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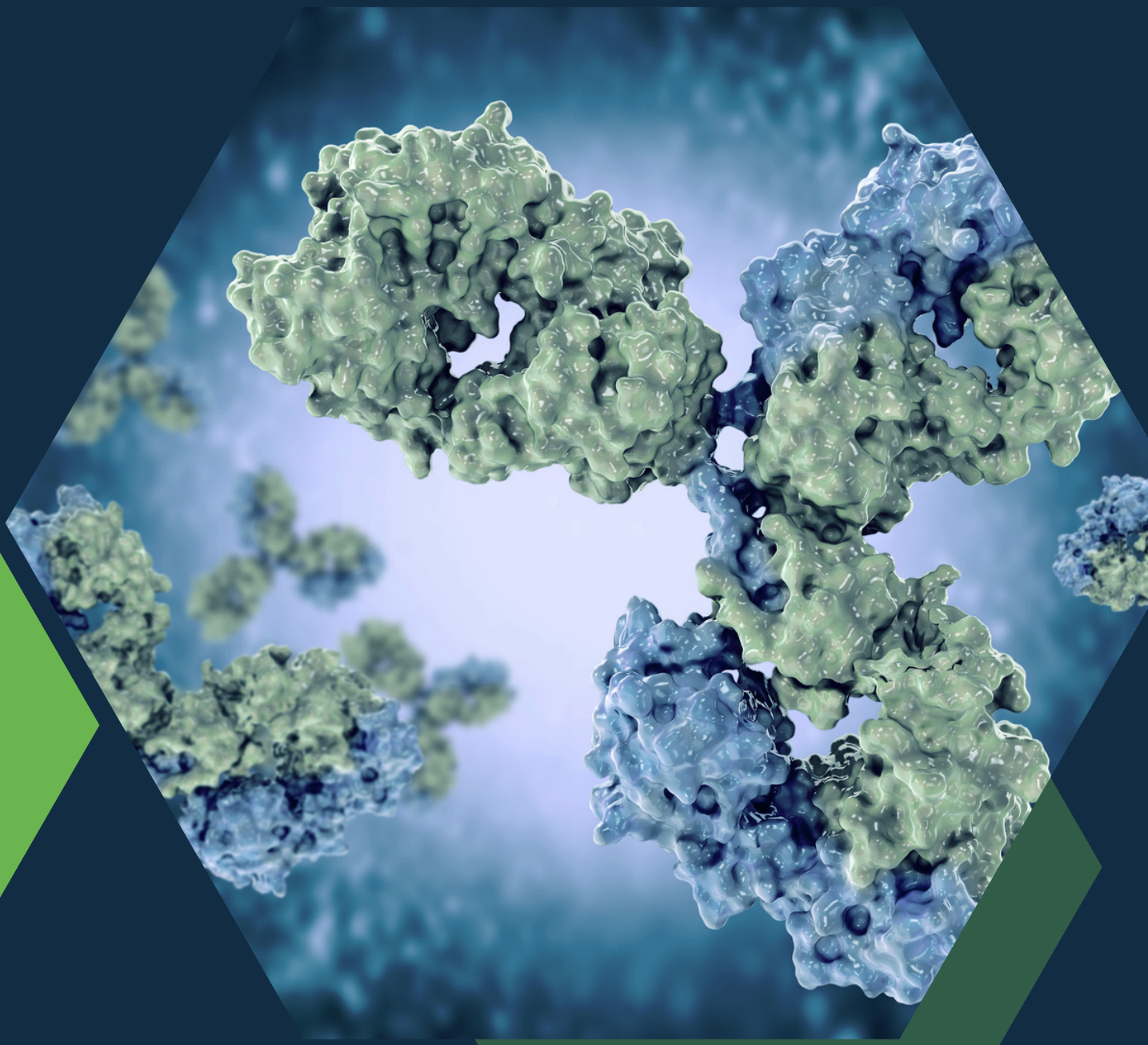

HCP detection and quantitation: a review of ELISA, MS and Antibody Affinity Extraction immunoaffinity chromatography

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Contents

HCP detection and quantitation: a review of ELISA, MS and Antibody Affinity Extraction™ immunoaffinity chromatography

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Keywords: Recombinant DNA; Recombinant proteins; Therapeutic antibodies, Vaccines; Cell culture; Host cell proteins (HCPs); Post-translational modification; Mass spectrometry; ELISA.

The use of recombinant proteins in biopharmaceuticals

The large-scale production of recombinant proteins intended for vaccines and antibody-based therapeutics necessitates the use of diverse, genetically modified host systems and advanced recombinant DNA technology. These host systems encompass both prokaryotic organisms such as bacteria as well as eukaryotic cells, which include mammalian, yeast and insect cells [1]. To produce the desired recombinant proteins, a vector carrying the target DNA sequence is administered into the host system, which is ultimately expressed by the host cell, producing the recombinant protein(s).

During recombinant protein production, the host cells also coproduce proteins vital for normal cell function such as cell growth, proliferation, survival, gene transcription and protein synthesis, among others. Additional cell apoptosis/death/lysis-related proteins may also be released into the cell culture. All endogenous proteins co-expressed by the host system, other than the recombinant protein of interest, are termed host cell proteins (HCPs). HCPs constitute a diverse array of proteins, which vary in their molecular mass, isoelectric point, hydrophobic properties and structure. An additional challenge is introduced when HCPs undergo post-translational modification by the host, which makes their quantification and characterization increasingly more difficult.

HCPs as contaminants

In the context of biological drugs, HCPs represent a heterogeneous group of process-related impurities. Whilst many are benign, some HCPs are immunogenic, meaning their presence in an administered biotherapeutic induces an unwanted immune response in the patient. Regulatory agencies and the broader industry generally accept a limit of 100ng/mg product protein for HCPs in biologics [2]. Some HCPs interact with the active drug component, whilst others such as proteases and lipases, can reduce effective product dosage. This is achieved through direct action on the drug or its stability by interfering with formulation buffers and these proteins would therefore be marked as high-risk [3].

Given that HCPs can pose a risk to patients and affect the efficacy and stability of biological drugs, the quantity and nature of residual HCPs in a drug substance are considered to be critical quality attributes (CQAs) industrywide [4]. However, HCP variety and complexity in

biological drugs, combined with limited publicly available knowledge of their effects (be those biological, physiological, pharmacological or toxicological) make HCPs a challenging component of drug development [5]. The primary concern for HCP quantitation is determining the threshold at which each type of HCP can be considered low-risk to patients. HCP specification limits rely on experience, process capabilities, clinical development stages and HCP test results from immunoassays supplemented with orthogonal methods. Despite these steps, guidance is limited on how to manage HCP-related risks during drug development [4].

It is generally agreed that HCPs must be understood, monitored and controlled as significant components of a biopharmaceutical drug developer's overall risk-management strategy. Managing the potential risks relating to HCPs is usually controlled by downstream processes, however, selected risks can be controlled upstream by genetically modifying the cell line. Using upstream processing to reduce one HCP may influence the production of other HCPs, thereby altering and creating new risks for the patient. Therefore, downstream processes are more commonly utilized, particularly when HCP impurities have not been previously identified or characterized. Various immunospecific and non-specific techniques have gained global recognition from regulatory agencies for HCP characterization and quantitation. In this Technology Digest, we review HCP Enzyme-Linked Immunosorbent Assays (ELISAs) and mass spectrometry (MS) as tools for detecting and qualifying HCPs, as well as the use of Antibody Affinity Extraction (AAE™) immunoaffinity chromatography as a supplemental sample preparation approach.

Methods for detecting and quantifying HCPs: HCP ELISA

ELISA is currently the most established HCP quantitation approach accepted by the FDA and, when automated, has a high-throughput capacity, making it a useful tool for supporting process development. Developing an HCP immunoassay requires a significant investment of time and resources, as there is a need for the generation of a proper HCP antigen and corresponding antibody [3]. HCP ELISA is the gold standard method for process monitoring and product release testing for HCPs, however, developers must ensure that selected HCP ELISA methods are fit for their intended use [6]. ELISAs facilitate the detection of a wide range of HCPs, particularly those eliciting significant immune responses. HCP coverage evaluations help developers assess the ability of HCP ELISA antibodies to recognize a broad range of HCPs — both those of a calibration standard and those that are present as part of in-process and drug substance samples. Nevertheless, ELISA's efficacy is bound by the antibodies it employs; disparity in the antibody's quantity and affinity for various antigens substantially impacts HCP quantitation [7]. It is advised that HCP immunoassays are complemented by alternative approaches, the simplest of which is 2D-polyacrylamide gel electrophoresis (2D-PAGE). However, PAGE methods are limited in their ability to detect low-abundance HCPs, resulting in many HCPs being overlooked.

Mass spectrometry (MS)

While there are numerous supplementary approaches to HCP ELISAs, MS is steadily becoming the major orthogonal technique [8]. Identification and quantification of HCPs require high sensitivity and specificity to detect HCPs at extremely low concentrations and a universal challenge of all HCP analytical methods is the significant dynamic range of protein concentrations in biopharmaceuticals, which can be between four and five orders of magnitude. MS approaches can rapidly monitor and identify multiple HCPs in a single sample and importantly, offer the advantage of precise HCP identification without reliance on antibodies. Furthermore, MS methods can also be coupled with chromatographic separations to monitor HCPs, and, utilizing multidimensional chromatographic separations, such as two-dimensional liquid chromatography (2D-LC), can increase the overall dynamic range of the method [9,10]. While MS instrumentation incurs significant costs and requires trained specialists for its use, most analytical laboratories now possess high-precision MS technology. With the above information considered, MS remains a powerful tool for HCP analysis.

Antibody Affinity Extraction (AAE™) immunoaffinity chromatography

Antibody Affinity Extraction (AAE™) immunoaffinity chromatography is an advanced orthogonal technique designed to assess the coverage of an HCP polyclonal antibody for HCPs present in a process stream, as well as its reactivity to downstream, process-specific HCPs that can copurify with a drug substance. Developed by Cygnus Technologies (NC, USA) in 2013, AAE utilizes an immobilized HCP polyclonal antibody covalently attached to a chromatography support [6]. The starting process stream and final AAE sample can be analyzed and compared using MS or 2D-PAGE. Combining AAE chromatography with mass spectrometry – referred to as AAE-MS™ – for HCP antibody coverage analysis facilitates the identification of all HCPs in harvest material, specifically those that are reactive with the antibody. AAE-MS also provides molecular weight and isoelectric point information. Alla Zilberman, PhD, Vice-President, Cygnus Technologies, explains:

> “We developed the Antibody Affinity Extraction method to overcome deficiencies of traditional orthogonal methods for assessing HCP coverage, e.g., two-dimensional (2D) Western blots and 2D differential-in-blot electrophoresis (2D-DIBE). Subsequently, the AAE coverage-assessment method was included in United States Pharmacopeia Chapter <1132> on the measurement of residual HCPs in biologics. When AAE is combined with MS, it provides valuable information not only on HCP antibody coverage but identities of all HCPs reactive with the HCP antibody. We should reframe the antibody coverage analysis question and ask not just what is the coverage percentage, but what are the specific HCPs your HCP ELISA detects and quantifies”.

Combining ELISA and MS approaches

As shown above, both HCP ELISAs and MS-based techniques possess distinct advantages and disadvantages for measuring HCPs in biotherapeutics but, when used in conjunction, can provide a comprehensive view of a biologic's impurity level to meet regulatory expectations. While HCP ELISA reports a 'total' value for HCPs within the final drug substance, MS can identify and quantify what that 'total HCP number' consists of, e.g., how many HCPs and in what quantities. There is still one caveat to be considered; the biotherapeutic substance is typically in the range of mg/ml, while HCPs exist in notably lower ng/mg concentrations. Therefore, to improve MS resolution to identify HCPs in the final biotherapeutic substance, Cygnus Technologies recommends using AAE to enrich HCPs and deplete the drug substance prior to MS analysis. Complete characterization of HCPs downstream is not part of current regulatory guidelines. Nonetheless, the potential for such information to aid biopharmaceutical companies in ensuring the safety and efficacy of their products makes it recognized as value-added data by proactive manufacturers and regulators.

Summary

HCP testing necessitates an approach that demonstrates a wide dynamic range, sensitivity, selectivity and high-throughput capacity. Immunospecific methods, such as ELISAs, offer simplicity for routine analysis and provide low-level quantitation when specific antibodies are available. Non-specific approaches such as multidimensional liquid chromatography coupled with MS are more intricate but provide a comprehensive overview of the HCP profile and, when used in conjunction with AAE chromatography, MS resolution can be optimized. Used collectively, these three approaches provide a much clearer depiction of the HCP profile and can help to establish meaningful thresholds for HCPs, ensuring patient safety and drug efficacy.

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Review

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Biopharmaceutical quality control with mass spectrometry

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Mass spectrometry (MS) is a powerful technique for protein identification, quantification and characterization that is widely applied in biochemical studies, and which can provide data on the quantity, structural integrity and post-translational modifications of proteins. It is therefore a versatile and widely used analytic tool for quality control of biopharmaceuticals, especially in quantifying host-cell protein impurities, identifying post-translation modifications and structural characterization of biopharmaceutical proteins. Here, we summarize recent advances in MS-based analyses of these key quality attributes of the biopharmaceutical development and manufacturing processes.

Tweetable abstract: MS is powerful for biopharmaceutical quality control. We review the status and opportunities of data independent acquisition, glycoproteomics, top-down MS and hydrogen-deuterium exchange MS for measuring host-cell protein contamination, post-translational modifications and protein structure.

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Keywords: biopharmaceutical quality control • host-cell proteins • mass spectrometry • protein post-translational modifications • protein structure

Biopharmaceuticals

Biopharmaceuticals (also known as biologics) are a category of medical products composed of nucleic acids, proteins or living cells that are produced through biotechnology. Most commonly, recombinant DNA technology is used to heterologously express protein biopharmaceuticals from mammalian cell lines [1]. Today, they are broadly used to treat disease indications including cancer, inflammatory and infectious diseases, wound healing, fertility, supplementation of hormone or cytokine deficiencies, modulation of immune function and replacement of enzymes. The general categories of biopharmaceuticals with corresponding examples and estimated current market values are shown in Table 1.

Biopharmaceuticals are safe and effective high-molecular weight drugs with few side effects compared with small-molecule drugs [10]. The chemical structure of many small-molecule drugs cannot be found in the human body, while the structures of biopharmaceuticals are often very similar to native human compounds because they are derived from a biological source [11]. This high complexity and structural similarity give them high specificity with few side effects, as well as the potential capacity to cure diseases instead of just treating the symptoms. However, this structural diversity and complexity, along with their high molecular mass, makes the manufacture of biopharmaceuticals comparatively complex [12]. Thus, quality control is extremely important during the whole manufacturing process including production, purification and packaging. This quality control includes assessment of the identity, purity and potency of the product. To precisely control biopharmaceutical quality, MS is a front-line tool for protein identification and characterization.

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Table 1. Classification of biopharmaceuticals with examples and market values.

Types	Examples	Annual revenue (US$)	Ref.
Coagulation factors	Factor VIII and IX	8.5 billion in 2017	[2]
Thrombolytic agents	Tissue plasminogen activator	5 million in 2012	[3]
Hormones	Insulin, growth hormone and gonadotropins	8 billion in 2019	[4]
Growth factors	Erythropoietin and colony stimulating factors	6 million in 2019	[5]
Interferon	IFN-α, -β and -γ	9 billion in 2019	[6]
Interleukin-based products	IL-2	6 billion by 2026	[7]
Vaccines	Hepatitis B surface antigen, varicella and HPV	61 billion by 2020	[8]
Monoclonal antibodies	Herceptin, alemtuzumab and rituximab	115.2 billion in 2018	[9]
Additional products	Tumor necrosis factor and therapeutic enzymes	–	

HPV: Human papillomavirus.

Overview of current biopharmaceutical markets

The global biopharmaceutical market has grown continuously in recent decades with increasing demand from geriatric populations, and investment in related research as well as manufacturing processes has effectively expanded the market by providing customers greater choice and biopharmaceuticals with improved potency. At present, biopharmaceuticals occupy nearly a quarter of newly introduced drugs to the market, and demonstrate high competitiveness and large potential partly because of their ability to treat previously incurable diseases.

In 1990, the total annual revenue from biopharmaceuticals was only around US$4.4 billion, while it has significantly increased to over US$275 billion at present with a growth rate of 61.5%, and is expected to continue to maintain an annual increase rate around 12–13% [13]. The year 2019 was an outstanding year for global biopharmaceutical markets with 7.32% of compound annual growth rate, which is well positioned for further stable increases [14].

Biopharmaceutical manufacturing process

The key conceptual steps in the biopharmaceutical manufacturing process are cell-line development, upstream processes and downstream processes (Figure 1). Microbial systems (bacteria, yeast, filamentous fungi and unicellular algae) and mammalian systems (CHO, NS0 and HEK 293 cells) are both widely used as host cells to produce protein-based biopharmaceuticals. *Escherichia coli* is a common and inexpensive bacterial system with fast growth that is used to produce first-generation biopharmaceuticals like insulin and growth hormone [15]. However, not all biopharmaceuticals can be produced in bacterial systems as these are not natively able to modify proteins after translation, which can lead to production of misfolded or inactive proteins [16]. Most therapeutic protein drugs require complex post-translational modifications (PTMs) such as glycosylation, acetylation, disulfide bonds or phosphorylation for desired drug stability and efficacy [17]. Thus, mammalian cells are widely used to produce the protein products that must be modified, such as monoclonal antibodies (mAbs) [18].

Upstream process can be divided into cell culture optimization, fermentation process optimization and application in large-scale bioreactors. Batch, fed-batch and continuous perfusion fermentation are common types of fermentation processes, with batch fermentation currently the most common with a 90% usage rate in industry [19]. During the fermentation process, fermentation conditions involving temperature, pH and oxygen concentration need to be monitored and regulated to guarantee optimal yield and production efficiency [20]. Additionally, sterile techniques or antibiotics can be used to protect the bioreactor environment from contamination.

Downstream processes refer to the process from after cell culture to the final biopharmaceutical product, and involve clarification, purification, polishing and viral inactivation to collect biomolecules of interest and remove impurities such as host cell debris and endotoxin [21]. Purified proteins can then be optionally modified by enzymatic conversion [22] or other methods depending on the specific biopharmaceutical, followed by formulation and/or lyophilization. Before packaging, product quality is controlled through a series of analyses to ensure the identity, purity, and quantity of the biopharmaceutical. MS is one of the most powerful analytical techniques for these purposes.

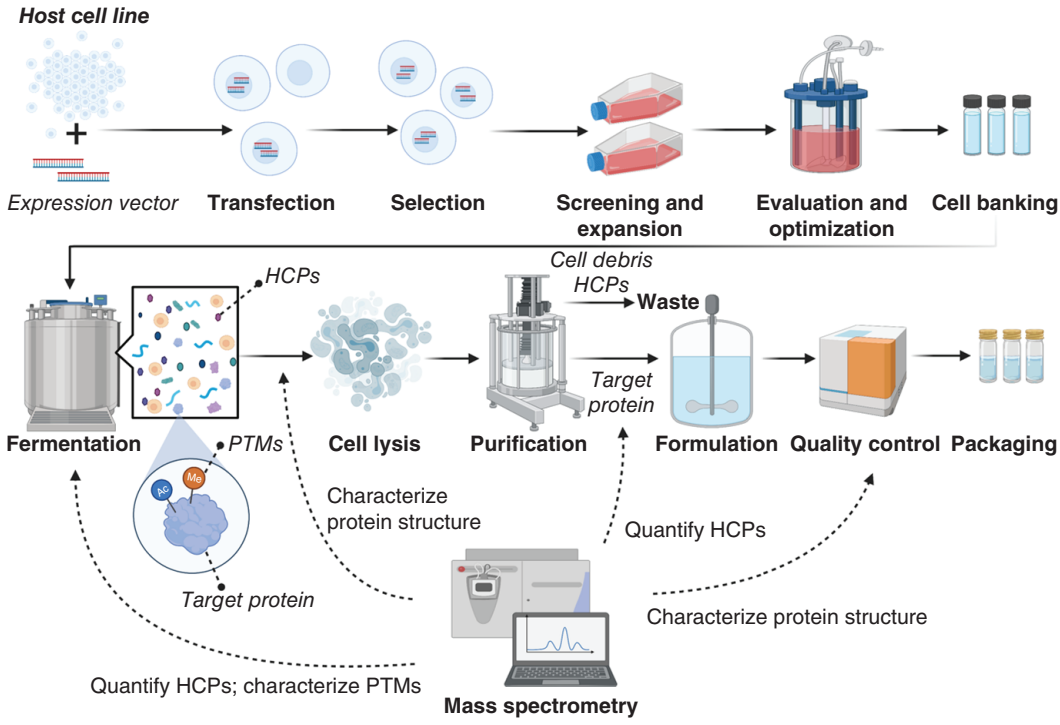

Figure 1. Biopharmaceutical manufacturing processes with MS applications. HCPs are contaminating proteins secreted from host cells along with the target protein. PTMs refer to the chemical modification of proteins through the removal or addition of functional groups that affect the structure and function of proteins. Created with BioRender.com.
HCP: Host-cell protein; PTM: Post-translational modification.

Mass spectrometry

MS is an indispensable analytical technique that is widely used in chemistry, biochemistry and pharmacy. It plays an essential role in the biopharmaceutical industry to identify, quantify and characterize proteins during production, purification and packaging processes to ensure the final biopharmaceutical products are pure, correctly folded and active proteins.

MS analysis of complex samples is commonly enabled by online or offline fractionation by various separation techniques. LC is particularly powerful and popular for this purpose. Separation of peptides or proteins typically uses reversed-phase LC. In addition to simplifying samples before MS analysis, LC of glycans or glycopeptides can separate different structures or isomers to improve identification [23]. Ion-exchange chromatography is suitable for fractionation and purification of charged compounds, including peptides and proteins [24]. Size-exclusion chromatography can separate biomolecules according to size, and can be combined with MS to characterize protein structural diversity or protein–protein interactions [25]. Hydrophobic interaction chromatography is a nondenaturing separation technique based on the hydrophobicity of the native analyte, and is becoming more popular to characterize the hydrophobicity, heterogeneity, sequence and structure of mAbs [26].

The three key components of an MS instrument are an ion source, a mass analyzer and a detector [27]. However, MS instruments can have many different and varied configurations. The basic theory of MS is to produce ions by one of the various ionization methods (depending on the characteristics of the sample), to separate these ions by virtue of their m/z, and to detect the ions to determine their m/z and abundance [28].

ESI and MALDI are the two most common methods used for analysis of proteins and peptides [29]. ESI ionizes analytes from a solution, and so is easily coupled to techniques that apply liquid-based separation such as LC. Integrated LC ESI–MS systems (LC–MS) are therefore commonly used to analyze complex samples. In contrast, MALDI uses laser pulses to sublimate and ionize molecules in samples from a dry crystalline matrix, and is usually used to analyze relatively simple peptide mixtures [30].

The mass analyzer is the central component of an MS instrument, and separates the ionized molecules based on their m/z ratios [31]. Mass analyzers are sensitive, high-resolution and capable of generating information-rich ion

mass spectra from proteins, peptides and their fragments. Quadrupole, TOF and Orbitrap are the most common types of analyzer, and these analyzers can be used singly or in combination to take advantage of the strengths of each [32].

MS/MS is a popular and powerful option in biomolecular analysis. In MS/MS, the ionized proteins or peptides are separated by the first mass analyzer (MS1) by their m/z ratio, and then ions of a certain m/z-ratio are selected to be further fragmented. After fragmentation, the smaller fragment ions are introduced into the second mass analyzer (MS2) which separates the fragments by their m/z ratio again and detects them [33]. As such, MS/MS can provide information on the composition or structure of complex molecules. Different fragmentation methods can provide complementary structural information, and are suitable for different types of biomolecules. For example, Collision Induced Dissociation (CID) or Higher Energy Collision Dissociation (HCD) are commonly used for analysis of peptides, as they result in efficient and predictable fragmentation at peptide bonds. Electron Transfer Dissociation (ETD) or Electron Capture Dissociation (ECD) provide complementary fragmentation patterns, and are particularly useful for assigning the site of modification in glycopeptides.

Other gas phase separation techniques can be combined with MS, including ion mobility spectrometry (IMS), which is increasing in popularity in modern MS instruments. The basic principle of IMS is that ionized molecules are separated through a cell filled with an inert 'drift gas' on a millisecond timescale according to their ion mobility, which is related to their mass, shape and charge [34]. IMS can be used between LC and MS as an additional intermediate fractionation technique for complex samples, and can also be used to obtain structural information by separating isomeric ions, revealing primary conformations and tracking dynamic changes in structure [35]. IM–MS can also be combined with complementary strategies such as fragmentation with ETD to obtain insights into protein conformation and modifications, or collision-induced unfolding to characterize protein dynamic structure and stability [36].

With the rapid development of MS instrumentation in recent years improving speed, accuracy, sensitivity and robustness, and offering diverse fragmentation options, this technology has become one of the most powerful analytical techniques for analysis and quantification in proteomics, glycoproteomics and detailed protein characterization. MS is therefore clearly a useful and versatile tool for many aspects of biopharmaceutical quality control which can quantify host-cell proteins (HCPs), identify PTMs and characterize the structure of biopharmaceutical proteins to guarantee the purity, safety and potency of biopharmaceuticals.

Recent advances

Quantification of HCPs

HCPs are contaminating proteins expressed and secreted from host cells that accompany the production of intended recombinant biopharmaceutical proteins [37]. The presence of HCPs is what necessitates additional purification steps to obtain pure biopharmaceutical protein product. HCPs must be removed during the purification process, as if some of them still remain as impurities in the final products they may result in reduction of biopharmaceutical efficacy or unintended immunogenic responses. The general guideline for acceptable levels of HCPs in biopharmaceutical products is less than 100 ng/ml (100 p.p.m.), and products with higher levels are generally not accepted by regulatory agencies [38]. Thus, quantification of residual HCPs in biopharmaceutical products is critical to ensure their adequate removal during the manufacturing process.

Traditionally, ELISA are commonly used to detect and measure HCPs during the biopharmaceutical manufacturing process [39]. ELISAs typically provide both high sensitivity and selectivity. However, ELISAs are only available for around 70% of all HCPs in typical samples [40], and development of new anti-HCP antibodies for use in ELISAs can be difficult and time consuming. In addition, HCPs may sometimes not be successfully detected even though the reagent contains the corresponding antibodies due to incompatible binding conditions or the accessibility of the relevant HCP epitopes [41].

As it is not possible for ELISA to identify all possible HCP contaminants, MS has emerged as an alternative technique for HCP analysis, as MS can monitor and identify multiple HCPs in a sample in one unbiased analysis. Moreover, even low quantities of HCPs are still able to be detected by MS; this is crucial for biopharmaceutical manufacture because even low levels of impurities can lead to adverse effects such as provoking immunogenicity.

HCP analysis requires both identification and quantification. MS can identify and quantify proteins, using either label-free or various chemical labeling strategies. In either approach, LC–MS/MS with rapid measuring speed as well as high sensitivity and selectivity has been widely applied to quantify HCPs in bottom-up proteomics workflows. In this method, proteins are digested with specific proteases, and the resulting peptides are desalted and analyzed

by LC–MS/MS. Proteins are identified by matching experimental MS/MS spectra to theoretical fragmentation patterns from predicted peptides. LC–MS/MS data can also be used for quantification of peptides and the proteins from which they originate. MS labeling strategies have been widely applied and demonstrate high accuracy. Such strategies include metabolic labeling such as stable isotope labeling with amino acids in culture [42] and chemical labeling of proteins such as 2D-difference fluorescence gel electrophoresis [43], or of peptides such as with isobaric tag for relative and absolute quantitation [44] or Tandem Mass Tag [45] systems. However, there are some drawbacks of labeling approaches, as they involve extra sample processing and are not possible for all sample types. In recent years, label-free quantification strategies have become an alternative popular and effective method used in MS proteomics [46–50]. Label-free quantification can use spectral counting or intensity-based measures. Spectral count is derived from identification from MS/MS spectra, or the total number of MS/MS spectra that correspond to a particular protein [51]. Generally, proteins with higher abundance in a sample will have more detectable peptides present after protease digestion and will therefore subsequently be represented by more MS/MS spectra [52]. Label-free quantification can also be based on peptide-ion intensity derived from LC–MS/MS data. Because the signal intensity of peptide ions is related to the peptide concentration, peptide abundance can be measured based on ion intensity through AUC or peak height. Data independent acquisition (DIA) LC–MS/MS workflows such as sequential window acquisition of all theoretical ions mass spectrometry (SWATH–MS) are powerful label-free approaches for deep, proteome-wide profiling with high-throughput and reproducible analysis [53]. Additionally, absolute quantification of proteins is an effective label-free strategy, in which stable isotopes are incorporated into synthetic peptides, imitating native peptides generated through proteolysis, and are added as internal standards to allow absolute quantification of targeted proteins [54,55].

A key challenge of HCP analysis is that the HCPs may be present at very low concentrations in the presence of a very high concentration of the biopharmaceutical product of interest. The analytical challenges posed by this difficulty can be overcome in several ways.

Sample preparation is key for all LC–MS/MS workflows, and can be used strategically to increase HCP detection. ProteoMiner technology has been used to increase detection of low abundance HCPs by reducing the dynamic range of peptides after proteolysis [56,57]. Depletion of the biopharmaceutical product before proteolysis has also been achieved with denatured HILIC fractionation [58]. The speed of sample preparation can be critical for the overall efficiency of HCP measurement. For example, sodium deoxycholate is a protein denaturant that does not need to be removed before trypsin digestion, and which can be easily removed by acidification after digestion to enable LC–MS/MS analysis [59].

After sample preparation, LC–MS/MS workflows can be tailored for HCP quantification. Targeted detection of known HCPs at very high sensitivity can be performed with multiple reaction monitoring [60,61]. However, multiple reaction monitoring relies on previous identification of HCPs which may be present. In contrast, DIA analysis can measure previously unpredicted proteins, and LC–MS/MS DIA–MS workflows also have excellent quantitative dynamic range and have been used for HCP quantification [62]. In a recent study that predicted yield and quality of the purified coagulation factor IX product through analysis of bioreactor supernatant, a set of LC–MS/MS DIA/SWATH workflows were established and used to quantify the factor IX product and HCPs, both during cell culture in bioreactors and after purification (Figure 2) [63]. In another study, a data independent liquid chromatography/mass spectrometry platform (2D-LC/MS^E) with Hi3 quantitation was used to measure HCPs in purified mAb samples to evaluate the impact of elution buffer choice for downstream purification, cell culture harvest time and additional downstream purification steps [64]. The high dynamic range of these DIA workflows allowed detection and quantification of low abundant HCPs in the presence of abundant biopharmaceutical product (Factor IX or mAb) without enrichment or depletion [65]. Another approach for increasing dynamic range, but using data-dependent acquisition (DDA), is the recently reported HCP-automated iterative MS workflow for identification and quantification of HCPs at extremely low levels (10 p.p.m.) without enrichment or pretreatment, in which samples were analyzed by LC–MS/MS multiple times, with precursor ions automatically excluded for selection for MS/MS in iterative replicates [66].

Recent years have seen the addition of IMS capabilities to MS instruments from several vendors. The additional online fractionation provided by IMS can allow deeper proteome profiling to increase the dynamic range of LC–MS/MS experiments. This is exemplified by the use of high-field asymmetric waveform ion mobility spectrometry on a Orbitrap Fusion Lumos Tribrid MS instrument, which increased the depth of HCP measurement [67].

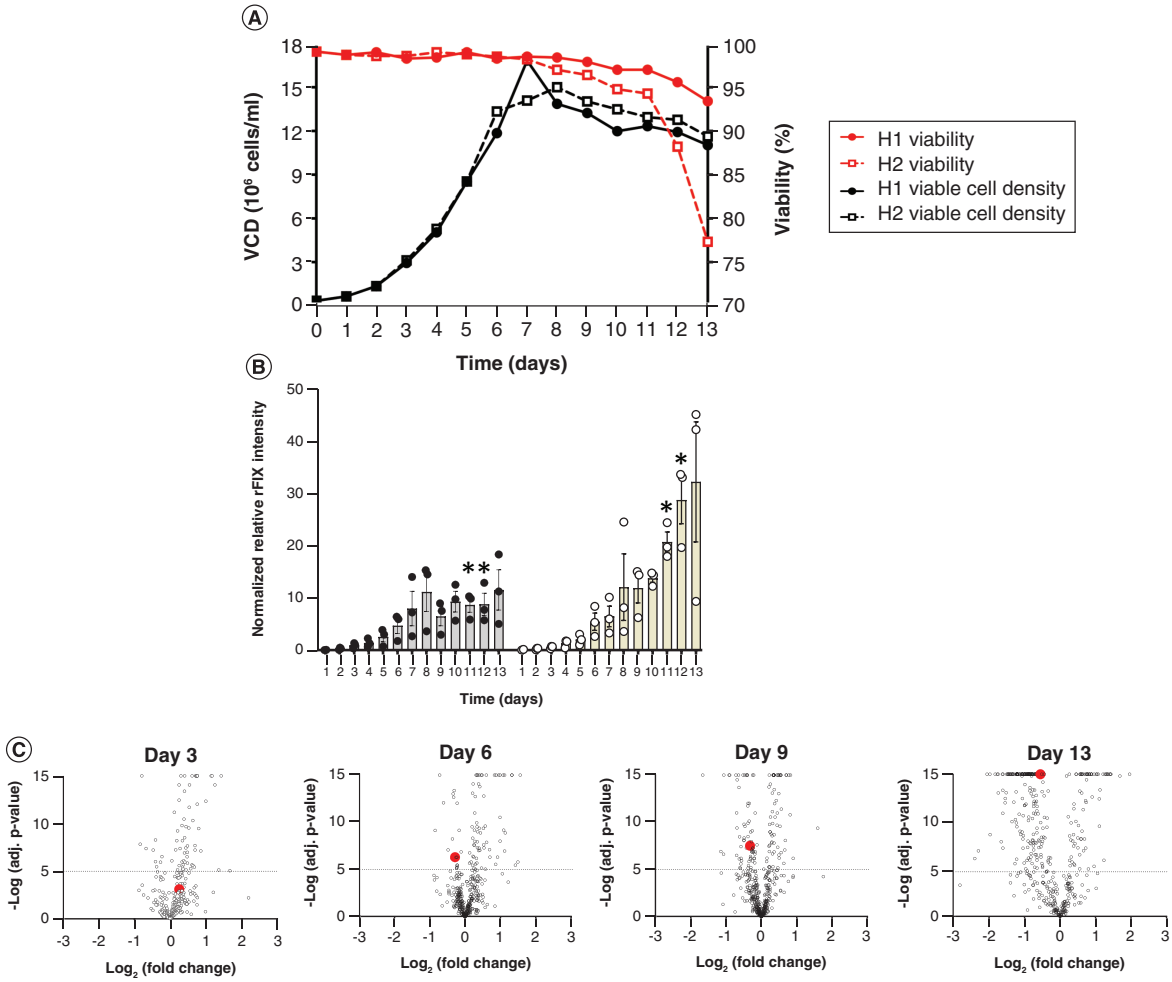

Figure 2. Viability, productivity and host cell protein profile of CHO cells expressing FIX in fed-batch conditions.
CHO cells co-expressing FIX and PACE/Furin were grown in fed-batch bioreactor mode with either EfficientyFeed A (H1) or EfficientFeed B (H2) as feeds. **(A)** Viability (red line) and (VCD; black line) in H1 (solid line, closed circle) and H2 (dotted line, open square); n = 1. **(B)** Relative FIX abundance (normalized to trypsin) in the bioreactor supernatants during operation (Mean ± SEM; multiple *t* test, n = 3 independent technical replicates; *p = 0.0072 and p = 0.0166 for day 11 and day 12 in H1 vs H2, respectively). Individual data points are indicated in black (H1 bioreactor, gray bars) or white circles (H2 bioreactor, yellow bars). **(C)** Volcano plots depicting log2 of the fold change in protein abundance versus -log10 of adjusted p-value for comparisons of culture media of bioreactor H1 versus H2 at days 3, 6, 9 and 13. The dotted horizontal line indicates the value above which the comparisons were significant (MSstats, $p < 10^{-5}$, n = 3 independent technical replicates). The red dots indicate rFIX at day 3 (adjusted p = 0.00078), day 6 (adjusted $p = 5.02 \times 10^{-7}$), day 9 (adjusted $p = 3.1 \times 10^{-8}$) and day 13 (adjusted p = 0) in H1 versus H2. Each open circle is a unique protein.
SEM: Standard error of the mean; VCD: Viable cell density.
Reproduced with permission from [63] © Zacchi *et al.* (2021).

The increased sensitivity and speed of modern MS instruments is enabling their use with rapid LC systems, while still maintaining deep proteome coverage. For instance, the Evosep ONE LC system can allow rapid robust online LC separation, for up to 60 samples per day [68].

HCPs are a major process-related impurity, and their sufficient removal (<100 p.p.m.) is crucial to obtain high-quality biopharmaceutical products. Thus, quantification of HCPs is necessary during and after purification. ELISA is an effective method for quantification of HCPs, but it has largely been replaced by LC–MS/MS due to the latter's rapid analysis time, high sensitivity and ability to measure all detectable HCPs in a sample in an unbiased manner. A variety of quantitative LC–MS/MS workflows are possible for this purpose, including labeled and label-free methods, depending on the precise experimental questions at hand.

Characterization of PTMs

Proteins can be modified with a highly diverse range of PTMs, including glycosylation, phosphorylation, proteolysis, acetylation, formylation, methylation, ubiquitination, carboxylation and many more. These PTMs increase the structural and functional diversity of proteomes [69]. Most PTMs are catalyzed by enzymes, allowing tight regulation of these functionally important features of proteins. As the correct presence and structure of PTMs are often critical for protein function, their detailed characterization is a necessary step in the quality control of biopharmaceuticals. For instance, antibodies, blood factors, erythropoietin, some IFNs and some hormones are glycosylated, which is important for their folding, stability, function, half-life and immunogenicity [70,71]. However, the diversity and structural complexity of PTMs on biopharmaceuticals can make their analysis complex and time-consuming.

Although PTMs are critical for biopharmaceutical quality, the measurement of various PTMs is challenging. Conventionally, Edman degradation, isotopic labelling, immunochemistry and amino acid analysis were commonly used techniques used to measure PTMs [72]. These approaches can be very sensitive, and are effective in single-site PTM detection, but their low-throughput makes them inappropriate for large-scale measurement of PTMs. This is a critical flaw, as many biopharmaceuticals are modified with multiple PTMs at many different sites. MS has emerged as the technique of choice for identifying and measuring PTMs. It has high sensitivity, and can identify specific-site PTMs, novel or unexpected PTMs, and PTMs in complex mixtures of proteins. None of the traditional methods has all of these abilities.

Generally, approaches for PTM identification by MS can be divided into bottom-up, middle-up and top-down strategies. Bottom-up analysis works at the peptide level, which means that the studied proteins are digested by proteases such as trypsin to produce peptides generally in the range of 500–3000 Da [73]. These proteolytically cleaved peptides tend to have few PTMs, which substantially simplifies their analysis. Bottom-up analyses are the most popular due to their high throughput and sensitivity, but they also have limitations. Specifically, not all proteolytic peptides resulting from digest with a given protease are normally able to be detected by MS, because some will be too large or too small [74]. This deficiency can be overcome, at least in part, by the use of independent treatment with different protease enzymes with complementary specificities. However, bottom-up analyses also lose any connectivity between sites of PTMs on the same protein molecule. To overcome these limitations, top-down approaches can be performed. In this approach, intact proteins are directly analyzed by LC–MS/MS without prior proteolytic digestion. This strategy is especially effective for characterization of essentially pure samples of small proteins without extensive or overly heterogeneous PTMs [75], and is inappropriate for high-throughput analyses owing to its low sensitivity and time-consuming data evaluation and interpretation [76]. Middle-down analyses, with restricted or limited proteolytic digestion, combine some of the benefits of top-down and bottom-up proteomics. These approaches aim to analyze protein fragments around 5–20 kDa in size, with intermediate PTM diversity [77].

Coagulation factor IX (FIX) is a biopharmaceutical with a very high number and diversity of PTMs, many of which are critical to its function. In particular, FIX is modified with many glycosylation events and γ-carboxylation of its N-terminal GLA domain. γ-carboxylation is a PTM mediated by γ-glutamyl carboxylase during protein biosynthesis, and complete γ-carboxylation is a key quality determinant of recombinant FIX [78]. The study mentioned above developed DIA LC–ESI–MS/MS methods to measure site-specific PTMs across FIX during bioreactor operation and after purification [63]. It was found that it is difficult to detect and identify fully γ-carboxylated GLA peptides in positive ion mode LC–ESI–MS/MS owing to the negative charge of the γ-carboxyl groups, and neutral loss of CO_2 upon CID fragmentation. However, standard bottom-up DIA LC–ESI–MS/MS could detect uncarboxylated or partially γ-carboxylated GLA peptides in positive ion mode, and could be used to infer the extent of site-specific γ-carboxylation. Derivatization of γ-carboxyl groups also allowed measurement of fully modified peptides, although this increased the complexity of the procedure [79]. These DIA–MS methods were used to monitor γ-carboxylation throughout bioreactor operation and compare differences in the extent of modification in the finished product with varied bioreactor operation. FIX is also modified by other heterogeneous PTMs such as proteolysis, *N*- and *O*-glycosylation, sulfation, phosphorylation, β-hydroxylation and disulfide bonds. To test the occupancy and structure of these PTMs, in-depth DDA–MS analysis was performed to identify and characterize PTMs, which was then used as the basis for DIA–MS quantification. The majority of the known PTMs on rFIX and several new PTMs (Figure 3) were observed and monitored in this study, highlighting the benefits of DIA–MS for PTM profiling of biopharmaceuticals.

Glycosylation is one of the most widespread, important and analytically challenging PTMs present on biopharmaceutical products. Glycoproteomic workflows are powerful and commonly used approaches for profiling the

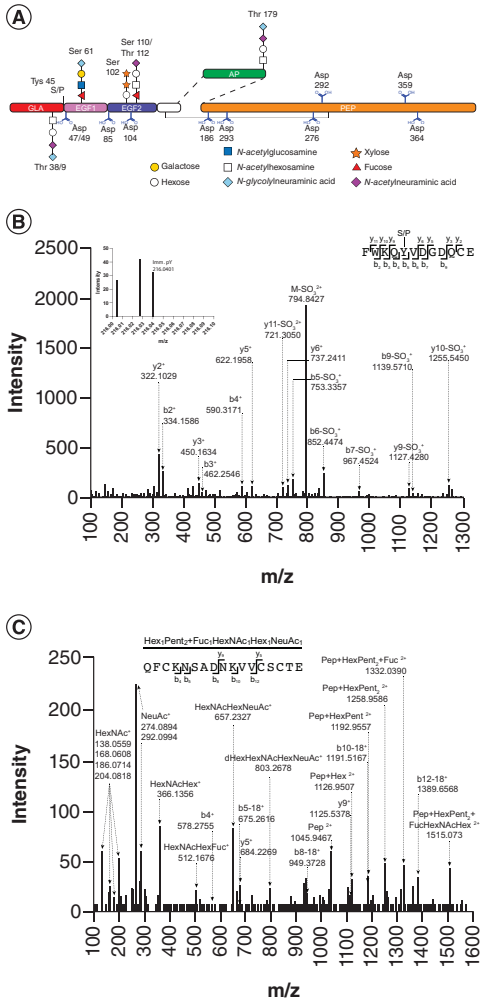

Figure 3. New post-translational modifications identified by DDA LC–MS/MS on recombinant Factor IX. (A) Schematics of FIX containing the new PTMs identified on rFIX. **(a, b)** CID fragmentation of select GluC FIX peptides. **(B)** F^{41}WKQYVDGDQCE^{54} peptide with sulfation/phosphorylation (S/P) at Y^{45} (observed precursor m/z 834.8189^{2+}, Δ2.4 p.p.m.). The inset shows the phosphotyrosine immonium ion (pY, 216.0401 m/z, Δ2.9 p.p.m.). **(C)** Q^{97}FCKN(+1)SADN(+1)KVVCSCTE^{113} glycopeptide with Hex_1Xyl_2 and $Fuc_1HexNAc_1Hex_1NeuAc_1$ *O*-glycans attached to $S^{102/110}$ and T^{112} (observed precursor m/z 1107.1067^{3+}, Δ7.56 p.p.m.). Pep, peptide.
CID: Collision-induced dissociation; PTM: Post-translational modification.
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site-specific glycosylation of biopharmaceuticals, as they use relatively standard proteomic bottom-up LC–MS/MS techniques [80–82]. CID or HCD fragmentation can provide substantial information on the peptide identity and glycan monosaccharide composition of glycopeptides [83], while ETD or ETD with HCD supplemental activation (EThcD) fragmentation is typically required for unambiguous localization of the site of modification, especially for *O*-glycans [84]. Data analysis pipelines must consider the additional structural complexity of glycopeptides compared with peptides, and many informatics solutions are currently available and under further development [85]. Glycoproteomic workflows can also be complemented with identification of the sites of *N*-glycosylation by deglycosylating biopharmaceuticals with enzymes such as peptide-N-glycosidase F (PNGase F), which leave a 'chemical scar' of deamidation of asparagine to aspartate at previous sites of glycosylation, prior to LC–MS/MS analysis [86,87]. Enzymatically released glycans can also be analyzed using glycomics workflows, which can provide detailed structural information about the glycans which can be difficult to obtain with glycoproteomic LC–MS/MS workflows alone [88]. Analysis of both released glycans and intact glycopeptides can also benefit from complementary separation methods such as LC, capillary electrophoresis and IMS [23,89], which can allow separation of glycan structural isomers [90].

A recent study characterizing the SARS-CoV-2 glycan shield demonstrated the power of mass spectrometric glycoproteomics to reveal the site-specific glycosylation of a recombinant SARS-CoV-2 S immunogen, including site-specific *N*-linked glycan composition and occupancy [91]. To maximize the coverage of the many *N*-glycosylation sites present on the S glycoprotein, three different proteases were used – trypsin, chymotrypsin and alpha-lytic protease. LC–MS/MS with high-energy HCD fragmentation was then used to analyze the glycopeptide pools and determine the glycan composition. Different glycosylation sites were found to vary in their site-specific *N*-glycosylation profiles (Figure 4). More specifically, three sites, N234, N709 and N801, were mainly oligomannose-type glycosylation; several sites, especially N657, possessed diverse hybrid-type glycans; and sites N61, N122, N165, N603, N657 and N1074 were occupied by a mixture of oligomannose- and complex-type glycans. The high confidence characterization of site-specific *N*-glycosylation structures and occupancy across the many modified sites of the S protein achieved by this analytical approach demonstrates its utility for glycoprotein biopharmaceutical quality control.

The previous examples highlight the power of bottom-up analysis for PTM identification and quantification at the peptide level. However, the peptide-centric focus of this approach means that most analyzed peptides have single PTMs, which hinders overall profiling of the entire protein. A recent study used an integrated strategy combining high-resolution native MS and middle-down proteomics to characterize co-occurring PTMs of human erythropoietin and human plasma properdin, enabling profiling of the structural micro-heterogeneity that often affects the functionality of biopharmaceuticals [92]. Native MS could measure the relative abundance and overall PTM composition of different proteoforms that could be distinguished by mass [93]. Middle-down analyses were then applied to characterize the site-specificity of these PTMs. The data from both approaches were then combined and compared to assess the completeness and reliability of PTM assignments. This combined integrated MS strategy provided a very complete profile of the measured glycoproteins and also discovered unexpected heterogeneity in three C-glycosylation sites on properdin. In theory, this integrative workflow could be used to quantitatively profile the site-specific molecular heterogeneity of PTMs on any protein, only limited by the resolution of the MS and the PTM heterogeneity of the protein.

Most biopharmaceuticals are proteins with diverse and complex PTMs that play important roles in their stability, function, half-life or immunogenicity. Detailed characterization of PTMs is therefore critical to guarantee high quality and effective potency of biopharmaceuticals. LC–MS/MS is a powerful technique for identifying and quantifying site-specific PTM structure and occupancy, particularly with a combination of bottom-up, middle-up, top-down or integrated analytical strategies.

Structural characterization of proteins

Protein function depends on correct folding and structure. Unfolding or misfolding can lead to unstable proteins with partial or total loss of function. Additionally, and of particular importance for biopharmaceuticals, disordered or misfolded proteins may aggregate, decreasing the effectiveness of the biopharmaceutical products and leading to other risks such as increased immunogenicity [94]. As correct protein structure is crucial for therapeutic proteins, the structural characterization of biopharmaceuticals is therefore necessary to ensure product quality by avoiding unfolded, misfolded or aggregated proteins.

X-ray crystallography and NMR are both classical tools for protein structural analysis, while hydrogen-deuterium exchange mass spectrometry (HDX–MS) has emerged as a highly complementary technique for mapping protein folding, protein–protein and protein–ligand interactions, and dynamic conformational changes in proteins. Additionally, HDX–MS is versatile and can be used to explore other systems including highly dynamic proteins, large biomolecular complexes and membrane-associated species [95]. In a typical HDX–MS analysis, proteins in H_2O-based solvent are diluted into D_2O-based solvent, which induces the liable hydrogens on the protein to exchange with deuterium in the solvents, with the exchange rate largely determined by surface accessibility, protein structure and dynamics, as limited solvent access and hydrogen bonding can protect hydrogens from exchange. Data on the extent of exchange are typically collected at several intervals, providing a profile of deuterium exchange versus time which reflects protein conformation and dynamics [96].

Bottom-up and top-down workflows are both available for HDX–MS analysis, with the former more common as it can be used for any protein without limitations on protein size. In this approach, proteins are rapidly digested with pepsin and LC–MS/MS data are collected, which can measure the extent of HDX at a peptide- or even amino acid-level. However, approximately 10–50% deuterium label loss can occur in this approach during enzymatic digestion and HPLC separation of the peptides [97]. In contrast, in top-down workflows, intact protein is directly analyzed

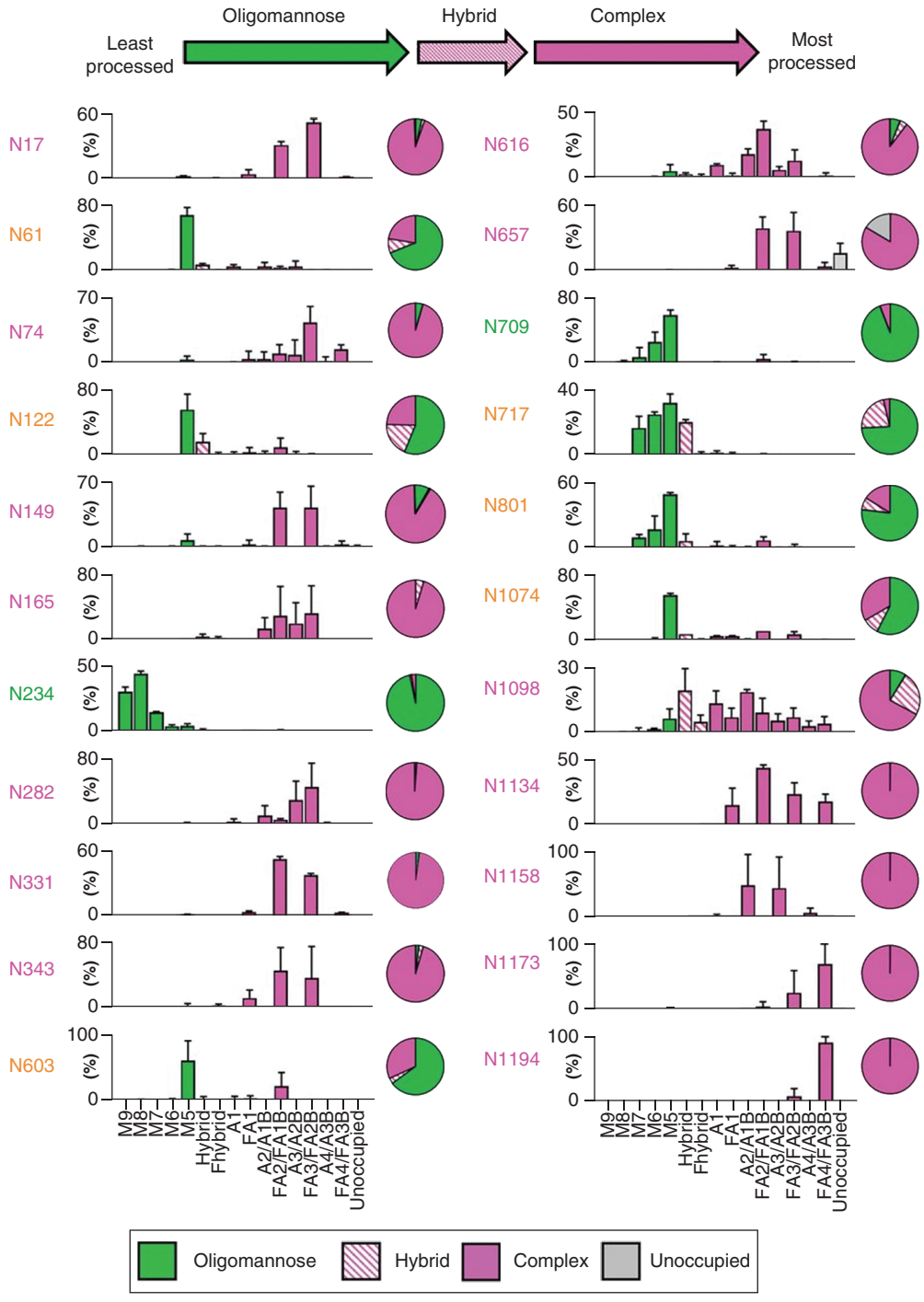

Figure 4. Site-specific *N*-linked glycosylation of the SARS-CoV-2 S glycoprotein. The schematic illustrates the color code for the principal glycan types that can arise along the maturation pathway from oligomannose- to hybrid- to complex-type glycans. The graphs summarize quantitative mass spectrometric analysis of the glycan population present at individual *N*-linked glycosylation sites simplified into categories of glycans. The oligomannose-type glycan series (M9 to M5; Man9GlcNAc2 to Man5GlcNAc2) is colored green, afucosylated and fucosylated hybrid-type glycans (hybrid and F hybrid) are dashed pink, and complex glycans are grouped according to the number of antennae and presence of core fucosylation (A1 to FA4) and are colored pink. Unoccupancy of an *N*-linked glycan site is represented in gray. The pie charts summarize the quantification of these glycans. Glycan sites are colored according to oligomannose-type glycan content, with the glycan sites labeled in green (80–100%), orange (30–79%) and pink (0–29%). The bar graphs represent the mean quantities of three biological replicates, with error bars representing the standard error of the mean.
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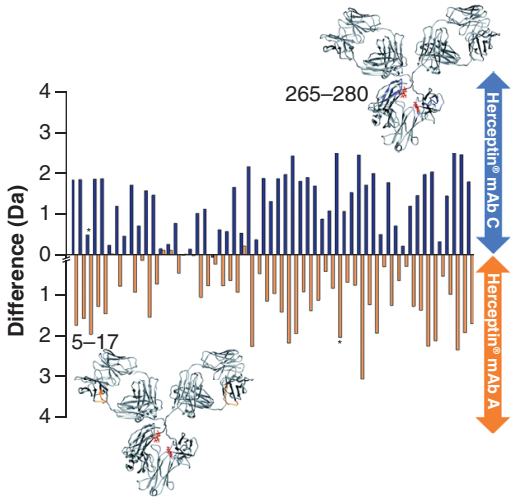

Figure 5. Deuterium uptake difference plots for heavy chain intact versus endoS2 treated Herceptin®; endoS2–intact. Each bar represents a different peptide (HC sequence coverage = 70.2%, 55 peptides). Blue bars represent the deuterium uptake differences for Herceptin lot C; orange bars represent deuterium uptake differences for Herceptin lot A. Labeled peptides 5–17 (VESGGGLVQPGGS) and 265–280 (VVVDVSHEDPEVKFNW) are those with significant uptake differences between the two Herceptin lots, i.e., a difference of >1 Da. The peptide locations for the two peptides with uptake differences >1 Da are highlighted on the mAb structures in blue and orange for lot C and lot A, respectively (PDB: 1IGY). A representation of endoS2 treated glycans are shown in red. Subtraction of intact mAb data from enzyme treated mAb data for individual lots, means that the intact data serves as a control to counteract any day-to-day variations in the HDX setup.
*Corresponds to the equivalent peptide in the other sample.
HC: Heavy chain; HDX: Hydrogen-deuterium exchange; mAb: Monoclonal antibody; PDB: Protein data bank.
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by LC–MS/MS, involving ionization and fragmentation. This minimizes loss of deuterium and potentially allows true site-specific HDX measurement [98]. However, the success of this method decreases with increasing protein size, and is only currently feasible with proteins less than 30 kDa [99,100]. Additionally, deuterium scrambling can occur during MS/MS fragmentation, where hydrogen or deuterium migrate along the peptide backbone leading to distortion or loss of the original labeling pattern [96].

A recent study combined the complementary approaches of bottom-up and top-down HDX-MS to characterize and compare the higher-order structure of an originator antibody drug and two batches of biosimilars [101]. Although the same samples were used in the two approaches, it is somewhat difficult to directly compare the results due to the different back-exchange rates and spatial resolution achieved with the two methods. Nonetheless, the structural data from the two approaches were consistent and complementary, with both approaches finding no structural differences between the three drug samples. Moreover, the sequence coverage for heavy chain and light chain was 87 and 74%, respectively with bottom-up analysis, and 50 and 100% with the top-down approach. This highlights the consistency and complementarity of the two methods. Overall, the combination of both methods provided high-quality complete structural information for the whole antibody without any missed regions or residues. HDX–MS is an effective technique to rapidly map binding epitopes in the early stage of biosimilar development, providing data to assess similarity [102–104]. Compared with more traditional HDX–MS, time-resolved ESI hydrogen-deuterium exchange MS (TRESI–HDX–MS) with ms time-scale deuterium labeling can detect more subtle changes in protein conformation and interactions [105–109]. This powerful tool was used in a recent study to compare the interactions of a commercial Avastin and its biosimilar ApoBev with their biological target, VEGF-A. Clear epitope mapping was obtained through TRESI–HDX–MS, which showed that the binding epitopes of Avastin and ApoBev for VEGF-A are very similar, but with subtle differences in VEGF dynamics [110]. Combinations of techniques can provide particularly informative descriptions of protein structure and dynamics. For instance, HDX–MS has been used together with IM–MS to identify batch-to-batch signatures of the mAb Herceptin that correspond with the impact of *N*-glycosylation on protein structure and dynamics (Figure 5) [111]. IM–MS can be particularly informative in combination with collision-induced unfolding, to characterize the structure, dynamics and interactions of proteins [36,112,113].

The potency of biopharmaceuticals depends on them having correct structures, so correct folding is a key quality requirement. HDX–MS can be applied as a rapid and unbiased technique to monitor the folding or aggregation status of diverse proteins, with bottom-up, top-down or integrated analytical workflows, while TRESI–HDX–MS is capable to achieve faster and more unambiguous detection. IM–MS also shows exciting potential for rapid and informative structural profiling of biopharmaceuticals.

Conclusion

MS is a mature and powerful technique that is applicable to many aspects of biopharmaceutical quality control. In particular, it is useful for identification and quantification of HCP contaminants, characterisation of complex PTMs and monitoring the structural integrity of biopharmaceutical products. Diverse MS workflows enable this wide range of applications, including DIA LC-MS/MS, DDA LC-MS/MS with diverse fragmentation techniques, IM-MS, HDX-MS and native MS. Future developments in sample preparation, instrumentation and data analysis will undoubtedly further extend the capabilities and utility of MS analyses in biopharmaceutical quality control.

Future perspective

LC–MS/MS technology and applications are expected to rapidly progress in coming years, with wider and more frequent application in biopharmaceutical process and product quality control. Modern MS instruments have incredible performance in sensitivity and resolution, and the amount of biopharmaceutical product required for analysis is not generally limiting. Instead, it is analytical through-put which limits the usefulness of MS for many applications. Current MS workflows including sample preparation, analysis, and data processing typically take 1–2 days, limiting their utility in time-sensitive applications such as process monitoring. We therefore see improvements in the speed, through-put, automation and robustness of LC–MS/MS analytic workflows as a critical opportunity, with rapid automated digestion and sample preparation for bottom-up strategies, or improved technology for top-down strategies. For instance, with such improvements it may prove feasible to use LC–MS/MS during fermentation to monitor desired or unwanted product PTMs, or to monitor the purification process for residual HCP impurities in real-time, increasing product quality and purification efficiency of target biopharmaceutical proteins.

Executive summary

- MS has become one of the key methods used in the characterization and quantitation of proteins in biopharmaceutical quality control during the past two decades owing to improvements in instrument sensitivity, resolution, specificity and selectivity.
- Detailed information is needed for quality control of biopharmaceuticals, including residual host-cell proteins (HCPs), site-specific post-translational modifications (PTMs) and protein folding status.
- LC–MS/MS can identify and quantify HCPs with high selectivity and sensitivity, as even low quantities of HCPs are detectable by MS, and many HCPs can be identified in one analysis.
- For characterization of PTMs, MS strategies include bottom-up, top-down and middle-down. Bottom-up analyses are the most common, providing high sensitivity and high-throughput peptide level measurements, but can be limited by incomplete coverage of a protein's sequence. Top-down analyses are suitable for analysis of small proteins with modest PTM heterogeneity, although data interpretation can be time-consuming. Middle-down approaches, or an integrated combination of all three strategies, have emerged as an effective approach for detailed global, site-specific PTM analysis.
- Hydrogen-deuterium exchange MS allows structural characterization of the folding and aggregation status of proteins with bottom-up or top-down analyses, with bottom-up approaches being applicable for proteins of all sizes.
- Improvements in the speed, automation and throughput of bottom-up LC–MS/MS, and of the resolution and data analysis workflows of top-down LC–MS/MS are expected to allow these techniques to be useful for real-time monitoring of product quality during fermentation, or of HCP impurities during purification, improving process efficiency and product quality.

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Editorial

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Bioanalysis

Analytical challenges and advancements in bioanalysis of therapeutic proteins

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Therapeutic proteins & associated challenges

Therapeutic proteins have been widely explored for the treatment of several diseases resulting into development of novel therapies. The protein therapeutics class comprises peptides, recombinant proteins, monoclonal antibodies, antibody–drug conjugates, antibody fusion proteins and antibody fragments [1]. In the past two decades, therapeutic proteins have gained significant success with clinical significance resulting into several US FDA approved therapies with improved patient care. Therapeutic proteins encounter critical challenges during their production and designing of formulations. The challenges can be attributed to the complexity of therapeutic proteins with respect to high molecular weight, inherent heterogeneity, requirement for post-translation modifications and production from broad range of organisms leading to the presence of a variety of biological matrices during production [2]. These macromolecules are prone to be affected by microheterogeneities resulting either from their intrinsic complex nature or chemical/enzymatic modifications subjected during their production or storage conditions, or interactions with host cell proteins [3]. While the developed microheterogeneities may demonstrate mild-to-moderate impact in most cases, they may also trigger structural or conformational changes in other cases resulting into severe consequences raising concerns about the safety of developed therapeutic protein [4].

Importance of characterization & analytical tools for macromolecular therapies

The production and manufacturing of therapeutic proteins is a complex and challenging process involving several critical intermediate steps. Therapeutic efficacy and adverse effects of these proteins are significantly affected by their structure and composition and in certain cases are modulated due to conformational changes [5]. Therefore, the exact structure and composition of therapeutic proteins are crucial as minor changes may lead to unwanted side effects and possible loss of therapeutic activity. Also, the detailed structural analysis of therapeutic proteins would help to facilitate development of successful biosimilars [6]. Owing to such vital requirements, it is essential to characterize the therapeutic proteins at every step of production and processing along with final end products.

Analytical approaches serving as bioanalytical tools

Remarkable advancements in analytical field over the last few decades have led to development of various technologies efficient in elucidating complex structures. The protein characterization techniques have evolved dramatically; from x-ray crystallography being used to determine the first protein structure to the widely used NMR [4]. However, these analytical methods are known to have limited abilities such as x-ray crystallography can only determine the conformation of the unfolded protein structures, while NMR involves molecular weight limitations and incompetency in determination of amino acid composition [4]. Other commonly used analytical techniques for protein characterization are size-exclusion (SE) chromatography and analytical ultracentrifugation; for determination of protein aggregates and fragmentation, circular dichroism and differential scanning calorimetry; for determination of protein folding, stability and interaction and microflow digital imaging for visualization of any visible particles [5]. These techniques provide limited information about the protein while the detailed constitutional information remains unrevealed.

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Numerous chromatographic approaches such as SE, affinity chromatography, hydrophilic/hydrophilic interaction and reverse phase have been indispensably used for characterization of therapeutic proteins [6]. Several modes of LC have been employed for assessing physicochemical characterization of therapeutic proteins, quantitative analysis in various biological matrices, determining pharmacokinetic profile of therapeutic proteins and determination of post-translation modifications [7]. MS has established success for characterization of macromolecules and has been widely exploited to analyze protein structures and determine molecular weight. Native MS is used for qualitative and quantitative determination of intact protein under non-denaturing environment. This approach is however known to suffer due to intensive manual work hampering its implementation to the automated advancements [8].

Electrophoresis is another routinely used technique for separation of complex therapeutic proteins with respect to their size along with assessment of impurities. Traditional SDS-PAGE is widely employed for separating contaminants and monitoring presence of protein aggregates. However, it is limited to only qualitative analysis and is also known to be have issues of non-reproducibility [9]. CE has gained significance as a distinct electrophoretic technique for determination of heterogeneity based on size and charge along with adequate monitoring of post-translational modifications [10]. CE such as capillary gel, capillary isoelectric focusing and capillary zone electrophoresis are known to possess advantages such as faster rate of separation, reduced run time, requirement of lower sample volume and enhanced resolution [11]. While efficacious, CE is known to be affected by adsorption of analytes which in turn impacts the sensitivity and reproducibility of the method, thereby limiting its clinical applications.

Implication of hyphenation techniques

Hyphenation approach involves coupling of a separation technique with a detection methodology for exploiting the advantages of both the methods and overcoming the existing limitations. MS is the most used detection technique coupled with chromatographic or electrophoretic separation for characterization of therapeutic proteins. LC–MS has been extensively used for determining mass distribution, presence of complexes and conformation of therapeutic proteins [12]. Furthermore, LC–MS/MS is exploited for assessing the presence of protein (top-down approach) and protein species (bottom-up approach), determination of partial sequences and molecular weight of intact species, identification of protein species and post-translational modifications [13]. These hyphenation methods are also useful in determining the secondary and tertiary structures of proteins along with composition of the protein species and identification of post-translation modifications [12]. However, these methods also face several challenges including intensive sample preparation prior to analysis, requirement of spectral libraries to determine the structure and composition of analyzed therapeutic protein and in some cases may require high amount of sample and provide limited information about the protein species [14]. Another hyphenation approach, SE–LC is employed for determining the purity, analyzing heterogeneity of charges and some specific post-translational modifications [15]. SE–LC can be further coupled with MS as an indirect interface since direct coupling is limited due to presence of high amount of salts, poor peak shape and impact on protein conformation [8].

The most recently used combinatorial approach of CE–MS has proven to be an indisputable strategy for characterization of therapeutic proteins resulting in development of fast, sensitive and specific techniques [16]. This strategy helps in determination of molecular weight of intact proteins, determining the amino acid composition as well as any post-translational modifications [17]. Consequently, to improve and miniaturize the electrophoresis technique, micro-chip electrophoresis has also been developed [18]. This technique has significantly reduced the time for analysis in addition to reduction in sample volume with increased efficiency of resolution, therefore making it a cost-effective technique [18,19].

Conclusion

The development of therapeutic proteins is a highly complex process wherein several critical attributes are required to be continuously monitored. Advances in the analytical field have improved the protein characterization significantly. However, each analytical method has its own limitations that impact the protein characterization in its own unique way. Existing limitations of analytical methodologies are being resolved using combinatorial/hyphenation approaches; wherein two technologies are coupled to overcome the limitations and impart synergistic effect for effective characterization of therapeutic proteins. These emerging hyphenation technologies impart clinical significance to the developed bioanalytical methods, help in development of biosimilars, provide high-throughput production setting, and improve the quality of therapeutic products.

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Review

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Bioanalysis

Analysis of regulatory guidance on antidrug antibody testing for therapeutic protein products

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Therapeutic proteins have the potential to induce unwanted immune responses. The potential impact of immunogenicity on pharmacokinetics, pharmacodynamics, safety and efficacy are well established. Here, we analyze key aspects of current US FDA and EMA guidelines on the development and validation of antidrug antibody assays. Although FDA and EMA guidance documents are in harmony on most points, EMA allows greater leeway for scientific judgement, while FDA recommends specific approaches that may not be appropriate in some situations. Many white papers suggest approaches different from the guidance documents, however, these can conflict with each other and are themselves only scientifically valid in certain situations. Here, we indicate when alternatives to guidance may be needed and what those approaches might be.

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Keywords: antidrug antibody • drug tolerance • European Medicines Agency • Food and Drug Administration Guidelines • immunogenicity • positive control • pre-existing antibody • sensitivity • therapeutic proteins

Therapeutic proteins have revolutionized the treatment of disease in the last 30 years. They are one of the fastest-growing classes of drugs and currently include more than 160 protein products approved for clinical use [1–3]. Unlike traditional small molecule drugs, therapeutic proteins have larger and more complex structures and the potential to induce an unwanted immune response [4,5]. The potential impact of immunogenicity on pharmacokinetics (PK), pharmacodynamics (PD), safety and efficacy are well established [6–9]. The resulting clinical consequences of an immune response range from no measurable effect to infusion reactions, hypersensitivities, secondary treatment failures and rarely even life-threatening events [10–12].

Unfortunately, an accurate prediction of harmful immune reactions is not yet possible due to the complexity of human immune system. The development of an immune response against a biologic drug is influenced by both patient-specific factors (gender, ethnicity, immunologic status allergy and HLA classification) and product-specific factors (origin, structure, degradation products, post-translational modifications, impurities, formulation, and container closure, dose level, delivery route – among others [7–10,13–16].

As the use of biologics has expanded, a number of industry white papers and regulatory guidelines have been published to chaperone the implementation of assays strategies to measure and analyze immunogenicity [12,14,15,17–21]. Only the US FDA and the European Medicines Agency (EMA) have published guidelines on the development and validation of assays for antidrug antibody (ADA) testing. Currently, no dedicated guidelines describing ADA assay development and validation exist within or outside the International Council for Harmonization regions (China, Japan, Brazil, etc.) [22]. Many pharmaceutical and contract research organizations are currently developing and performing ADA assays according to FDA and EMA guidelines and certain white papers [7,12,17,20].

In this review, we discuss current regulatory guidance and analyze parameters such as the selection of appropriate positive controls (PC), ADA method formats, sampling time and frequency, determination cut points with pre-existing antibodies (pre-Abs), sensitivity, minimal required dilution and drug tolerance (DT) which influence the

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detection of immune response. In this analysis, we focused on the inconsistency between the agency guidance and general practice in industry. We also provided our view and recommendations in areas where the guidelines did not provide adequate direction to current practices. This analysis will highlight the need for further harmonization of the respective guidelines and need for additional clarification that will lead to improved immunogenicity assessment.

Overview of FDA & EMA immunogenicity guidance for therapeutic proteins

The FDA released its first draft guidelines on immunogenicity testing for therapeutic proteins in 2009, followed by a revised draft in 2016, and subsequently, they issued final guidance for industry in January 2019 [8]. The EMA released its first guideline on immunogenicity testing for recombinant therapeutic proteins in 2006, followed by a revised draft in 2015, and final guidance in May 2017 [9]. For both agencies, the final guidance was issued based on experience from regulatory submissions, scientific information in the literature and numerous discussions between industry and regulators. The FDA provided detailed guidance for product development, with comprehensive instructions for Investigational New Drug (IND) and Biological License Application (BLA) filings. The EMA's approach allows greater flexible, especially in early development, unless regulatory guidance is requested [23].

The revised FDA guideline recommends that the sponsor should include an integrated summary of immunogenicity in the application including a risk assessment. Sponsors should start creating integrated an immunogenicity summary report early in development and update it at regular intervals through IND, BLA and even post approval [8,9,22]. Both FDA and EMA focus on clinical trials and provide limited recommendations for pre-clinical ADA assessment. This prioritization is logical because although preclinical ADAs may complicate the toxicokinetic interpretation, the immunogenicity of a human protein in animals does not predict immunogenicity in humans [24] except in special cases, for example, bacterial protein immunogenicity in animals is often predictive for humans. The FDA is more receptive to preclinical studies, while the EMA seems more aggressive in attempting to reduce animal studies [25]. Both agencies encourage the adoption of novel *in vivo, in vitro* and *in silico* tests for the selection and design of lead compounds. Whichever tools are used, a risk-based approach should be used in evaluating and managing immune responses to immunologically related events in the clinic, including PK, pharmacodynamic, safety and efficacy [6,8,9].

Both agencies suggest that the immunogenicity testing strategy should be in place and assay development should take place before clinical testing is needed. A complete validation package would be provided at BLA submission. Both regulatory guidelines also advise a multitiered ADA assessment approach including an initial screening test, followed by confirmation, and then the titration, neutralizing antibody and any other characterization assays of screen-positive samples. Depending on the stage of development, a neutralizing antibody assay would be developed to further characterize the neutralizing capacity of the ADA-positive samples. The new FDA guidelines also discuss the sample collection plan for clinical studies and include a recommendation for the appropriate storage of preclinical samples. ADA detection methods should be developed and validated to meet the requirements of a drug program, but no assay format or technology is preferred over others. The agency proposes that the ADA assays should be designed to detect Abs that could mediate unwanted clinical consequences such as neutralization of the biological function of the drug, or hypersensitivity responses. Table 1 summarizes the comparative analysis of FDA and EMA guidelines for the requirement of developing and validating ADA assays for therapeutic proteins.

Guidance on PC & negative control for developing ADA assays

PCs

The FDA and EMA recognize the importance of selecting appropriate positive and negative controls (NC) in the development and validation, of 'fit-for-purpose' immunogenicity assays for study sample analysis. ADA assays are initially characterized, and the performance monitored using a PC that typically is prepared by combining an ADA with the NC matrix. Key assay parameters such as sensitivity, specificity and DT are relative values determined by the choice of PC which can vary in species, clonality, isotype and affinity [26,27]. These aspects make comparisons of immunogenicity between different therapeutics protein of limited value, even when the therapeutics are similar yet sufficiently different so that the same PC does not bind similarly to both [10,26,28].

It is thought that patient-derived ADAs would be ideal PCs that might represent the full range of a clinical ADA response. Unfortunately, subject-to-subject differences mean that no one ADA would represent all patient responses, even if obtaining and maintaining a consistent supply of patient-derived ADA PC throughout assay validation and the phases of clinical analysis were practical. Thus, both agencies recognize the necessity and wide use of other sources of affinity-purified polyclonal and monoclonal antibodies (MAbs) as PCs. These surrogate

Table 1. A summary comparing US FDA versus European Medicines Agency guidance on immunogenicity testing for therapeutic protein products.

Criteria	FDA guidance (released in 2019)	EMA guidance (released in 2017)
Regulatory approach	Provides detailed guidance for immunogenicity testing, with comprehensive instructions for IND and BLA filings	More flexible guidance especially in early stage of development unless the sponsor requests additional guidance
Integrated immunogenicity summary report	Recommends that the sponsor should include an integrated summary of immunogenicity in the application	Same
Nonclinical studies	Encourages the use of a novel *in vivo, in vitro* and *in silico* tests for the selection and design of lead compounds	Same
Testing strategy	A risk based and multitiered approach	Same
Screening assay	Approximately a 5% false-positive rate in the screening assay is acceptable without compromising assay sensitivity	Same
Confirmatory assay	A 1% false-positive rate is calculated of the confirmatory cut-point	No false-positive rate is specified
PC	Recognizes the importance of selecting an appropriate PC in the development and validation	Same
NC	The NC should match the characteristics of the study samples	Same
Low, medium and high positive control	The LPC should be set to achieve a 1% assay failure rate Recommends including a medium PC in the validation of ADA assays, but this is not required for the routine sample analysis	Low, medium- and High-PC should be included during assay characterization and validation of ADA assay and later to monitor assay performance No such recommendation
Sensitivity	Recommends that the sensitivity of the assay should be calculated by interpolating the linear portion of the dilution curve to the assay cut point Clinical ADA assays should achieve a sensitivity of at least 100 ng/ml	No such recommendation No sensitivity value is specified
Drug tolerance	The DT should be determined in presence of expected concentration of on-board drug	The DT of the assay must be higher than the expected drug levels in the clinical samples
MRD	Suggests performing recovery experiment (ADA response in serum and assay diluent) in 10 individuals to determine MRD, should not exceed 1:100	No guidance
Pre-Abs	Suggests some methods to calculate cut points for populations having a high incidence of pre-Abs Separate analysis of safety in patients recommended if samples have pre-existing Ab	No detailed instruction about cut-point calculation Same
Sampling for ADA assay	Provide some recommendation but no detailed instructions on the sampling schedule Pre-treatment samples from all subjects and more frequent sampling for molecules anticipated to have higher immunogenicity Sampling for ADAs should be scheduled at time when the level of the therapeutic protein is lowest	Same Same, routine sampling, however additional samples collected when the incidence of an unwanted immune response is suspected Same
MDTs	Guideline recommends multiple assays may needed so that an immune response to all domains of an MDT can be detected	Same

ADA: Antidrug antibody; BLA: Biological License Application; DT: Drug tolerance; HPC: High positive control; LPC: Low positive control; MDT: Multi-domain therapeutic; MPC: Medium positive control; MRD: Minimum required dilution; NC: Negative control; Pre-Abs: Pre-existing antibodies; PC: Positive control.

antibodies are not expected to represent the spectrum of immune responses that might be observed in patients, however, using ADAs from immunized animals is considered the acceptable practice in the industry by regulatory agencies. Polyclonal antibodies (PAbs) from drug-immunized animals are frequently used, although reduction in the use of animals, especially nonhuman primates, is increasingly encouraged particularly in the EU [25]. MAbs derived from phage-display libraries or hybridomas are advantageous because they allow for unlimited production of a consistent and well-characterized PC while using few or no animals. Additionally, technological advancements have allowed for the humanization and veneering of nonhuman MAbs [29,30] in order to engineer such PCs into a structure that closely represents subject ADAs.

Another consideration is to select a suitable PC based on the drug epitope(s) to which it binds. For therapeutic MAbs, FDA prefers PCs that are anti-idiotypic antibodies with 'reactivity against variable regions' over those that bind to the immunoglobin framework. Because those epitopes include the pharmacological activity and are the domains most likely to be identified as immunologically foreign, they are highly relevant epitopes. All of these novel epitopes have the potential to induce ADA responses [31]. FDA also recommends that ADA assays should detect all components of a therapeutic proteins. The generation of PCs that allow detection and characterization of potential

ADAs against these additional domains incurs another level of difficulty. Ideally, assays should map ADA binding to all critical drug domains.

The agencies also note that assay format is another area that impacts PC selection. In the commonly used bridging immunoassay format, the species of the PC is generally irrelevant. However, nonbridging formats are sometimes necessary, and these generally require a secondary antispecies antibody as the detection reagent, in which case the PC must be of an appropriate isotype and species. If a nonhuman PC is used in a sandwich assay for human ADA, then nonhuman and human secondary detection antibodies are needed to monitor the PC and detect patient ADA, respectively. Although briefly mentioned in FDA or EMA guidance, one can assume that using different secondary antibodies for PC and samples is to be avoided because characterization of the PC cannot provide key information about the assays ability to detect patient ADA.

The selection of a PC influences the apparent sensitivity and DT of an ADA assay in many ways. For example, one can select PC antibodies that tolerate acid or other harsh assay conditions and thereby inflate the apparent DT of an assay. A PC of low purity will inaccurately indicate the ADA assay has poor sensitivity, while a PC that retains or enriches matrix-derived impurities may have an unpredictable impact on an assay. Although PAbs are mentioned favorably in regulatory guidance, monoclonal PCs allow one to characterize affinity, isotype, epitope specificity and other properties of ADAs. It has been shown in the literature that the use of a high-affinity PC can produce better apparent sensitivity than a lower affinity PC, in the same assay [32–34]. However, we have found that affinity may have the inverse effect on sensitivity when acid dissociation is used to enhance DT [28]. In any case, if both high- and low-affinity PCs are not characterized, care should be taken so as not to select PC simply for high affinity when this leads to over-estimation of assay sensitivity [20]. Each type of surrogate ADA has limitations that should be considered during the PC selection.

Although health authorities state that PAbs best represent an endogenous ADA response and favor their use for assay characterization experiments, they also state that high-affinity MAbs are acceptable for monitoring assay performance [35]. Industry representatives agree that the choice of PC is important as it is used to define key assay parameters. Most labs select only a single PC however the preferred method is to use at least one low and one high-affinity PC during clinical assay development and validation in order to capture the range of affinities seen in polyclonal responses.

Both guidance documents recommend that sponsors should generate and store enough PC antibodies to monitor quality control during routine performance of the assay. The EMA endorses including PCs at low, medium and high concentrations (in addition to NC) to characterize/validate the ADA assay, and later to monitor assay performance. The FDA also recommends including a medium PC in the validation of ADA assays, but this is not required for the routine sample analysis (Table 1). The FDA suggests that the low PC should be set low enough to produce an estimated 1% assay failure rate, meaning that the low PC assay signal should fall below the cut point about 1% of the time. The rationale behind this is to ensure that the low PC helps maintain assay performance at the claimed sensitivity. From our experience, the amount of data generated during development and validation is insufficient for a statistically robust determination of a 1% failure rate. One might suggest up-dating the low PC as study data accumulate but a primary purpose of the low PC is to ensure consistent assay sensitivity over the life of an assay and changing the low PC would prevent this. Currently, some labs select a medium PC randomly while other lab does not include it. ADA results are not derived from a standard curve, and the PC is not equivalent to each subject's ADA, therefore the medium PC serves no apparent purpose, and we believe it should be removed from guidance. The purpose of the high PC is to cover expected study data at the high end of the dynamic range of the assay and to determine if a hook effect is present. While it is informative to monitor the assay dynamic range, and for this reason we agree with the need for a high PC during validation, and possibly at intervals during sample analysis, we point out that ADA responses are reported as titer values then the hook effect can be defined once during validation and continuing to run a high PC beyond validation is of limited value.

Negative controls

Regulatory guidance given by the agencies regarding the NC is less extensive than that for the PC. However, both agencies recognize the importance of an appropriate NC to establish a baseline for the assay response near but below the assay screening cut point. The FDA and EMA recommend that if possible, the NC should represent the study samples with regards to age, gender and medication status and that it should be handled in the same manner as samples. As study samples are frequently not available during assay development, and there are challenges associated with obtaining bulk quantities of matrix from patients (especially from those who are very young or very ill or if

the disease is rare or highly infectious), it is general practice in industry to prepare an NC from drug-naive pooled matrix, and in so far as possible, to characterize and compare with matrix from a limited patient population during validation in order to demonstrate equivalence or to justify the need for using a patient-derived NC.

Methods for detection of ADA

Assay methodologies have evolved over the years and a variety of analytical platforms are used to measure ADAs. These include ELISA, radioimmunoassay, radio-immunoprecipitation assay, surface plasmon resonance (SPR), Gyros, biolayer interferometry (BLI) and electrochemiluminescence (ECL). Each of these methods has its strength and weaknesses in throughput, sensitivity and ability to investigate various aspects of an ADA [27,28]. Ligand-binding assays have been the primary method used to detect ADAs in biological samples [27] due to their robustness, reliability, sensitivity and ease of automation. ECL detection in a bridging format became popular way to detect human antibodies against therapeutic antibodies. The bridging format is also easily adapted to high throughput, however, without further modification it is particularly prone to interference (especially for high-affinity Abs) from the drug (and sometimes the drug target) and it can be less sensitive (especially to low-affinity Abs) than nonbridge formats. The sandwich assay format cannot be used to detect human antibodies against human therapeutic antibodies, but in most other situations it performs well but requires samples and controls to be species matched.

Unless appropriate steps are taken, all assay formats can give false negative or positive results particularly when samples contain high concentrations of drug or soluble target protein. Some labs produce data to show that one technology is more sensitive than others, but this can often be dismissed, first because ADA assays of adequate sensitivity can be designed in all the technologies mentioned, and second because assay conditions with these technologies greatly influence the sensitivity one achieves from the technology. As no single assay format is appropriate for evaluating immunogenicity in every biologic, ADA assays must be thoroughly validated to ensure reproducible, consistent and definitive results. Previously published recommendations for ADA assays, described in detail the various validation parameters (Table 2) that should be tested to ensure that the method is suitable for its intended purpose [8,17,21,32]. For example, Shibata [36] screened the anti-erythropoietin monoclonal antibody reference panel that was developed by the WHO to compare the analytical performance of three ligand-binding ADA assay methods, ECL, SPR and BLI. The SPR and BLI methods gave similar results, however, the binding of low-affinity anti-erythropoietin antibodies could not be detected by the ECL method. Similarly, Li [33] compared three ADA assay platforms and determined that the sensitivity for a polyclonal ADA to be 1, 6 and 130 ng/ml, respectively by ECLIA, ELISA and Octet (BLI) methods. However, for low-affinity MAb ADAs, the Octet provided the best sensitivity. These examples shows that no one single technology is best suited to detect all type of ADA. Therefore, understanding the analytical performance of ADA assays is very important for accurate assessment of ADAs. It is important to use an immunogenicity risk assessment to define the critical ADA attributes and then chose an appropriate method(s).

Current regulatory practice to determine sensitivity, minimum required dilution & DT

In this section, we analyze sensitivity, minimum required dilution (MRD) and DT together as they are closely related to each other. The sensitivity of an ADA assay is defined as the lowest concentration of ADA which consistently produces a positive result that is equal to or greater than the assay cut point. The FDA advises that ADA assay sensitivity should be determined in the presence of the expected concentration of drug in order to be relevant [37]. The original FDA draft guidance recommended a clinical ADA screening assay sensitivity of 250–500 ng/ml [38], however, current FDA draft guidance has lowered this to 100 ng/ml [8]. Assays developed to assess IgE ADA should have sensitivity in the high pg/ml to low ng/ml range. The EMA has not defined a uniform standard sensitivity value (see Table 1) [9].

In the FDA guideline, it is recommended that the sensitivity of the assay should be calculated by interpolating the linear portion of the dilution curve to the assay cut point. However, some sponsors use alternative approaches. Interpolation typically generates a sensitivity result that is less than the tested calibrators and is misleading because it suggests quantitative precision for methods that are semiquantitative and based on a surrogate analyte that will differ in various ways from the ADA generated by each test subject and sample collection time. We suggest the guideline should be modified to reflect of these limitations.

Many biological matrices (serum, plasma, saliva, etc.) contain factors (soluble target, Fc receptors, rheumatoid factors, etc.) that could cause assay interference leading to false positive or negative results. The FDA recommends that sponsors examine matrix interference by spiking the PC antibody (low, medium and high) in diluent and

Table 2. Definitions and parameters for the validation of an antidrug antibody assay.

Parameter	Definitions	Validated method
Controls	A substance that is tested to assess acceptable assay performance ADA assays are characterized and later monitored using a PC sample that is prepared by combining ADA with the negative control matrix	Clinical: specific PCs (e.g., anti-IDs) and negative controls from relevant population Non-clinical: Same as above or anti-human Fc (for applicable projects)
Cut point analysis:		
– Screening	To establish appropriate data format for study phase bioanalysis and screening method cut-point that distinguishes negative from potentially positive ADA samples	Balanced experimental design: minimum of 20 samples per population (50 preferred)
– Titration	To establish the titer method cut-point for the greatest sample dilution that generates a potentially positive ADA result	Evaluation of diluent controls and naive samples
– Specificity	To establish the method cut-point that confirms the specificity of ADA to drug	At least 2 operators and 20 samples
Drug tolerance	Drug tolerance is defined as the sensitivity of the assay in the presence of expected levels of the therapeutic agent	Assess with LPC and other concentrations of ADA. For a clinical assay, the method is optimized to meet expected drug concentrations For a nonclinical assay, DT is characterized and possibly optimized
Other interference	The property of a specific factor that may impact assay results (e.g. soluble drug target, unrelated biologics)	Test soluble drug target, other unrelated biologics, etc. (as relevant)
Sensitivity	The sensitivity of ADA assay is defined as the lowest concentration of ADA which consistently produces a positive result that is equal to or greater than the assay cut point	Clinical assay: 100 ng/ml Non-clinical assay: 500–1000 ng/ml
Dilutability	To evaluate the dilutability in assay diluent and naive serum	Clinical and nonclinical assay: gest dilutability in relevant buffers and naive serum
Recovery	To evaluate the effect of matrix on the ability to detect PC ADA	Clinical assay: test recovery in diluent, pooled nNHS, in five individuals (normal and disease), five hemolyzed and five lipemic samples Nonclinical assay: not required
Robustness	To characterize the assay performance relative to a change in the assay method	Test different lots of reagents, incubation times, batch size, etc. (as relevant)
Stability (reagents)	To evaluate the impact of storage conditions on stability of assay plates and reagents	Test storage stability of reagents under the appropriate storage conditions
Stability (samples)	The stability of surrogate samples comprised of positive consistency controls in naive serum	Evaluate the effect of storage temperature, and freeze/thaw cycles on the assay performance of the controls. Test short-term and long-term stability for up to 14 days and 3 years, respectively
Precision	To characterize intra-assay precision for the screening method and inter-assay reproducibility for the screening, and specificity methods	Intra- and inter- assay precision assessed; a minimum of 20 runs*

ADA: Antidrug antibody; DT: Drug tolerance; LPC: Low positive control; nNHS: Naive normal human serum; PC: Positive control.

matrix and then comparing ADA recovery to indicate the degree of interference. If interference is present, further diluting samples may help to reduce matrix interference. However, in order to maintain enough sensitivity FDA recommends that MRD should not exceed 1:100. The FDA also suggests testing the matrix from ten individuals to evaluate recovery in order to determine an appropriate MRD. There is no similar recommendation in the EMA guidance or any white paper we could find [8,9]. The guidance does not address that changing MRD can have unequal effects on sensitivity and DT which has led to a lack of consensus in industry for prioritizing MRD versus the sensitivity or DT. Some sponsors conduct the MRD experiment as recommended by the FDA while others argue that sensitivity and DT must take priority over MRD because some matrix effect can be tolerated in a qualitative assay.

Therapeutic proteins can form immune-complexes with ADAs, which can inhibit ADA detection and reduce assay sensitivity in the presence of drug [21,39]. High drug levels are a greater issue in multiple-dose studies where the increased concentrations may persist for a significant period of time [40]. If an ADA assay does not have enough DT, it will give false-negative results and will underestimate ADA incidence. EMA guidance requires that ADA assays should have higher DT than the drug levels in the clinical samples. It is also recommended by FDA and EMA that DT should be evaluated early in development. Sometimes, it is not possible to prospectively predict the concentration range of drug that will be present in samples from early clinical studies, and this is further complicated by such things as subject-to-subject variability, new routes of administration, dosing schemes, how the indication is defined and co-medications. Therefore, as clinical data is gathered, new or modified ADA assays may need to be developed at each phase of clinical trials. Regulators are currently asking that new assays to be developed and studies to be reanalyzed if the prospectively estimated DT is not enough to cover all study samples. This is not

a simple matter because at high circulating drug concentrations (greater than ~10 μg/ml), small improvements to DT often require large development efforts that cause delays and drive up costs. We suggest that an alternative approach might be to set the DT goal at twice the maximum concentration of drug predicted to be found in patient ADA samples. We believe further discussion is needed to set a scientifically based DT criterion. It is reasonable to assume that strong immune responses are most readily detected either because they clear drug or persist until drug concentrations declines to drug-tolerant levels. The challenge is to detect less robust immune responses. A well-considered risk assessment should indicate whether failure to detect a weak but persistent ADA response might have a hidden impact. Examples might include ADAs that impact a nonredundant pathway [41,42] or trigger a serious allergic response [43].

Methods for improving DT have advanced over time. The most straightforward method to improve assay DT to increase the dilution of the test sample, but, sensitivity often will be compromised as the MRD increases [32]. Therefore, more complex approaches have been used to improve DT for example, acid treatment to dissociate immune-complexes, affinity capture elution (ACE) and ACE bridging formats (ACE-bridge), solid-phase extraction with acid dissociation and the precipitation and acid method [44–50]. In some instances, the ACE-bridge format exhibits excellent sensitivity and DT compared with an unmodified bridging method [50]. In the situation where it is not possible to achieve the required DT, sponsors should discuss the issue with regulatory agencies as early as possible.

Consideration to set ADA cut point for samples containing pre-existing Ab

The assay cut point is the level of response that defines a sample as positive or negative. The Shankar *et al.* White Paper [20] describes a statistical approach to the cut point calculation and has been an industry standard for many years, but it does not cover every situation. Also, the latest guidance suggests changes to the specificity cut point that increases the incidence of false positives.

Pre-Abs are endogenous antibodies that react with epitopes on a therapeutic protein and are present prior to the first administration of the protein. Pre-Abs may result from previous exposure to similar natural or xenobiotic environmental antigens other than the drug [51]. Pre-Abs to therapeutics that have been described include antibodies to; rheumatoid factor, polyethylene glycol, antibodies (especially nonhuman antibodies), recombinant proteins, human proteins having nonhuman glycosylation patterns, peptide linkers, oncolytic viruses and viruses that might be used in various forms of gene therapy [43,52–56]. Reports of pre-Abs indicate a high incidence in autoimmune disease patient samples (85%) and a low incidence in cancer patients (8%) and individuals with metabolic disorders (3%) [57]. The clinical consequences of pre-Abs can vary from no known impact on patient safety to diminished efficacy and even serious adverse effects [58], and a high incidence may not indicate a high impact, as seems to be the case for pre-Abs to polyethylene glycol for which the incidence appears to be near 100% [59] but fewer than 10% of these have ever been linked to any clinical impact. The titer and affinity of some pre-Abs are insufficient to cause clinical consequences while others pose the same risks as postadministration ADAs.

While the FDA suggests some methods to calculate cut points for populations having a high incidence of pre-Abs, the EMA guidance provides less detail about this. However both agencies recommend that if samples have pre-Abs, there should be a separate analysis of safety in these patients and these samples should be removed from the cut point analysis [8,9,23]. As per FDA guidance, if the prevalence of pre-Abs is high, it may be necessary to distinguish between pre-Abs and drug-induced ADA responses based on the subject's change in titer following drug exposure. This type of immune response increase is also known as treatment-boosted or treatment-emergent ADA.

Currently, industry has no widely accepted approach for cut point evaluation when there is a high incidence of pre-Abs, although several approaches have been suggested [12,51,55,58]. During ADA assay development and/or validation, a small number of samples may contain pre-Abs, and these are removed as biological outliers during cut point analysis. When a high incidence of pre-Abs is observed it can be challenging to determine which samples to exclude and which to include in cut point calculations. Some of the approaches that have been suggested [58] for addressing pre-Abs include:

- Identify and eliminate samples with pre-Abs;
- Remove statistical outliers. Identification of samples with pre-Abs may be difficult so removal of outliers ensures they are not part of the cut point analysis;
- When a large portion of samples contains pre-Abs, a pseudo-negative population can be prepared by spiking drug into the sample;

- A titer-based approach can be used to distinguish between treatment-induced and treatment emergent pre-Abs. Samples with treatment-induced antibodies are those that show titer increases when comparing predose and postdose values (per FDA guidance, the fourfold difference is considered significant);
- Preparing immunoglobulin-depleted drug-naive sera for setting a cut-point;
- Determining subject-specific cut-points in the confirmatory assay. This is the most common method of characterizing antibodies.

As no standard recommendation exists to determine an ADA assay cut-point when pre-Abs are encountered, selection of the appropriate method becomes challenging, especially when the incidence of pre-Abs is high. In our experience, for any study which has a limited number of samples with pre-Abs, the most common method used to determine a cut-point is to remove biological outliers and those determined to be drug-specific based upon drug competition in the confirmatory assay (≥50% inhibition of signal). However, when a high incidence of pre-Abs is observed, conventional approaches may not give a useful cut point, leading us to try several different strategies to ensure a cut point supported by a valid statistical justification. Before clinical trials are completed, the risks posed by pre-Abs are unknown. When an association is found between immunogenicity and some clinical impact, retrospectively setting cut points would allow one to set a useful cut point that is relevant to patient safety, PK or efficacy, for example when prospectively setting difficult cut points, it may be necessary to consult with regulators.

Agency guidance on sampling for ADA assays

An appropriate sampling schedule is crucial to monitoring immunogenicity to therapeutics. A sampling schedule should capture relevant information about ADA response and the relationship to PKs and adverse events. The FDA and EMA provide recommendations to this end but neither agency gives detailed instructions on the sampling schedule (Table 1). Appropriate sample collection time points should lead to determination of the ADA incidence and persistence in patients. A baseline collection is crucial to ascertain pre-Abs. A 2014 white paper [12] recommends post-treatment sampling at "2 weeks (optional); 1, 2, 3, 6, 9, 12, 18 and 24 months; and every year thereafter during treatment, including an end-of-study sample." It is our experience that FDA wants every study to include ADA collection 2 weeks after the initial administration. However, as with other immunogenicity considerations, one may present the merits of a risk-based approach [8,9]. Drug-related factors such as dosing frequency, route of administration, product half-life, immunosuppression, DT of the assay and the severity of ADA risk need to be weighed when determining sampling times. Additional considerations for sampling may include the ADA isotype response [8] and HLA type which can vary with region and ethnicity [32].

A detailed sampling schedule should attempt to capture information about ADA formation whether it occurs rapidly or gradually and transiently or persistently. Regardless of the schedule, both agencies indicate sampling should be driven by a risk-based strategy intended to understand whether there is a relationship between immunogenicity and adverse events. ADA samples are collected throughout the clinical program. In the early part of a clinical study more frequent sampling is recommended, however, sampling frequency may be reduced later during prolonged treatment [6,35].

Although increasingly sophisticated methods can significantly reduce drug interference, circulating drug concentrations sometimes exceed the predicted higher limits. In such cases, it is more accurate to report a result as ADA-inconclusive rather than negative [32]. Sampling at trough concentrations is most practical. Concurrent ADA and PK sampling can yield useful information about the effect of immunogenicity on PKs. In general, sampling schemes should also consider ADA kinetics (onset, duration and cessation) and allow for differentiation between transient versus persistent ADA [12,60]. Both the FDA and EMA endorse a washout period to reduce drug interference based upon drug half-life. The FDA guidelines specifically state a washout period of five half-lives post-treatment to ensure minimal drug interference with long-lived therapeutics, however. in reality if the method is sufficiently drug tolerant there may be no inconclusive ADA sample results.

While the agency documents provide guidance on sampling to ensure the accurate detection of ADA, less attention is focused on the reporting of immunogenicity data. Three papers in particular [6,12,61] provide additional information on the sampling, evaluation and reporting of immunogenicity data. In general, sampling schedules would be determined on a case-by-case basis and a thorough immunogenicity risk assessment could help to ensure a strong sample collection strategy.

Regulatory guidance for developing ADA assays for multi-domain therapeutics

Multi-domain therapeutics (MDTs) may incorporate molecules of different origins in order to; direct the drug to a certain location, altering the drug half-life or impart a drug with more than one pharmacological activity. MDTs may have multiple and sometimes complex mechanisms of action. Development of MDTs has seen significant growth in recent years [2,62,63], and several have received regulatory approval. Like any biotherapeutic molecule, MDTs can also evoke an immune response and consequently require thorough immunogenicity assessment [58]. It is recommended that for a low immunogenicity risk MDT, the incidence of treatment-induced ADA development should be reported to the whole drug. However, for an MDT, one must also determine the incidence of treatment-induced ADA to each of its functional domains, and perhaps additional novel regions such as linkers [38,64].

The regulatory pathway for evaluation of less complex biotherapeutics has been well established, but there is a lack of clear industry guidance for products that target more than one antigen or carry multiple distinct activities. Due to the complexity of these molecules, investigators are currently developing individualized strategies for these molecules. One group recommends that the confirmatory cut-point should be evaluated in an appropriate manner for each part of a MDT and if required separate cut points should be assigned [65]. Guidance and white papers suggest that the whole drug be included in these confirmatory assays [8,9], but the guidance also notes that multiple assays may need to be developed to assess immune responses to different domains of an MDT and that all components of the therapeutic protein product, including the antibody, linker and new epitopes should be measured for ADA (see Table 1). Two approaches being used by industry are to develop assays for each domain while using intact drug to confirm, or to screen and confirm using intact drug while using individual domains to map antidomain specificity.

Conclusion

This review compares several aspects of the current FDA and EMA guidelines on the development and validation of ADA assays and notes areas for further debate. One of the first undertakings in the development of an ADA assay is to determine the appropriate assay format and select positive and negative controls. These two activities are linked as one affects the other. Key assay parameters such as sensitivity, DT and selectivity/recovery are defined by the positive control, so its selection is critical in the development of an effective assay. The myriad of potential immune responses to a therapeutic protein cannot be mimicked by one MAb or PAb PC, however, one might list potentially important and representative characteristics of ADAs that may be generated by the study population and attempt to select a control(s) having similar qualities, such as affinity, specificity, isotype and species (if assay detection is species specific).

Once the PCs have been selected, establishing assay sensitivity in the absence or presence of drug can be determined. Depending upon the assay format, the PC affinity can greatly influence the apparent DT of the assay. Many strategies have been devised to improve the ability to detect ADAs in the presence of drug. As previously mentioned, the direct means of increasing the MRD may increase DT slightly but may simultaneously lead to an unacceptable decrease in sensitivity. Other, more involved strategies include acid treatment, ACE-bridge, solid-phase extraction with acid dissociation and the precipitation and acid method method. We increasingly employ more and more complex strategies to maximize DT, while keeping in mind that each strategy includes a risk that the detection of certain types of ADA may be altered.

Another factor contributing to the challenge of ADA assay development is the increasing molecular complexity of therapeutics. The common therapeutic MAb has been joined by bi-specific antibodies, BiTEs, fusion proteins and conjugates of proteins/oligonucleotides/artificial bio analogs/nonbiomolecules among others. MDT may require assays designed to allow domain mapping. This can be done by developing separate assays for each domain or by incorporating domain mapping into the confirmatory assay. Complexity is increased when many individuals have pre-existing Abs, and just like induced immune responses, pre-Abs have the potential to influence patient safety and efficacy.

Although FDA and EMA guidance documents are in harmony on most points, there are differences between the two. One of the main differences is that EMA allows greater leeway for scientific judgement while FDA tends to give specific recommendations which may be helpful but may not be appropriate in every situation. Several white papers give detailed approaches on these issues, however, some of these approaches conflict with each other and are only scientifically valid in certain situations. It is always up to the individual investigator to devise an appropriate analysis plan and communicate with regulators as necessary.

Future perspectives

It is possible that future guidance documents will be further harmonized and include additional clarifications that will lead to more scientifically relevant ADA assays. We suggested several topics that might benefit from revision. First, it is important to generate good surrogate PCs because human polyclonal ADAs are not available during development and validation and may not be practical even if they eventually become available. Some industry leaders and regulatory guidance suggest that a polyclonal Ab is always the best surrogate for a clinical ADA assay, but we do not believe this is always true. Nonhuman PAbs generated via immunized animals may be of high affinity and primarily target human protein epitopes, whereas human subjects often generate lower affinity antibodies to nonhuman epitopes. Also, if there is a need to isotype ADAs, animal PAbs do not make useful clinical assay controls. With advances in technology, non-MAb biotherapeutics are gaining traction and so best practices must address more than just human therapeutic MAbs. If we list some key attributes of human ADAs (for example, specificity, affinity, isotype, serum recovery and ability to neutralize drug) it is then possible to screen for MAbs having those properties. From there a human PC MAb can be engineered without too much difficulty and a consistent well characterized standard maintained throughout the product life cycle. One recent review (Myler 2019) suggested a compromise position in which assay parameters such as sensitivity, DT, selectivity and recovery be validated with a PAb and then a MAb would be used for monitoring assay performance, but we would argue that a carefully selected MAb(s) is as good and in some ways a better choice.

Once we have identified the most appropriate PC, we need to agree upon how to use it in the characterization of our ADA assays. For example, there is significant variability in how assay sensitivity is determined. Some labs interpolate sensitivity, but we think this is misleading for semiquantitative assays intended to report titers. Another area in which there is room to expand upon current recommendations is how to handle a high incidence of pre-existing ADA. In the area of DT, there is continued debate about how it should be determined and there have been requests for assays able to tolerate very high levels of drug prior to washout, which does not seem aligned with current guidance, so clarification on this subject would be helpful. Finally, we see that technology and knowledge continue to grow and evolve, and so industry and regulatory agencies must continue to share knowledge and information so that future recommendations can keep pace.

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Executive summary

- All biologics, including therapeutics protein when administered to patients have the potential to induce an unwanted immune responses response. The potential impact of immunogenicity on pharmacokinetics, pharmacodynamics, safety and efficacy are well established.

Overview of US FDA & EMA immunogenicity guidance for therapeutics protein

- The US FDA provides detailed guidance for product development, with comprehensive instructions for Investigational New Drug and-Biological License Application filings. The EMA approach is more flexible, especially early in development unless regulatory guidance is requested.
- FDA and EMA are focused more on clinical trials, providing limited recommendations for pre-clinical ADA assessment.
- Both guidelines recommend that sponsor should include an integrated summary of immunogenicity in the application, including a risk assessment.
- Both regulatory guidelines also suggest a multitiered approach including an initial screening step, followed by confirmation and titration assays of screen-positive samples.

Agency guidance on positive & negative controls for developing ADA assays

- Key assay parameters such as sensitivity, drug tolerance (DT) and selectivity/recovery are relative value determined by the with the positive control, so its selection is critical in the development of an effective assay.
- Health authorities state that polyclonal antibodies best represent an endogenous ADA response and should be used for assay characterization experiments, however high-affinity monoclonal antibodies are acceptable for monitoring assay performance.
- The guideline for PC should be more defined and there should be increased harmonization among the industry.

Methods for detection of antidrug antibody

- ADA assay methodology has evolved over the years and a variety of formats are used to measure ADAs. Each of these methods has their strengths and weaknesses including throughput, sensitivity and ability to investigate various aspects of an ADA.
- As no single assay format is appropriate for evaluating immunogenicity, ADA assays must be thoroughly validated to ensure reproducible, consistent and definitive results.

Current regulatory practice to determine sensitivity, minimum required dilution & DT

- The regulatory guidance recommends that ADA assay sensitivity be determined in the presence of the expected concentration of drugs in order to be meaningful.
- The FDA and EMA recommend by that DT should be evaluated early in development. In the situation where it is not possible to achieve the required DT, sponsors should discuss the issue with agencies as soon as possible.

Consideration to set ADA cut point for samples contain pre-existing Ab

- Determining if pre-existing antibodies are present in a population is important as they can influence patient safety, efficacy and immunogenicity potential.
- Pre-existing Abs impact the calculation of cut points and several strategies on how to manage cut point calculation in their presence have been described in guidance documents and white papers.
- As no standard recommendation exists to determine an ADA assay cut-point, selection of the appropriate method becomes challenging when the incidence of pre-existing antibodies is high.

Agency guidance on sampling for immunogenicity assays

- In general, sampling schedules should be determined on a case-by-case basis and a thorough immunogenicity risk assessment could help to ensure a strong sample collection strategy.
- The timing of clinical sample collection can influence the detection of ADAs. Regulators and clinicians must evaluate these aspects of study design carefully when considering immunogenicity data.

Regulatory guidance for developing ADA assays for multidomain therapeutics

- Multidomain therapeutics may require assays designed to allow domain mapping. This can be done by developing separate assays for each domain or by incorporating domain mapping into the confirmatory assay.
- Due to the complexity of these molecules, investigators are currently developing individualized strategies for these molecules.

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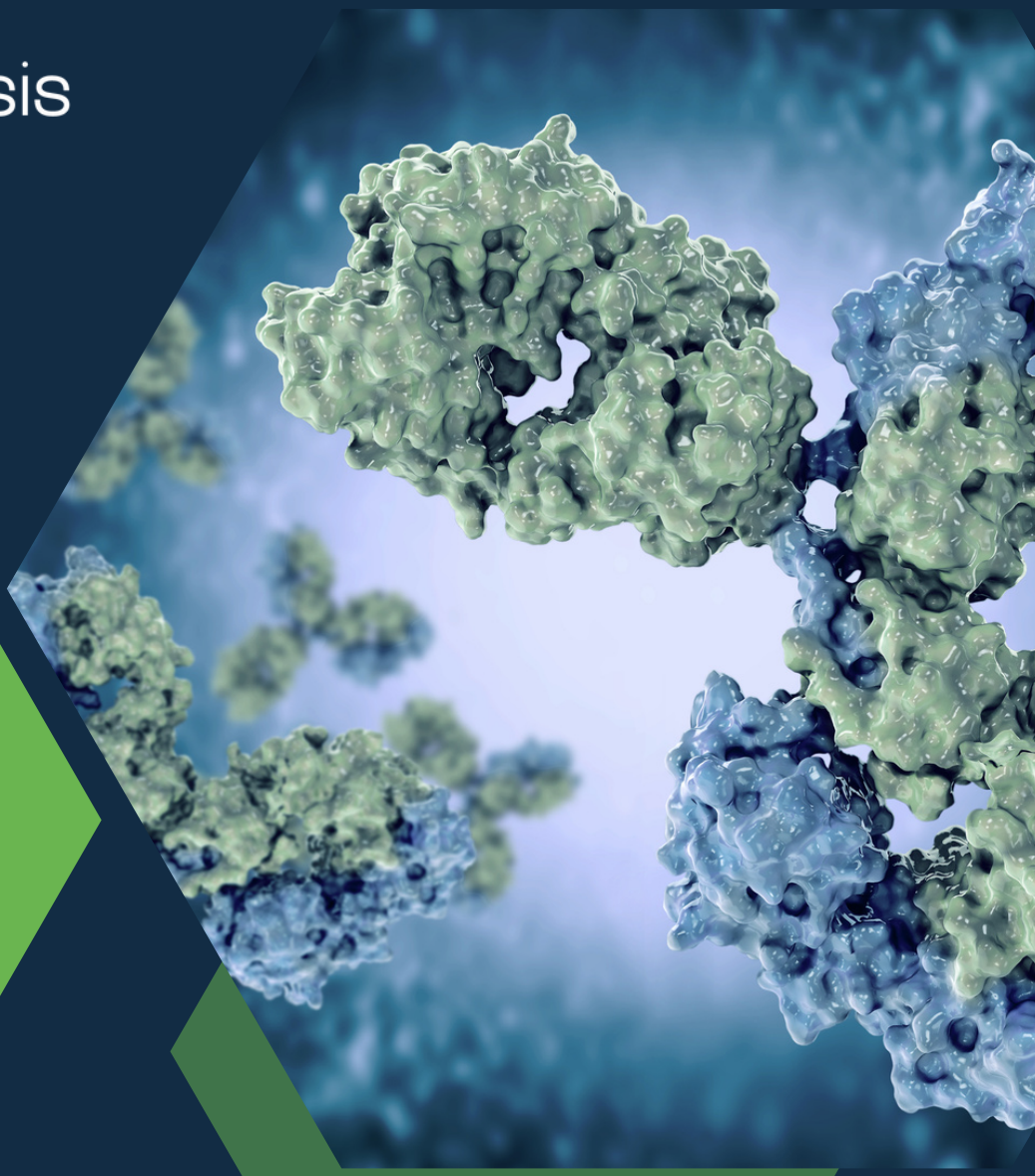

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