biotherapeutic molecules must undergo a wide range of testing regimes to determine identity and purity. Particular attention is paid to impurities that could impact the safety and efficacy of a drug candidate. Many analytical tests are based on LC and can be conducted under native, intact, non-denaturing conditions, which are largely composed of aqueous buffer conditions at near-neutral pH. Unfortunately, such conditions are generally not directly compatible with MS, so that adjustments to the mobile phase are required before MS interfacing. Other LC techniques involve organic solvents and are performed under denaturing conditions that commonly result in a

denatured and perhaps fragmented sample. Such techniques are typically more compatible with MS detection.

SEC columns for bioanalysis. SEC is seeing increased use for characterization of both native and denatured biomolecules. Because of its versatility, there are several columns available for bioanalysis that cover a broad range of molecular weights and sample types. Agilent has introduced the AdvanceBio 1.9 µm 120 Å and 200 Å columns to address the need for a sub-2 µm column for small proteins and polypeptides as well as larger proteins such as monoclonal antibodies. The coated silica stationary phases in these columns reduce the level of non-specific interactions to achieve high data integrity. The quest for higher column efficiencies is the driving force behind the use of smaller and smaller particles. A 50% reduction in particle size doubles the efficiency, which can help resolve closely eluting species. Moreover, peaks are much sharper and better resolved, producing an increase in sensitivity. However, the consequence of a smaller particle size is a dramatic increase in operating pressure. This is alleviated somewhat by today's modern LC instruments, which are capable of operating at much higher pressures than traditional chromatography. Another potential concern is that these smaller particle-sized columns can become clogged more easily or could trap large species present in a sample.

As such, it is important to recognize that choosing the correct combination of particle size and pore size is essential. Although column data and specifications are represented by a molecular weight resolving range, the column is actually separating molecules by differences in their hydrodynamic radius. If a molecule has a different shape than typical globular proteins, it will elute at a different time. For example, a calibration curve of oligonucleotides will produce a significantly different result than that of proteins. Oligonucleotides elute much earlier than expected based on their molecular weight alone when compared with globular proteins. This is because elongated molecules have a larger hydrodynamic radius.

Most separations are performed under non-denaturing conditions using mobile phases that are aqueous and at neutral pH. Phosphate buffered saline (PBS), or simply 150 mM sodium phosphate, at pH 7.0 will yield optimum results. Nonetheless, some methods, for historical reasons, have required the use of much harsher mobile phase

"SEC is seeing increased use for characterization of both native and denatured biomolecules."

"Most good analytical methods include some form of system suitability test; it is advisable to regularly monitor the performance of a column across its entire working range."

conditions. In the case of insulin, it was necessary to use acidic conditions with a mobile phase containing an organic modifier and arginine. Aggregate analysis of insulin using the AdvanceBio SEC 120 Å 1.9 µm column in these conditions has demonstrated excellent resolution between the monomer and high molecular weight species.

The manufacturing techniques developed for the AdvanceBio SEC columns ensure that non-specific interactions are minimized. A coating on the stationary phase enables high-quality separations over a wide variety of sample types, with superior recovery and peak shapes compared to uncoated stationary phases. In addition to quantifying dimers and higher order protein aggregates, researchers are interested in measuring low molecular weight species, such as misfolded proteins or a monoclonal antibody (mAb) with a missing light chain. The small particle sizes with proprietary coatings provide high efficiency separations with sufficient resolution to facilitate detection of these challenging constituents.

The improved column robustness means that columns can be operated at even higher flow rates for faster separations and higher throughput. The new AdvanceBio SEC 200 Å 1.9 µm 4.6 x 150 mm column can be operated at flow rates as high as 0.7 mL/min. When Agilent introduced the AdvanceBio SEC 300 Å 2.7 µm 15 cm column, its higher flow rate capability could achieve sample throughput of up to 280 samples in 24 hours. The new AdvanceBio SEC 200 Å 1.9 µm can handle up to 480 samples in the same amount of time, representing a 71% increase in productivity.

Best practices. Most good analytical methods include some form of system suitability test; it is advisable to regularly monitor the performance of a column across its entire working range. For this reason, vendors often recommend a standard containing a mixture of suitable molecules covering the resolving range of a column. It is important to use a standard that is appropriate for the pore size of the column, and this will allow any problems to be detected early in the characterization process.

When using sub-2 µm SEC columns, efficiency can be maximized by minimizing dead volume, ensuring secure connections, moderating the flow rate, and using high data collection

rates. The use of low dispersion LC with minimal tubing ID and length is key to optimizing the performance of the entire system. Employing 0.12 mm ID tubing will reduce the extra column volume by 50% compared with 0.17 mm ID tubing. In addition, dead spots, leakage, peak tailing, and other problems caused by bad connections can be eliminated by using the spring-loaded design of Agilent's A-Line Quick Connect fittings that achieve secure, reliable connections even at high pressures.

As with all SEC columns, to extend column lifetime, changes in the mobile-phase flow rate should be made slowly. This can sometimes be done automatically in the LC instrument and software. Methods should start at a low flow rate, such as 0.1 mL/min, and gradually increase flow in increments of no more than 0.1 mL/min until the intended operating flow rate is reached.

The high efficiency of small particles calls for faster data collection rates to avoid missing critical peaks. Data collection rates of 10–20 Hz could result in a 4–5% reduction in column efficiency compared with 40 or 80 Hz. Faster rates accommodate the sharp peaks generated by the sub-2 µm particles.

SEC can also be used for very large molecules; with an increase in hydrodynamic radius comes a need to use larger pore sizes. Larger particle sizes may also be necessary to reduce the risk of the sample clogging the column or being sheared by forces generated during flow. Flow rate and other parameters would likely require adjustment as well. In some cases, it may be beneficial to run two columns in series for best results.

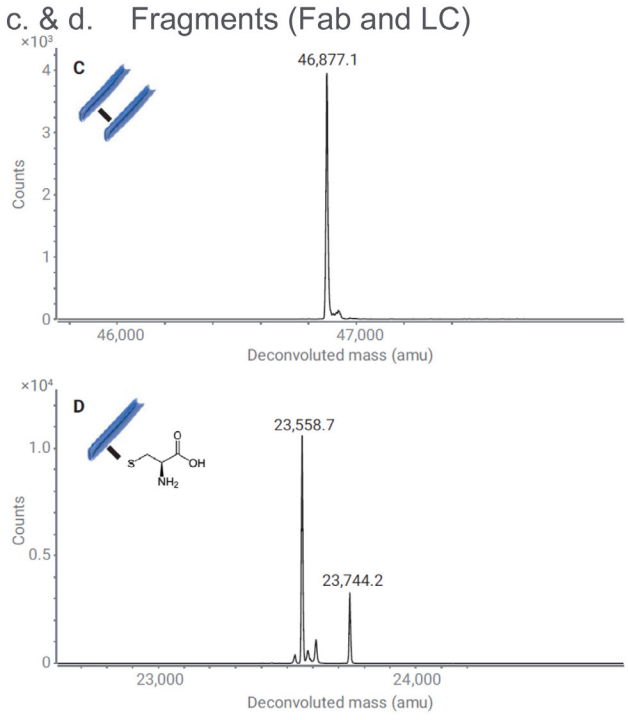
Applications

Valuable information provided by MS has resulted in the increased popularity of SEC–MS for biotherapeutic analysis. Since SEC mobile phases generally consist of relatively high salt and non-volatile buffers, it is not possible to directly couple SEC to mass spectrometry using this kind of mobile phase. Serious ion suppression and possible MS instrument contamination would result. Thus, SEC-MS is usually run under either denaturing conditions or native mode with MS-compatible mobile phases.

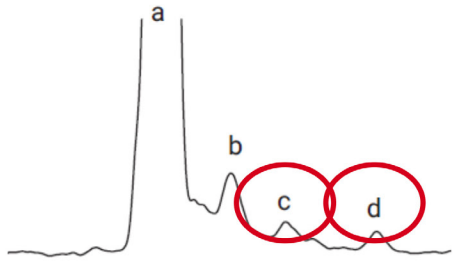
Denaturing SEC–MS. Denaturing mobile phase conditions, such as aqueous acetonitrile with formic acid and trifluoroacetic acid (TFA) ion pair reagents, could unfold proteins and help with ionization of the molecules in the MS electrospray (ESI) source. These conditions increase the hydrodynamic size of the molecule so that it may elute earlier than expected. Choosing the correct SEC column, such as the AdvanceBio SEC 200 Å 1.9 µm, ensures that the background spectrum will be very

Native and Denaturing Mode Size Exclusion Analysis of Biotherapeutics

Figure 1: Conducting SEC under denaturing conditions: intact trastuzumab.



LC Conditions	1290 Infinity LC System + 6540 Q-TOF
Column Dimensions	Agilent Bio SEC-3 300Å 3µm, 7.8 x 300 mm
Mobile phase	20% ACN, 0.1% FA, 0.1% TFA
Temperature	25 °C
Sample	Trastuzumab (intact)
UV detection	214 & 280 nm



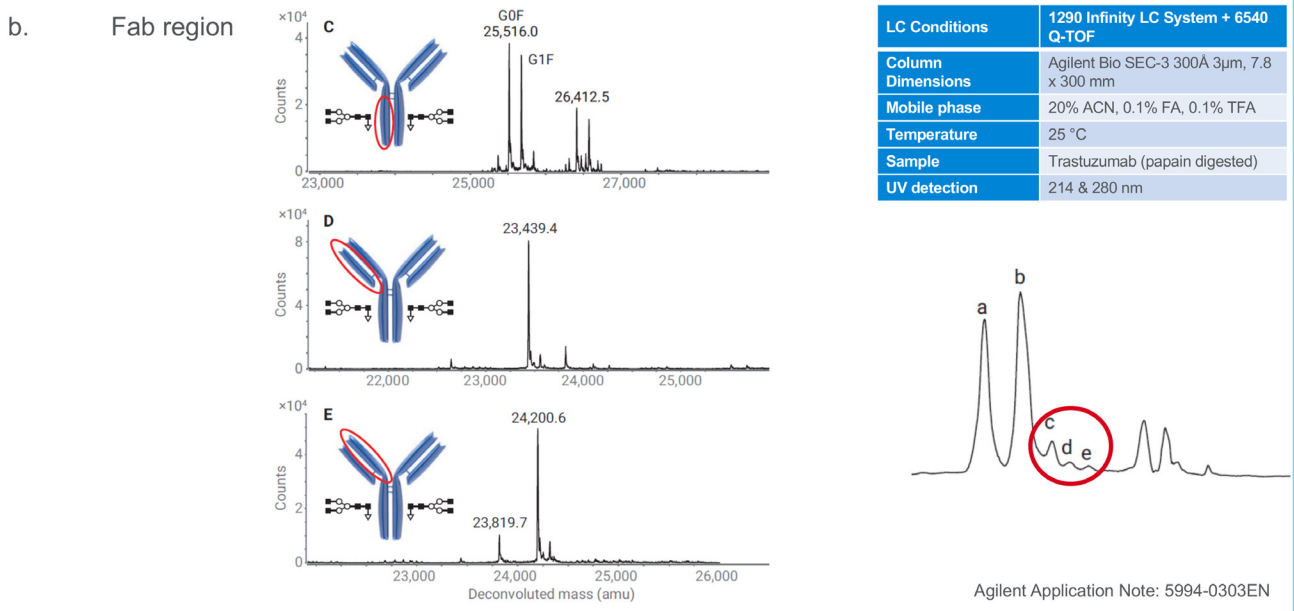
Agilent Application Note: 5994-0303EN

clean. This is important because there will be many multiple-charged ionized species under denaturing conditions which reduce the overall sensitivity as the count level for each species is much lower.

One of the strengths of MS detection is the identification of small impurities. For example, SEC LC–UV–MS of intact trastuzumab under denaturing conditions (20% acetonitrile and 0.1% FA and 0.1% TFA) revealed three impurities, as shown in the lower right of **Figure 1**. The main peak, labeled a, was easily identified as the intact monomer (with associated heavy chain glycan pattern), and peak b was a "half" antibody comprising a single heavy chain and single light chain, including the N-linked glycan structure associated with the asparagine (Asn) side chain. The second and third impurity peaks, c and d, were lower molecular weight smaller species associated with antigen-binding (Fab) and light chain (LC) fragmentation of the intact molecule.

By treating the sample with papain to form Fab and crystallizable (Fc) fragments, additional information could be acquired. Shown in **Figure 2**, four impurities were evident in the resulting chromatogram. Peaks c, d, and e were attributed to partial fragments similar to those observed in the analysis of the intact molecule, where

Figure 2: Conducting SEC under denaturing conditions: papain-digested trastuzumab.



LC Conditions	1290 Infinity LC System + 6540 Q-TOF
Column Dimensions	Agilent Bio SEC-3 300Å 3µm, 7.8 x 300 mm
Mobile phase	20% ACN, 0.1% FA, 0.1% TFA
Temperature	25 °C
Sample	Trastuzumab (papain digested)
UV detection	214 & 280 nm

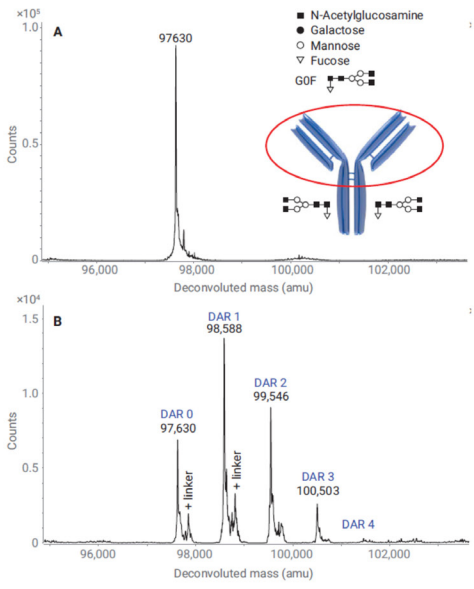
Agilent Application Note: 5994-0303EN

peak c represented the Fc fragment, d the light chain fragment, and e the heavy chain fragment from the Fab region. The molecular weight of these species is extremely similar and yet they were clearly resolved by the Bio SEC-3 300 Å column. It is possible that these molecules have slightly different structures (from more rod-like to more globular) even under these denaturing conditions, or perhaps some secondary interactions were occurring that helped to resolve these otherwise similar mass species.

Also under denaturing conditions, IdeZ-treated trastuzumab and IdeZ-treated adoTrastuzumab emtasine antibody drug conjugate (ADC) were compared by SEC LC–UV–MS. ADCs generally have increased hydrophobicity, which can lead to problems with peak shape as a result of secondary interactions that are not typically observed with unconjugated mAbs. Furthermore, there are multiple locations around the ADC molecule that can become conjugated. This leads to multiple variants containing none, one, or several copies of the cytotoxic drug-linker. By interfacing the Agilent Bio SEC-3 300 Å 3 µm column with MS, the different species were identified without complications.

The first peak to elute for each sample, the $F(ab')_2$ fragment, is shown in **Figure 3**. The native mAb yielded a single mass corresponding to the correct molecular weight. In the lower trace,

Figure 3: Conducting SEC under denaturing conditions: IdeZ-treated mAb and ADC—F(ab')$_2$ region.



Trastuzumab F(ab')$_2$

adoTrastuzumab Emtasine F(ab')$_2$

LC Conditions	1290 Infinity LC System + 6540 Q-TOF
Column Dimensions	Agilent Bio SEC-3 300Å 3µm, 7.8 x 300 mm
Mobile phase	20% ACN, 0.1% FA, 0.1% TFA
Temperature	25 °C
Sample	Trastuzumab (IdeZ digested) Ado-Trastuzumab Emtasine (IdeZ digested)
UV detection	214 & 280 nm

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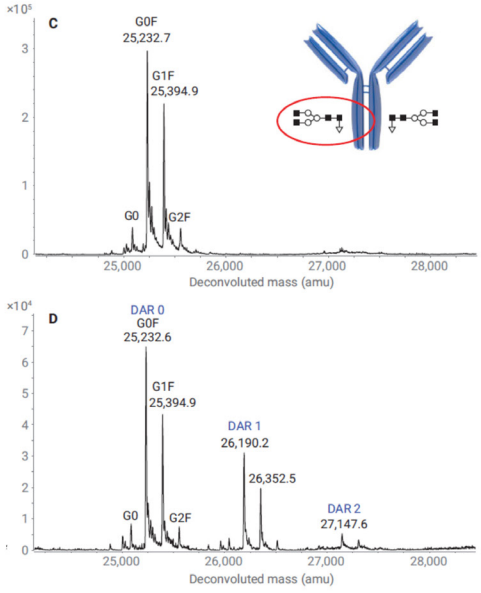
there were clearly several species with increasing numbers of drug-linker attached; DAR refers to the drug to antibody ratio and is a vital measure in the analysis of these compounds.

The other major peak corresponded to the Fc portion of the molecules. The familiar glysoylation pattern is evident in the top trace of **Figure 4**, which is repeated in the Fc portion of the ADC, but with the addition of the DAR1 and DAR2 species. There were up to four DAR species identified in the previous figure and there are four more shown in **Figure 4** (two for each half of the Fc region), giving the possibility of up to eight copies of drug per antibody. The value of accurate SEC–MS is unambiguously demonstrated in this application.

Native mode SEC-MS. The case for implementing native mode separations may be made by examining myoglobin under both denaturing and native conditions. **Figure 5** illustrates that under denaturing conditions, the MS data from the monomer peak of myoglobin show similar results on the AdvanceBio SEC 1.9 µm 120 Å versus a column from another vendor. However, the TIC (ion chromatograms) indicates an improved separation of the dimer peak from the

> "The other major peak corresponded to the Fc portion of the molecules."

Figure 4: Conducting SEC under denaturing conditions: IdeZ-treated mAb and ADC—Fc region.



LC Conditions	1290 Infinity LC System + 6540 Q-TOF
Column Dimensions	Agilent Bio SEC-3 300Å 3µm, 7.8 x 300 mm
Mobile phase	20% ACN, 0.1% FA, 0.1% TFA
Temperature	25 °C
Sample	Trastuzumab (IdeZ digested) Ado-Trastuzumab Emtasine (IdeZ digested)
UV detection	214 & 280 nm

Agilent Application Note: 5994-0303EN

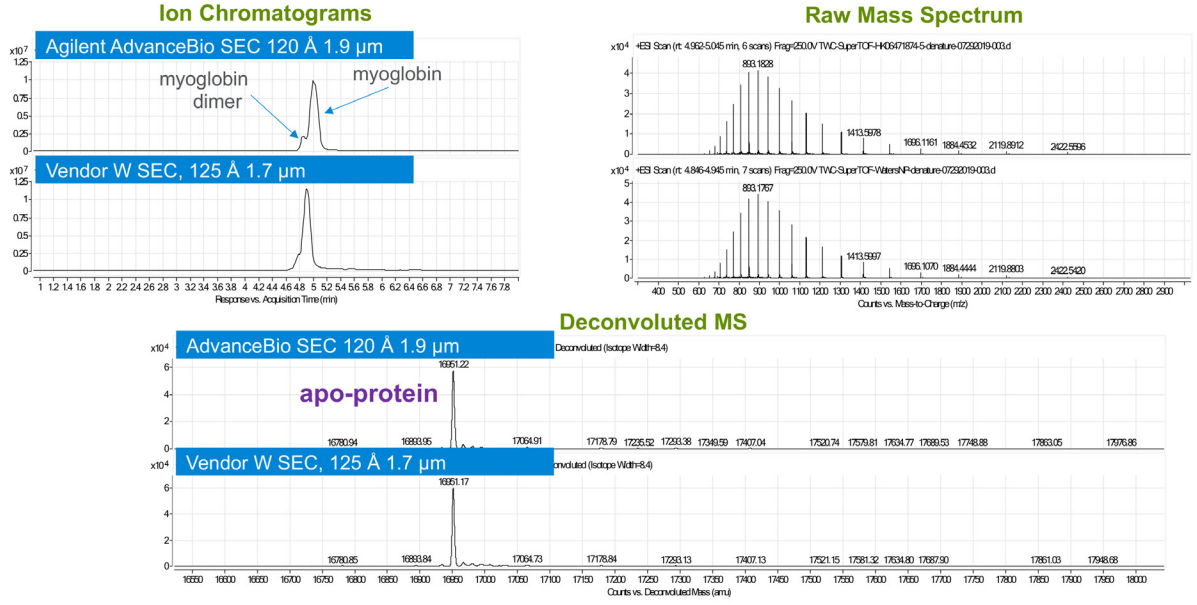
monomer peak on the AdvanceBio column. Under denaturing conditions, noncovalent complexes are disrupted so that only the apo-protein, missing its heme group, is observed; the holo-protein, which has the heme group intact, is not evident. The deconvoluted MS shows the correct mass for apo myoglobin (horse) at 16951 Da.

The same separation under native mode conditions with 50 mM ammonium acetate, shown in **Figure 6**, reveals that the protein elutes from the column as expected, but the peak shape on the column from another vendor is now showing signs of secondary interactions with a broader peak shape and later elution time. This illustrates the advantage of using Agilent's coated particles for more reliable separations. The benefit of using SEC–MS under native mode conditions is that not only is the apo-protein observed, but significant amounts of the holo-protein are also evident, with the heme group intact. In addition, notice that the raw spectrum appears very different for the apo-protein compared with the holo-protein.

It is important to note that not all biotherapeutic proteins are mAbs and ADCs. Numerous companies are

> "The deconvoluted MS shows the correct mass for apo myoglobin (horse) at 16951 Da.

Figure 5: Denaturing SEC/MS—Myoglobin (30% ACN, 0.1% FA, 0.02% TFA, 5 µg loading).



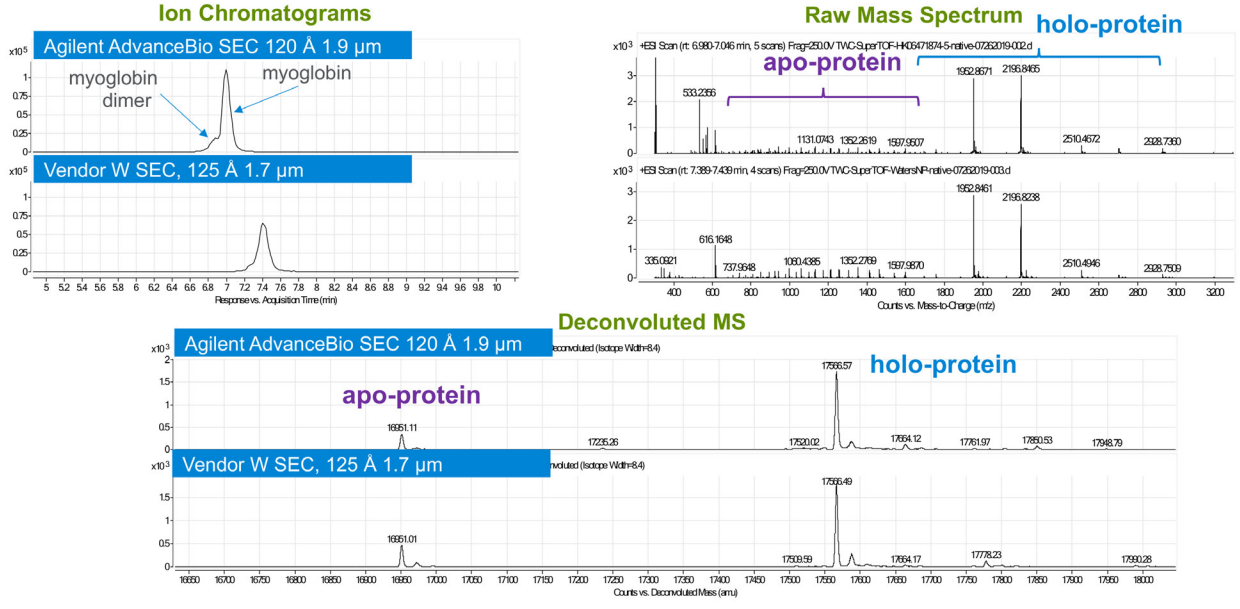
working on much smaller parts of these molecules or with different antibody structures such as those found in camelids, which are much smaller and easier to manufacture. Interestingly, these smaller molecules often behave extremely well on a SEC column under a wide range of mobile phase conditions. Even very low salt or very high salt concentrations do not adversely affect the peak shape or retention time. This enables the use of native mode SEC–MS with lower concentrations of ammonium acetate to reduce the level of ion suppression and improve the signal.

Conclusion

The utility of SEC lends itself to a broad assortment of applications for biotherapeutics analysis. This technique is amenable to both native mode and denaturing conditions, in addition to being compatible with MS for deeper characterization of drug candidates. With Agilent's suite of SEC columns, high-efficiency separations can be achieved with various sample types for analysis of impurities, fragments, aggregates, mAbs, ADCs, polypeptides, and proteins. Selection of an appropriate column and standards suitable for

> "Even very low salt or very high salt concentrations do not adversely affect the peak shape or retention time."

Figure 6: Native mode myoglobin LC/MS (50 mM ammonium acetate, 50 µg inj.).



the column pore size are essential for optimal measurements. Sub-2 µm coated particles allow faster separations and higher throughput with a significant reduction in non-specific interactions. The widespread applicability of SEC to diverse sample modalities has led to an escalation of its use for biopharmaceuticals.

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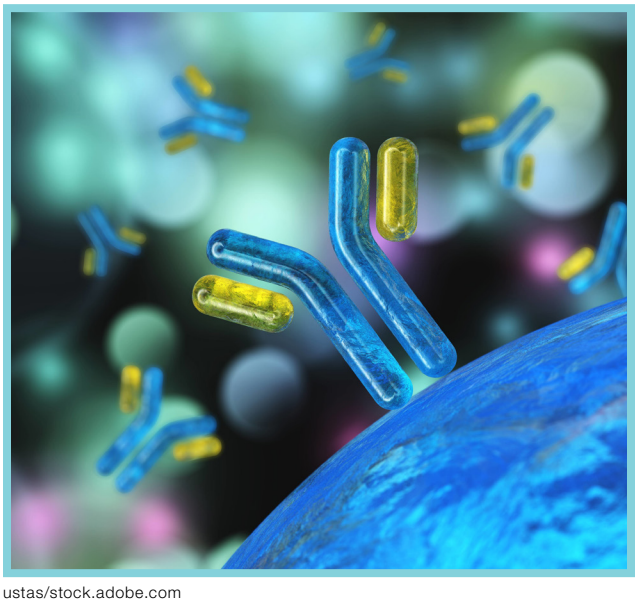

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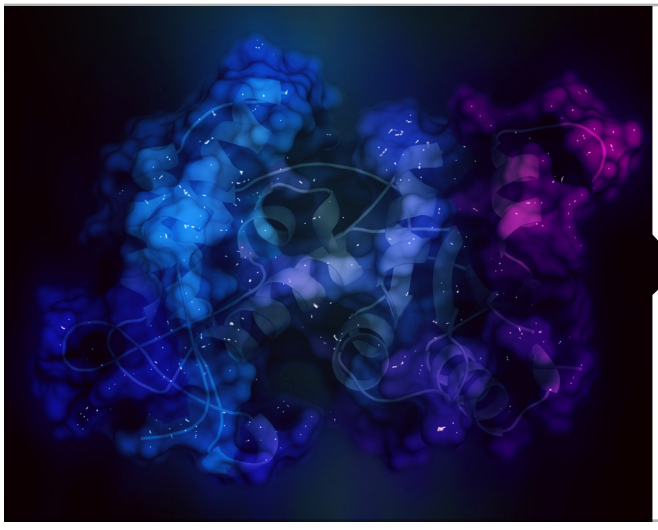

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High-Resolution Size Exclusion Chromatography Analysis of Small Therapeutic Proteins

Sandeep Kondaveeti, Te-Wei Chu, and Andrew Coffey

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) and high performance liquid chromatography (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 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

The method conditions are listed in **Table I.**

Table I: HPLC conditions.

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

High-Resolution Size Exclusion Chromatography Analysis of Small Therapeutic Proteins

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

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

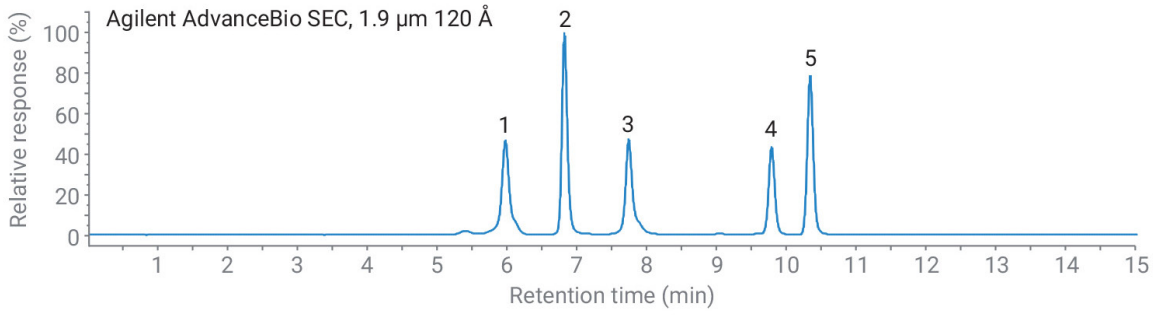
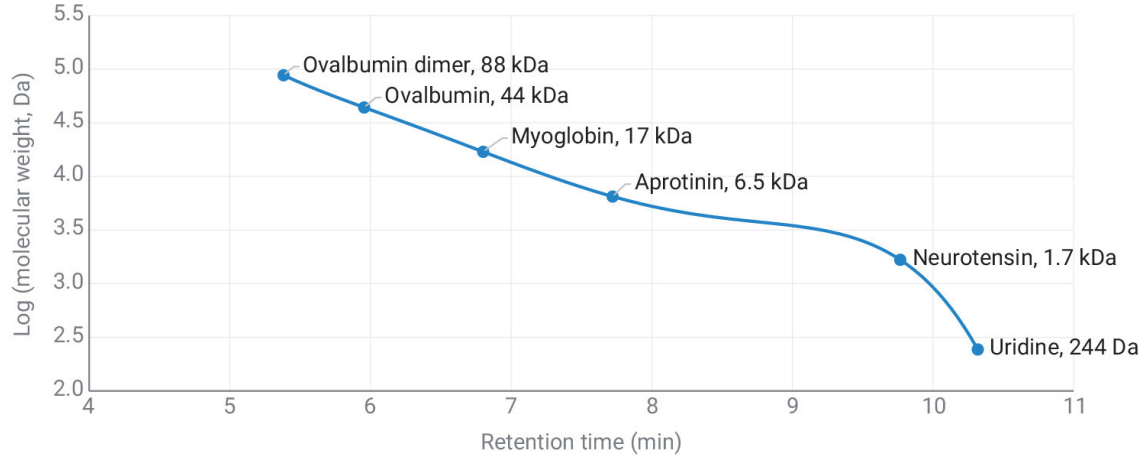


Figure 2: AdvanceBio SEC 1.9 µm 120 Å calibration curve of low molecular weight protein and peptide standards.



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 1–80 kDa.

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 provides 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.

"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."

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

High-Resolution Size Exclusion Chromatography Analysis of Small Therapeutic Proteins

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

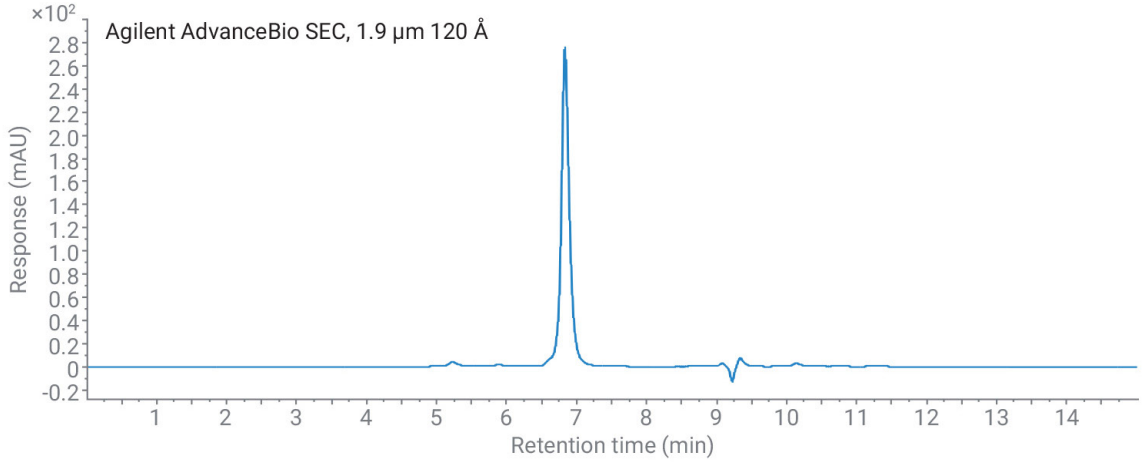
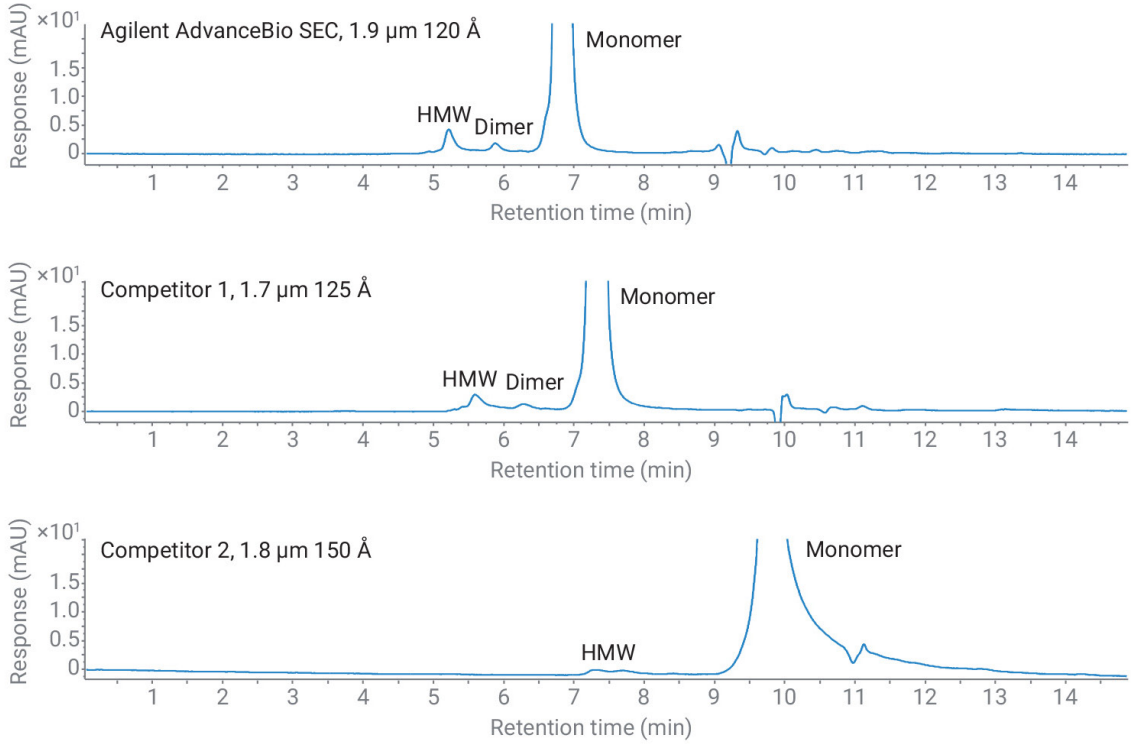


Figure 4: Close up of size-exclusion chromatograms of hG-CSF at 0.35 mL/min.

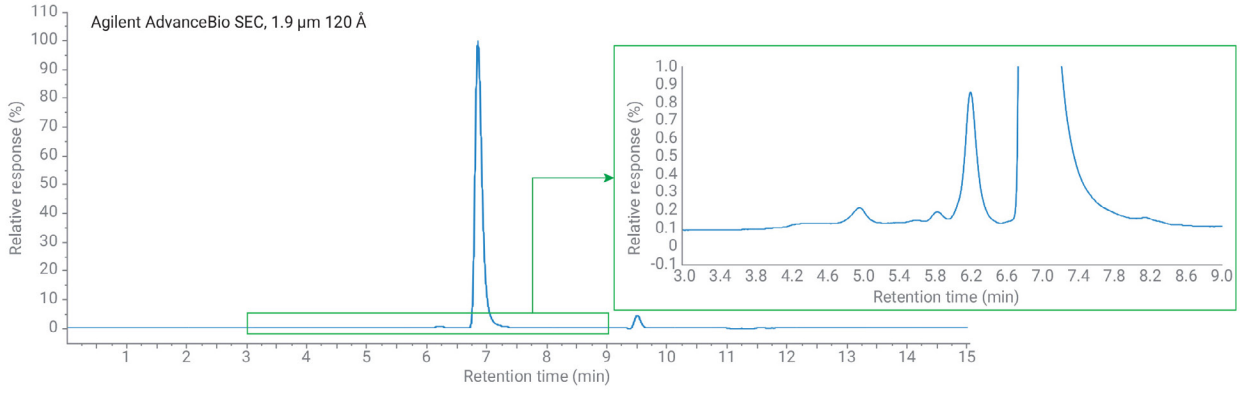


High-Resolution Size Exclusion Chromatography Analysis of Small Therapeutic Proteins

Table II: 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).



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).

Many other biotherapeutic proteins have similar molecular weights and are therefore also suitable for analysis on the same AdvanceBio SEC 1.9 µm

High-Resolution Size Exclusion Chromatography Analysis of Small Therapeutic Proteins

Table III: 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

"Other proteins may require further method development to obtain the optimum peak shape and resolution."

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 III**.

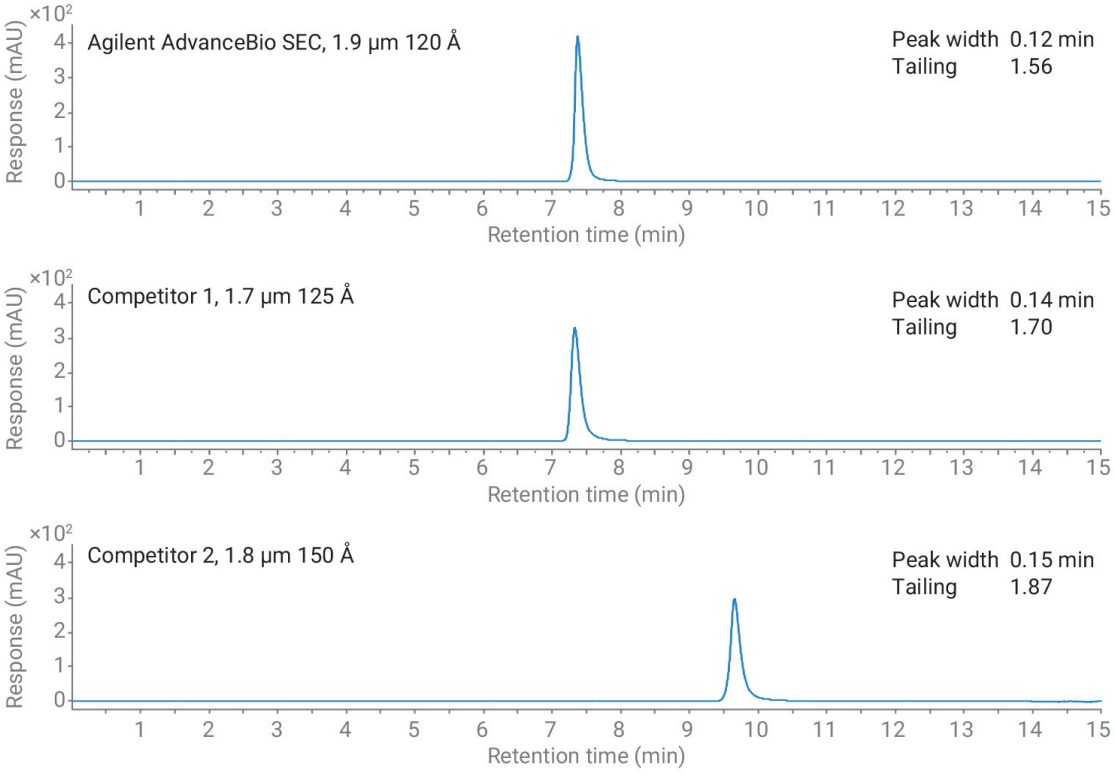
The size-exclusion chromatograms of interferon alpha-2b reference material run on three different sub-2 µm SEC columns is shown in **Figure 6**, along with the retention time and peak tailing data. The difference in column performance may lead to a difference in resolution when separating IFN α-2b impurities 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 molecules (4). The choice of cell line for

High-Resolution Size Exclusion Chromatography Analysis of Small Therapeutic Proteins

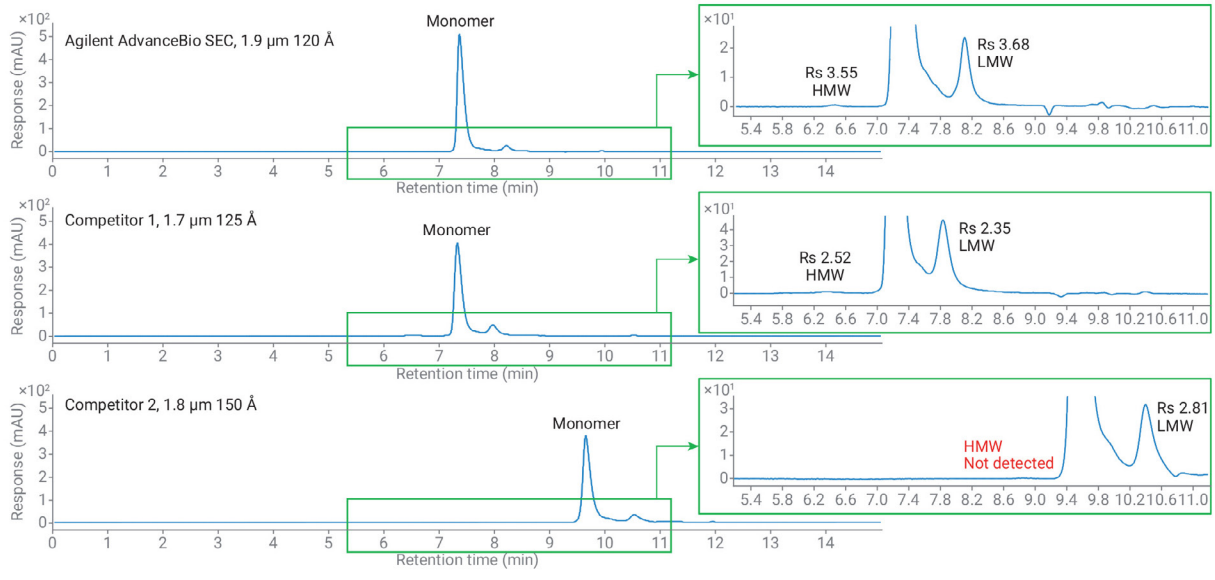
Figure 6: Size-exclusion chromatograms of interferon α-2b.

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



High-Resolution Size Exclusion Chromatography Analysis of Small Therapeutic Proteins

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



> **"In turn, corrective action can be taken ultimately reducing system downtime and improving productivity."**

recombinant protein manufacture is a critical parameter since *E. coli* cell lines do not introduce glycosylated variants.

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

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.

High-Resolution Size Exclusion Chromatography Analysis of Small Therapeutic Proteins

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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2. V.K. Sharma and D.S. Kalonia, *Pharm. Res.* 2003(20) 1721–1729 (2003).
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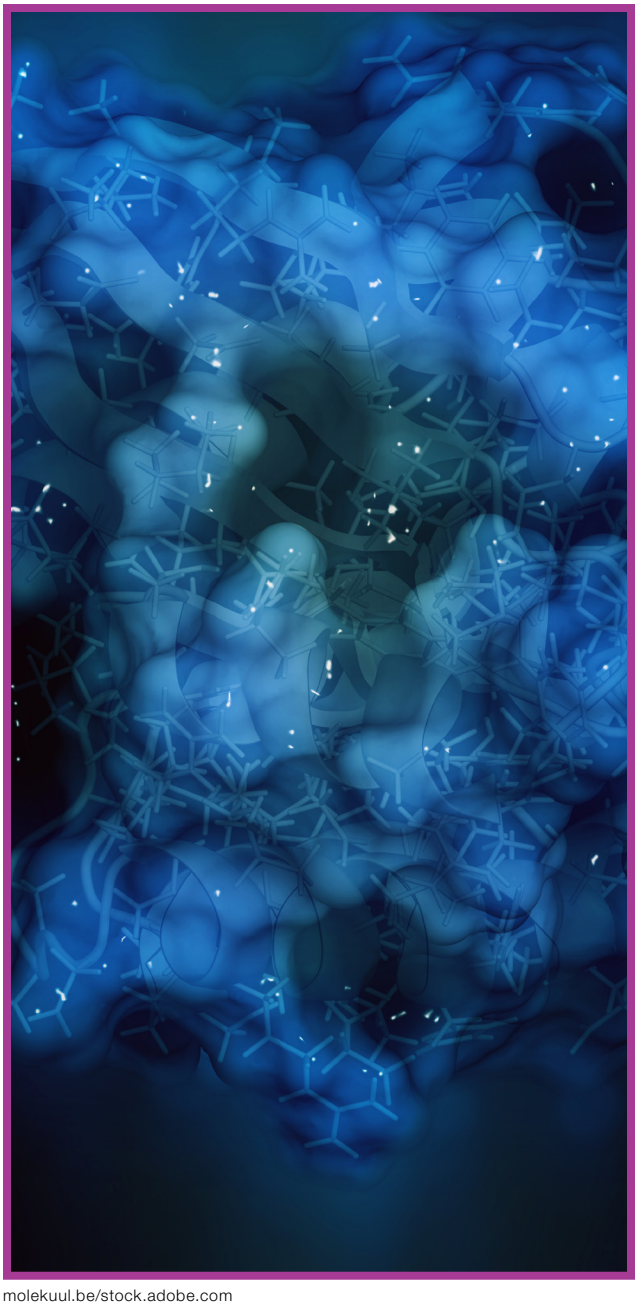

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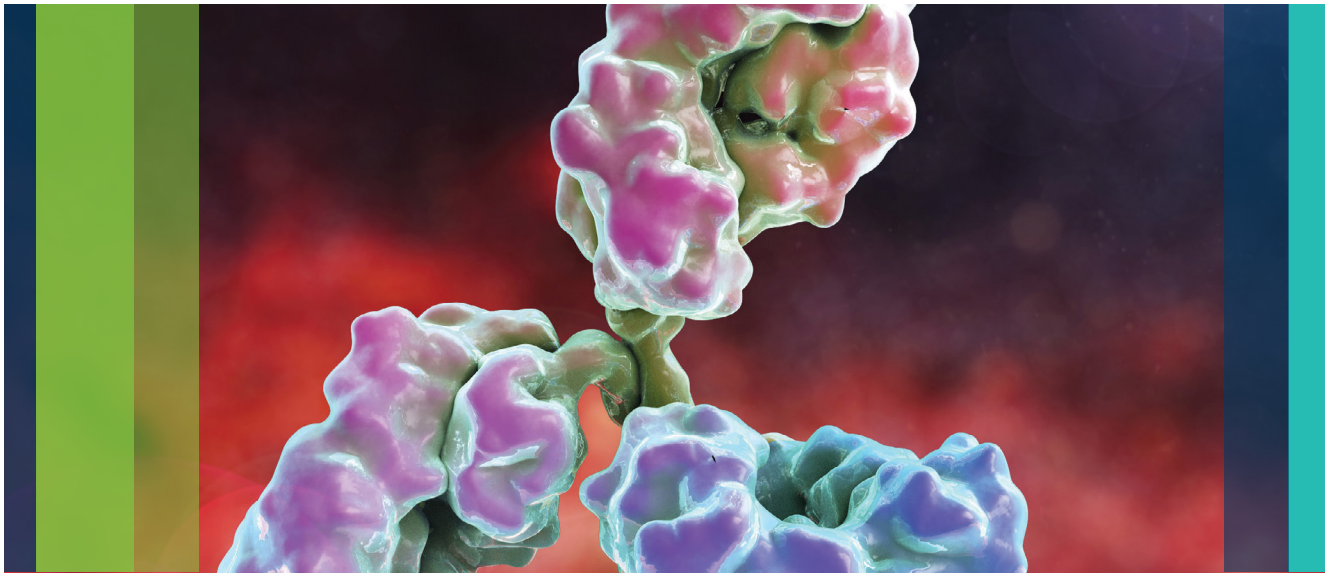

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