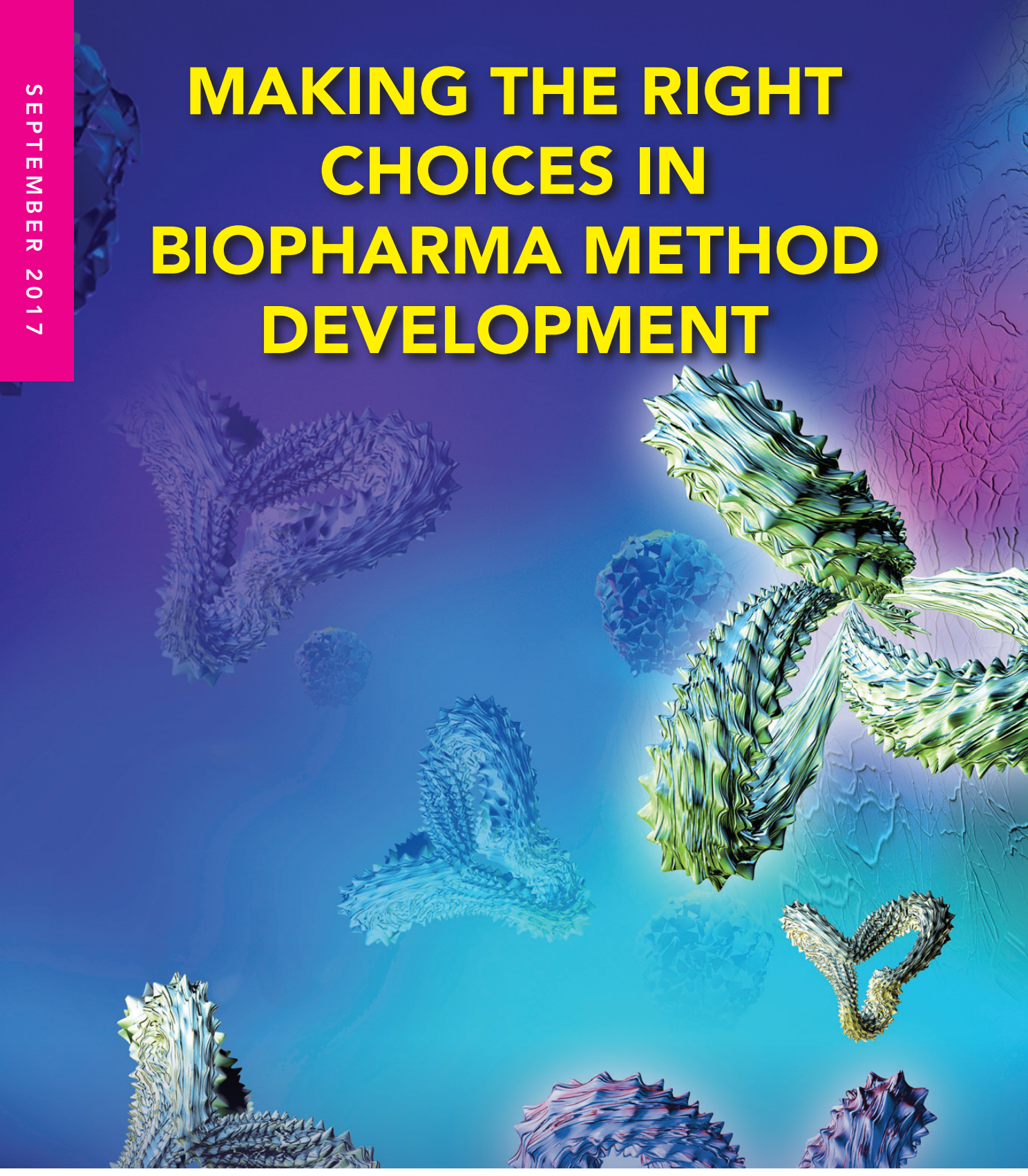
SEPTEMBER 2017

MAKING THE RIGHT CHOICES IN BIOPHARMA METHOD DEVELOPMENT



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INTRODUCTION

As the importance of biopharmaceutical therapies grows, there is increasing interest in analytical techniques that can improve the analysis of the compounds, the complexity of which has increased in recent years. For instance, bispecific monoclonal antibodies (mAbs), glycoengineered mAbs, fusion proteins, and antibody–drug conjugates are especially difficult and require modern tools and solutions for in-depth analysis.

In *Making the Right Choices in Biopharma Method Development*, an ebook with materials sponsored by Agilent and presented by *LCGC*'s CHROMacademy, analysts will learn about some important ways to ensure analytical methods for biopharmaceutical analysis are accurate and reproducible.

Andrew Coffey, a senior applications chemist at Agilent, starts the discussion by covering how critical variables in size-exclusion chromatography (SEC) can affect the quality and efficacy of the final biotherapeutic product. He details several parameters that can affect SEC separations of biopharmaceuticals, including pore size, column dimensions, temperature, flow rate, and mobile-phase composition.

Next, Dr. Coffey discusses column and mobile-phase selection and different approaches to mass-spectrometry analysis, including special considerations for analyzing ADCs.

Last, Tony Taylor, the technical director at Crawford Scientific Limited and *LCGC*'s CHROMacademy, interviews Koen Sandra, Dr., the scientific director of the Research Institute for Chromatography in Belgium, about the most challenging aspects of biopharmaceutical analysis and what solutions are available to address them.

The best practices and solutions highlighted in this ebook are sure to be very helpful to analysts involved in complicated biopharmaceutical analysis.

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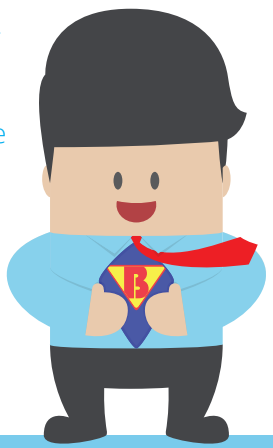

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MAKING THE RIGHT CHOICES IN SEC METHOD DEVELOPMENT FOR BIOTHERAPEUTIC SAMPLES

Andrew Coffey

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A Comprehensive Workflow To Optimize and Execute Protein Aggregate Studies

When developing optimum size-exclusion chromatography methods for biotherapeutic samples, it is important to understand how critical separation variables can affect the quality of the final product.

The Importance of Biotherapeutics

Monoclonal antibodies (mAbs) are the fastest-growing class of biopharmaceutical drugs. mAbs are of clinical importance because of their effectiveness in treating some of today's most devastating diseases such as cancer, rheumatoid arthritis, and multiple sclerosis. Scientists can engineer mAbs to trigger an autoimmune response so that our bodies can target these disease states and begin to heal themselves.

mAbs were originally produced from murine cell lines, which presented potential risks. Genetic engineering led to the development of chimeric cell lines, followed by humanized and, now, fully human protein sequences expressed in mammalian cell lines to create glycosylated

structures similar to those generated in our own cells.

Theses advancements have led to other adaptations including:

- Antibody–drug conjugates (ADCs, i.e., mAbs that act as highly specific drug delivery vehicles),
- Bispecific mAbs (i.e., mAbs that recognize two different antigens),
- mAbs that can cross the membrane into the brain, and
- Glycoengineered mAbs in which the glycan structure is further modified to improve efficacy and longevity.

Many of these molecules no longer fall under patent protection, which has spurred activity in developing biosimilars and biobetters. *Biosimilars* have similar properties, efficacy, and safety as the original molecule and are often more affordable. Although, it is not possible to create an identical version of the original biopharmaceutical due to the complexity of these drugs, in many cases, the molecules involved can be slightly different but still possess similar therapeutic performance.

Biobetters are modified molecules that demonstrate improved efficacy over previous therapies. These improvements, in some cases, are sufficient to lead to drug approval and patents. Biobetters are often produced by improving the glycosylation profile of the mAb. Scientists are now able to modify cell lines so that they perform the glycosylation and further enhance drug performance. **Table I** shows a list of biosimilars and biobetters that are currently in development, many of which are mAbs.

Table I: Biosimilars and biobetters currently in development (1).

Biopharmaceutical	Brand	Sales ($b)	Biosimilars	Biobetters
Humira	Adalimumab	$9.27	13	7
Remicade	Infliximab	$8.90	9	9
Enbrel	Etanercept	$7.87	21	8
Rituxan	Rituximab	$7.29	30	17
Herceptin	Tansuzumab	$6.40	24	12
Lantus	Insulin glargine	$6.40	5	2
Avastin	Bevacizumab	$6.26	14	9
Neulasta	Pegfilgratim	$4.10	14	9
Lucentis	Ranibizumab	$3.72	2	2
Aransep	Darbepoetin alfa	$3.00	4	2
Epogen/Procrit	Epoetin alfa	$3.73	69	26
Novoseven	Coagulation factor	$1.50	8	12
Neupogen	Filgrastim	$1.44	52	17

In Europe and the United States, biosimilars and innovator reference mAbs must have an identical amino acid sequence. From a regulatory standpoint, antibodies that are not highly similar must undergo greater preclinical and clinical evaluation. Abbreviated clinical programs are generally suitable for biosimilar mAbs showing molecular profiles and characteristics within the range of originator versions. Changes of quality attributes are acceptable only if they do not alter safety and efficacy.

Assessing the Quality and Efficacy of Biotherapeutics

Many variants can arise during the manufacture of a biotherapeutic. A mAb comprises more than 1,300 individual amino acids. The complexity of the molecule and the sources of the possible variations mean that a biotherapeutic comprises a mixture of hundreds or thousands of closely related compounds.

The biopharmaceutical characteristics typically investigated include:

- amino acid sequence
- amino acid composition
- structural integrity
- higher-order structures
- aggregation
- S-S bridges
- N- and O-glycosylation
- N- and C-terminal sequence
- charge variants
- deamidation/isomerization
- oxidation
- clipping

Identifying and quantifying these variants is challenging and can be achieved only by using a range of chromatographic techniques and mass spectrometry. Reversed-phase HPLC is used for protein identification and impurity profiling. Ion-exchange chromatography is used to look at charge variants. Hydrophilic interaction liquid chromatography (HILIC) is useful for glycan analysis. Aggregate analysis is performed using size-exclusion chromatography (SEC), which will be the focus of this discussion.

A range of quality attributes should be considered in the therapeutic molecule, such as product variants relating to the therapeutic molecule itself or others that can arise from the fermentation process (e.g., DNA, HCP, and cell culture components). Impurities such as buffer components or Protein A from the purification media can be introduced during isolation and purification. Drug attributes such as clarity, pH, and potency also must be assessed.

A critical-quality attribute (CQA) list is developed to assess what effect each quality attribute (e.g., a physical, chemical, or biological property) has on the final product. A scoring system is used to rank factors such as biological activity, pharmacokinetics, pharmacodynamics, and safety. Uncertainty is also rated relative to the likelihood that attributes could affect the final product.

The range of scores allows prioritization of the CQAs from high to low regarding their potential impact on the product as a basis for taking the steps needed to understand and monitor these influencing factors.

Aggregated proteins generally carry a higher risk in terms of efficacy and safety. An aggregated protein may be ineffective because the aggregates are deactivated, but it could also result in adverse effects including potentially life-threatening anaphylactic shock.

Consequently, dimers and higher-order aggregates must be resolved from the product and quantified and the product monitored closely during batch release and storage. SEC is the preferred technique to separate these species, although other techniques such as capillary electrophoresis (CE) should also be considered. In some cases, aggregates may be so large that SEC cannot be used to separate them.

SEC Method Development

SEC is a straightforward isocratic technique. Separation occurs by means of diffusion into and out of pores. Molecules are eluted based on their size in solution, with the largest molecules being eluted first. Interaction of sample molecules with the stationary phase must be suppressed, so pore size and eluent selection are important. Proteins can undergo aggregation when exposed to changes in pressure and temperature, so the SEC process itself can affect the level of aggregation and the results of the analysis.

Several parameters are important to consider when developing robust separations through SEC because of their potential effect on aggregation: pore size, column dimensions, temperature, flow rate, and mobile phase composition.

Pore size. Selecting the optimum pore size for the molecule being analyzed is important. The size of the molecule in solution should be considered relative to pore size. Protein monomers and dimers must be able to permeate the pore structure. Pore structure that is too small will affect the accuracy of the quantitative data.

A rule of thumb is that the pore size of stationary phase should be three times the diameter of the molecules of interest.

Ideally, peaks will be spread across a wide range of the chromatogram and not crowded at the beginning, as with thyroglobulin in **Figure 1a** which illustrates a separation on a 300-Å column. Thyroglobulin is a large molecule with a lot of dimer and higher-order aggregates present. IgG shows nearly a baseline separation of the dimer and monomer peaks. BSA is a smaller molecule showing good peak shape.

Figure 1b shows the same three proteins separated on a 200-Å column, which clearly is too small for thyroglobulin and marginal for IgG. Note that the IgG monomer (150 kDa) is eluted slightly later than the BSA dimer (132.8 kDa), indicating nonideal behavior that will require further method development.

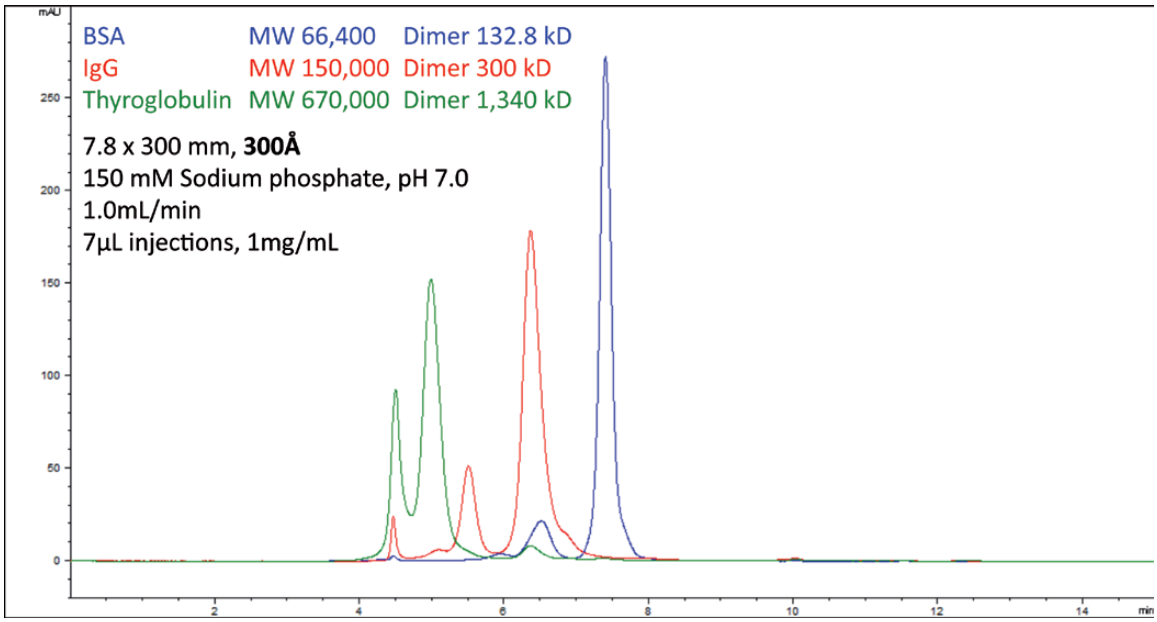

Figure 1a: Selecting the optimum pore size SEC column: 300-Å column example.

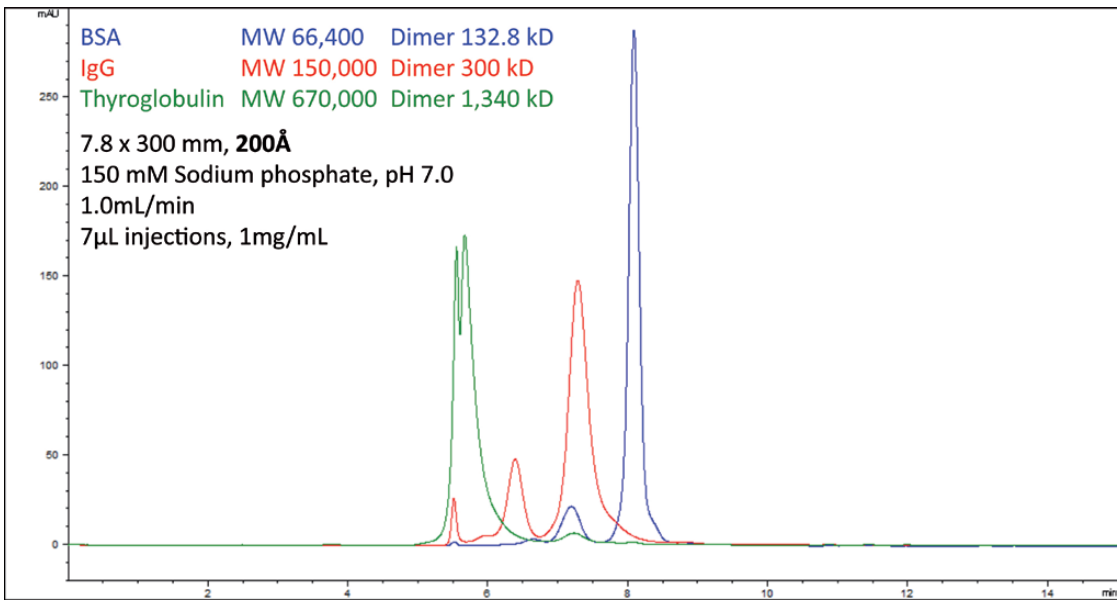

Figure 1b: The same three proteins shown in Figure 1a separated on a 200-Å column, which clearly is too small for thyroglobulin and marginal for IgG. Note that the IgG monomer (150 kDa) is eluted slightly later than the BSA dimer (132.8 kDa), indicating nonideal behavior that will require further method development.

On a 450-Å column (not shown), thyroglobulin is well-resolved, but the overall resolution of the peaks of interest is not adequate. Baseline separation is lost, and the proteins are eluted in a narrow retention time window.

In summary, the first step in SEC method development is to select a column with the appropriate pore size for the molecules of interest.

Column dimensions. Typical column dimensions for SEC are 4.6-mm or 7.8-mm i.d. and a length of 30 cm. Because the separation mechanism is based purely on diffusion into and out of the pores of the stationary phase, the greatest separation results from larger column sizes. Slow flow rates give the molecules time to diffuse in and out of the pool of mobile phase within the pore structure.

The normal flow rate for a 7.8-mm i.d. SEC column is 1.0 mL/min. This flow rate translates to 0.35 mL/min for a 4.6-mm i.d. column, which means that the amount of sample injected can be reduced by ~33%. If sample is limited, a 4.6-mm i.d. column may be called for. Because the separation depends on available pore volume, using longer columns or multiple columns in series increases the available pore volume and, therefore, the resolution. One precaution to note is that operating a 4.6-mm i.d. column at 0.35 mL/min on a non-optimized LC instrument can lead to extracolumn band broadening and, thus, a reduction in resolution.

Going from a 30-cm column to a 15-cm column cuts the run time in half. As long as resolution is maintained, using a shorter column can increase sample throughput, which is important for certain applications. Again, if more resolution is needed, more pore volume is required, which can be achieved by coupling columns in series. Analysis time will double, however.

As can be seen, the selection of column dimensions depends on the amount of sample to analyze and the analysis time required.

Temperature. The role of temperature is often overlooked in SEC. Many methods specify "ambient" temperature; however, temperature control using a column oven will ensure good reproducibility. Laboratory temperatures can easily vary by more than 10 degrees over a 24-hour period. A 10-degree change in temperature can alter mobile phase viscosity significantly, which, in turn, affects the column operating pressure and the diffusion process within the pore structure.

Increasing the temperature reduces the viscosity of the mobile phase and the column pressure, resulting in faster diffusion, sharper peaks, and better resolution. High temperatures, however, can result in problems with the sample such as aggregation and, ultimately, precipitation.

Flow rate. Chromatographers who work with reversed-phase HPLC separations are accustomed to operating at high flow rates and achieving optimum plate count

for small molecules, for example, 1.1–1.2 mL/min on a 7.8-mm i.d., 300 x 7.8-mm column. For larger molecules such as proteins, or even a molecule as small as vitamin B12 (cobalamin), the optimum flow rate is much lower (i.e., 0.6 mL/min). As flow rate is reduced, resolution improves, but run times are longer, so a balance must be found relative to the goals of the method.

Mobile phase. Because mobile phase ionic strength, pH, and buffer composition can have profound effects on some proteins, it is important to understand the effect that even small changes in these variables can have on method optimization and robustness. **Figure 2,** for example, illustrates a dramatic shift in retention times and changes in peak shape when using a 150-mM sodium phosphate buffer, pH 7.0 versus 20 mM Tris, 90-mM NaCl, pH 8.0 buffer on the same column. Some proteins are more affected by these changes than others.

Detergents and other denaturants used in the mobile phase can cause proteins to unfold and become larger in solution or can bind to such an extent that molecular weight and size in solution increase, leading to shorter retention times.

Manually preparing batches of buffer while paying attention to variables such as ionic strength and pH is time-consuming. The liquid chromatography instrument

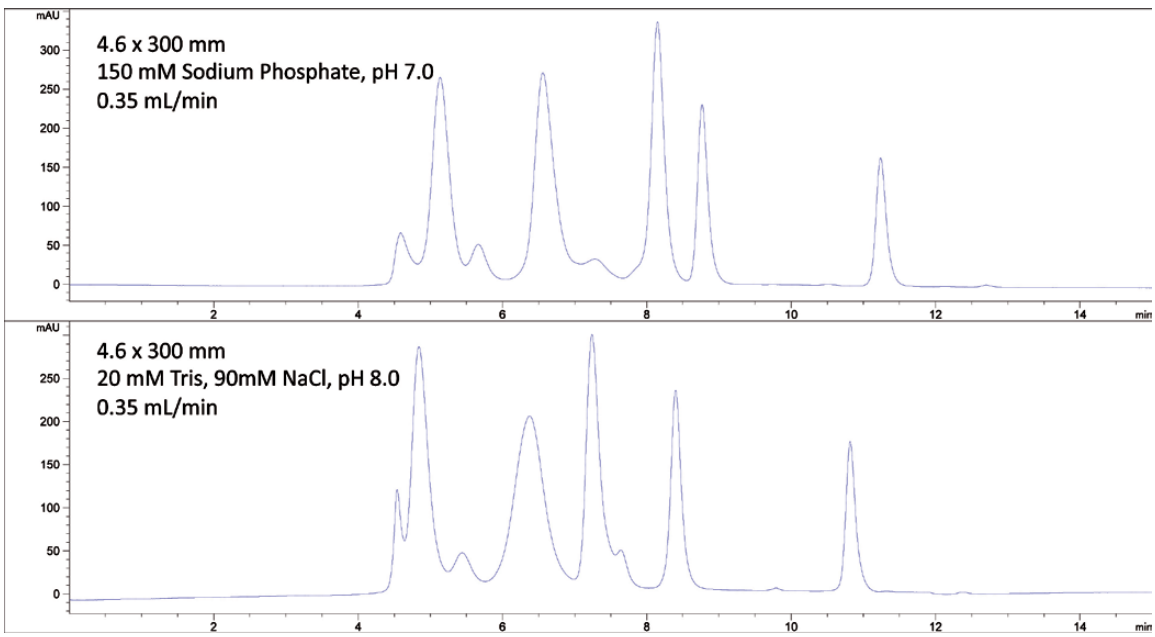

Figure 2: The effect of mobile phase variables on some proteins.

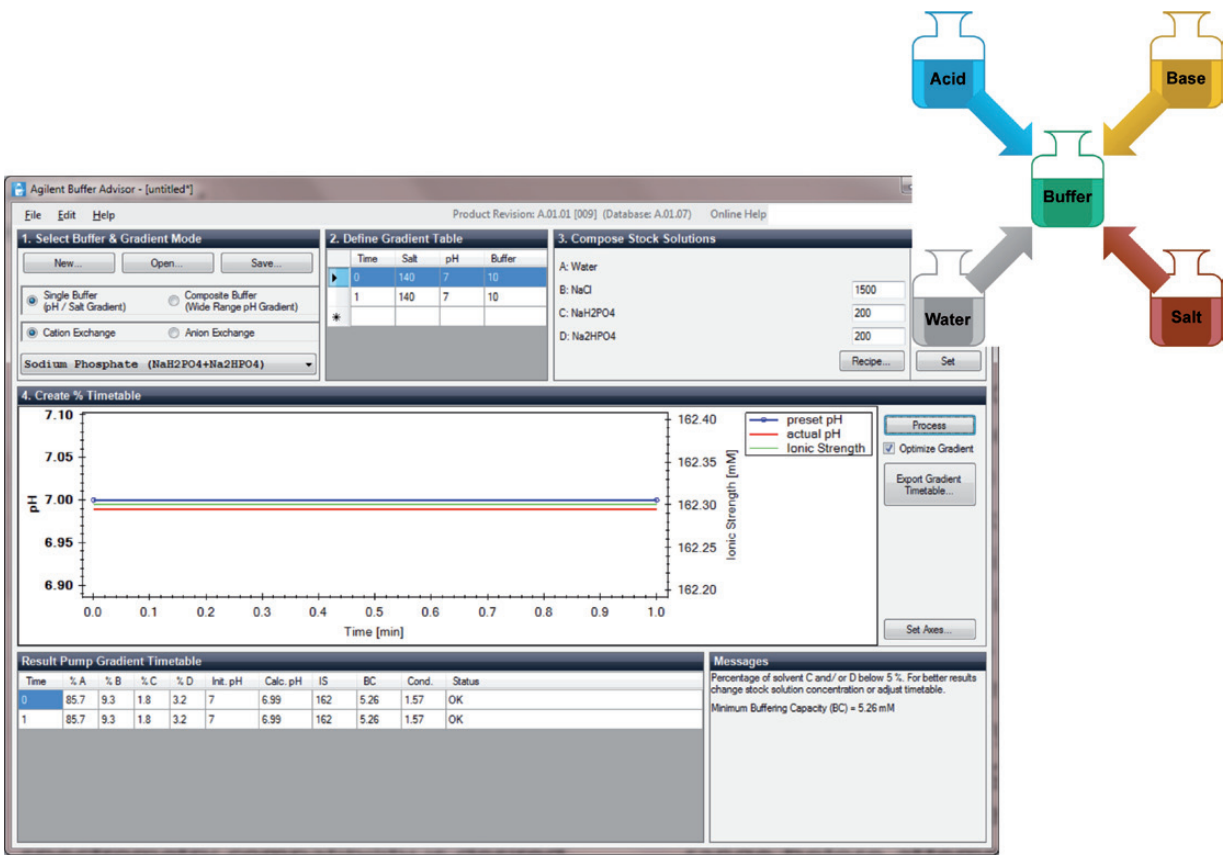

Figure 3: Buffer Advisor from Agilent.

and column must be flushed with each buffer change, which adds to the time requirements.

Software is available to automate much of this process. For instance, the Buffer Advisor software from Agilent (see **Figure 3)** uses the same principles used in manual eluent preparation by mixing the correct proportions of acidic and basic components with sodium chloride (NaCl) and water to create a buffer of the desired buffer strength and pH. This information is imported into the OpenLAB CDS Chemstation Edition software (Agilent) to instruct the quaternary pump to mix and run the correct proportions. Eluent composition is easily adjusted with the software, and the analyst does not need to replace the buffer between runs.

The Agilent Buffer Advisor software application makes use of the quaternary pump's capabilities and enables exploration of a wide range of mobile phase conditions for method development and investigation of method robustness for both SEC and IEX applications.

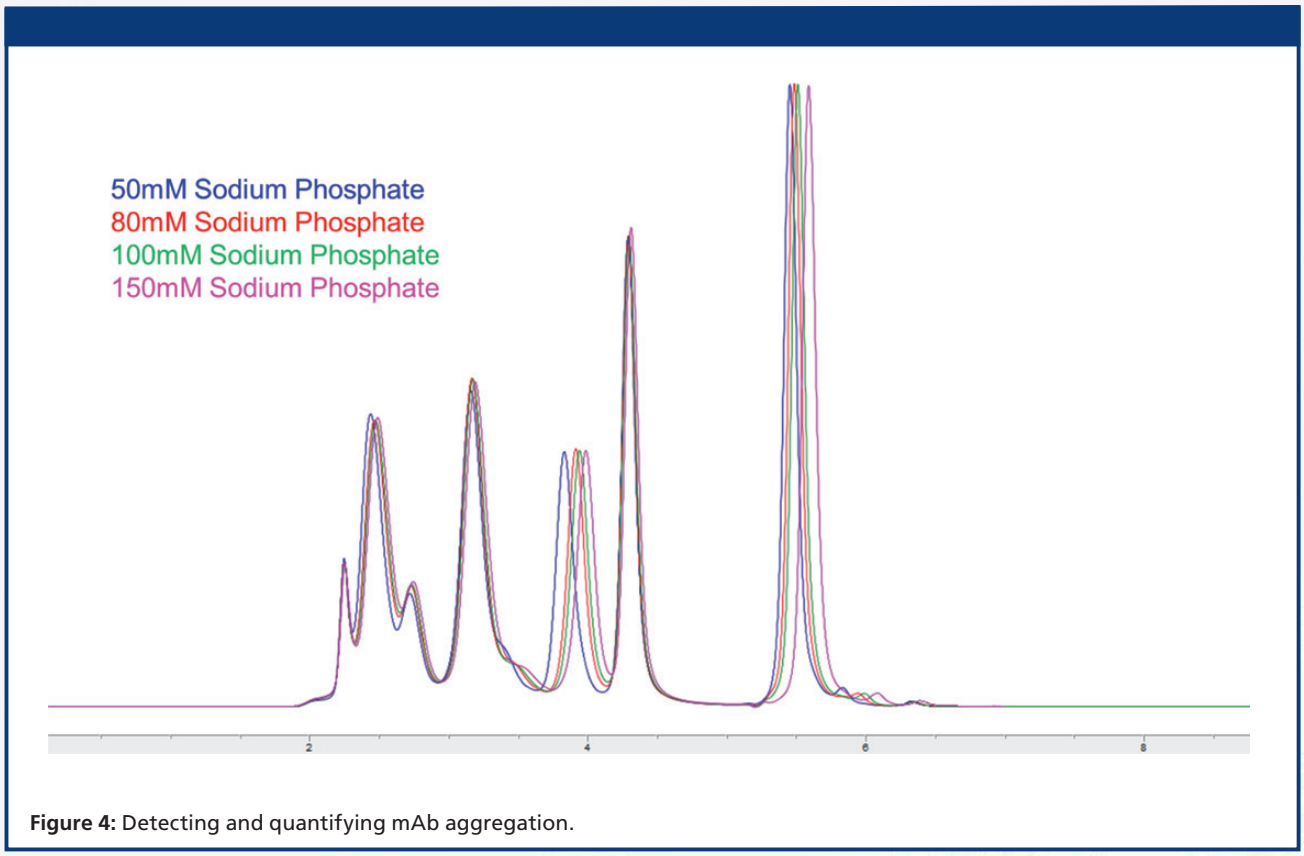

Figure 4: Detecting and quantifying mAb aggregation.

Figure 4 shows how simply changing sodium phosphate concentration at constant pH affects some proteins more than others. The sample was run at 50 mM, 80 mM, 100 mM, and 150 mM sodium phosphate. Some of the proteins (e.g., myoglobin and IgG) are stable with the buffer concentration changes. Others (i.e., ovalbumin and vitamin B12) move around quite a bit.

Adding NaCl to the mobile phase also affects the separation. If phosphate-buffered saline (PBS) is used, the composition is 10 mM phosphate and 140 mM sodium chloride, but many vendors recommend the use of very high ionic strengths to overcome some of the issues they face with SEC columns when interactions occur. Most columns are silica-based, and it is common to see undesirable interactions occurring at low ionic strength. As ionic strength is increased, however, other effects such as hydrophobic interactions may start to occur.

Eliminating clones. SEC is the ideal method for separating monomer species from higher molecular weight species (e.g., dimers and higher-order aggregates) during the development of biotherapeutics. Because the presence of aggregates and smaller fragments will cause the loss of yield or present difficulties during subsequent purification,

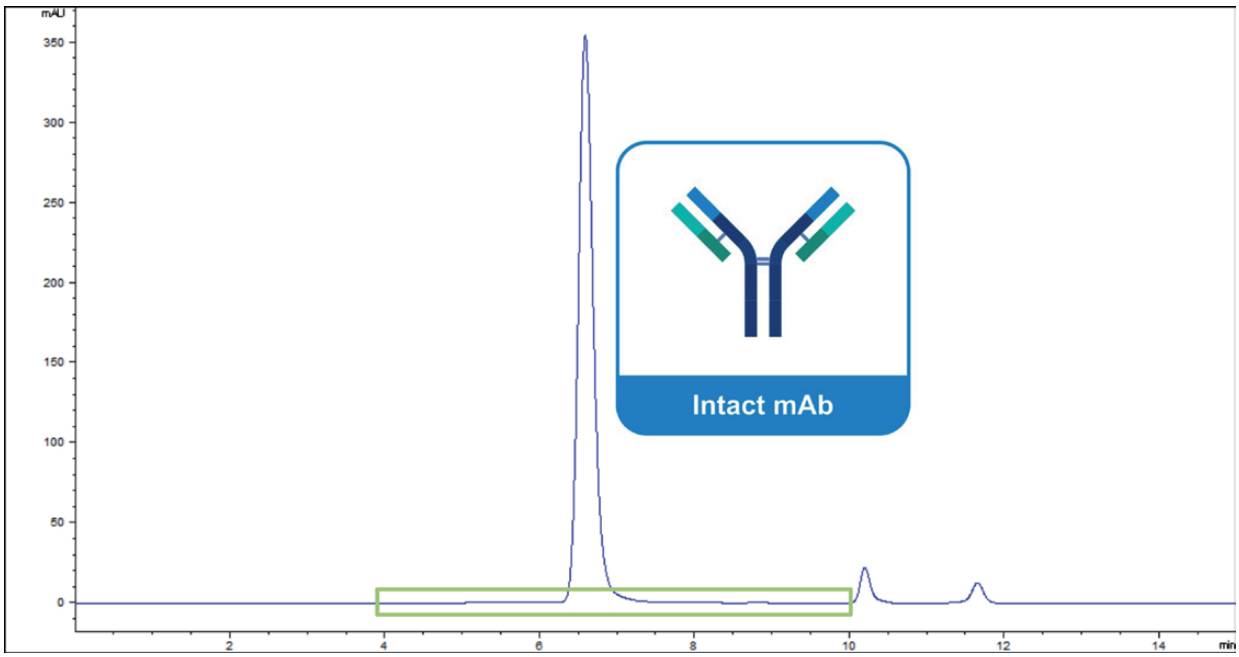

Figure 5: A mAb separation in which a closer look at the baseline reveals impurities.

it is desirable to eliminate or reduce problematic clones at this stage.

Quantifying and controlling aggregation of mAbs and other biotherapeutics is extremely important. The full-scale deflection of a therapeutic mAb is shown in **Figure 5**, with few impurities appearing to be present. A closer look the baseline in **Figure 6**, however, shows that small amounts of aggregate and lower-molecular-weight impurities are present. These peaks require quantification and accurate integration.

In an isocratic run such as this, the baseline should be horizontal with no baseline drift occurring. It is important to check that the integration parameters are suitable and are not measuring valley-to-valley. The amount of dimer and higher-order aggregates typically should be <1.0% (relative to the monomer peak only). The smaller fragments are not included in the quantification in this case.

For biosimilars, eliminating clones that do not give the desired profile is also essential. When comparing originator and biosimilar, retention time reproducibility and quantification of dimers and higher-order aggregates can be examined to assess their similarity.

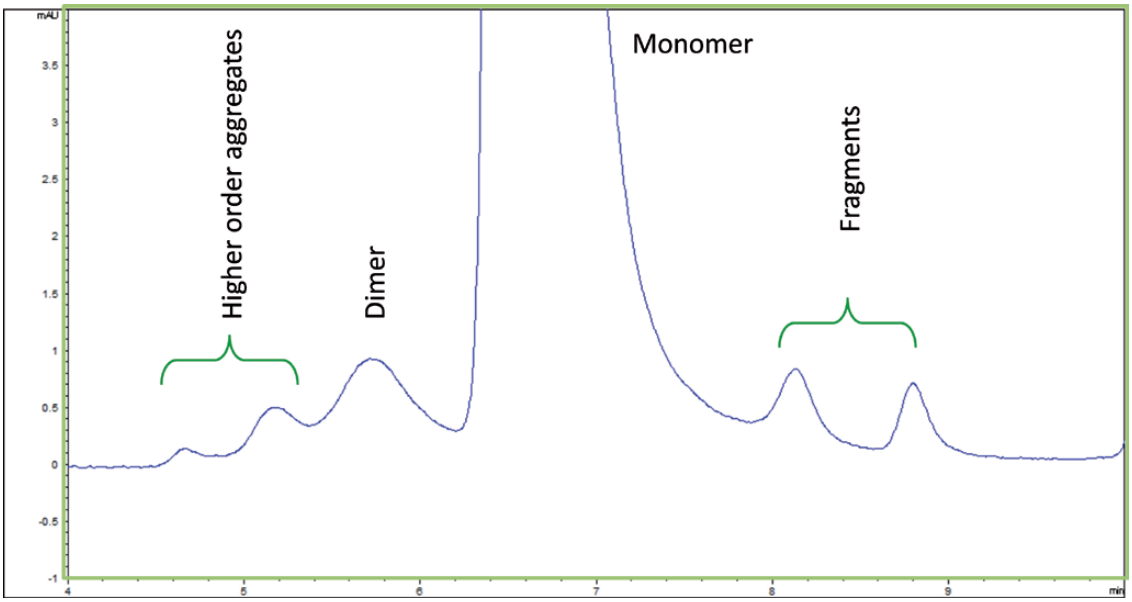

Figure 6: Enlarged view of mAb aggregation example.

Conclusion

SEC is the method of choice for the quantification of aggregates in biotherapeutic samples, but variants can come into play that are not detectable by SEC. Other techniques, such as reversed-phase HPLC, HILIC, and ion-exchange chromatography are parts of the complete toolkit needed to demonstrate similarity and identify other critical quality attributes of biotherapeutic molecules.

Andrew Coffey
Senior Applications Chemist
Agilent

Reference

1. R. Rader, *BioProcess Int.*, 11(6s), (2013).

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CHOOSING THE CORRECT COLUMN AND MOBILE PHASE FOR LC–MS CHARACTERIZATION OF BIOLOGICS

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Enhancing the Quality of Peptide Mapping Separation for the Analysis of PTM

Mass spectrometry is a powerful tool for exploring the heterogeneity of large molecules like monoclonal antibodies and antibody–drug conjugates.

Overview

Biopharmaceuticals such as monoclonal antibodies (mAbs) and antibody–drug conjugates (ADCs) are the fastest growing therapeutic agents in today's market. Their large size, structural complexity, and heterogeneity pose many challenges in their analysis. It is essential to choose the right column and mobile phase conditions for successful biologics characterization, as there is no single solution. Breaking down the large molecule and using mass spectrometry (MS) identification can be a powerful way to determine the origin and cause of their inherent heterogeneity.

Biopharmaceutical Marketplace

The human immune system is capable of

recognizing and fighting infections in the body. This complex system, however, breaks down in the case of autoimmune diseases or cancers. Cancer cells often show over-expression of certain antigens on their surface. Advances in protein engineering have made possible the design of antibodies with specific binding regions that recognize this overexpressed antigen site and, therefore, trigger an immune response to fight the disease.

Starting from the murine cell lines in the early days, the latest generation of mAbs features mostly human amino acid sequences, where possible, to avoid an immunogenic response. Variations such as bispecific mAbs, antibody fragments, glycoengineered mAbs, and ADCs are further reshaping the field of biologics. Once envisioned as the "magic bullet" by German physician, scientist, and Nobel laureate Paul Ehrlich, ADCs deliver cytotoxic small-molecule drugs directly to cancer cells with highly selective antibodies, minimizing damage to healthy cells.

Protein-based biotherapeutics have gained widespread popularity in recent years and have shown unprecedented benefits in patient outcomes. Patent expiry for several high-profile biopharmaceutical drugs (e.g., Humira) has led to a race to develop their biosimilar versions. In certain cases, efforts are being made to develop a "biobetter," a generic version of the innovator drug with enhanced efficacy and increased bioavailability by altering the post-translational modifications.

Unlike the small-molecule drugs, it is impossible to create an exact copy of the biologic drug due to variations associated with fermentation methods and protein modifications. Although biosimilars have shown efficacy similar to that of the innovator drugs, the variations in their sequence and glycan structure can lead to a different efficacy and safety profile. It is important for biosimilar developers to identify the differences and prove they do not cause differences in pharmacokinetic activities. The great complexity of large biomolecules makes their analysis especially challenging. Conjugating biomolecules with small-molecule drugs or modifying glycosylation can further contribute to the complexity of analysis. Mass spectrometry is an essential tool in biologics characterization.

Complexity of Biopharmaceutical Analysis

Key analyses of biologics include the identification of the intact protein and sequences, impurity profiling, potency assay, and post-translational modifications, among others (see **Figure 1**). In addition to the mAb characteristics listed in Figure 1, further characterization is required to determine the drug-to-antibody ratio (DAR), drug distribution, drug conjugation sites, and amount of residual small molecule drug for ADCs.

A combination of techniques such as size-exclusion (SEC), ion-exchange, reversed-phase chromatography, and mass spectrometry are used for analysis. However, SEC and ion-

Typical biopharmaceutical characteristics investigated

- Amino acid sequence
- Amino acid composition
- Structural integrity
- Higher order structures
- Aggregation
- S-S bridges
- N- and O-glycosylation
- N- and C-terminal sequence
- Charge variants
- Deamidation / isomerization
- Oxidation
- Clipping

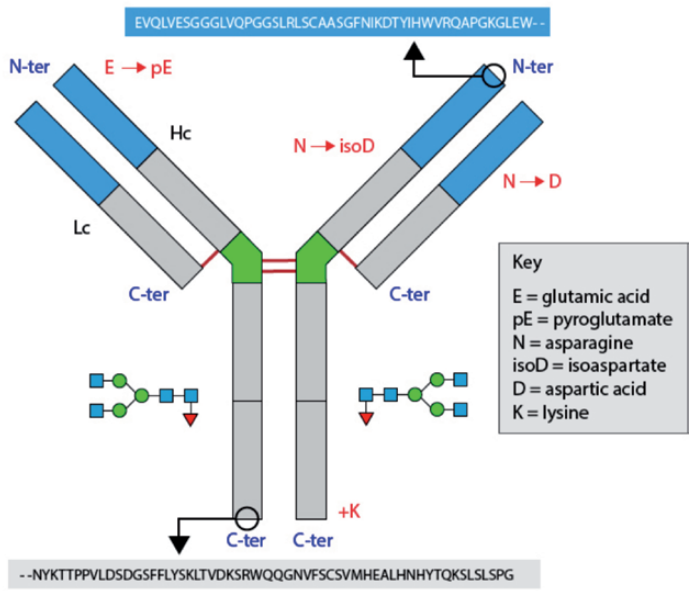

Figure 1: Typical characteristics of biopharmaceuticals that must be analyzed.

exchange chromatography mobile phase conditions that do not denature the proteins are typically not compatible with MS instruments. They require offline desalting or the use of two-dimensional liquid chromatography to go from SEC–ion-exchange chromatography to a compatible mobile phase to make the analysis amenable to MS detection.

In a typical workflow, ultraviolet (UV) or fluorescence detection can be used for quantification while performing identification with an MS instrument. Owing to the size, complexity, and heterogeneity of biologics, their characterization presents several challenges. Using trastuzumab (Herceptin) as an example, one may expect the following challenges when attempting mAb characterization.

- **Isotopes:** The very large molecular weight of ~150,000 comprising over 6,000 carbon and 44 sulfur atoms would result in a significant number of isotopes being detected in MS systems.
- **Lysine truncation:** The lysine on the C-terminus may be cleaved by carboxypeptidase, resulting in a lysine-truncated variant that has a mass difference of 128 and a different charge. Identifying a mass difference of 128 from a molecule that is over 145,000 is quite difficult.

- **Glycosylation:** The N-linked glycosylation, typically occurring on the asparagine 197 position, can add 1,300–1,800 to the overall molecular weight of the mAb.
- **Dehydration:** At the N-terminus, it is possible for glutamine to convert to pyroglutamic acid releasing a water molecule. Accurately detecting a mass difference of 18 on such a large molecule can be difficult.
- **Oxidation:** Methionine oxidation adds 16 mass units of oxygen with 12 possible sites.
- **Deamidation:** Deamidation of asparagine to form aspartic acid only results in a change of one mass unit, with a total of 48 asparagine sites on the antibody. This can have a significant impact if it occurs in the binding region.
- **Isomerization:** The aspartic acid can isomerize to form isoaspartic acid (total 52 positions) which, in fact, does not result in change of the mass.

In addition to these issues, the following are additional challenges faced when characterizing an ADC.

- **DAR:** ADCs with various DAR numbers can be chromatographically resolved and will exhibit mass differences that relate to the addition of the linker and the drug. This separation can be complex depending on lysine versus cysteine conjugation.
- **Drug conjugation sites:** A drug molecule attached at different sites produces variants but with the same mass and DAR. These variants can make the chromatographic separation quite complex.
- **Residual drug:** It is important to determine the level of residual potent drug that does not bond to the mAb to form ADC.

Method Development

Several factors must be considered when beginning to develop a method for biologics separation. For the column, traditional reversed-phase chemistries such as C18, C8, and C4 are typically used in addition to alkylated (e.g., diphenyl) or polymeric (e.g., PLRP-S) phases with large pore sizes for separating large molecules.

For intact protein analysis, an alkylated reversed-phase liquid chromatography column (e.g., RP-mAb diphenyl) may provide different selectivity through secondary interactions (e.g., π-π interactions) and show additional or different levels of detail compared with traditionally used bonded C4 or C8 phases. Smaller particle sizes should be preferred on an ultrahigh-pressure liquid chromatography (UHPLC) column to obtain higher efficiencies and better separations. Superficially porous particles (SPPs) are viable alternatives that have modest back pressure and can also provide high efficiencies on a 400/600-bar HPLC instrument.

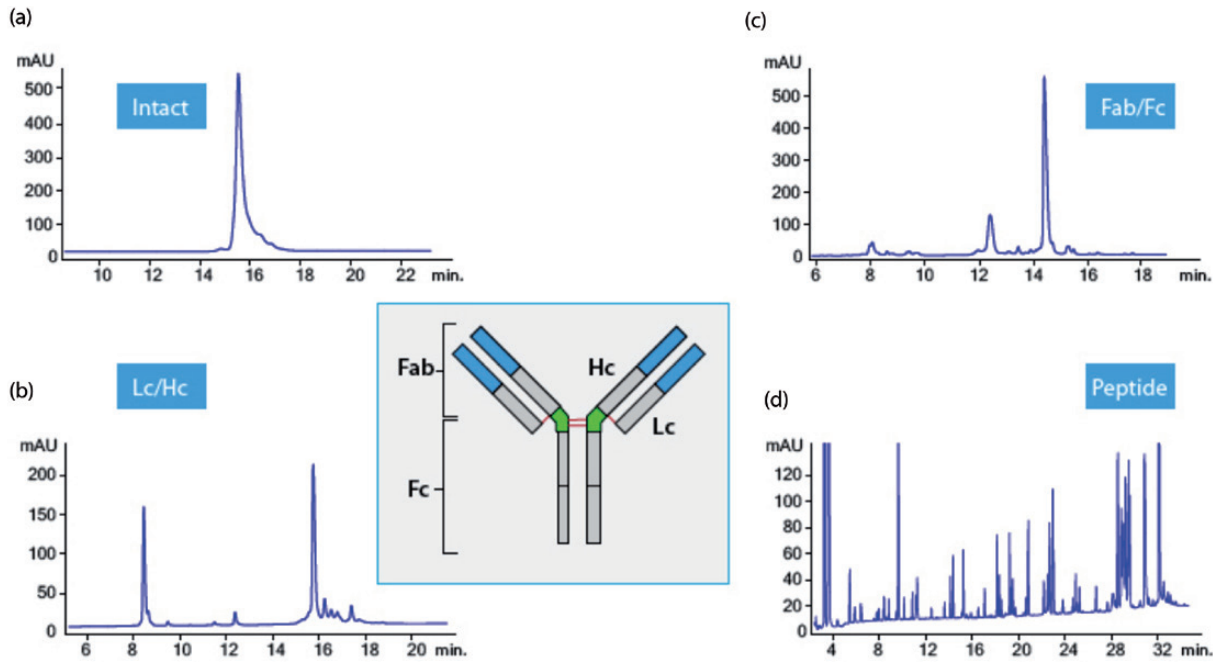

Figure 2: Intact versus fragmented versus peptide map.

Their shorter diffusion path and faster mass transfer can enable improved performance for large molecules resulting in sharper peaks and faster separations. Note that compared to fully porous particle based columns, SPP columns have slightly reduced loading capacity and can be overloaded more quickly. Column geometry is another factor to be considered depending on whether a faster analysis is desired for increased productivity or a higher resolution and complete characterization are required.

Method development choices can depend on whether only UV detection is being used for concentration or mass spectrometry compatibility is desired. The choice of the ion pairing agent can depend on whether an MS system is being used since formic acid may provide better ionization efficiency, but trifluoroacetic acid may provide better peak shapes. One may choose to include isopropanol in the mobile phase for improved recovery, but it can be difficult to use due to its high viscosity. As no generic method works for all situations, the column and mobile phase should be carefully chosen in a manner that satisfies the analysis criteria. Large-molecule separations are quite sensitive to changes in the organic modifier, so it is advisable to begin with a shallow gradient over a wider percent organic range before attempting to refine and

optimize the gradient. Additionally, common instrument optimizations such as low extra-column dead volume and high detector sampling frequency are necessary for intrinsic characterization.

Characterization of Antibodies

When analyzing an antibody for impurity profiling or identification, a greater emphasis is placed on peak capacity and improved resolution over faster analysis time. **Figure 2** shows an overview of one of the types of analyses performed for mAb identification and purity determination. While intact mAb analysis with reversed-phase liquid chromatography-UV (Figure 2a) can provide a straightforward impurity detection, mass spectrometry coupled fragmented mAb analysis (Figure 2b) uncovers a greater level of details to pinpoint the location and cause of underlying heterogeneity. The digestion of mAb into antigen-binding (Fab) fragments (Fab) and fragment crystallizable (Fc) regions via papain or mAb reduction to decouple the light and heavy chain (Figure 2c) can simplify the analysis and allow for easier detection of modifications based on mass difference. Going a step further,

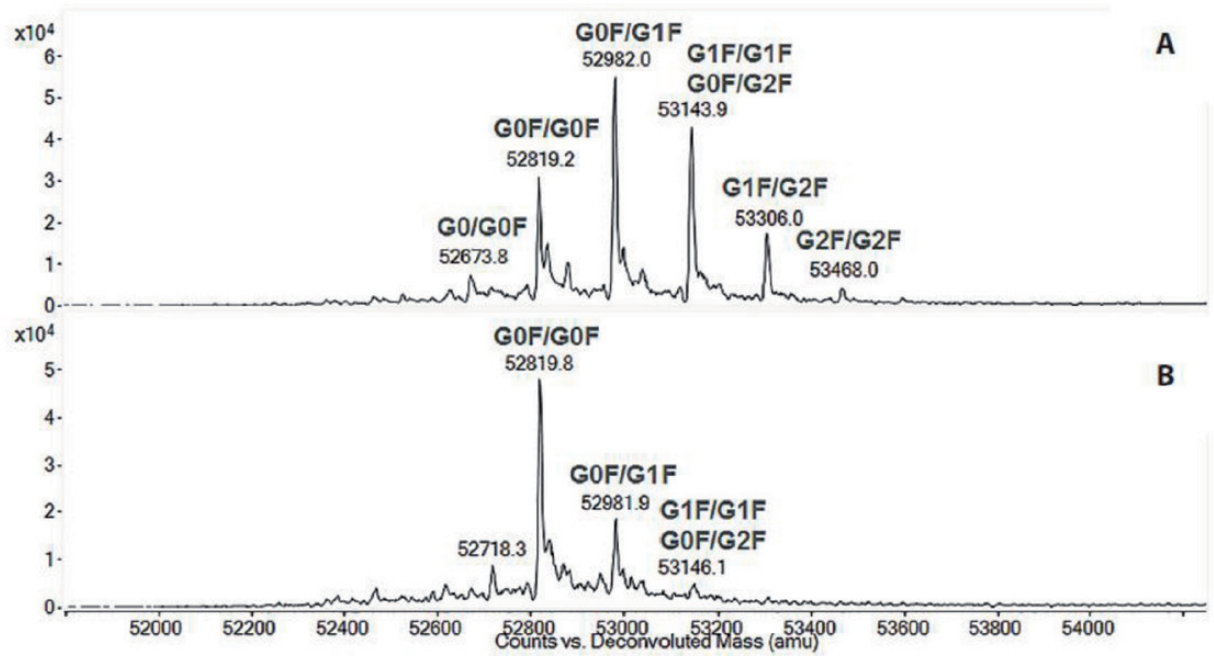

Figure 3: A deconvoluted mass spectra of separations of an originator mAb (a) and a biosimilar (b). Separation of the Fab/Fc fragments of the mAb was obtained from a papain digest. Differences between the twp products are readily apparent and the MS spectra from the Fc region peaks show the undergalactosylation of the biosimilar.

a complete characterization of the mAb can be performed with peptide mapping (Figure 2d).

When comparing an innovator and a biosimilar, fragmented mAb analysis with MS instruments can allow a detailed comparison of post-translational modifications such as glycosylation profiles. Certain protein modifications may be more prevalent in the originator or biosimilar and their detection and quantification can require a high-efficiency chromatographic separation of species with the use of smaller particle size silica or SPP-based columns. Comparing the intact originator and biosimilar mAbs with high-resolution reversed-phase liquid chromatography and MS detection can help deconvolute the source of heterogeneity, such as differences in N-glycan structures. The change in mass difference can be assigned to specific sugar units or to other modification events such as deamidation. To identify the location, however, the mAbs must be fragmented or reduced and their subsequent chromatographic separation can pinpoint the heterogeneity differences in the light chain region versus the heavy chain region.

Figure 3 shows a deconvoluted mass spectrum comparison of an originator and a biosimilar's Fab and Fc fragments, as obtained from papain digestion. It is apparent that the biosimilar (bottom) shows under galactosylation in the Fc region compared with the originator that may lead to a difference in its biological activity.

ADCs

ADCs are a new class of biotherapeutics that comprise an antibody attached to a highly potent drug via a linker covalently conjugated through lysine or cysteine residues on the mAb. When dealing with cancer, small-molecule drugs typically have a small therapeutic window (i.e., the minimum dosage of drug necessary to have the intended therapeutic effect is very close to the dosage that would harm normal cells). ADCs leverage the specificity of mAbs to deliver the drug to a desired site, limiting collateral damage to healthy cells, and provide a larger therapeutic window. The small-molecule drug attached to the mAb is generally a cytotoxic agent with a validated mechanism. The linker that attaches the drug molecule to the mAb is stable in plasma and is designed to release the drug molecule upon internalization of the ADC by the host cell.

With ADC characterization, heterogeneity of additional linker and small molecule drug is added to the existing challenges of mAb analysis. Because there are multiple lysine and cysteine linking sites on the mAb, multiple drug molecules can be attached to a single antibody. In addition to existing techniques of mAb analysis for size, identity, aggregation, and charged variants, it is also essential to determine

	Intact	Reduced
Column	Agilent PLRP-S 1000 Å, 2.1 x 50 mm, 5 µm	
Mobile phase A	0.1% formic acid in water	0.1% formic acid in water
Mobile phase B	70% IPA/20% MeCN/10% water with 0.1% formic acid	99.9% MeCN with 0.1% formic acid
Gradient (segmented)	0 minutes - 15 %B, 4 minutes - 20 %B, 5 minutes - 75 %B, 10 minutes - 100 %B, 10.1 minutes - 15 %B	0 minutes - 20 %B, 5 minutes - 20 %B, 6 minutes - 75 %B, 10 minutes - 90%B
Post time	4 minutes	0 minutes
Column temperature	60 °C	85 °C
Flow rate	0.4 mL/min	0.4 mL/min

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Figure 4: Two methods for ADC determination using LC–MS.

the DAR and residual drug. Too many drug molecules attached to an ADC may make it more hydrophobic and cause it to clear from the body more quickly, thus reducing its bioavailability. Additionally, depending on the drug's toxicity, there generally is an optimum DAR for intended therapeutic effects because too few drug molecules may fail to kill the cancer cell while too many drug molecules have the potential to harm normal cells. Unfortunately, not all techniques used for ADC characterization are MS compatible.

Figure 4 shows two LC–MS-compatible methods to analyze intact and reduced ADC DAR using the Agilent PLRP-S polymeric stationary phase.[1,2] However, the intact ADC analysis is only feasible for lysine-conjugated molecules as the structural integrity of the cysteine-conjugated ADCs is compromised due to partial or complete reduction of disulfide bridges. Cysteine-conjugated ADCs typically result in an even number DAR value such as 0, 2, 4, 6, and 8, due to cysteine pairs being chemically equivalent. Lysine-conjugated ADCs

are even more complex (**Figure 5**), as there are numerous conjugation sites (90 lysine residues in trastuzumab). The top panels (A–E) in Figure 5 show the reduced glycosylated analysis, which remains complex even after spectrum deconvolution. Prior removal of the n-linked glycans (using PNGase F) can simplify the analysis (Figure 5 panels E–H) and provides a cleaner deconvoluted spectrum for easier identification of DAR on light chain and heavy chain.

Polymeric columns typically provide improved peak shapes over bare silica columns as the former do not contain any silanol groups that tend to induce tailing due to their strong interactions with amines.

Peptide Mapping

Peptide mapping can provide a vast amount of information on a protein's primary structure as well as post-translational modifications for a comprehensive characterization. By reducing the intact mAb to de-couple the light and heavy chain followed by a tryptic digest, the mAb can be broken down into smaller peptide fragments. With many peptide fragments of varying polarities from very hydrophilic to very hydrophobic, optimizing a chromatography method to separate all species can be arduous. Using specially endcapped columns (e.g., AdvanceBio Pepide Plus from Agilent) over traditional reversed-phase chemistries such as C18 may be beneficial in retaining the

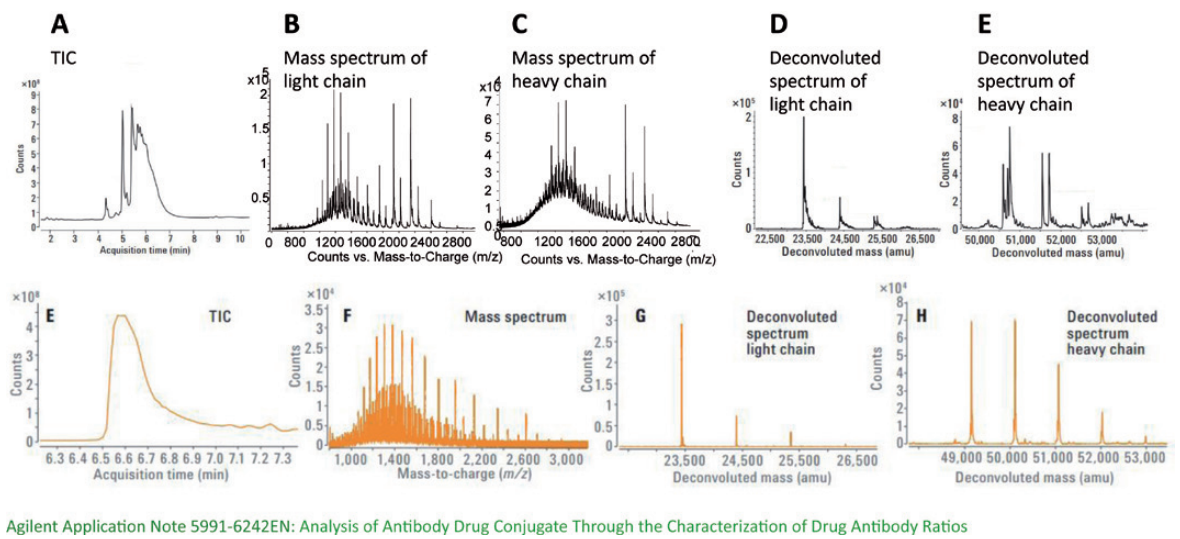

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Figure 5: TIC, mass spectrum, and deconvoluted spectra for light and heavy chains of the reduced glycosylated (top panels A–E) and deglycosylated ADC (panels E–H). Deglycosylating the conjugate reveals heterogeneity due to conjugation.

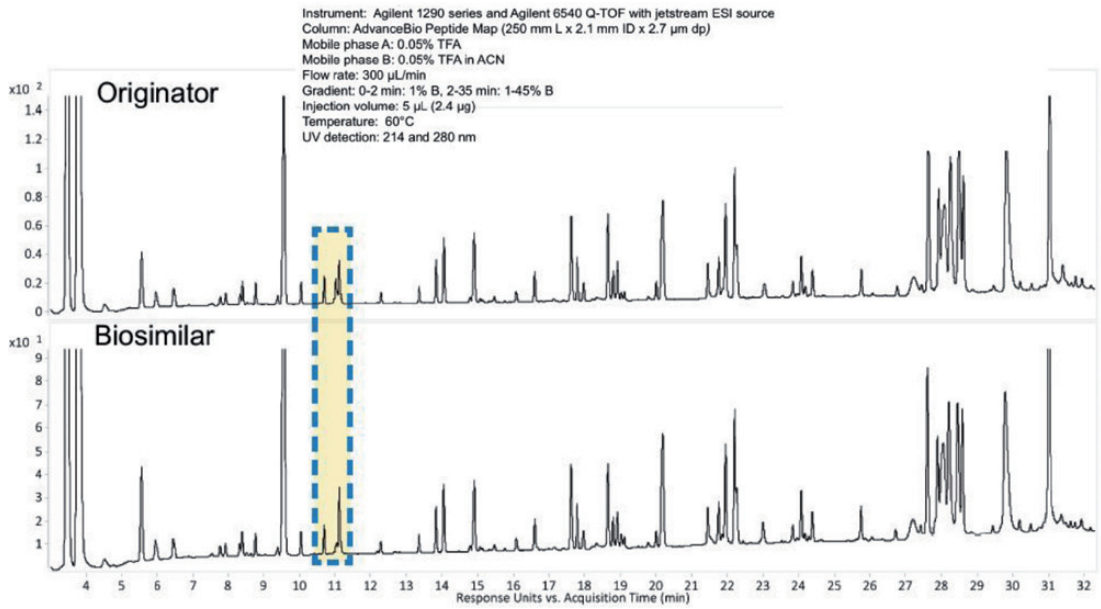

Figure 6a: Example of tryptic digest separation compared for an originator ADC and a biosimilar. The separation provides an enormous amount of detail on the primary structure and post-translational modifications allowing extensive characterization and comparison between and originator and the biosimilar.

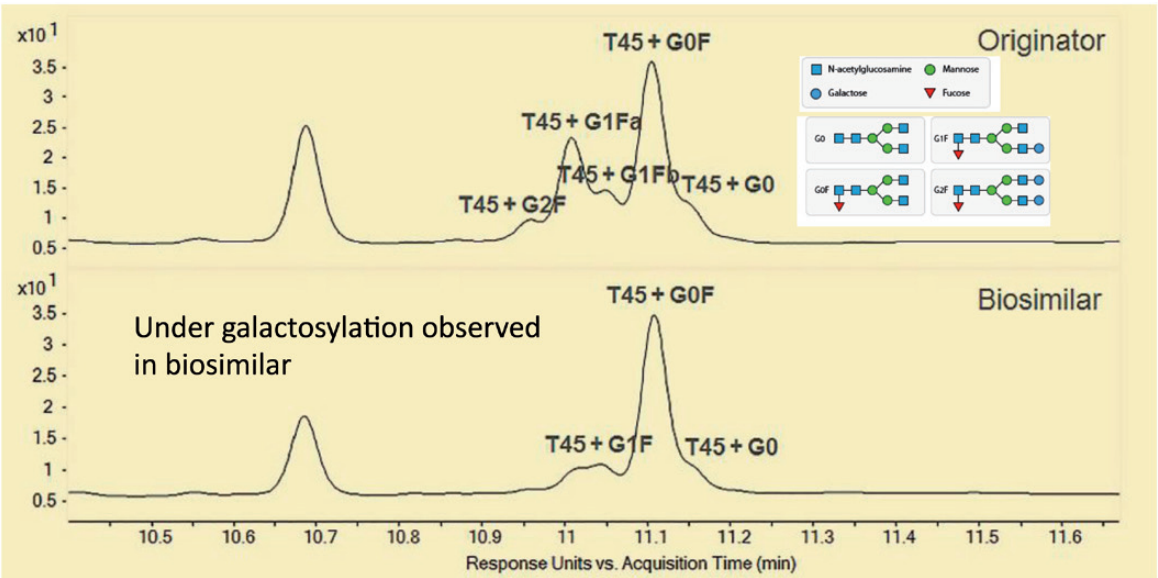

Figure 6b: Separation and identification of peptide fragments of an ADC can reveal differences in post-translational modifications.

highly polar fragments and obtaining improved peak shapes. For a tryptic digestion of trastuzumab, more than 60 fragments are obtained, which can be resolved chromatographically and their mass determined by mass spectrometry. If the complete amino acid sequence of a protein is already known, software workflow can assign an identity to peptide fragments to determine if the experimental results are as expected and where the deviations in the sequence occur, if any.

Figure 6a shows an example of a tryptic digest separation for an originator ADC and a biosimilar. Separation and identification of peptide fragments with high-efficiency chromatographic separation and MS detection can reveal the finer differences in post-translational modifications between originator and a biosimilar, such as differences in galactosylations (see **Figure 6b**). Changes in the peptide sequence from lysine truncation, methionine oxidation, and asparagine deamidation, among other modifications, can also be identified with relative ease using peptide mapping with mass spectrometry. It can help reveal modifications such as deamidation of asparagine to aspartic acid and even the extent of this modification in an innovator versus a biosimilar (**Figure 7**). Although the relative change may be minor

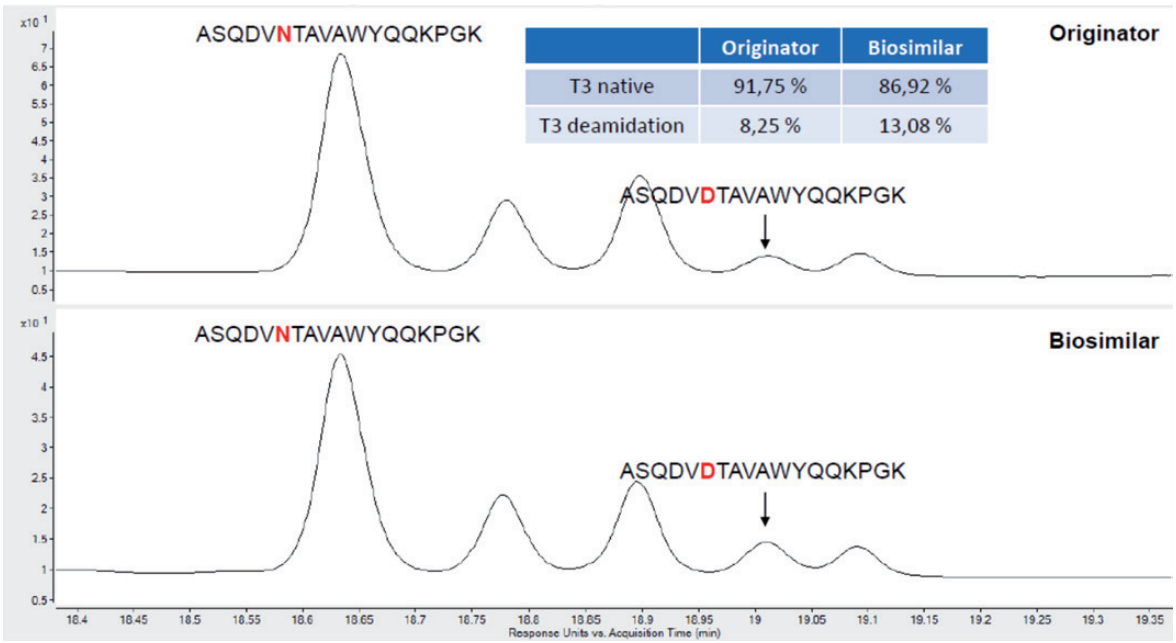

Figure 7: For comparing an originator ADC and a biosimilar, peptide mapping can reveal modifications such as deamidation.

compared with the entire molecule, these modifications can be vital to the potency and safety of the mAb and should be critically evaluated.

Conclusions

The field of biologics is complex and the characterization of antibodies and ADCs can pose many challenges. Analysis of intact molecule, fragmented molecule, and peptide mapping are essential to completely uncovering the details of a protein, for example, in the case of comparing an originator to biosimilars. Using a high-efficiency column with the right chemistry and the appropriate ion pairing agent to maximize resolution and sensitivity is key to successful biologics characterization. Mass spectrometry is essential to identifying the location and cause of protein heterogeneity. Peptide mapping is the most comprehensive approach to identifying the similarities and differences between innovators and biosimilars, down to the changes at single amino acid levels.

References

1. S. Babu, "Application Note 5991-7163EN: PLRP-S Polymeric Reversed-Phase Column for LC/MS Separation of mAbs and ADC," Agilent Technologies Inc., 2016; *5991-5493EN Prepping Biosimilars for a Big Play,* GEN eBook. Acknowledgment: Special thanks go to Koen Sandra, Research Institute for Chromatography, Belgium for providing invaluable experimental data and content.
2. R. Gudihal and J. Chen, "Application Note 5991-6242en: Analysis of Antibody Drug Conjugate Through the Characterization of Drug Antibody Ratios," Agilent Technologies Inc., 2015.

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ADDRESSING THE CHALLENGES OF BIOPHARMACEUTICAL CHARACTERIZATION

An interview with Koen Sandra

It is undoubted that the proliferation of biopharmaceutical therapies over the past 10 years presents new and complex challenges for analytical science. Traditional peptide and protein characterization techniques are tested as the range of critical quality attributes (CQAs) demanded by regulators expands and new biopharmaceutical modalities present additional issues for qualitative and quantitative analysis. Here, Tony Taylor, the technical director at Crawford Scientific Limited, interviews Koen Sandra, the scientific director at the Research Institute for Chromatography (RIC) in Kortrijk, Belgium, about current and future challenges of protein and peptide analysis, as well as some solutions to the more demanding aspects of biopharmaceutical analysis.

Taylor: What has brought the biggest advance in the analysis of biologics during your career?

Sandra: These are exciting times to be involved in biopharmaceutical analysis. In the last 15 years, we have seen an enormous evolution in instrumentation (e.g., ultrahigh-pressure liquid chromatography [UHPLC], two-dimensional liquid chromatography [2D LC], high-resolution mass spectrometry [MS]), LC column technology (e.g., fully porous sub-2-µm and superficially porous sub-3-µm particles, and

widepore columns applied to different LC modes), reagents, and software (e.g., data analysis), which really allows an in-depth analysis of biologics such as monoclonal antibodies (mAbs) and antibody–drug conjugates (ADCs). Fifteen years ago, we had to work with columns that were less efficient and robust as well as mass spectrometers that were less accurate and resolving. Moreover, data interpretation was predominantly a manual task. For the latter, there is still a need for manual interpretation and verification, but a large part of the work is taken over by the computer. When we now analyze blockbuster mAbs that were developed and characterized 20 years ago (e.g., in the context of biosimilar development), we pick up more product and process-related impurities.

Taylor: What is the biggest challenge currently facing the field of biologics analysis?

Sandra: One of the biggest challenges we are confronted with nowadays is the ever-increasing complexity of the molecules. Analyzing next-generation products such as fusion proteins, bispecific mAbs, or ADCs really pushes state-of-the-art analytics to its limits and also demands the best analysts. This immediately brings us to an important bottleneck. It is not so easy to find well-trained analysts experienced in biologics.

The analytical field should also be ready for the next wave of biopharmaceuticals such as cell and gene therapy products that will again require new technologies and skills.

Taylor: What technologies will take the analysis of biologics further?

Sandra: As mentioned, in the last 15 years, advances in mass spectrometry, liquid chromatography, reagents, and informatics have taken the analysis of biologics to the next level. We expect technologies like 2D LC, native MS, ion mobility, and hydrogen–deuterium exchange (HDX) among others, to be more widely applied in the coming years. Mass spectrometers will furthermore be introduced in routine biologics analysis (i.e., in the context of quality assurance and quality control in an environment governed by good manufacturing practices) and one single LC-MS method will replace various HPLC release methods (multi-attribute monitoring). We also should not forget the electrophoretic techniques that clearly have their place in the analysis of biologics. With renewed interest and more research efforts, it should be possible to take more profit of this technology.

Taylor: What CQAs are important?

Sandra: This needs to be studied on a case-to-case basis. A particular post-translational modification (PTM) can be important for one product and yet not at all affect safety or efficacy for another. The location in the molecule is also important. We often observe that aspartate isomerization in complementarity-determining regions (CDRs) affects mAb target binding. Indeed, the formation of isoaspartate brings an extra carbon in the protein backbone, thereby changing its higher order structure. Aspartate

isomerization in other regions in the mAb is potentially less problematic.

Aspartate isomerization is typically studied by peptide mapping, ion exchange chromatography, or capillary isoelectric focusing (cIEF). For mAbs, glycosylation is a CQA that can affect product safety and efficacy, so characterizing these glycan structures is an essential requirement. Afucosylated glycans (e.g., G0) on the conserved Fc glycosylation site give rise to increased antibody-dependent cell-mediated cytotoxicity (ADCC), the Gal-α-1,3-Gal glycotope and N-glycolylneuraminic acid might lead to immunogenicity, and the appearance of high-mannose glycans on mAbs can result in faster clearance. Glycosylation is highly dependent on cell type and cell culture conditions, thereby making it challenging to produce mAbs with consistent glycan profiles.

Aggregation of mAbs and other therapeutics has the potential to induce immune responses and must be tightly controlled.

While glycosylation, oxidation, deamidation, isomerization, and aggregation are the usual suspects, one also encounters more unusual CQAs such as mannose-6-phosphate (M6P), which is required for targeting therapeutic enzymes to the lysosomal compartment of the cell; formylglycine, which is important for the enzymatic activity of sulfatase; or the drug-to-antibody ratio (DAR) of an ADC, which defines the payload that is delivered to the tumor cell.

Taylor: Is there a place for 2D LC and GC in biopharmaceutical characterization?

Sandra: Yes. There is definitely a place for 2D-LC. With the introduction of commercial instrumentation, 2D LC has in recent years seen an enormous evolution and one of the fields where 2D LC is being widely adopted is in the analysis of biologics such as mAbs and ADCs. These molecules come with a structural complexity that drives state-of-the-art chromatography and mass spectrometry to its limits. 2D LC instruments are ideal tools to tackle this analytical complexity. 2D LC systems, either in comprehensive or heart-cutting mode, have various applications throughout the entire mAb lifecycle, ranging from clone or lead selection to process optimization, detailed characterization, and comparability assessment. Its use in a QC environment for stability and release testing has even been suggested. Furthermore, 2D LC instruments have applications in the characterization and quantification of host-cell proteins and in studying the pharmacokinetic behavior of biologics in serum, plasma, or cerebrospinal fluid.

Gas chromatography, evidently, is not applicable to the biologics, but can be valuable in media analysis (e.g., process optimization) and excipient analysis, among others applications.

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