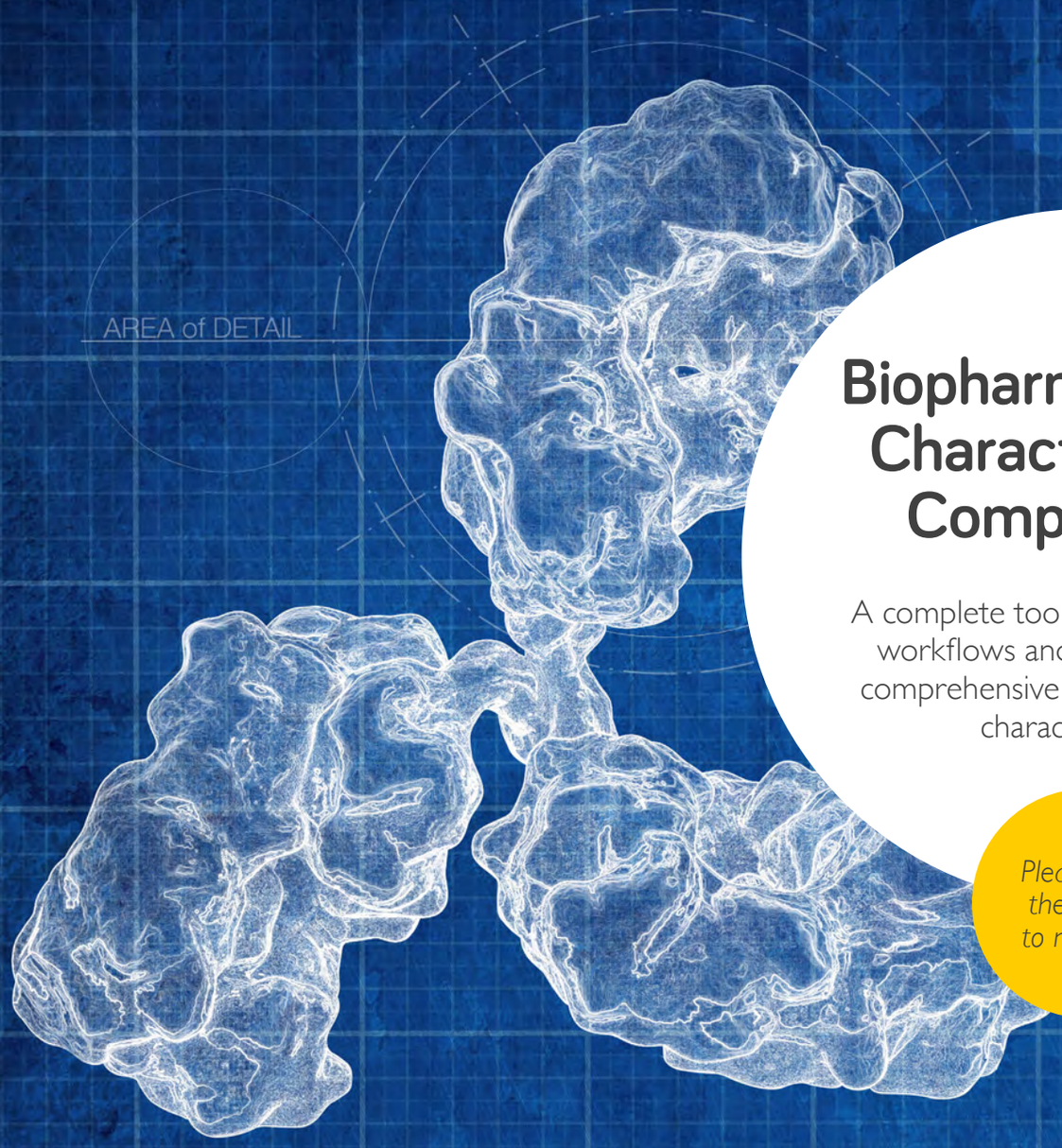
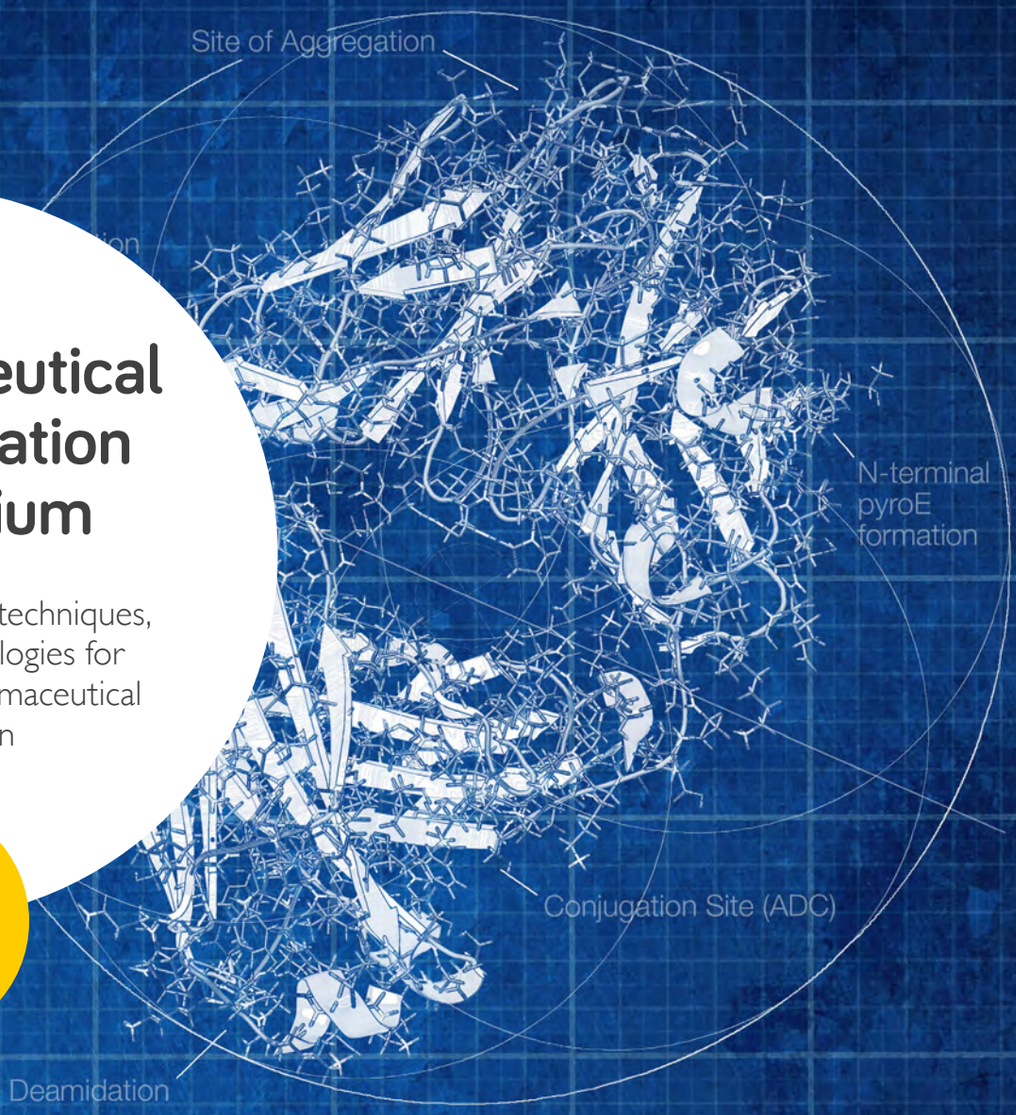


Biopharmaceutical Characterisation Compendium

A complete toolbox of techniques, workflows and technologies for comprehensive biopharmaceutical characterisation

Please click the circles to navigate



Immunoglobulin protein | ca. 150,000 Daltons | participates in the immune reaction as the antibody for a specific antigen | There are five main types: IgA, IgD, [illegible] IgM

Humanized IgG antibody fragment (Fab) | 50,000 Daltons | VH, CH1 and VL, CL regions, linked by an intramolecular disulfide bond.

INTRODUCTION

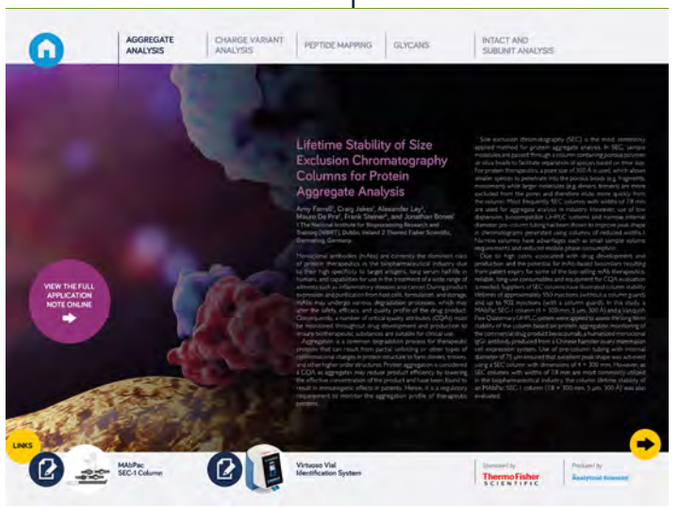
AGGREGATE ANALYSIS

CHARGE VARIANT ANALYSIS

PEPTIDE MAPPING

GLYCANS

INTACT AND SUBUNIT ANALYSIS



The resources collected in this eBook stem from collaboration between Thermo Fisher Scientific and Ireland's National Institute for Bioprocessing Research and Training (NIBRT). An independent center of excellence, based near Dublin, NIBRT is world-renowned for the training and research support it provides to the bioprocessing industry.

The partnership saw NIBRT develop workflows on the Thermo Scientific™ biomolecule column range with its associated consumable portfolio, combined with advanced Thermo Scientific liquid chromatography systems and Thermo Scientific™ Orbitrap™ high-resolution mass spectrometers. NIBRT scientists used this powerful suite of tools to gain a deeper understanding in five key areas:

- Aggregate analysis
- Charge variant analysis
- Peptide mapping
- Glycans
- Intact and subunit analysis

The application notes and resources available within are designed to provide complete workflows for a range of challenging analyses in biopharma. Just follow the links on each page. You can even download solutions direct to your instrument, via the cloud-based Thermo Scientific AppsLab library of analytical applications.

By working with Thermo Fisher Scientific and gaining access to cutting-edge technology, the team at NIBRT were better able to serve their partners in the pharmaceutical industry. This project demonstrates, once again, that instrument manufacturers are a vital part of the analytical science ecosystem – and proves that collaboration is the beating heart of the field.

A Universal Chromatography Method for Aggregate Analysis of Monoclonal Antibodies

Amy Farrell[1], Jonathan Bones[1], and Ken Cook[2]
[1] NIBRT, Dublin, Ireland; [2] Thermo Fisher Scientific, Hemel Hempstead, UK

The biopharmaceutical industry continues to develop mAb-based biotherapeutics in increasing numbers. Due to the complexity of these biotherapeutics, there are several key quality attributes (CQAs) that need to be measured and controlled to guarantee their safety and efficacy. The presence of aggregates in a formulated drug product must be assessed to avoid potential issues with immunogenicity.

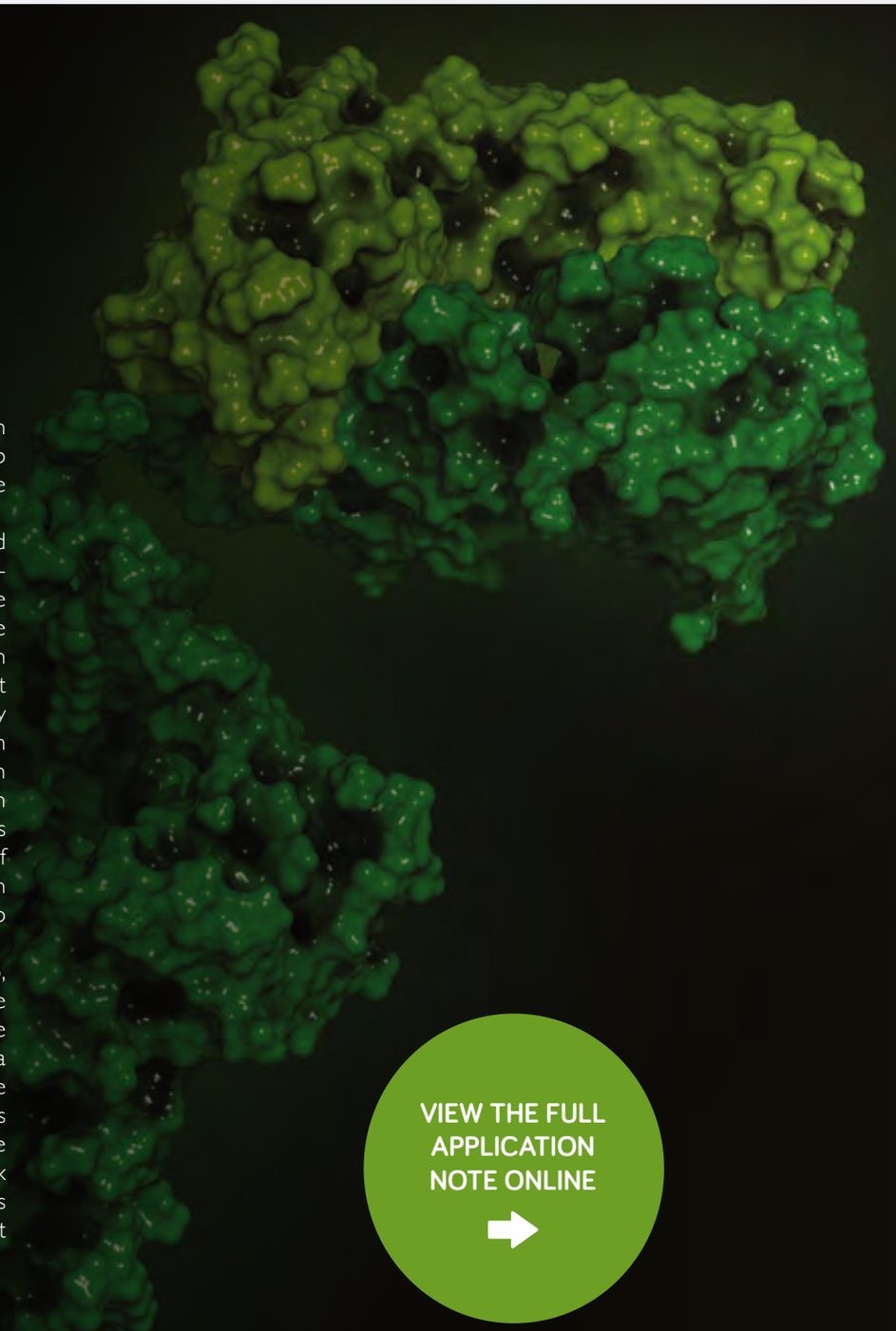
Aggregates are typically dimers, trimers, or larger order structures of antibody molecules. They are formed at the following stages:

- Product expression during fermentation
- Product purification in downstream processing of the
- Drug substance
- Storage or mishandling of the drug prior to patient administration.

Protein aggregation has been implicated as the cause of adverse immunological reactions that result in serious safety and efficacy issues. Aggregation must be monitored throughout the production process and during storage of the formulated biotherapeutic. MAb fragments that are smaller than the expected molecular weight elute after the parent peak and can also be determined.

Size-exclusion chromatography (SEC) is the standard method for this important analysis, but the compounds can show non-specific binding to the columns, which leads to retention time shifts, peak tailing, or even complete loss of protein peaks. The Thermo Scientific™ MAbPac™ SEC-1 column is silica based with a proprietary, covalently bonded diol hydrophilic layer to prevent secondary interactions. Even so, the mobile phase eluents usually contain high salt concentrations to prevent ionic interactions, which can lead to corrosion of metal components. For this reason, an inert UHPLC system is recommended. The MAbPac SEC-1 column separates by size and the pore size for this column (300 Å) was chosen to give a good separation in the molecular weight range of the monomer and dimers of a typical 150 kDa mAb. This column therefore serves as a good, broadly applicable column for mAb aggregate analysis.

Five important biotherapeutic mAbs (bevacizumab, cetuximab, infliximab, rituximab, and trastuzumab) were selected to investigate column performance. The monoclonal antibodies chosen are structurally diverse to investigate secondary interactions over a wide range of the physicochemical space. They cover a pI range between 7.6 and 8.7 and have widely different glycosylation patterns from very simple (bevacizumab) to highly complex (cetuximab). The MAbPac SEC-1 column and Thermo Scientific™ Vanquish™ Flex Quaternary UHPLC system were applied to the aggregate analysis of five important biotherapeutic mAbs using a common high salt buffer mobile phase at pH 6.8.



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LINKS

MAbPac SEC-1 Column

A Universal Chromatography Method for Aggregate Analysis of Monoclonal Antibodies

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The Importance of Correct UHPLC Instrument Setup for Protein Aggregate Analysis by Size-Exclusion Chromatography

Amy Farrell[1], Jonathan Bones[1], and Ken Cook[2]
[1] NIBRT, Dublin, Ireland; [2] Thermo Fisher Scientific, Hemel Hempstead, UK

The industry-standard size-exclusion chromatography (SEC) column dimension for protein aggregate analysis is 7.8 mm internal diameter (i.d.), running at 1.0 mL/min. This is usually carried out using older HPLC systems, which perform well enough with these relatively high flow rates. The introduction of UHPLC, with much lower dispersion, has allowed the use of lower flow rate columns and higher resolution stationary phases. It is commonly believed that smaller dimension SEC columns are difficult to pack and this accounts for the reduction in performance seen with these columns compared to higher flow rate 7.8 mm i.d. columns. A more likely cause for apparent reduced performance is that the UHPLC system used for the comparison does not have the correct tubing or low dispersion flow path required to maintain peak integrity, an essential factor when using SEC at lower flowrates.

A Vanquish Flex Quaternary UHPLC system was applied for the SEC analysis. This is a low dispersion, inert UHPLC, which can be used successfully for this type of application. For this analysis, pre-column dispersion was intentionally introduced to show the effects of dispersion in front of the column at different flow rates. Buffer mobile phase at pH 6.8.

The separation was performed on MabPac SEC-1 columns of differing internal diameters and flow rates.

The Vanquish UHPLC system was used to show the applicability of the MAbPac SEC-1 column for monoclonal antibody aggregate analysis. TheMAbPac SEC-1 column is silica based and has been covalently modified with a proprietary diol hydrophilic layer to prevent secondary interactions which can hinder the chromatography of certain proteins. SEC is one of the few chromatography methods that exhibits no 'on-column' focusing. Due to this, the pre-column dispersion on the system used is extremely important, especially at reduced flow rates on smaller i.d. columns, as there will be no focusing of broad peak volumes at the head of the column. In adsorption chromatography, even under isocratic elution conditions, one would expect some focusing of the injection volume at the head of the column. In SEC, the volume in which the sample is presented to the column will only get larger in volume as it moves through the column. Therefore, many columns do not attain their expected resolution when using older HPLC systems with inherent dispersion on-injection.

The effect of pre-column dispersion has been the subject of several reviews and can easily lead to up to 50% increase in peak widths on dispersive HPLC systems. The low dispersion Vanquish UHPLC system was applied for the SEC analysis to control and study the effects of dispersion. The separation was performed on MabPac SEC-1 columns.

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LINKS

Importance of UHPLC Instrument Setup for Protein Aggregate Analysis by Size-Exclusion Chromatography

Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) Software

Sponsored by ThermoFisher SCIENTIFIC

Produced by the Analytical Scientist

Lifetime Stability of Size Exclusion Chromatography Columns for Protein Aggregate Analysis

Amy Farrell[1], Craig Jakes[1], Alexander Ley[2], Mauro De Pra[2], Frank Steiner[2], and Jonathan Bones[1]
[1] The National Institute for Bioprocessing Research and Training (NIBRT), Dublin, Ireland [2] Thermo Fisher Scientific, Germering, Germany

Monoclonal antibodies (mAbs) are currently the dominant class of protein therapeutics in the biopharmaceutical industry due to their high specificity to target antigens, long serum half-life in humans, and capabilities for use in the treatment of a wide range of ailments such as inflammatory diseases and cancer. During product expression and purification from host cells, formulation, and storage, mAbs may undergo various degradation processes, which may alter the safety, efficacy, and quality profile of the drug product. Consequently, a number of critical quality attributes (CQAs) must be monitored throughout drug development and production to ensure biotherapeutic substances are suitable for clinical use.

Aggregation is a common degradation process for therapeutic proteins that can result from partial unfolding or other types of conformational changes in protein structure to form dimers, trimers, and other higher order structures. Protein aggregation is considered a CQA as aggregates may reduce product efficiency by lowering the effective concentration of the product and have been found to result in immunogenic effects in patients. Hence, it is a regulatory requirement to monitor the aggregation profile of therapeutic proteins.

Size exclusion chromatography (SEC) is the most commonly applied method for protein aggregate analysis. In SEC, sample molecules are passed through a column containing porous polymer or silica beads to facilitate separation of species based on their size. For protein therapeutics, a pore size of 300 Å is used, which allows smaller species to penetrate into the porous beads (e.g. fragments, monomers) while larger molecules (e.g. dimers, trimers) are more excluded from the pores and therefore elute more quickly from the column. Most frequently, SEC columns with widths of 7.8 mm are used for aggregate analysis in industry. However, use of low dispersion, biocompatible UHPLC systems and narrow internal diameter pre-column tubing has been shown to improve peak shape in chromatograms generated using columns of reduced widths. Narrow columns have advantages such as small sample volume requirements and reduced mobile phase consumption.

Due to high costs associated with drug development and production and the potential for mAb-based biosimilars resulting from patent expiry for some of the top-selling mAb therapeutics, reliable, long-use consumables and equipment for CQA evaluation is needed. Suppliers of SEC columns have illustrated column stability lifetimes of approximately 550 injections (without a column guard) and up to 902 injections (with a column guard). In this study, a MAbPac SEC-1 column (4 × 300 mm, 5 µm, 300 Å) and a Vanquish Flex Quaternary UHPLC system were applied to assess the long-term stability of the column based on protein aggregation monitoring of the commercial drug product bevacizumab, a humanized monoclonal IgG1 antibody produced from a Chinese hamster ovary mammalian cell expression system. Use of pre-column tubing with internal diameter of 75 µm ensured that excellent peak shape was achieved using a SEC column with dimensions of 4 × 300 mm. However, as SEC columns with widths of 7.8 mm are most commonly utilized in the biopharmaceutical industry, the column lifetime stability of an MAbPac SEC-1 column (7.8 × 300 mm, 5 µm, 300 Å) was also evaluated.

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LINKS

Vanquish Flex Quarternary UHPLC System

Thermo Scientific™ Virtuoso™ Vial Identification System

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Produced by the Analytical Scientist

High-Throughput Protein Aggregate Analysis of Monoclonal Antibodies Using a Novel Dual-Channel UHPLC Instrument

Sara Carillo[1], Martin Samonig[2], Jonathan Bones[1]
[1] National Institute for Bioprocessing Research and Training (NIBRT), Dublin, Ireland [2] Thermo Fisher Scientific, Germering, Germany

In the current scientific environment, there is an increased need for the rapid and robust high-throughput analysis of biotherapeutics, in particular monoclonal antibodies (mAbs). This is due to the rise in the use of mAbs for the treatment of diseases. As a result, standardized chromatographic methods and excellent reproducibility are essential for sample analysis in quality control (QC) laboratories. Although standard UHPLC systems can analyze samples simply and rapidly, there is typically only the option to use one stationary phase at any given time. As a result, analysts are limited regarding the number of injections that a chromatography system can perform, leading to less efficient sample analysis and additional costs. In the biomanufacturing pipeline, biopharmaceutical handling and storage can cause a number of unintentional size variants of the original product that are potentially harmful for human health. Size-exclusion chromatography (SEC) is considered a gold standard for monitoring the formation and level of mAb aggregates and fragments and is probably one of the most frequently performed analyses in QC laboratories.

In this study, the Vanquish Flex Duo system for Dual LC was used for the high-throughput analysis of a mAb. One hundred injections of bevacizumab were performed on two identical MAbPac SEC-1 size exclusion columns. SEC is commonly used for the highresolution separation of mAb aggregates and fragments.

A number of chromatographic parameters were determined—including analyte retention time, peak width, area, symmetry, and efficiency—highlighting the similar results between each of the channels of the Vanquish Flex Duo system for Dual LC and the low % RSD values obtained. These results were also obtained on a standard Vanquish Flex Quaternary UHPLC system for direct comparison.

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LINKS

MAbPac SEC-1 Column

Vanquish Duo UHPLC System

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Simultaneous Analysis of Monoclonal Antibodies Using A Novel Dual-Channel UHPLC Instrument and Orthogonal Chromatography

Nicola McGillicuddy[1], Amy Farrell[1], Sara Carillo[1], Martin Samonig[2], Jonathan Bones[1]
[1] National Institute for Bioprocessing Research and Training (NIBRT), Dublin, Ireland [2] Thermo Fisher Scientific, Germering, Germany

Although standard UHPLC systems can analyze samples simply and rapidly, typically only one stationary phase can be used at any given time, although there are multiple features to monitor and each requires its own method. As a result, analysts are limited regarding the number of injections that a chromatography system can perform, leading to less efficient sample analysis and additional costs. The Vanquish Flex Duo system for Dual LC can address these problems using two simultaneous LC channels for the same sample.

In this study, we focused on the flexibility of the Vanquish Dual LC workflow. Orthogonal analyses were performed on the same sample at the same time. The two analyses use different elution solvents and different instrument methods, varying in data collection wavelengths, gradient, or isocratic conditions and different run times.

A number of chromatographic parameters were determined—including analyte retention time, peak width, area, symmetry, and efficiency—highlighting the similar results between each LC channel of the Vanquish Flex Duo system for Dual LC and the low % RSD values obtained.

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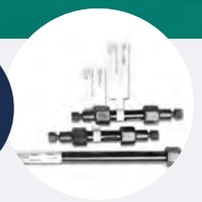
LINKS



MAbPac SEC-1 Column

MAbPac RP Column

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Using the NISTmAb Reference Standard to Demonstrate a Simple Approach to Charge Variant Analysis

Silvia Millán, Amy Farrell, and Jonathan Bones
Characterisation and Comparability Laboratory, NIBRT – The National Institute for Bioprocessing Research and Training, Dublin, Ireland

The characterization of monoclonal antibodies (mAbs) during biopharmaceutical development involves the identification, monitoring, and analysis of charge variants. Antibodies can exhibit changes in charge heterogeneity during production and purification caused by amino acid substitutions, glycosylation, and other post-translational or chemical modifications. Not only can these changes impact stability and activity, they can also cause adverse immunological reactions. Identification of charge variants in development, and their monitoring throughout manufacturing is therefore critical.

Ion-exchange chromatography (IEX) is widely used for the characterization of therapeutic proteins and can be considered a powerful reference technique for the qualitative and quantitative evaluation of charge heterogeneity. IEX separates charge variants by differential interactions with a charged support.

Numerous variants are commonly observed when mAbs are analysed by charged-based separation techniques. These variants are generally referred to as acidic or basic species, compared with the main isoform, and are defined based on their retention times relative to the main peak. Acidic species are variants with lower pI that elute before the main peak by cation exchange (CEX), and basic species are variants with higher pI that elute after the main peak by CEX. Deamidation of asparagine residues and sialic acid content have been widely reported to contribute to the formation of acidic species. Other modifications have also been shown to result in the generation of acidic species such as the non-classical disulfide linkages or high mannose oligosaccharides content. So far, basic species can be fully explained by known modifications including N-terminal glutamine, N-terminal leader sequences, C-terminal lysine, C-terminal amidation, or succinimide.

State-of-the-art and emerging analytical and biophysical methodologies provide very detailed process and product information; however, their accuracy, precision, robustness, and suitability are also of critical importance. The NIST monoclonal antibody IgG1K (NISTmAb) is intended to provide a well-characterized, longitudinally available test material that is expected to greatly facilitate analytical development applications associated with the characterization of originator and follow-on biologics for the foreseeable future.

The NISTmAb reference material is a recombinant humanized IgG1K expressed in murine suspension culture, which has undergone biopharmaceutical industry standard upstream and downstream purification to remove process related impurities. It is an approximately 150 kDa homodimer of two light chain and two heavy chain subunits linked through both inter- and intra-chain disulfide bonds. The molecule has a high abundance of N-terminal pyroglutamination, C-terminal lysine clipping, and glycosylation of the heavy chain sub-units. The protein also has low abundance post-translational modifications including methionine oxidation, deamidation, and glycation.

This application note presents the benefits of using pH gradient elution for separation of charge isoforms of NISTmAb reference material 8671 (Lot No. 14HB-D-002) using the combination of the MAbPac SCX-10 strong cation exchange (SCX) column and the Thermo Scientific CX-1 pH Gradient Buffers on a Vanquish Flex UHPLC for optimum performance. Additionally, NISTmAb charge variants were characterized following the chromatographic conditions described in the recently published book 'State-of-the art and emerging technologies for therapeutic mAb characterization. Volume 2', which serves as both a foundational body of NISTmAb product knowledge as well as an evaluation of its suitability as an industry-appropriate reference material (RM), containing representative methods and associated data for the NISTmAb of which extent and quality is comparable to that in a Biologics Licence Application (BLA).

LINKS

Using the NISTmAb Reference Standard to Demonstrate a Simple Approach to Charge Variant Analysis

Thermo Scientific™ ProPac™ Elite WCX Column

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Evaluation and Application of Salt- and pH-Based Ion-Exchange Chromatography Gradients for Analysis of Therapeutic Monoclonal Antibodies

Amy Farrell, Craig Jakes, and Jonathan Bones
Characterization and ComparabilityLaboratory, NIBRT – The National Institute for Bioprocessing Research and Training, Dublin, Ireland.

Monoclonal antibodies (mAbs) are well-established pharmacological therapeutics with high specificity to target antigens, long serum half-life in humans, and capabilities for the treatment of a wide range of ailments, such as inflammatory diseases and cancer. Recombinant mAbs are highly heterogeneous biomolecules that may undergo a wide range of enzymatic and chemical modifications during bioprocessing and storage, which have the potential to alter the safety and efficacy profile of mAbs by impacting protein binding, reducing drug activity, and giving rise to adverse toxicological and immunological responses in vivo. Hence, it is essential to detect, characterize, and quantify mAb variants and modifications (i.e. their critical quality attributes (CQAs)), to ensure the safety, quality, and efficacy of therapeutic biomolecules and thereby enable regulatory approval of mAbs for clinical use. Charge-related microheterogeneity of mAbs is frequently observed following bioprocessing due to both enzymatic processes and non-enzymatic degradation of therapeutic proteins.

Determination of a mAb charge variant profile provides information regarding commonly encountered product CQAs. Bioprocess-related protein modifications may impart a positive or negative charge on mAbs, resulting in the generation of modified mAb variants with different isoelectric point (pI) values. Chemical modifications, such as deamidation, may give rise to a negative charge on mAbs, thereby decreasing their pI value and resulting in the formation of acidic mAb variants.Similarly, C-terminal cleavage of lysine residues results in the loss of positively charged amino acids on the mAb also creating acidic protein variants. Other modifications that may affect the charge of mAbs include cyclisation of N-terminal glutamine to form pyroglutamate and peptide bond cleavage.

Ion-exchange chromatography (IEX), using either a salt or pH separation gradient, is the gold standard for determination of the charge variant profile of therapeutic mAbs.8 Traditionally, salt-based gradients, wherein the pH is maintained at a constant value while the ionic strength is increased over the duration of the gradient, were most frequently used for IEX. However, optimization of a salt gradient for charge variant analysis of individual mAbs often requires significant effort. Salt gradient buffer optimization may include the choice of salt reagent, salt concentration, and the pH value of the buffers. Use of pH gradients for IEX separation has been demonstrated as an excellent alternative to salt-based gradients for IEX of mAbs. pH-gradient-based ion-exchange chromatographic methods are reported to have greater resolving power and peak capacities when compared to ionic strength elution ion-exchange chromatography.9A protein's pI value corresponds to the pH value at which the protein has no net charge. In pH gradient SCX, the charge on the protein is reduced as the pH increases; elution occurs when the protein has no electrostatic interaction with the SCX stationary phase. Unlike a salt gradient, optimization of a pH gradient for IEX does not require alteration of the mobile phase composition and hence facilitates more rapid method optimization. Furthermore, reproducible batch-to-batch preparation of salt-based buffers may be challenging when using certain buffer reagents. In this application note, the reproducibility of different salt buffers prepared across multiple preparation dates by different analysts is evaluated for both salt- and pH-based gradient buffers. In addition, two approaches to the generation of a pH gradient were investigated. One approach consisted of quaternary pump mixing of an acid, a base, a salt solution, and water to produce a pH separation gradient. The second approach was generation of a pH gradient using a Thermo Scientific cation-exchange pH gradient buffer platform consisting of a low pH buffer at pH 5.6 (CX-1 pH Gradient Buffer A) and a high pH buffer at pH 10.2 (CX-1 pH Gradient Buffer B). Using a gradient from 100% of CX-1 pH Gradient Buffer A to 100% of CX-1 pH Gradient Buffer B, a linear pH gradient from pH 5.6 to pH 10.2 may be generated. Since the majority of mAbs have pI values in the range of pH 6 to 10, pH-gradient-based separation methods based on the pH buffer platform and MAbPac SCX-10 columns may serve as a generic platform for mAb charge variant analysis. Furthermore, the described platform enables rapid, simple method optimization by merely using a shallower pH gradient over a narrower pH range following initial separations in the typical mAb pI range of pH 6 to 10.

Currently, six of the top 10 selling biopharmaceutical products are therapeutic mAbs, all of which will have come off patent by 2019.10 As a consequence, many biosimilars corresponding to these innovator mAbs are currently being marketed or are under development. Thus, the availability of a simple, readily optimized, widely applicable platform for charge variant analysis is of great benefit to producers of innovator therapeutic mAbs and biosimilars. The charge variant profile of the commercial mAbs bevacizumab, cetuximab, infliximab, and trastuzumab were also determined in this application note using a MAbPac SCX-10 column on a Vanquish Flex UHPLC with CX-1 pH gradient buffers. Minimal method optimization in <1.5 hours facilitated excellent separation of mAb variants. This approach could be easily applied to multiple mAbs and mAb biosimilar products.

VIEW THE FULL APPLICATION NOTE ONLINE

LINKS

Evaluation and Application of Salt- and pH-based Ion-Exchange Chromatography Gradients

MAbPac SCX-10 Column

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Simple Charge Variant Profile Comparison of an Innovator Monoclonal Antibody and a Biosimilar Candidate

Silvia Millán, Anne Trappe, Amy Farrell, and Jonathan Bones

Characterization and Comparability Laboratory, NIBRT – The National Institute for Bioprocessing Research and Training, Dublin, Ireland.

Therapeutic proteins are large, heterogeneous molecules that are subject to a variety of enzymatic and chemical modifications during their epression, purification, and long-term storage. These changes include several possible modifications, such as oxidation, deamidation, glycosylation, aggregation, misfolding, or adsorption, leading to a potential loss of therapeutic efficacy or unwanted immune reactions.

Biosimilars are therapeutic proteins that are similar to originator protein therapeutics but are obtained using a different bioprocess. The two products can vary due to the cell line in which the monoclonal antibody is expressed, from small changes in the purification process or from a different composition of the final formulation. As a growing number of biosimilars have been introduced to the market, robust and reliable analytical techniques to confidently evaluate similarities and differences between the biosimilar and its originator are required. For biosimilar approval, regulatory bodies require detailed therapeutic protein characterization including lot-to-lot and batch-to-batch comparisons, stability studies, impurity profiling, glycoprofiling, determination of related proteins and excipients, as well as determination of protein aggregates.

Charge heterogeneity analysis is critical for monoclonal antibody (mAb) characterization as it provides valuable information regarding product quality and stability. Heterogeneity is complex and can be caused by such molecular adaptions as C-terminal lysine modification, deamidation, and other post translational modifications (PTMs).

Protein charge depends on the number and type of ionizable amino acids present. Lysine, arginine, and histidine residues are basic, whereas glutamic acid and aspartic acid residues are acidic. Each ionizable group has its own pKa; therefore, the number and type of ionizable amino acid groups dictates the overall number of charges on a particular protein at a given pH. Considering the structure in its entirety, each protein has a pI value corresponding to the pH value where its surface has no net charge.

The number of possible charge variants increases with the molecular weight of the protein and changes in charge may be additive or subtractive, depending on any PTMs. Numerous variants are commonly observed when mAbs are analyzed by charged-based separation techniques. These variants are generally referred to as "acidic" or "basic' species when compared with the main protein isoform.

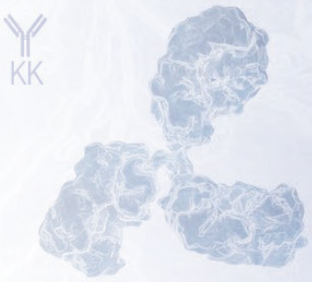
Ion-exchange chromatography (IEX) is a widely used, powerful reference technique for the characterization of therapeutic proteins. It is applied to both the qualitative and quantitative evaluation of charge heterogeneity. IEX separates charge variants by differential interactions on a charged support. Cation-exchange chromatography (CEX) is considered the gold standard, but method development is often complex as parameters such as column type and mobile phase composition (pH and salt concentration) are often required to be optimized for each individual protein analyzed.

CEX chromatographic separation may be performed using either a salt- or pH-based gradient. While both approaches may be optimized to provide good peak resolution, use of a pH gradient is advantageous due to the simplicity of using commercially available mobile phases, which allows easy generation of a linear pH gradient. Indeed, buffers used for salt gradient elutionare usually difficult to prepare in a reproducible way.

Salt gradients can cause corrosion to metal HPLC systems and care must be taken to avoid stainless steel or regularly passivate the HPLC system to avoid this.

With good buffer choice to develop a linear pH change across the HPLC gradient, the use of a pH gradient facilitates more reproducible mobile phase preparation resulting in consistent method selectivity and greater retention time precision when compared to salt gradient buffers. In addition, a pH gradient is readily optimized and can be applied more universally for mAb variant analysis of diverse proteins. Nevertheless, for an optimum separation of monoclonal antibodies acid and basic variants, the possibility to obtain a steady pH change across the gradient is important to improve selectivity and resolution.

Monoclonal antibodies typically possess pI values between 6 and 10. When the pH is at the pI of the protein, there will be no ionic interaction between the protein and the IEX stationary phase; when using a pH gradient there will be no interaction between the protein and the stationary phase so the protein will elute from the column. This requires the starting pH of a mobile phase to be below the pI of the lowest pI proteoform, while the final pH has to be somewhat higher than the pI of the highest pI proteoform. Considering the behavior of charge variants in cation-exchange chromatography, acidic variants elute before the main peak and basic variants after the main peak.



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LINKS

CX-1 pH Gradient Buffers

MAbPac SCX-10 Column

High-Precision, Automated Peptide Mapping of Proteins

Amy Farrell[1], Jonathan Bones[1], Ken Cook[2], Suraj Patel[2], Alexander Schwahn[2], Jon Bardsley[2]

[1] Characterisation and Comparability Laboratory, NIBRT – The National Institute for Bioprocessing Research and Training, Dublin, Ireland [2] Thermo Fisher Scientific, Reinach, Switzerland; Runcorn, UK; Hemel Hempstead, UK

The biopharmaceutical industry continues to develop protein-based biotherapeutics in increasing numbers. Due to their complexity and biotechnological production, there are many attributes that need to be analyzed to guarantee their safety and efficacy.

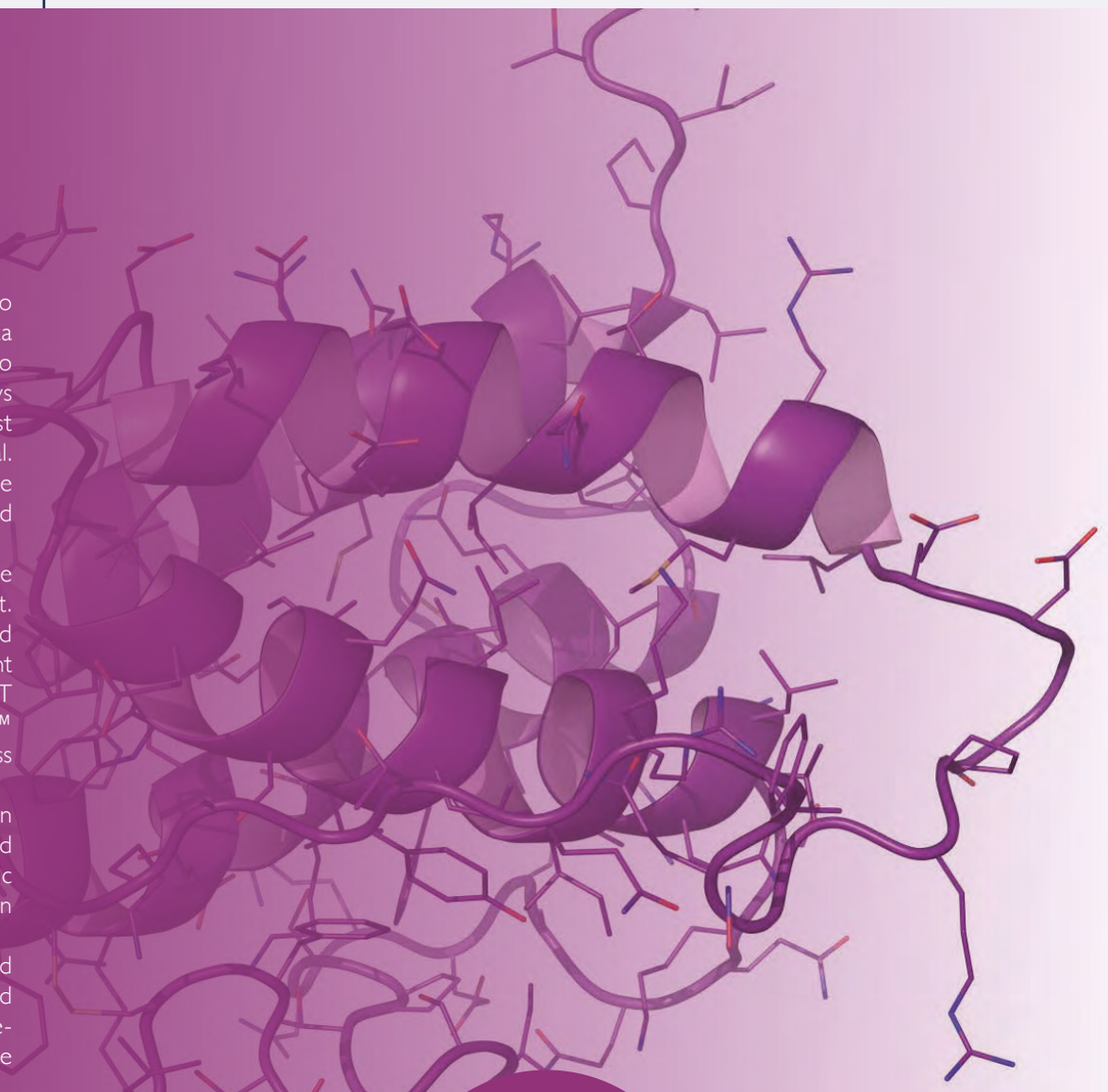
Peptide mapping is used to measure several critical quality attributes (CQA) required for the characterization of any biotherapeutic protein. The analysis is used to confirm that the correct sequence has been expressed for the protein and to check for post-translational and chemical modifications.

Mass spectrometry (MS) is coupled to liquid chromatography (LC) for peak identification and confirmation of the sequence. However, many quality control (QC) methods use detection by ultraviolet (UV) absorption only after the peaks identities have been confirmed. Trypsin is the enzyme most commonly used for proteolytic digestion due to its high specificity. Although a widely accepted technique, in-solution trypsin digestion protocols required for sample preparation are labor intensive and prone to manual errors. These errors affect the quality of the analytical data compromising the ability to reproducibly characterize a protein to the required standard. In the most critical cases where workflows only employ UV detection without confirmation by MS, robust and stable sample preparation and separation methods are critical. The digestion must be reproducible and chromatography must be extremely stable to allow unambiguous peptide identification based on chromatographic retention time.

This work details the automated peptide mapping of cytochrome c, recombinant somatotropin, and infliximab drug product. These proteins were chosen to investigate the applicability and reproducibility of the automated digestion protocol and subsequent analysis. The combination of the Thermo Scientific™ SMART Digest™ magnetic beads and the Thermo Scientific™ KingFisher™ Duo purification system was used to automate the digestion process to produce high quality, reproducible peptide mapping data.

Magnetic beads are a proven support used for many purification and sample preparation approaches in life science research and biotechnology. The KingFisher purification system enables robotic handling and easy automation of any magnetic bead based application resulting in superior performance and reproducibility.

The Vanquish Horizon UHPLC system was subsequently used to analyze the samples by UHPLC-UV and, additionally, coupled to a Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer for MS confirmation of the peptide sequence.



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LINKS

High-Precision, Automated Peptide Mapping of Proteins

KingFisher Duo Prime Purification System

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Robust and Reproducible Peptide Mapping and Intact Mass Analysis Workflows on a Single Instrument Platform

Amy Farrell[1], Kai Scheffler[2], Ken Cook[2], Martin Samonig[2], David Munoz[2], Alexander Schwahn[2], and Jonathan Bones[1]

[1] Characterisation and Comparability Laboratory, NIBRT – The National Institute for Bioprocessing Research and Training, Dublin, Ireland [2] Thermo Fisher Scientific

The biopharmaceutical industry continues to develop protein-based biotherapeutics in increasing numbers. Due to their complexity and biological production by living cells, there are many attributes that need to be analyzed to guarantee their safety and efficacy. This can involve multiple analytical techniques based on several different instrument platforms. There is an industry desire to simplify the processes, produce multi-attribute methodologies, and increase reproducibility between laboratories. Here we use a single instrument platform and software with multiple characterization workflows that generate data for multiple quality controlled attributes.

Peptide mapping is one of the most important assays in the characterization of any biotherapeutic protein. The analysis is used to confirm that the correct sequence has been expressed for the protein and as a check for several post-translational modifications. High-resolution mass spectrometry is coupled to chromatography for peak identification and quantitation. Many QC methods use solely UV detection after the peak identities have been confirmed to simplify the method for a QC environment. However, UV-only data limits the attributes that can be measured and does not give absolute positive identification of the peptides. Here we show the possibility to use a simple, easy-to-implement LC-MS method with evidence of the benefits of such a system over UV-only detection.

Trypsin is the most commonly used proteolytic digestion enzyme due to its high specificity. However, many trypsin-based protocols and kits that have been developed for proteolytic digestion of proteins are labor intensive, prone to manual errors and may also introduce post-translational modifications during digestion. Reproducible digestion is imperative for peptide mapping sample preparation, yet often leads to difficulty during method transfer. The digestion method used here is simplified and improved using immobilized heat stable trypsin.

Intact protein analysis confirms that product with the correct molecular weight has been expressed and is an important characterization step for biotherapeutic proteins. High-resolution, accurate-mass (HRAM) Orbitrap mass spectrometry has been shown to be essential for this technique. The same instrument platform, incorporating an easily automated change of columns and separation gradient, was used for the peptide mapping analysis. The intact molecular weight analysis was performed under native and denaturing conditions at high resolution to give isotopically resolved mass spectra.

Somatotropin is a small recombinant biotherapeutic protein used here as a model protein to describe the use of a new improved single instrument platform for extensive protein characterization analysis.

It is essential to detect, characterize, and quantify any undesirable modifications and confirm the correct product identity of recombinant proteins. In this application note, we demonstrate that typical protocols used for this type of characterization can be simplified and made more reproducible with new workflows performed on the same system. Peptide mapping is the most common analytical method employed for this purpose and delivers a wealth of information from correct amino acid sequence to the presence, location, and quantification of several post-translational modifications. Multiple quality attributes can be defined by peptide mapping analysis. Although a widely accepted and powerful technique, the digestion protocols for sample preparation are labor intensive and prone to manual errors and unwanted modifications. This can affect the quality of the analytical data and creates a source of irreproducibility. Incomplete digestion may render the accurate quantification of modifications impossible, however, a small amount of missed cleavage may enable 100% sequence coverage. A careful balance of digestion completion needs to be maintained in a very reproducible manner.

The SMART Digest™ Kit was used for the sample preparation for peptide mapping analysis. This protocol greatly simplifies the digestion process and increases reproducibility. Intact protein analysis is a complimentary technique used to ensure the correct molecular weight of the protein biotherapeutic. Both these techniques can be performed on the same analytical platform with no change in the eluents used for chromatography. The Thermo Scientific™ BioPharma Finder™ software combines the identification and quantitation tools for peptide mapping with the deconvolution software used for intact protein analysis. Use of the described workflows on a single platform for this extensive characterization easily

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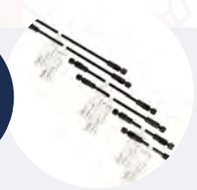
LINKS

SMART Digest Kit



MAbPac RP Column

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Investigating Process-Related Post-Translational Modifications in NISTmAb RM 8671 Using High-Throughput Peptide Mapping Analysis

Silvia Millán, Craig Jakes, Noemí Dorival, Sara Carillo, Jonathan Bones
Characterisation and Comparability Laboratory, NIBRT – The National Institute for Bioprocessing Research and Training, Dublin, Ireland.

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Monoclonal antibodies (mAbs) and related products are the fastest growing class of human therapeutics. They are a class of recombinant proteins that are susceptible to a variety of enzymatic or chemical modifications during expression, purification, and long-term storage. Unlike small molecule drugs, protein therapeutics are made via DNA expression techniques. This is a highly complex process; hence, protein therapeutics require a close monitoring of their structural characterization and evaluation of their quality in each step to ensure drug safety and efficacy. Therefore, it is vital that biopharmaceuticals are comprehensively characterized.

Peptide mapping is a critical workflow in biotherapeutic protein characterization and is essential for elucidating the primary amino acid structure of proteins. For recombinant protein pharmaceuticals, such as mAbs, peptide mapping is used for proof of identity, primary structural characterization, and quality assurance (QA). Global regulatory agencies, including U.S. Food and Drug Administration (US FDA) and European Medicines Agency (EMA), look to harmonized guidelines from the International Council for Harmonisation (ICH). ICH Q6B5 covers the test procedures and acceptance criteria for biologic drug products and specifies the use of peptide mapping as a critical quality test procedure for drug characterization used to confirm desired product structure for lot release purposes.

To generate a peptide map, a bottom-up strategy is generally used. The therapeutic protein must first be digested into its constituent peptides via a chemical or enzymatic reaction. Robust separation and identification of the resultant peptides then provides insight into a protein's full sequence information, displaying each amino acid component and the surrounding amino acid microenvironment. Structural characterization at this level highlights PTMs such as site-specific glycosylation, amino acid substitutions (sequence variants), and/or truncations, which may result from erroneous transcription of complementary DNA.

A peptide map is a fingerprint of a protein that provides a comprehensive understanding of the protein being analyzed. Consequently, it is a routine analysis for the characterization of mAbs. Although modern peptide-mapping procedures adequately perform their primary function, they typically consist of many laborious steps, which can vary due to differences among techniques or operators, or even across laboratories. As such they are susceptible to changes that affect reproducibility, reduce assay sensitivity and significantly increase analysis times. These procedures are not easily automated, which can reduce data confidence and potentially introduce sample artefacts due to manual sample processing. This variation can be especially challenging when it is necessary to compare different product batches across months or years. As data quality is imperative, variation in results might jeopardize product quality, ultimately affecting patient safety. All this raises the need to adopt a new method that can overcome all these drawbacks and offer a simple, robust and reliable alternative.

The SMART Digest Kit provides a simple alternative for peptide mapping sample preparation. It is a fast and simple procedure that greatly improves intra- and inter-laboratory data reproducibility, assuring absolute confidence in analytical results. In this study, peptide mapping experiments were performed using the NISTmAb (NISTmAb RM 8671) provided by the National Institute of Standards and Technology (NIST). The NISTmAb was chosen because it is a well-characterized, commercially available test material that is expected to greatly facilitate analytical development applications associated with the characterization of originator and follow-on biologics for the foreseeable future.

The NISTmAb is a recombinant humanized IgG1κ expressed in murine suspension culture, which has undergone biopharmaceutical industry standard upstream and downstream purification to remove process related impurities. It is a ≈150 kDa homodimer of two light chain and two heavy chain subunits linked through both inter- and intra-chain disulfide bonds. The molecule has a high abundance of N-terminal pyroglutamination, C-terminal lysine clipping, and glycosylation of the heavy chain sub-units. The protein also has low abundance PTMs including methionine oxidation, deamidation, and glycation.

In this study, both light and heavy chain sequence coverage was assessed, as well as the identification and relative quantification of a specific set of PTMs: oxidation, glycosylation, and deamidation.

After digestion with the SMART Digest kit was completed, separation of peptides was performed using the Vanquish Flex Binary UHPLC system with the Thermo Scientific™ Acclaim™ VANQUISH™ C18 analytical column. MS data was interrogated using BioPharma Finder software. A list of peptides was generated from the highly complex data and subsequently matched against the corresponding mAb sequence with a sequence coverage map generated. The use of the SMART Digest kit resulted in a simple, easy to use, fast method, emphasizing its high reproducibility, lower tendency to generate PTMs, higher enzyme stability, and high amenability to automation.

LINKS

SMART Digest Kit

Q Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer

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Produced by the Analytical Scientist

Automated Chymotrypsin Peptide Mapping of Proteins by LC-MS

Amy Farrell[1], Jonathan Bones[1], Ken Cook[2]
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The biopharmaceutical industry continues to develop protein-based biotherapeutics in increasing numbers. Due to their complexity and biotechnological production, there are many attributes that need to be analyzed to guarantee their safety and efficacy.

Peptide mapping is used to measure several critical quality attributes (CQAs) required for the characterization of any biotherapeutic protein. The analysis is used to confirm that the correct sequence has been expressed for the protein and to check for post-translational and chemical modifications (PTMs). Mass spectrometry (MS) is coupled to liquid chromatography (LC) for peak identification and sequence confirmation.

Trypsin is the most commonly used proteolytic digestion enzyme due to its high specificity for cleavage at arginine and lysine residues. However, there are proteins that do not digest well with trypsin due to too many or too few of the specific trypsin cleavage sites in the sequence. There are also cases where the cleavage sites can be too close together, producing very short hydrophilic tryptic peptides that do not retain on the reversed-phase HPLC columns and are therefore difficult to detect.

Chymotrypsin is a protease that has alternative cleavage specificity to trypsin. Chymotrypsin will cleave primarily at the hydrophobic aromatic amino acid residues of tryptophan, tyrosine, and phenylalanine. However, chymotrypsin is also known to additionally cleave at other sites such as leucine, histidine, and methionine, but with a lower level of specific activity. The consequence of chymotrypsin's selectivity is that the aromatic amino acids are among the first peptide bonds to be broken with other sites being cleaved at a slower rate.

Unlike trypsin, which has a high specificity for only two amino acid residues, the chymotrypsin digestion pattern alters as the digestion time increases due to a slower rate of activity at its alternative digestion sites. Unless the time of digestion is carefully controlled, errors can occur that will compromise the ability to reproducibly characterize a protein to the required standard. This is especially true where workflows only employ UV detection without peptide confirmation by MS. The digestion must be reproducible and chromatography must be extremely stable to allow unambiguous peptide identification based on chromatographic retention time.

This work details the automation of a chymotrypsin digestion of recombinant somatotropin as a model protein. The applicability and reproducibility of an automated chymotrypsin digestion protocol and subsequent analysis was investigated. In addition, this work also shows the effects of digestion time on chymotrypsin activity.

Magnetic beads are a proven support used for many purification and sample preparation approaches in life science research and biotechnology. The Thermo Scientific™ KingFisher™ Duo purification system enables robotic handling and easy automation of any magnetic-bead-based application resulting in superior performance and reproducibility. The combination of Magnetic SMART Digest beads and the KingFisher Duo purification system was used to automate the digestion process to produce high quality, reproducible peptide mapping data using an alternative protease.

The Vanquish Horizon UHPLC system was subsequently used to analyze the samples and coupled to a Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer for MS confirmation of the peptide sequence.

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LINKS

Vanquish Horizon UHPLC System

Acclaim Vanquish C18 Column

Sponsored by ThermoFisher SCIENTIFIC

Produced by the Analytical Scientist

Comparison of Alternative Approaches to Trypsin Protein Digestion for Reproducible and Efficient Peptide Mapping Analysis of Monoclonal Antibodies

Silvia Millán-Martín, Craig Jakes, Noemi Dorival-García, Nicola McGillicuddy, Sara Carillo, Amy Farrell, Jonathan Bones

National Institute for Bioprocessing Research and Training (NIBRT), Dublin, Ireland.

Due to their long half-life in humans and high specificity to target antigens, monoclonal antibodies (mAbs) are the leading biotherapeutics used for the treatment of multiple disease states, including cancer, inflammatory, autoimmune, cardiovascular, and infectious diseases. Sales of €125 billion worldwide by the year 2020 are predicted at the current approval rate. However, due to their complexity and the possibility of protein degradation, extensive characterization of mAbs must be carried out prior to clinical trials and for routine quality control assessments.

Peptide mapping is a "gold standard" tool in biotherapeutic characterization, used to measure critical quality attributes (CQAs) of mAbs to ensure product quality, efficacy, and safety. Peptide mapping consists of the enzymatic or chemical treatment of a protein, resulting in peptide fragments that are then separated, detected, and interpreted. The protease trypsin is most frequently employed to digest therapeutic mAbs for peptide mapping analysis due to its specificity for cleavage at lysine and arginine residues. This results in the production of peptides with the preferred mass range for mass spectrometry detection.

Various approaches to peptide mapping have been developed, utilizing different protein denaturation procedures and digestion times. However, there can often be a lack of reproducibility and confidence in results with commonly used digestion protocols for peptide mapping. This leads to the incurring of excessive cost and reduced productivity due to the laborious sample handling procedures and long digestion times (up to several hours). Furthermore, modifications such as deamidation and oxidation may be induced by sample preparation methods, thereby distorting results. Hence, a simple, robust and reproducible method is essential for peptide mapping.

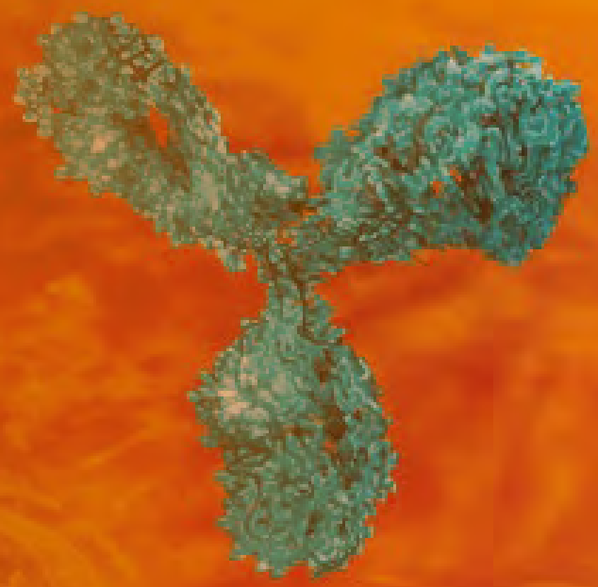
In this study, a traditional overnight trypsin digestion method and a recently applied rapid digestion protocol were compared to two SMART Digest Kit options (the standard kit and the magnetic bulk resin option). These kits contain thermally stable, immobilized trypsin that enables high temperature protein denaturation without a requirement for addition of denaturants. The SMART Digest trypsin kit (magnetic bulk resin option) was used in conjunction with the KingFisher Duo Prime purification system, which allows easy automation of magnetic bead based applications and is therefore suitable for high-throughput, reproducible sample analysis.

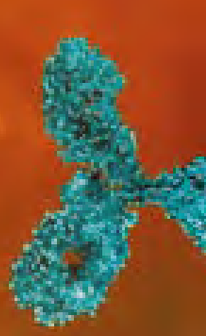
Two monoclonal antibodies were used to assess the alternative digestion methods: adalimumab, the world's top selling biotherapeutics product2 and the reference monoclonal antibody NISTmAb RM 8671 (NISTmAb), a standard commonly applied for evaluation of analytical method performance.7 All samples were analyzed on a high-resolution analytical platform consisting of a Vanquish Flex Binary UHPLC and a Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer with BioPharma Option.

Four sample preparation methods for peptide mapping were investigated to evaluate the impact of sample preparation on reproducibility and PTMs detected. For each preparation method evaluated (reference experimental section), samples were prepared by two different analysts, with varying sample preparation experience, over three different days and analyzed by liquid chromatography-mass spectrometry (LC-MS) detection. Data analysis was performed using BioPharma Finder software. The analytical platform enabled excellent reproducibility of chromatographic data enabling 100% sequence coverage of mAbs.

Peptide reproducibility and PTMs induced by sample preparation were investigated, highlighting the benefits of using a heat stable immobilized trypsin for sample preparation compared to other trypsin formulations that require high pH buffers for protein denaturation.

In addition, sample reproducibility was found to be further improved using trypsin immobilized on magnetic beads in combination with an automated handling system, e.g. KingFisher Duo Prime purification system.





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LINKS

SMART Digest Kit

Q Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer

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Produced by the Analytical Scientist

An Automated High-Throughput Workflow for Peptide Mapping to Monitor Post-Translational Modifications (PTMS) of Monoclonal Antibodies

Silvia Millán-Martín, Craig Jakes, Giorgio Oliviero, Sara Carillo, Jonathan Bones
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Complex glycoproteins, specifically monoclonal antibodies, are currently the most prevalent type of biotherapeutics in development. Monoclonal antibodies (mAbs), which offer high specificity and low side effects, are used to treat many types of cancer, autoimmune and inflammatory diseases, infections, and metabolic disorders, yielding their impressive success as human medicines. These large proteins have molecular weights near 150 kDa and are composed of two identical

~50 kDa heavy chains (HC) and two identical ~25 kDa light chains (LC). They also contain at least 16 disulfide bonds that maintain three-dimensional structure and biological activity. Although they share similar secondary protein structures, different mAbs vary greatly in the sequence of variable regions.

Since the commercialization of the first therapeutic mAb product in 1986, this class of biopharmaceutical products has grown significantly so that, as of December 1st, 2017, seventy-six mAb products have been approved in the US or Europe for the treatment of a variety of diseases, and many of these products have also been approved for other global markets. Rituximab (Rituxan®), cetuximab (Erbitux®), bevacizumab (Avastin®), trastuzumab (Herceptin®), and adalimumab (Humira®) were five of the top-ten selling mAbs in 2012.

Post-translational modification (PTM) refers to the covalent and generally enzymatic modification of proteins during or after protein biosynthesis. Proteins are synthesized by ribosomes translating mRNA into polypeptide chains, which may then undergo PTM to form the mature protein product. PTMs are important components in cell signaling and can occur on the amino acid side chains or at the protein's C- or N- termini. They can extend the chemical repertoire of the 20 standard amino acids by modifying an existing functional group or introducing a new one. PTMs affect structural and functional aspects of therapeutic proteins, and the effects can be detrimental, that is, heterogeneity and immunogenicity, even though the modification may originally be required for functional activity of the polypeptide.

Most therapeutic proteins approved or in development bear at least one or more PTMs. Variants of proteins produced for medicinal purposes can occur during manufacturing, handling, and storage, and can impact the activity and stability of the biotherapeutic. Their characterization is challenging due to their size and inherent heterogeneity caused by PTMs, among which glycosylation is probably the most prominent.

Peptide mapping is used routinely to study PTMs and is capable of pinpointing the amino acid residue within the sequence at which the modification has occurred. Peptide mapping is commonly used in the biopharmaceutical industry to establish product identity by confirming the primary structure of a product.8,9 For recombinant protein pharmaceuticals, peptide mapping is used for the initial "proof of structure" characterization. It confirms expression of the desired amino acid sequence and characterizes any PTM, for subsequent lot-to-lot identity testing, in support of bioprocess development and clinical trials. It is also used as the current method of choice for monitoring the "genetic stability" of recombinant cell lines.

This application note describes a full workflow solution for peptide mapping of five different monoclonal antibodies using the Magnetic SMART Digest resin option on a KingFisher Duo Prime purification system, in combination with the high-resolution, accurate-mass (HRAM) capabilities of theQ Exactive Plus mass spectrometer, and high-resolution chromatographic separation with the Vanquish Flex UHPLC system. BioPharma Finder software was used to interrogate the high-quality data sets.

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LINKS

SMART Digest Kit

Vanquish Flex Binary UHPLC System

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Fast Profiling of the N-Glycan Population in Biotherapeutic Antibodies by UHPLC-FLD with MS Confirmation

Silvia Millán, Stefan Mittermayr, Amy Farrell, and Jonathan Bones
Characterisation and Comparability Laboratory, NIBRT – The National Institute for Bioprocessing Research and Training, Dublin, Ireland

Glycans play an essential role in many biological processes, including cell development and differentiation, cell-cell or cell-matrix communication, and pathogen-host recognition. The glycan components of biotherapeutics can be important determinants of biological activity and therapeutic efficacy and hence characterization of the glycan profile of a biomolecule is required under regulatory guidelines (ICH Q5E/Q6B and USP 129). Therapeutic antibodies must be demonstrated to meet applicable quality requirements to ensure continued safety, purity, and potency of a drug product. An intensive biochemical characterization of the antibody itself is required, which includes a thorough examination of glycan distribution and potential impacts of glycoforms on mAb function. On the other hand, differences in glycan profiles between healthy and diseased states are utilized for clinical diagnosis8, providing targets for many novel classes of therapeutics including cancer chemotherapy, diabetes treatment, and antibiotic and anti-viral medicine.

The 'gold standard' for studying IgG glycosylation relies on enzymatic N-glycan release, subsequent fluorescent labeling by reductive amination, and analysis of the labeled glycans by high-performance liquid chromatography with fluorescence detection (HPLC-FLD), using hydrophilic interaction liquid chromatography (HILIC) with fluorescence detection. In addition to characterization of the sugar sequence, the analysis must elucidate linkages and separate all isomeric, charge, and branching variations of glycans.

HILIC columns commonly used for glycan analysis are based on amide, amine, or zwitterionic packing materials. These columns separate glycans mainly by hydrogen bonding, resulting in separations based on size and composition. The Thermo Scientific™ Accucore™ 150-Amide-HILIC HPLC column is a solid particle core phase designed for the separation of hydrophilic biomolecules in HILIC mode, which offers an excellent choice for glycan separation.

This application note presents a step-by-step method for release, labeling, separation, and exoglycosidase-based structural elucidation of N-glycans from human serum IgG and commercial chimeric IgG1 mAb (infliximab) using Vanquish Horizon UHPLC-FLD and confirmation of the structures by a Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer.

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LINKS

Accucore 150 Amide HILIC LC Columns

Vanquish Horizon UHPLC System

Sponsored by ThermoFisher SCIENTIFIC

Produced by the Analytical Scientist

An Ultrafast, Batch-to-Batch Comparison of Monoclonal Antibody Glycosylation

Silvia Millán, Stefan Mittermayr, Amy Farrell, and Jonathan Bones
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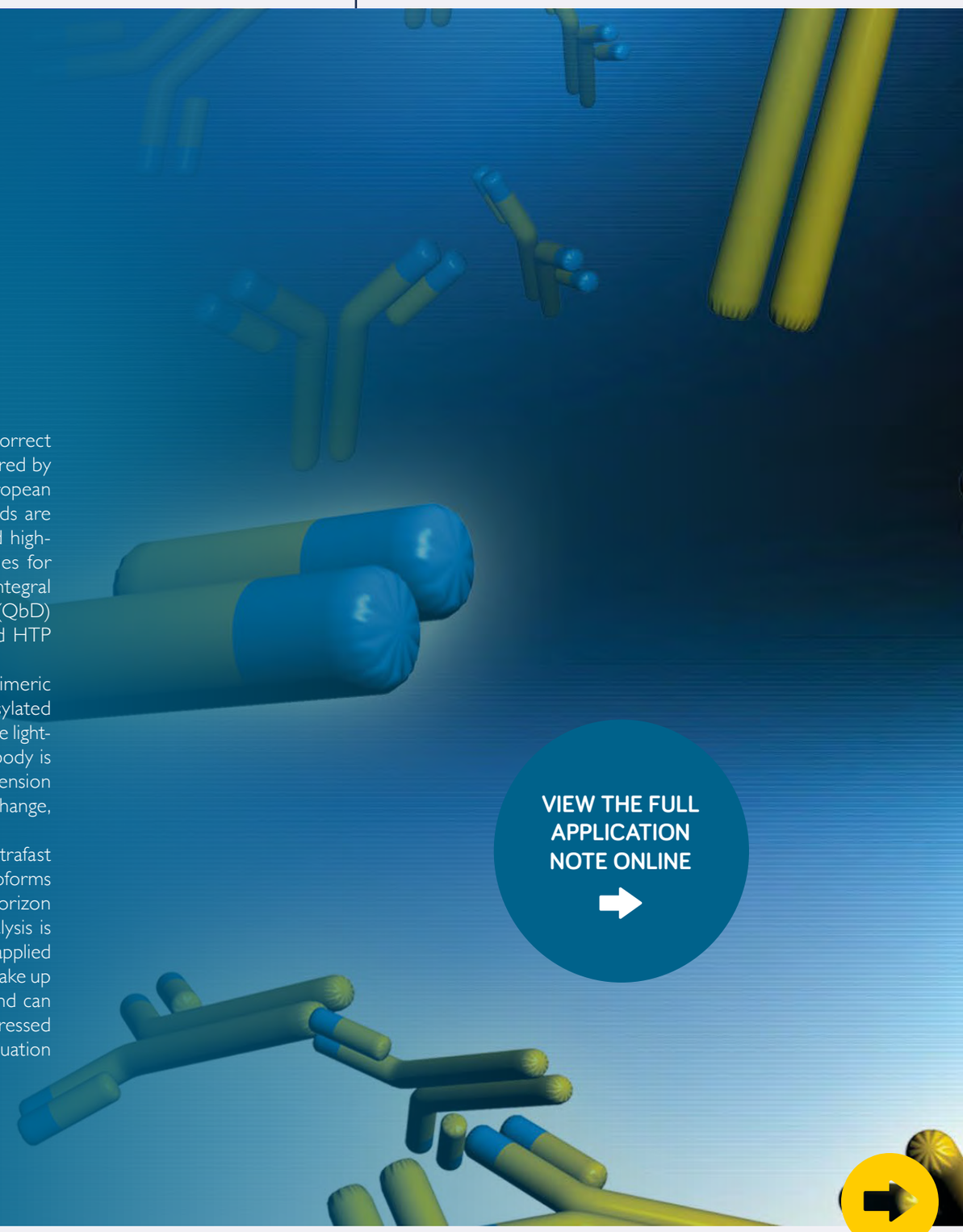
One of the fastest growing fields in the pharmaceutical industry is the market of therapeutic glycoproteins (glycosylated proteins), which are produced by living cell systems. These include monoclonal antibodies (mAbs) and other recombinant protein products (e.g., fusion proteins, growth factors, cytokines, therapeutic enzymes, and hormones), which are approved or under development as therapeutics. Glycosylation is a critical quality attribute (CQA) for development and manufacturing of therapeutic mAbs in the biopharmaceutical industry and, therefore, needs to be assessed to ensure desired product quality, safety, and efficacy. Different glycosylation variants have been shown to affect stability, pharmacokinetics, serum half-life, immunogenicity, and effector functions.

Glycoforms on biopharmaceutical glycoproteins are affected by the culture conditions as well as the cell type by which they are produced. Biopharmaceutical glycosylation monitoring for correct structure during production and for quality control is required by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMEA). Current glycoanalytical methods are laborious and time-consuming; therefore, more rapid and high-throughput (HTP) methods are required. HTP techniques for in-depth monitoring of glycoform distributions must be integral components for the implementation of quality by design (QbD) approaches. To meet this need, novel approaches toward HTP monitoring are required.

Rituximab (MabThera®) is a genetically engineered chimeric mouse/human monoclonal antibody representing a glycosylated immunoglobulin with human IgG1 constant regions and murine light-chain and heavy-chain variable region sequences. The antibody is produced by mammalian (Chinese hamster ovary) cell suspension culture and purified by affinity chromatography and ion exchange, including specific viral inactivation and removal procedures.

This application note presents a proof of concept for an ultrafast N-glycan analysis approach to glycoprofiling of the main glycoforms of mAbs by HILIC chromatography and the Vanquish Horizon UHPLC system with fluorescence detection. Time for analysis is reduced considerably in comparison to the standard method applied for full detailed characterization (the standard methods can take up to 55 minutes). The analysis is completed in 2.5 minutes and can be applied for the comparison of mAbs glycosylation expressed under various cell culture conditions, as well as for the evaluation of antibody culture clones and various production batches.



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LINKS

Accucore 150 Amide HILIC LC Columns

Ultrafast, Batch-to-Batch Comparison of Monoclonal Antibody Glycosylation

Sponsored by ThermoFisher SCIENTIFIC

Produced by the Analytical Scientist

Comprehensive Protein Glycosylation Comparison of an Innovator Monoclonal Antibody to a Candidate Biosimilar by HILIC UHPLC Analysis

Silvia Millán, Anne Trappe, Amy Farrell, and Jonathan Bones
Characterisation and Comparability Laboratory, NIBRT – The National Institute for Bioprocessing Research and Training, Dublin, Ireland

Biosimilars are biologic products that receive authorization based on an abbreviated regulatory application containing comparative quality, nonclinical and clinical data that demonstrate similarity to a licensed biological product (ICH Q5E/Q6B, USP 129). Regulatory authorities have generally reached the consensus that extrapolation of similarity from one indication to other approved indications of the reference product can be permitted if it is scientifically justified. Monoclonal antibody (mAb) products are extraordinarily heterogeneous due to the presence of a variety of enzymatic and chemical modifications, such as deamidation, isomerization, oxidation, glycosylation, glycation, and terminal cyclization. The modifications in different domains of the antibody molecule can result in different biological consequences.

Therefore, characterization and routine monitoring of domain-specific modifications are essential to ensure the quality of the therapeutic antibody products.

Glycosylation is considered a critical quality attribute (CQA) and therefore, should be within an appropriate limit, range or distribution to ensure desired product quality, safety and efficacy. Different glycosylation variants have been shown to influence the pharmacodynamics and pharmacokinetic behavior, while other glycan structures may be involved in adverse immune reactions. Comprehensive glycan profiling may be achieved using a variety of techniques, including use of oligosaccharide standards, enzymatic digests and lectin affinity in combination with liquid chromatography, or capillary electrophoresis coupled with fluorescence detection and mass spectrometry.

Trastuzumab (Herceptin®) is a commercially available recombinant IgG1 kappa, humanized monoclonal antibody biotherapeutic produced in Chinese hamster ovary (CHO) cell culture. The early stage development biosimilar candidate has been produced using transient expression in a CHO cell line (Gibco™ ExpiCHO™ expression system). In this work, comprehensive glycan profiling of 2-AA labelled mAbs was achieved using HILIC chromatography coupled to a Vanquish UHPLC system with fluorescence (FLD) detection and subsequent structural confirmation by exoglycosidase digestions and high-resolution, accurate-mass mass spectrometry.

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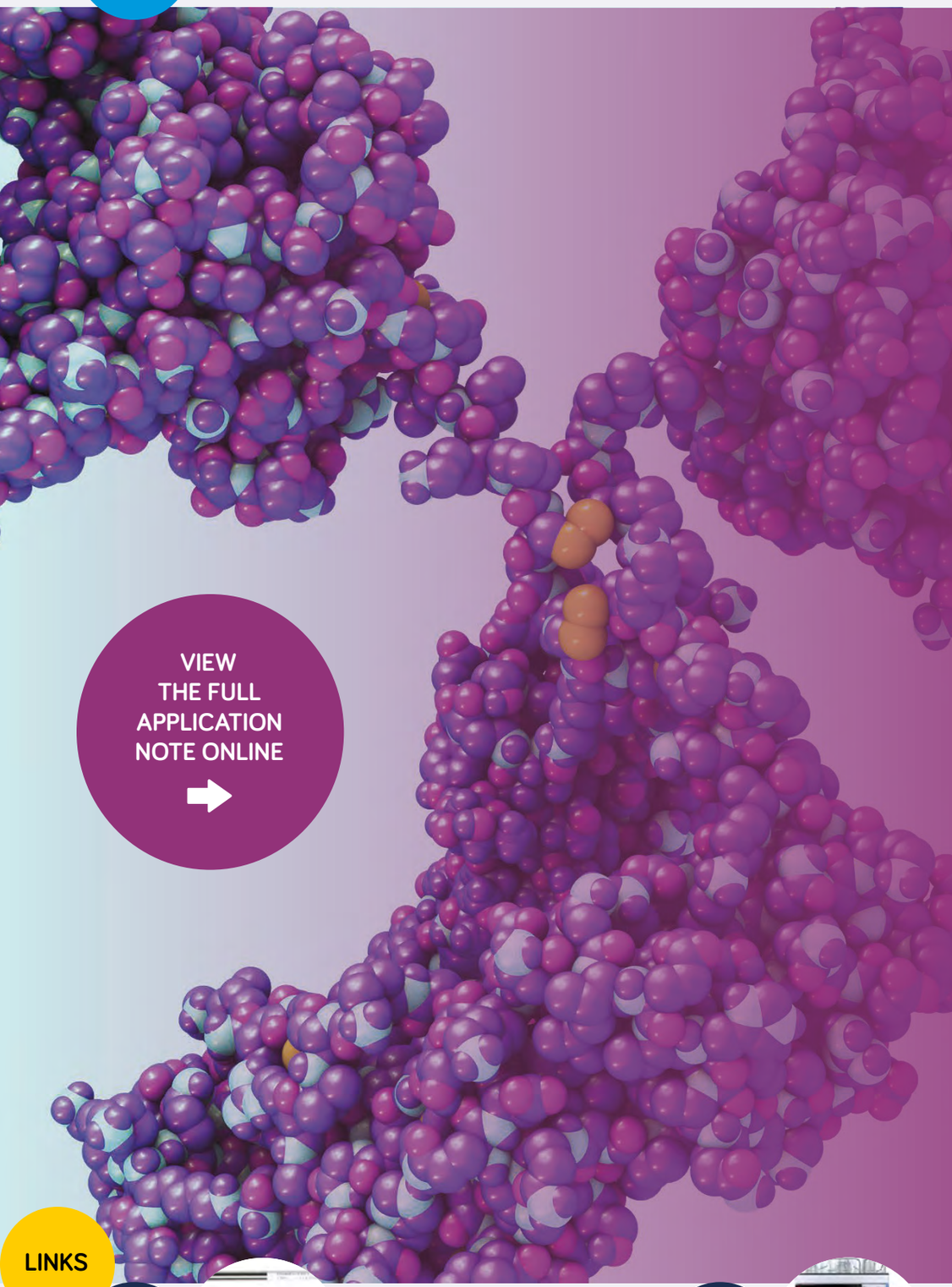
LINKS

Comprehensive Glycosylation Comparison Of Innovator and Candidate Biosimilar mAbs via HILIC UHPLC

Vanquish Horizon UHPLC System

Sponsored by ThermoFisher SCIENTIFIC

Produced by the Analytical Scientist



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Use of Alternative Chromatographic Phases and LC-MS for Characterization Of N-Glycans From NISTmAb RM 8671

Amy Farrell, Silvia Millan Martin, Csaba Váradi, and Jonathan Bones
National Institute for Bioprocessing Research and Training (NIBRT), Dublin, Ireland

N-linked glycosylation is an important post-translational modification (PTM) that imparts structural heterogeneity to recombinant monoclonal antibodies (mAbs). Oligosaccharides (glycans) attached to the CH2 domain of an IgG1 can impact the pharmacodynamic and pharmacokinetic behavior of therapeutic proteins and are often considered to be critical quality attributes (CQAs). Detailed characterization of the glycosylation profile of mAbs is a regulatory requirement to ensure the safety, quality, and efficacy profile of therapeutic proteins. Characterization of the glycan profile of biosimilar products is also required to demonstrate the comparability of an innovator therapeutic protein and related biosimilar candidate. Analysis of glycans attached to mAbs is challenging as N-glycosylation is heterogeneous as a result of the activity of the individual enzymes that construct the oligosaccharides at each glycosylation site. Consequently, extensive sample handling and full detailed analysis, often including orthogonal technologies, are necessary. These requirements have resulted in an increased demand for reliable and robust analytical technologies for the comprehensive characterization of protein glycosylation.

A common strategy for N-glycan analysis usually involves removal of N-glycan species from therapeutic proteins using peptide N-glycosidase F (PNGase F), followed by modification of the free reducing terminus of released glycans with an appropriate fluorophore to improve detectability, chromatographic retention, and/or ionization efficiency. Hydrophilic interaction (HILIC) liquid chromatography is the most frequently applied separation strategy for glycan analysis during which glycan retention increases with increasing glycan size and associated reduction in hydrophobicity. Introduction of an additional and complementary separation dimension has been employed to increase resolution of oligosaccharides. Several hybrid chromatographic phases are available, such as the Thermo Scientific™ GlycanPac™ AXH-1 column, which has both weak anion exchange and HILIC properties, enabling separation of molecules based on charge, polarity, and size. After liquid chromatography (LC) or capillary electrophoresis separation, labeled glycan species are detected using fluorescence (FLD) or mass spectrometry (MS) detection to enable identification and relative quantitation of oligosaccharides.

Glyco-analytical standards are of critical importance in evaluating the suitability of analytical methods for glycosylation analysis of therapeutic mAbs throughout a therapeutic protein product life-cycle. The NISTmAb reference material 8671 (NISTmAb) is a recombinant humanized IgG1K that may be used as a reference standard for system suitability or analytical method evaluation for mAb characterization. NISTmAb is a ~150 kDa homodimer of two light chain and two heavy chain subunits linked through both inter- and intra-chain disulfide bonds, which has undergone biopharmaceutical industry standard upstream and downstream purification to remove process-related impurities.

During production, NISTmAb undergoes various PTMs including glycosylation, and thus it is a suitable analytical standard for glycan analysis methodologies.

In this application, the glycosylation profile of NISTmAb was analyzed using liquid chromatography-mass spectrometry (LC-MS). Released and labeled glycans were analyzed using an LC-MS platform composed of a Vanquish Flex UHPLC system with FLD, coupled to a Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer with the BioPharma option. Separation of oligosaccharides was achieved using a Accucore Amide-150 HILIC, 2.1 × 150 mm (HILIC) column or a Thermo Scientific GlycanPac AXH-1 (AXH), 1.9 µm, 2.1 × 150 mm column. Excellent reproducibility of the sample preparation and resulting chromatographic separation of NISTmAb glycans was observed on both columns. Subsequent identification of glycan moieties was performed based on the mass of the species detected upon LC-MS analysis. Use of a high-resolution, accurate-mass MS enabled high-confidence identification of oligosaccharides, with < 3 ppm error in mass accuracy for all N-glycan species determined.

LINKS

GlycanPac AXH-1 Column

Vanquish Flex Quarternary UHPLC System

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Evaluation of Chromatographic Phases for Separation of Differentially Labeled Glycans from Erythropoietin and Trastuzumab

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Glycosylation is a critical quality attribute that may affect the pharmacodynamic and pharmacokinetic behavior of therapeutic proteins. Hence, detailed characterization of glycans attached to therapeutic drug substances is a regulatory requirement. Furthermore, characterization of biosimilar products is required to demonstrate the comparability of an innovator therapeutic protein and related biosimilar candidate. Combined, these requirements have resulted in an increased demand for reliable and robust analytical technologies for the comprehensive characterization of protein glycosylation.

Analysis of glycan moieties is commonly performed subsequent to their release from a therapeutic protein and derivatization with an appropriate fluorophore, followed by liquid chromatography (LC) or capillary electrophoresis separation and fluorescence (FLD) or mass spectrometry (MS) detection. Hydrophilic interaction liquid chromatography (HILIC) is the most frequently applied separation strategy for glycan analysis during which glycan retention increases with increasing glycan size and associated reduction in hydrophobicity. While HILIC columns are an excellent choice for separation of glycan pools, especially if the majority of glycans present are neutral, they often result in inadequate separation for glycans harboring two or more charged moieties are present (e.g. sialylated glycans). Furthermore, the structural complexity of glycan mixtures including closely related isomeric species represents a major analytical challenge with commonly occurring partial or incomplete liquid phase separation.

Introduction of an additional and complementary offline separation dimension has been employed to increase the separation of oligosaccharides; however, the inclusion of extended sample handling steps may introduce selective sample alterations or loss of glycoforms. Hybrid phases, such as anion exchange-HILIC or anion exchange-reversed phase chromatography, have been developed that combine multiple separation strategies on a single column. The GlycanPac AXH 1 column has both weak anion exchange and HILIC properties, which enables the separation of molecules based on charge, polarity, and size, creating an added dimension for separation of highly charged glycoforms. The GlycanPac AXR-1 mixed mode column also offers an alternative separation strategy to traditional HILIC columns. Separation is achieved by first resolving glycans into different charge groups and then subsequently separating glycans in each group based on size and isomerization. Both alternative strategies may increase resolution of complex glycan mixtures enabling separation and identification of glycan structures that are not observed by more traditional methodologies.

In addition to consideration of the chromatographic separation phases, multiple fluorophores are now available for labeling of released N-glycans species by reductive amination prior to UHPLC analysis with FLD. Different fluorophores impart different properties on labeled glycan samples: chemical derivatization with 2-aminobenzoic acid (2-AA) imparts one negative charge to glycan moieties; labeling with 9-aminopyrene-1,4,6-trisulfonic acid (APTS) imparts three negative charges onto glycan species; derivatization with 2-aminobenzamide (2-AB) has no impact on the charge of glycan species; while derivatization with 2-aminoacridone (2-AMAC) changes the hydrophobicity of oligosaccharides. Hence, choice of fluorescent label may have a large impact on selectivity depending on the mode of chromatographic separation employed. Therefore, in addition to evaluation of the column for N-glycan analysis, it is also imperative to consider the choice of label used in combination with the chromatographic stationary phase as both the properties imparted on glycan samples from derivatization with different fluorophores and separation capabilities of different analytical columns may be manipulated to enable better separation of oligosaccharides.

In this application, N-glycans released from erythropoietin (EPO) were used to evaluate the effect of labeling and chromatographic chemistry on the separation of glycan species. EPO was chosen as a model protein as it is highly glycosylated and exhibits high heterogeneity due to the inherent inclusion of various highly branched sialylated N-glycan structures.1 O-glycosylation was not examined herein due to the reported minor biological significance of O-glycans on EPO. EPO was derivatized using three different labels, namely 2-AA, 2-AB, and 2-AMAC. Subsequently, labeled glycans were analyzed using a Accucore Amide-150 HILIC, 2.1 × 150 mm (HILIC), a GlycanPac AXH-1 column (AXH), and a GlycanPac AXR-1 column (AXR). In addition to sample analysis using a Vanquish Flex UHPLC system with fluorescence detection, identification of individual glycan species was performed using a Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer with the BioPharma option. Due to the prevalence of monoclonal antibody (mAb) drug substances in the biopharmaceutical industry, released glycans from the therapeutic mAb trastuzumab were also analyzed using the three chromatographic columns. Due to the neutral nature of the glycans present on this mAb, they were only derivatized with the commonly used 2-AA label.

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LINKS

GlycanPac AXR-1 Column

GlycanPac AXH-1 Column

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Subunits Analysis Approach for The Determination of Fucosylation Levels in Monoclonal Antibodies Using LC-HRAM-MS

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There is a growing interest in the analysis of recombinant monoclonal antibodies (mAbs) and in the study of their biological interaction mechanisms. N-glycans present on the Fc region of the monoclonal antibody can affect monoclonal antibody stability but can also interfere with the intended mechanism of action of the drug. It has been demonstrated that when the monoclonal antibody presents an afucosylated chitobiose core, the affinity of the mAb for the FcγRIII expressed on natural killer cells is enhanced leading to an antibody-dependent cellular cytotoxicity (ADCC). As a consequence, antibody drug engineering has made much effort to enhance this feature, and an accurate quantification of glycan core fucosylation is often required.

In this study, high-resolution, accurate-mass spectrometry (LC-HRAM-MS) was used in a middle-up approach for the quantification of core afucosylation on three mAbs. Samples were digested with IdeS and EndoS enzymes in non-reducing conditions. IdeS enzyme action cleaves the monoclonal antibody in the hinge region and generates two polypeptides (F(ab')2 and 2 x scFc regions). EndoS enzyme specifically cleaves the chitobiose core leaving on the polypeptide an N-acetyl glucosamine with or without the core fucose (Figure 1). Moreover, EndoS showed a specific activity for complex glycan and does not digest high mannose type glycans. The use of LC-HRAM-MS allows an accurate quantification of core afucosylation.

All the analyses have been performed on a high-resolution analytical platform consisting of a Vanquish Flex Binary UHPLC and Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer. Since scFc region (~25 KDa) and intact F(ab')2 region (~100 KDa) are generated, the analysis will involve obtaining MS information from two polypeptides with very different mass range; consequently, different mass settings and tune files need to be used (Tables 2 and 3). In this way, high-quality, high-resolution data is obtained on the scFc region, without losing information from Fab' region.

For the three mAbs (rituximab, bevacizumab, and trastuzumab), both originator and an in-house produced biosimilar were analyzed. Deconvoluted spectra from the six samples were obtained using BioPharma Finder 3.0 software for intact mass analysis.

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LINKS

MAbPac RP Column

Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer

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IdeS-Cleaved Mab Subunit Analysis with LC-HRAM-MS: A Quick and Accurate Comparison of Biosimilar and Originator Biotherapeutics

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Recombinant monoclonal antibodies (mAbs) are the fastest growing class of human therapeutics. Their success in the biopharmaceutical industry and the introduction of a growing number of biosimilar therapeutics go along with the need for reliable and fast characterization methods to establish drug quality and safety. Indeed, a biosimilar is a drug presenting minimal variations from its originator due to several factors like the expression system and growing conditions, the purification steps, or the final formulations. It is of critical importance to monitor and quantify these variations to correlate them to any potentially different in vivo activity, as different clearance time or other interactions within the patient.

To spot these differences, full sequence coverage and post-translational modifications (PTMs) are usually obtained with a bottom-up approach employing a combination of several LC-MS/MS datasets derived from different and orthogonal enzymatic digestions of the protein. Top-down or middle-up approaches have the potential to minimize sample handling and artefacts and to give quicker or complementary information.

In this study, liquid chromatography hyphenated with high-resolution, accurate-mass spectrometry (LC-HRAM-MS) was used in a middle-up approach for the comparison of three commercially available mAb drug substances (DS) and their respective biosimilar obtained in house (BS). Samples were digested with IdeS enzyme, cleaving the monoclonal antibody in the hinge region and generating, after reduction of disulfide bonds, two pairs of polypeptides from the heavy chain along with the light chain portion.

Analysis was performed on a high-resolution analytical platform consisting of a Vanquish Flex Binary UHPLC and Q Exactive™ Plus Hybrid Quadrupole-Orbitrap mass spectrometer. Data were analyzed using the platform for intact mass analysis within BioPharma Finder 3.0 software. The quality of the data together with the quick analysis allowed a confident identification and sequence verification of light chain and Fd' region and a rapid analysis of Fc region variants, including glycoform and N-terminal lysine loss. Moreover, BioPharma Finder 3.0 software is able to rapidly provide a comparison with previously acquired data used as reference; this provides a rapid and confident way for the determination of batch-to-batch variations in analytical laboratories.

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MAbPac RP Column

Vanquish Flex Binary UHPLC System

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Summary

We do hope you have enjoyed this collection of application notes developed by NIBRT and Thermo Fisher Scientific that offer a comprehensive toolbox of technologies for comprehensive biopharmaceutical characterisation. Further application notes are in development stemming from this collaboration and if you are interested in viewing these as they become available we would like to direct you to the NIBRT Collaboration Information webpage where new documents will be posted.

In addition, many of these applications are available on the Thermo Scientific AppsLab Library of Analytical Methods, where you can view the method, hardware and consumable requirements to run the application as well as download the eWorkflow which can be directly executed in Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) Software.