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Glycosylation of biosimilars: Recent advances in analytical characterization and clinical implications

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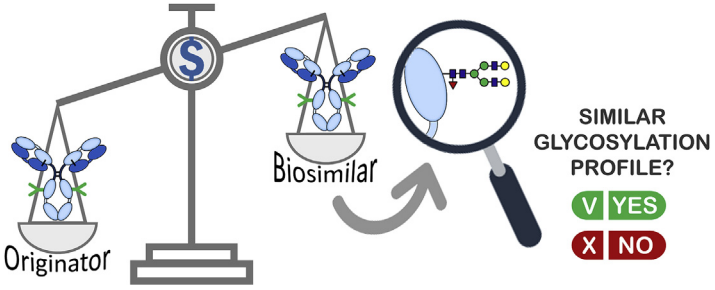
HIGHLIGHTS

- Multiple biosimilar products have become available for single originator biologics.
- Limitations in biological assays for the comparison of glycosylation of biosimilars.
- Novel analytical techniques for glycan analysis in biosimilar development.
- Clinical implications of glycan heterogeneity among multiple infliximab biosimilars.

GRAPHICAL ABSTRACT



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ABSTRACT

Over the past few years, loss of patent protection for blockbuster monoclonal antibody (mAb) drugs has caused a significant shift in the pharmaceutical industry towards the development of biosimilar products. As a result, multiple biosimilar mAbs are becoming available for a single originator drug. As opposed to small-molecular drugs, protein biopharmaceuticals do not have fully defined and reproducible structures, making it impossible to create identical copies. Therefore, regulators demand biosimilar sponsors to demonstrate similarity with the reference product to prevent safety and efficacy issues with the proposed product. Protein glycosylation is considered a crucially important quality attribute, because of its major role in immunogenicity and clinical efficacy of therapeutic proteins. However, the intrinsic biological variability of glycan structures creates a significant challenge for the current analytical platforms.

In this review, we discuss the importance of glycan characterization on therapeutic proteins, with a particular focus on the analytical techniques applied for glycan profiling of biosimilar mAb products. In

Abbreviations: mAb, monoclonal antibody; FDA, Food and Drug Administration; CHMP, Committee for Medicinal Products for Human use; EMA, European Medicines Agency; PHS, Public Health Service; PTM, post-translational modifications; CQA, critical quality attribute; MoA, mechanism of action; Fc, crystallisable fragment; ADCC, antibody dependent cell-mediated cytotoxicity; CDC, complement dependent cytotoxicity; IgG, immunoglobulin G; BLA, Biologic License Application; MS, mass spectrometry; Fab, antigen-binding fragment; MS/MS, tandem mass spectrometry; HTS, high-throughput screening; PNGase F, peptide N-glycosidase F; PAD, pulsed amperometric detection; CE, capillary electrophoresis; APTS, 8-aminopyrene-1,3,6-trisulfonic acid; 2-AB, 2-aminobenzamide; 2-AA, 2-aminobenzoic acid; HILIC, hydrophilic interaction liquid chromatography; RFMS, RapiFluor-MS; FLD, fluorescence detection; SPE, solid-phase extraction; RPLC, reversed phase liquid chromatography; IEX, ion exchange chromatography; QbD, quality by design; ADC, antibody-drug conjugate; 1D, one-dimensional; 2D, two-dimensional; HIC, hydrophobic interaction chromatography; SEC, size exclusion chromatography; LCxLC, comprehensive two-dimensional liquid chromatography; LC-LC, heart-cutting two-dimensional liquid chromatography; ASM, active solvent modulation; IM-MS, ion mobility mass spectrometry; CCS, collision cross section; MRM, multiple reaction monitoring; MAM, multi-attribute monitoring; NK, natural killer cells; RA, rheumatic arthritis; IBD, irritable bowel disease; AS, ankylosing spondylitis.

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addition, we present a case study on infliximab biosimilars to illustrate the potential clinical implications of differences in glycan profile between originator and biosimilar mAb products.

1. Introduction

Over the past few decades, protein biopharmaceuticals have emerged as one of the most important and innovative fields of human therapeutics, since these drugs are able to fulfill the increasing demand for clinical efficacy and target specificity better than small-molecule drugs [1]. This has enabled the treatment of multiple diseases, such as arthritis, cancer, diabetes and cardiovascular diseases [2,3]. With no apparent signs of slowing down, the field of protein therapeutics is expected to occupy 30% of the total pharmaceutical market by 2020 [4]. However, the increasing expenditures on biologic drugs create a major burden for many healthcare systems, which are forced to introduce rationing of high-cost treatments and prevent patients from accessing the correct treatment [5,6]. Prices for biopharmaceutical therapies greatly exceed the costs for conventional small molecule therapies [7,8]. Even though the raw material costs consist only of $2/gram of product, a recent price comparison of all U.S. Food and Drug Administration (FDA) approved monoclonal antibodies (mAbs) in the last 20 years (1997–2016) showed that the mean annual price for an antibody therapy was $96,731 [9,10]. This is the result of high research and development costs and large phase III clinical trials that significantly define the price for antibody therapies. In addition, high attrition rates throughout the drug development process further increase the overall costs of development and create a financial burden for biopharmaceutical companies [11]. Moreover, the complex manufacturing process of protein therapeutics in living systems (e.g., mammalian cell lines) by recombinant DNA technology further increases the price of antibody treatments compared to small molecule therapies [10].

Patent expiration of therapeutic proteins allows the development and manufacturing of biosimilar versions by other pharmaceutical companies. The recent loss of patent protection of numerous blockbuster biopharmaceuticals (e.g., infliximab, trastuzumab and adalimumab) has caused a significant shift in the pharmaceutical industry towards the development of lower-cost alternatives [12]. Therefore, biosimilars could make these life-changing treatments more accessible for a larger group of patients and potentially reduce costs for the overall healthcare system [7].

In Europe, the regulatory framework for biosimilar approval was already established in 2005, with the collaboration of the Committee for Medicinal Products for Human use (CHMP) and the European Medicines Agency (EMA) [13]. Therefore, the first approved biosimilar (Omnitrope by Sandoz) entered the market in 2006, 3 years prior to the development of the FDA regulatory framework and almost 10 years before the FDA-authorization of the first biosimilar [14,15]. As a result of the pioneering role in regulation of biosimilar medicines, the number of authorized biosimilars in Europe has increased rapidly compared to the number of biosimilars currently approved for the US market. To date, the FDA authorized 19 biosimilar products on the US market under the Public Health Service act (PHS) [16]. The EMA, in contrast, has authorized 56 biosimilar products for use throughout Europe via the European Union-wide authorization procedure (Table 1). Clearly, a higher number and a higher diversity of biosimilars have been approved by the EMA compared to the FDA to date.

In order to develop cost-effective alternatives, biosimilar sponsors benefit from an abbreviated approval procedure that allows the use of prior knowledge on the reference drug to extrapolate the biosimilar product to an indication for which the originator has shown to be safe and efficacious without the need of supplementary clinical data [17–20].

For patients, this should lead to lower therapy costs and improved access to appropriate treatments. However, analysis of the biosimilar sales in Europe have shown only an average price reduction of 15–30% compared to originator products [7]. Major price reductions are hampered by the substantial investments

Table 1
Overview of the biosimilar market in the EU and US.

Active substance	# approved biosimilars in EU	# approved biosimilars in US	Product class
Filgrastim	8	2	G-CSF
Adalimumab	8	3	Anti-TNF antibody
Rituximab	6	1	Cancer antibody
Pegfilgrastim	6	2	G-CSF
Trastuzumab	5	4	Cancer antibody
Infliximab	4	3	Anti-TNF antibody
Epoetin alfa	3	1	ESA
Insulin glargine	2		Insulin
Enoxaparin sodium	2	1	Anticoagulant[a]
Epoetin zeta	2		ESA
Etanercept	2	1	Anti-TNF antibody
Follitropin alfa	2		FSH
Teriparatide	2		Growth hormone
Bevacizumab	2	1	Cancer antibody
Insulin lispro	1		Insulin
Somatropin	1		hGh
Total # of biosimilars	**56**	**19**	

Only authorized biosimilar products were selected. ESA: erythropoiesis-stimulating agent; FSH: follicle-stimulating hormone; G-CSF: granulocyte-stimulating hormone; hGg: human growth hormone.

[a] First approved as biosimilar, but later considered small molecule. Last updated on May 15th· 2019 using the CDER list of Licensed Biological products (FDA) and publicly available EPAR reports (EMA).

required for the development and marketing of a biosimilar product, compared to a generic product. On average, the cost of generic drug development is between $1–4 million. In comparison, costs for biosimilar development are considerably higher and often exceed $250 million, during the 7–8 years of development [8].

One of the current limitations in developing and manufacturing biosimilars is the structural complexity of biological products, which is inherently related to their biological expression in living systems. As opposed to small generic molecules with fully defined and reproducible structures, protein pharmaceuticals are large heterogeneous molecules prone to numerous enzymatic and chemical post-translational modifications (PTMs) during production, formulation and storage [15,21]. Therefore, minor differences in manufacturing conditions and cell-line differences could result in major structural differences, making it impossible to create structurally identical copies of the originator recombinant protein. This generates a problem for regulatory agencies with the intention to approve interchangeable medicinal products. To circumvent this intrinsic problem, the biological product can be approved after conducting an extensive comparability exercise to demonstrate that there are no clinically meaningful differences in terms of safety, purity and potency with the reference product [15,22,23].

To demonstrate biosimilarity, manufacturers are expected to perform a comprehensive structural and chemical characterization of both the proposed product and reference product [15,17,18,24]. These studies should include the use of state-of-the-art methods, to compare the primary amino acid sequence, higher order structures (e.g., aggregation) and PTMs (e.g., glycosylation, oxidation and deamidation) of the proposed product and reference product. Data from the structural analysis is supported by functional assays, to demonstrate that there are no differences in biological activity and/or potency of the proposed product. Together, these studies aim at reducing the residual uncertainty in the assessment of biosimilarity. Moreover, the combination of the biological and structural critical quality attributes (CQAs) determines to what extent *in vivo* toxicological and clinical evaluation is required [25]. This is contrary to new biological products where extensive clinical safety and efficacy studies (phase II) are required prior to market authorization [26]. By shifting the focus towards the analytical characterization instead of costly and time-consuming clinical testing, biosimilar developers aim to create less-expensive treatment options and introduce market competition [6].

For the first wave of biosimilar products, the drugs were homologues of human protein products and therefore it was relatively easy to determine function of the drug after administration and CQAs that could impact the drug's potency, safety and efficacy [27]. However, approval of biosimilar-mAbs proved to be more difficult due to their non-physiological disease-modifying functions in often poorly defined pathophysiology of the disease. This proposes significant challenges for biosimilar sponsors and regulators to define the exact CQAs, based on the drug's structure and mechanism of action (MoA), that relates to both the safety and efficacy of the product [28]. The importance of extensive knowledge on the relationship between the attribute and product's clinical performance is demonstrated by the extrapolation of multiple infliximab biosimilars over several indications with a different disease MoA (see case study).

Glycosylation is considered as one of the most important CQAs because of its major role in immunogenicity and clinical efficacy of therapeutic proteins [29,30]. Glycans are oligosaccharide structures of the high-mannose, hybrid or complex type structure, depending on the cellular expression system. Most IgG-type mAbs contain glycans linked to the conserved N-glycosylation site near the asparagine-297 residue located in the crystallisable fragment (Fc) that is responsible for immune mediated effector functions such as antibody dependent cell-mediated cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC) [31]. It has been shown that the wide variety of glycan motifs created via differences in core fucosylation, terminal galactose and sialic acid content can significantly influence the Fc-mediated effector functions and subsequently affect the therapeutic efficacy of the drug [32–34]. In addition, distinct pharmacokinetic and pharmacodynamics (PK/PD) effects of glycosylation patterns have been described in the literature [35,36]. Unfortunately, glycosylation is among the PTMs causing the most heterogeneity in therapeutic protein products [37,38]. Moreover, changes in the production organism or the manufacturing process may substantially affect the glycosylation profile of the final product. Therefore, extensive characterization of the present glycan species is required from research and development to industrial-scale bioprocessing, to ensure manufacturing consistency and product safety [39,40].

In order to keep up with the increasing complexity of developed (biosimilar) protein therapeutics, there is an emerging need for novel complementary analytical techniques. Strong analytical techniques can provide a potent basis during biosimilar development and help to reduce the residual uncertainty during the comparability exercise of the approval process. Whereas multiple biosimilar products become available for a single originator product, it is of pivotal importance to accurately monitor the variability and ensure safe and efficacious treatments for patients. This was clearly demonstrated by a recent evaluation of glycosylated biosimilars approved in the EU and Japan. It was shown that differences in N-glycosylation not only exist between a biosimilar and a single originator product, but also exists among the different biosimilar products [41].

In this review article, we will highlight the importance of analytical characterization of glycosylation on therapeutic proteins, with a focus on immunoglobulin G (IgG) mAbs. Next, we will discuss emerging techniques that are of interest for the characterization of glycans during biosimilar development. To conclude, we present a case study demonstrating the importance of glycan characterization of biosimilars and the potential clinical implications of structural heterogeneity among multiple biosimilars for a single originator product.

2. Limitations in the biological characterization of glycans

In parallel to the structural analytical characterization of biosimilar products, functional assays have to be performed. Such studies can be subdivided in two different approaches: (1) assays focusing on receptor/target binding of the potential biosimilar product and (2) assays focusing on the biological effect of the product after target/receptor binding. Both approaches are used to elucidate the effect of structural variants on the effector functions and/or differences with the reference product. There is a significant effect of glycosylation on the Fc domain with the Fc receptor binding and the subsequent effector functions [42]. Therefore, assays focusing on Fc/Fc-receptor interaction are particularly well suited to analyze the effect of distinct glycan motifs on the *in vitro* biological activity. Recently, Cymer et al. published a comprehensive overview of the *in vitro* methods to evaluate the effect of glycosylation on receptor interaction [31]. In general, functional assays can be subdivided in three different categories with increasing *in vivo* resemblance: (1) cell-free binding assays, (2) cell-based binding assays and (3) functional cellular assays (Fig. 1).

However, parallel to the increase in *in vivo* resemblance of cell-based assays is the increasing assay complexity and complicated data interpretation from assays including multiple events (e.g. receptor binding and effector functions) in the read-out. Furthermore, the discussed biological assays are often hampered by assay

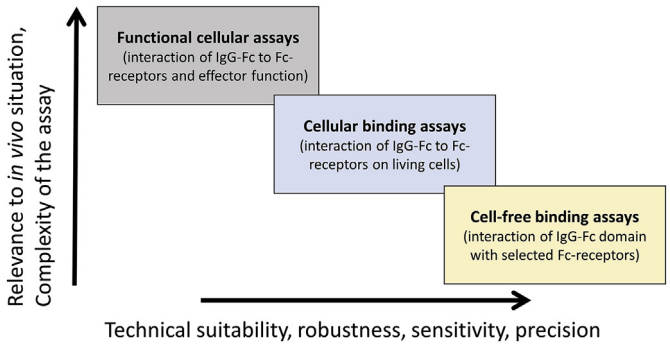

Fig. 1. Overview of IgG-Fc/Fc-receptor assays. Schematic representation of the complexity and application area for the different biological assays. Readapted from Cymer et al. [31].

heterogeneity, as a result of structural differences in the studied receptor and/or variability in the cellular systems. Further variability is introduced by heterogeneity of the analyzed IgG mixture including different glycan variants as well as other relevant PTMs [43]. Given the assay complexity and variability, caution should be taken when interpreting parameters (e.g., binding kinetics) derived from biological assays. Therefore, current biological assays have some major limitations when used for the comparison of a potential biosimilar product and reference product [44]. The reduction in assay sensitivity, caused by the intrinsic assay heterogeneity, potentially reduces the chances of finding differences in biological activity between the proposed biosimilar product and the reference product. Furthermore, the type of functional assay is dependent on the MoA of the IgG-drug [45]. Therefore, different assay formats and different receptors (e.g., TNFR, FcγR) are generally used to analyze specific biological products depending on their MoA [29]. The determination of the appropriate biological assay on a case-by-case basis, provides a major hurdle for regulatory agencies when creating harmonized methods for the functional evaluation of biosimilarity [44,46].

All the above mentioned limitations clearly emphasize the need for comprehensive analytical techniques to accurately monitor the glycosylation variability during biosimilar development and reduce the residual uncertainty in the comparability exercise.

3. Analytical characterization of glycosylation of therapeutic proteins

Due to the intrinsic heterogeneity of the expression systems, controlling the glycosylation pattern remains a major hurdle for biosimilar developers as well as innovator companies [47,48]. To ensure batch-to-batch consistency during manufacturing, health authorities demand a mandatory comparability exercise that has to be performed when manufacturing changes occur. Process improvements, scale changes and site transfers can influence the CQAs of the biological products, but the variations can be accepted if they do not alter the safety and efficacy of the product. When manufacturers fail to justify differences between the product from pre- and post-manufacturing changes, they are required to file a new Biologic License Application (BLA) [49,50]. This was experienced by innovator company Genzyme, when after an attempt to upscale the production of acid-α-glucosidase (Myozyme), the glycosylation profile had significantly changed and the product was not considered comparable by the regulators. Subsequently, Genzyme marketed the new product under the name Lumizyme, which after approval was added under the label of Myozyme by the FDA, creating an important regulatory precedent [51].

With respect to glycosylation, both FDA and EMA biosimilarity-guidelines require comprehensive analysis of the glycan composition, site-specific profiles and site occupancy [13,24]. In order to define the similarity acceptance criteria, biosimilar manufacturers should compare a sufficient number of reference product lots with a minimum of 10 biosimilar product lots [52]. The selected reference and biosimilar product lots should account for all potential variability (e.g., differences in shelf life, US- or EU-sourced material and different manufacturing scales) and therefore play a key role in the analytical similarity assessment. Recently, Amgen Inc. published the results of their analytical similarity assessment of ABP 980 biosimilar product to trastuzumab, following the stepwise approach recommended by both the FDA and EMA [53]. In their study, the glycosylation profile of 13 lots of ABP 980 were compared to 21 lots of trastuzumab (US) and 33 lots of trastuzumab (EU) to demonstrate analytical similarity following the regulatory guidelines. In order to keep up with the increasing regulatory and industrial requirements, novel fast, sensitive and cost-effective approaches have been developed in the past decades [54,55]. However, the characterization of glycosylation profiles remains to be among the most difficult features of biological products, due to the absence of a direct genomic blueprint [56].

The analysis of glycosylation can be easily sub-divided into three main approaches: intact (top) and subunits protein level (middle-up), glycopeptides (bottom-up) and released glycans, corresponding to the size level of the analyte during analysis (Table 2). Recent reviews have extensively described the conventional techniques for the analytical characterization of glycans on therapeutic proteins [37,54,55,57–60]. Here, we will provide a brief overview of the state-of-the-art approaches and their advantages and limitations when applied to the characterization of mAbs and more specifically in the development of biosimilar mAb products.

3.1. Top and middle-up level

Analysis at the intact protein level (top level: ~150 kDa) is performed by using a broad range of chromatographic, electrophoretic and mass spectrometry (MS) techniques. This is usually performed during multiple steps of the development process and provides information on the molecular mass of the protein and major PTMs, such as glycosylation [1]. In addition to the top level approach, analysis can be performed by using a middle-up approach that deals with protein subunits (25–100 kDa) obtained after chemical reduction of the disulfide bonds and/or enzymatic digestion using specific proteases (e.g., *IdeS,* Papain, FabALACTICA) [61,62]. Decreasing the molecular size of the IgG-proteins has the benefit to obtain a better separation of the protein variants in separation fronts and an improved MS sensitivity at the cost of introducing sample preparation.

Both the top and middle-up strategies provide an attractive approach for fast and robust analysis of batch-to-batch variability in major glycoform species, with the benefit of simple sample preparation and analysis [63]. This is of specific interest during biosimilar development, to rapidly screen for differences in major glycan species between the originator product and proposed product. Moreover, middle-up strategies allow to determine the levels of glycan pair symmetry or asymmetry on the heavy chain and study, e.g., the effects of glycan pairing on the antibody clearance rates [64]. However, Reusch et al. showed that these approaches are not suitable for detailed characterization of low-abundant glycoforms [57].

The information provided by MS is dependent on the resolution and mass accuracy of the instrument. However, improving the mass resolution of the MS does not necessarily provide new information, due to the broad isotopic distribution for large proteins and

Table 2
Analytical techniques used for glycan characterization of therapeutic proteins.

Level of analysis	Method	Obtained information	Advantages	Disadvantages	Ref.
Intact and subunit protein level	Lectin microarray	Glycoform determination with known standards	Great potential for high-throughput screening at intact level	Limited binding-affinities for broad range of glycans	[163,164]
	RPLC-MS	Glycan heterogeneity	Fast and robust technique for routine analysis at subunit level	Limited resolving power for glycoforms and introduction of artifacts by MS analysis	[63,165,166]
	CE/cIEF MS (MALDI or ESI)	Sialylation heterogeneity Glycoform determination	High resolution due to the electro-driven separation Mass identification of glycans	Strong protein adsorption to capillary wall The broad isotopic distribution of large proteins can compromise the accurate mass determination.	[3,167] [1,57,67]
Glycopeptides	LC-ESI-MS or MALDI-MS	Glycoform determination	Information on site specific glycosylation and other PTMs	Limited interaction with small polar peptides and introduction of artifacts by MS analysis	[38,57,74]
	CE-MS	Glycoform determination	Complementary to RPLC to achieve complete sequence coverage	Introduction of experimental artifacts by MS analysis	[148,168]
Released glycans	HPAEC-PAD	Glycoform determination with known standards	No labelling procedure required	Need for dedicated instrumentation	[58,89]
	HILIC-FLD (2-AB)	Glycoform determination with known standards	Easily integrated in QC/GMP laboratories and established database for data analysis	Only characterization of pre-determined glycans	[58,93,169]
	CE-LIF (APTS)	Glycoform determination with known standards	Ability to separate isomeric glycan variants	Biased separation towards sialylated glycans	[58,170–172]
	HILIC-MS (Rapifluor)	Glycoform determination	Commercially available platforms with integrated informatics for glycan identification	No site-specific information and introduction of artifacts	[60,94,102]
	ESI-MS/MS	Glycoform determination	Rapid screening technique of major glycoforms	Limited information on carbohydrate linkage and reduced sensitivity due to ion suppression	[37,173]
	MALDI-MS	Glycoform determination	Highly automated procedure with limited hands-on time and linkage specific information on terminal sialic acid	Additional stabilizing of sialic acids required prior to analysis	[57,174,175]

Increasing sample preparation complexity →

compromised sensitivity for detection of the monoisotopic peak. Therefore, only the average mass of the antibody can be determined, while considering that this can easily vary by a few ppm as a result of the isotopic abundance of atomic elements in the protein. This can hamper the identification of small-differences of PTMs and the exact identification of glycoforms at intact level which is hampered by the many possible mass isoforms. This is clearly demonstrated when observing a mass increase of +162 Da, which could correlate to glycation (Lys-glucose attachment) or glycoform differences (+162 Da difference of a hexose unit) [54,65–67].

Furthermore, top level strategies do not provide the site-specific information on the glycan composition that is obtained in middle-up level strategies. For most mAbs this is less important, since they only contain glycosylation in their Fc domain [68]. However, recently more complex products have entered the market with multiple glycosylation sites on, for example, the antigen-binding fragment (Fab) domain. Therefore, site-specific glycosylation is a valuable feature to study in the analysis of biosimilar products [29,69].

3.2. Bottom-up level

The analysis of glycopeptides is referred to as bottom-up approach and provides important information on the amino acid sequence, glycosylation and minor chemical and enzymatic modifications [3,70]. In bottom-up analysis the intact protein is enzymatically digested by using proteases (e.g., trypsin) to generate peptides of approximately 0.5–5 kDa. After proteolytic cleavage with trypsin, the obtained peptides and glycopeptides are analyzed by MALDI-MS or ESI-MS, either directly or preceded by a chromatographic or electrophoretic separation technique.

Because of competitive ion suppression between unmodified peptides and glycopeptides, the use of separation techniques prior to MS detection can greatly increase the sensitivity and confidence towards identification of low-abundant glycoforms. Moreover, the addition of a separation technique has the potential to discriminate between important isobaric glycoforms such as G2F and G1F with a α1,3-bound galactose bound to the galactose subunit, which has an important role in adverse immune reactions [31,34]. Therefore, multiple methods have been developed to separate the glycopeptides in the complex mixture and improve the elucidation of the microheterogeneity present at the glycosylation site [71,72]. However, MS methods are susceptible to experimental artifacts, e.g., as a result of in-source fragmentation. This could lead to compromised results in glycan identification. The effect of experimental artifacts should be limited by the use of suitable internal standards and careful method development. As previously reported, in-source decay is of specific importance when analysing sialic acid-containing glycans, which are linked to anti-inflammatory effects and reduced ADCC [34,57,73].

However, MS approaches are crucial for comprehensive analysis of the IgG proteins glycosylation on a peptide level. Indeed, mass information allows the detection of unidentified glycan species, which is impossible with solely separation-based methods. Furthermore, by using tandem MS (MS/MS), the attached peptide sequence as well as the glycan composition can also be successfully characterized. This provides, in contrast to the intact approach, important information on the site-specific glycosylation (variants) of the protein [74,75]. Site-specific glycan patterns can provide complementary information on the safety and efficacy of the therapeutic product, but is often missing in the literature [76,77]. Moreover, site-specific glycosylation is considered a crucial feature in the development of biosimilars for mAbs and fusion proteins with multiple glycosylation sites [78,79].

It has been shown that glycopeptides analysis has great

potential for high-throughput screening (HTS) of N-glycans of mAbs by automation of the sample preparation procedure and improved analysis throughput [80,81]. Besides the improvement of instrumentation, there is also an increasing demand for software platforms that allow fast, accurate and confident MS interpretation [82]. Therefore, the current limitations are in the extensive database building that has to be implemented to perform MS interpretation in a HTS manner.

Glycopeptides analysis using MS-based methods has great potential for the assessment of batch-to-batch consistency and for glyco-engineering purposes, owing to the fast, site-specific and accurate identification of the glycosylation profile. However, introduction of MS-based methods in QC environments remains a major hurdle in industry [83]. Nevertheless, recent evaluation of electronically submitted BLAs showed a consistent increase in use of MS for analysis of glycosylation of mAbs (Fig. 2). It must be mentioned that analysis of glycosylation across other, more complex, product types (e.g., fusion proteins, antibody-drug conjugates (ADC)) was lower compared to the average [84].

3.3. Released glycan level

The analysis of released glycans is a well-established approach in both academic and industrial settings and is often used as reference technique in the development of new methods [57,58,85]. Intact glycans can be enzymatically and chemically released from the intact protein for in-depth characterization. Enzymatic cleavage is preferred for most N-linked glycans using an amidase, such as peptide N-glycosidase F (PNGase F), that cleaves the bond between the GlcNac core and the asparagine residue of the protein [86]. When analyzing proteins containing N- and O-glycosylation, chemical release is the favored approach using hydrazinolysis and reductive β-elimination methods [87,88].

In general, there are three ways to analyze the released glycans: (1) HPLC (anion exchange chromatography mode) with pulsed amperometric detection (PAD) analysis, (2) direct-MS analysis or (3) HPLC/capillary electrophoresis (CE) separation coupled to either fluorescence detection or MS detection [58,89,90]. Since direct spectroscopic detection of glycans is not possible, due to the absence of chromophores in the carbohydrate structure, most analytical workflows for released glycan analysis contain a fluorescent labeling procedure. In addition, CE analysis with laser induced fluorescence detection of N-glycans requires the introduction of a permanent charge to the carbohydrate structures. In this context, derivatization with 8-aminopyrene-1,3,6-trisulfonic acid (APTS) is among the mostly used labeling agents prior to CE analysis. The use of 2-aminobenzamide (2-AB) or 2-aminobenzoic acid (2-AA) is a widespread derivatization procedure prior to hydrophilic interaction liquid chromatography (HILIC) [91,92].

The characterization of glycans by LC is based on internal standards or retention time libraries, including a variety of column dimensions. Publicly available databases are accessible for identification of the detected glycans and new analytical platforms with integrated informatics are developed [93–95]. Released glycan analysis can be easily integrated in most QC/GMP laboratories, because there is no requirement of introducing complex MS-based platforms. Furthermore, in a recent comparison by Reusch et al., the HILIC(2-AB) was considered as best suited approach for routine analysis because of its robustness, accuracy and reproducibility [57]. This was confirmed by an inter-laboratory study showing consistent repeatability and reproducibility of the HILIC(2-AB) method among 12 different laboratories in North America, Europe and Asia [96]. Despite the above-mentioned advantages, spectroscopic detection is restricted to the characterization of only predetermined glycans and is dependent on baseline separation for accurate identification.

MS analysis allows glycan identification without labelling procedures with MS or MS/MS [97,98]. However, sample preparation can also be beneficial for MS detection to improve ionization efficiencies, reduce in- and post-source decay (e.g., sialic acids) and improve the glycan identification in MS/MS approaches, by creating more informative fragments [99,100]. Szarka et al. demonstrated the possibility to perform simultaneous quantitative (optical) and qualitative (mass) analysis of APTS-labeled glycans using a novel imaging-LIF (iLIF) detector coupled to a CE-ESI-MS system [101]. By placing the iLIF detector at the Taylor cone of the electrospray

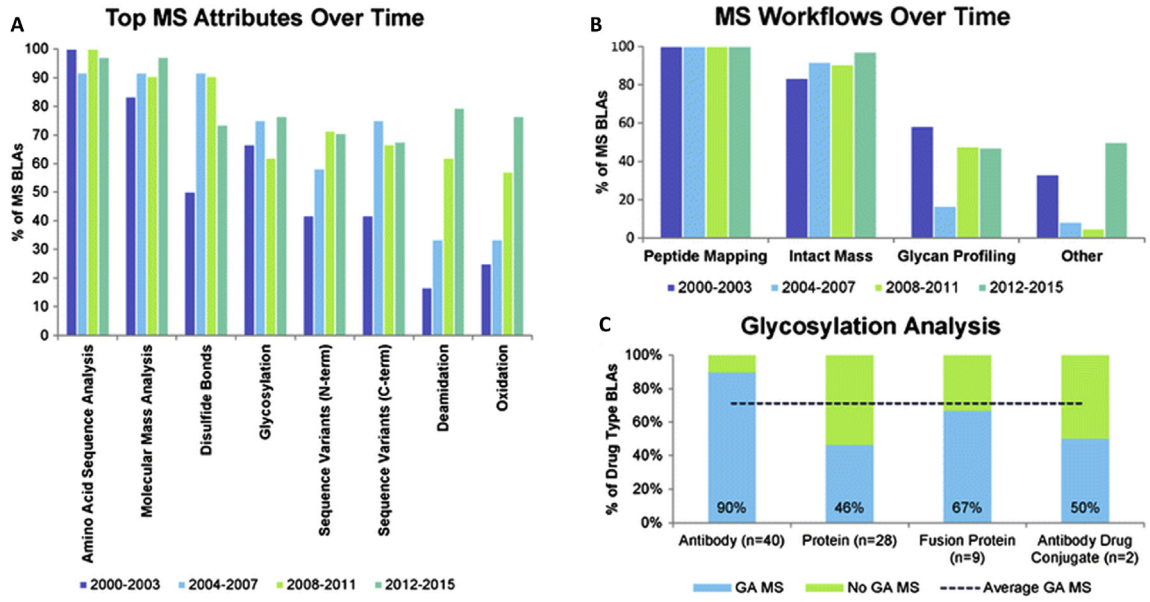

Fig. 2. Overview of the use of mass spectrometry (MS) for the characterization of protein therapeutics in FDA Biologic License Applications (BLAs). (**A**) Most used MS attributes in the BLAs in the period 2000–2015 ($n = 79$). (**B**) Distribution of MS workflows over time, portraying that released glycan profiling with MS did not increase in the period of 2008–2015, whereas the use of MS for intact mass analysis steadily increased over time. (**C**) Overview of glycosylation analysis for different therapeutic protein types, illustrating that MS is mostly used for antibody drugs and to a lesser extent for proteins, fusion proteins and antibody-drug conjugates. Readapted from Rogstad et al. [84].

interface it provides a quantitative fluorescence signal, prior to the MS-detection that is used for the structural analysis of the labeled glycans. Moreover, the iLIF detection functions independently of the ESI interface and therefore could be applied to other separation techniques, such as nanoLC and microchip electrophoresis.

Recently, Zhou et al. evaluated the most common derivatization techniques in combination with LC columns, to create guidance in selecting the appropriate derivatization agents for LC-MS/MS analysis of N-glycans [102]. It was shown that sample preparation procedures can take from 1 h up to 48 h for RapiFluor-MS (RFMS) and permethylation, respectively. In addition, RFMS provided the highest MS signal for neutral glycans, but was not able to overcome the sialic acid loss and rearrangement that was only prevented by the permethylation method.

Therefore, the additional benefit of MS in routine analysis could be considered questionable, since the detection of glycoforms is often hampered by the many different modifications that can occur during ionization and detection [57]. Indeed, a recent evaluation of all electronically submitted BLAs (years 2000–2015) showed that the use of MS-based glycan profiling remained consistent (47%) over the last 8 years in the study period (Fig. 2). In contrast, the use of MS for intact mass analysis increased from 83% to 92% and MS was used for peptide mapping in all BLAs [84]. However, MS-based glycomics will significantly promote the glycan data accumulation and therefore requires the development of strong software platforms to keep up with the current analytical tools [82,100,103].

In contrast to the colloquial vision that sample preparation methods are laborious and not interesting for HTS purposes, new fast and fully automated N-glycomics platforms have recently been developed. As example, Stockman et al. developed a robotic liquid-handling workstation, including the entire workflow for IgG purification from serum or cell culture samples to glycan release, labelling and quantification by UHPLC-fluorescence detection (FLD) analysis of 96 samples in 22 h [104]. Adaption of the initial workflow allowed the authors to further increase the throughput to 786 serum samples in a single automated platform with an accompanying reduction of 50% in analysis time. By switching to a new and more sensitive fluorescence labelling technique, an intermediate solid-phase extraction (SPE) procedure was eliminated, which reduced the workflow by 4–6 h. Parallel to the increase in throughput, the removal of the SPE procedure also allowed the removal of SPE-induced selectivity towards sialylated N-glycans [105]. However, faster fully automated workflows have been reported in literature but are often designated for the specific analysis of either N-linked glycans or O-linked glycans and therefore restricted in use for biosimilar analysis where comprehensive characterization of the entire glycosylation profile is required [106–108]. Additionally, with the use of only fluorescence detection, the identification of unknown species is impossible and thus further restricts the application in biosimilar development.

Several more limitations exist for the released glycan approach compared to intact and glycopeptides analysis. The released glycan approach could be modified to detect both N- and O-glycans for complex fusion proteins and bispecific antibodies. However, when site-specific information is of pivotal importance, released glycan analysis does not provide sufficient information with either FLR of MS detection. At last, it is worth mentioning that both intact and glycopeptides analysis are capable of detecting major glycoforms as well as a large number of other PTMs, making these approaches more attractive for the analysis of biosimilars.

4. Trends in analytical characterization of N-glycans for biosimilar mAb products

A plethora of different techniques (Table 2) are available for the glycosylation profiling of therapeutic proteins [60]. However, it is important to focus on specific fit-for-purpose techniques that answer to relevant analytical questions. In this section, new trends and novel approaches for glycan analysis will be discussed, with a specific focus on techniques that are of particular interest for biosimilar analysis (see Table 3 for a complete overview).

4.1. Middle-up analysis of glycoforms using hydrophilic interaction chromatography (HILIC)

After 20 years of HILIC being the core module in analyzing fluorescently labeled released glycans, the introduction of new sub-2 μm and widepore (300 Å) stationary phases has opened new possibilities for separations of released glycans, glycopeptides and intact glycoproteins [109]. Separation in HILIC is based on hydrophilic interactions (mostly through hydrogen bonds) and therefore provides orthogonal information to reversed phase liquid chromatography (RPLC) in terms of elution order and selectivity [63]. Periat et al. were first to show the potential of HILIC for the characterization of biopharmaceuticals, by demonstrating the separation of major mAb glycoforms at the middle-up level, which was not obtained with RPLC and ion exchange (IEX) separation modes [110]. To rapidly reach the optimal performance of HILIC, a quality by design (QbD) based method development approach was recently presented. By using chromatographic modeling software, the optimum conditions for mAb-subunit analysis could be determined within only one working day [111].

The main benefit of the middle-up analysis is the reduction in sample preparation time/steps, in comparison with the bottom-up approach, while still providing sufficient resolving power for glycan characterization (Fig. 3). Therefore, HILIC-based middle-up analysis is considered as an interesting approach to compare originator and biosimilar glycosylation profiles in routine analysis. This was demonstrated in a recent comparison of originator mAbs (i.d., infliximab, trastuzumab and cetuximab) with their biosimilar products at protein level. As shown by D'Atri et al., HILIC-MS analysis provided qualitative information on the glycosylation pattern, and allowed the direct comparison between originator and biosimilar product [112]. Additionally, chromatographically resolved glycan profiles promoted easier MS integration, owing to the more accurate peak deconvolution compared to RPLC-MS.

Furthermore, the capability of HILIC to characterize an ADC was demonstrated by middle-up analysis of brentuximab vedotin [113]. In a single chromatographic analysis, both drug payload and glycosylation variants were characterized. Therefore, the presented workflow is able to analyze the current marketed mAbs and their biosimilars, but is also readily applicable when complex biosimilars for ADCs will make their way onto the drug-market.

4.2. Multidimensional chromatographic approaches for glycan analysis of biosimilars

While current one-dimensional (1D) separation techniques are reaching their optimal performance, novel two-dimensional (2D) separation techniques provide an interesting alternative approach for the challenging mAb materials [114,115]. 2D approaches allow the combination of two chromatographic techniques, such as IEX, HILIC, RPLC, hydrophobic interaction chromatography (HIC) and size exclusion chromatography (SEC) in a single analytical platform. Common 2D approaches include the coupling of IEX to RPLC, HILIC to RPLC and HIC to SEC. The addition of a second orthogonal column can enhance the peak capacity as result of the increased resolving power [116]. Moreover, the use of a complementary dimension provides 2D peak patterns that facilitate the identification of unknown protein variants. Additionally, a second dimension can be

Table 3
Novel analytical strategies for glycan analysis in biosimilar mAb development.

Method	Level of analysis	Obtained information	Site-specific information	Multi-attribute monitoring (MAM)	Comments	Ref.
HILIC-MS	Middle-up	Glycoform determination	No	Limited	Limited sample preparation allows the direct comparison of biosimilars.	[110,112]
2D-LC-MS	Middle-up	Glycoform determination	No	Multiple CQAs	Increased resolving power from multidimensional approach. Complex data analysis and high technical requirements.	[119,120]
IM-MS	glycopeptide	Isobaric glycopeptide and glycoform differentiation	Yes	No	Increased throughput by analysis of glycans and peptides directly after PNGase F release	[124,127]
	Intact	Glycan heterogeneity	No	No	Direct comparison of biosimilars on glycan heterogeneity and HOS differences on intact level. Limited resolving power.	[131,132]
site-specific enzymatic digestion	Peptide	Limited glycoform determination	Yes- Quantitative	No	Only differentiation between high-mannose- or complex-type glycans possible. However, site-specific glycan occupancy information is available.	[141]
	glycopeptide	Glycoform determination	Yes	No	Allows qualitative site-specific glycan determination and total glycan occupancy levels.	[75]
MAM	glycopeptide	Glycoform determination	Yes	Multiple CQAs	Fully ICH-validated platforms available for MAM-monitoring. Essential for the implementation of QbD approaches	[144,146,176]

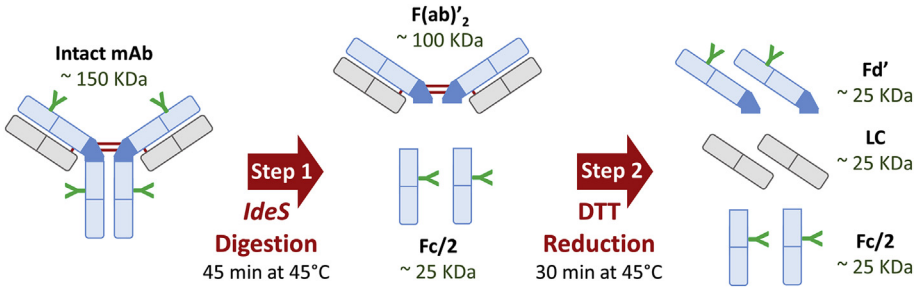

Fig. 3. Sample preparation procedure for middle-up analysis of mAb products. Reprinted with permission from D'Atri, Fekete, Beck, Lauber, Guillarme. Hydrophilic Interaction Chromatography Hyphenated with Mass Spectrometry: A Powerful Analytical Tool for the Comparison of Originator and Biosimilar Therapeutic Monoclonal Antibodies at the Middle-up Level of Analysis. *Anal. Chem.* 2017, 89, 2086−2092 [112]. Copyright 2017 American Chemical Society.

used to increase the compatibility of separation techniques with non-volatile mobile phase components in the first dimension to MS by providing a rapid on-line desalting procedure [117].

In general, on-line 2D-LC consists of two main approaches, i.e., comprehensive (LC × LC) and heart-cutting (LC-LC) or multiple heart cutting (mLC-LC) 2D-LC, which both have different strengths in the application for mAb characterization. In LC × LC, the two selected columns are directly coupled to each other and the entire effluent of the first column is loaded on the second column [116]. Thus, to maintain the separations obtained in the first column, the second separation should be performed very rapidly (<1 min). The heart-cutting or multiple heart-cutting approaches enable to selectively transfer one or more segments from the first to the second column [118]. Hence, the second dimension has no time constraints and the full separation performance can be exploited.

Sorenson et al. demonstrated the potential of IEX-RPLC for routine analysis of biosimilars by analyzing 3 pairs of originator-biosimilar products, i.e., cetuximab, infliximab and trastuzumab [119]. The proposed method allowed the direct comparison between originator and biosimilar products in a middle-up approach. Moreover, the coupling of RPLC to time-of-flight MS in the second dimension enabled the identification of differences in glycosylation patterns and amino acid level based on the 2D-chromatograms as well as the deconvoluted masses.

More recently, Stoll et al. developed a new comprehensive HILIC × RPLC approach for rapid and deep characterization of mAbs [120]. By introducing active solvent modulation (ASM), the peak distortion effect caused by the large acetonitrile proportion during the transfer of the HILIC to RPLC separation could be reduced. ASM allows the addition of water to the 1D-effluent, via a valve-switching procedure. This allowed larger 1D effluent volumes to be introduced in the 2D column and improved the detection sensitivity and peak shapes. The main benefit in this approach is attributed to the HILIC selectivity in the first dimension that reveals the extent of glycosylation of the mAb subunits. As can be seen from Fig. 4, the HILIC × RPLC approach resolved glycoforms of cetuximab that coeluted in the previously mentioned IEX × RPLC approach. Therefore, the proposed method has the potential to become a core module for the rapid and deep characterization of mAb samples and for the rapid evaluation of biosimilar glycosylation. However, introduction in QC/GMP environments would provide a significant challenge, because of the complex data analysis and extensive technical requirements.

4.3. Ion-mobility mass spectrometry for glycan analysis and structural information

Besides the introduction of multiple chromatographic

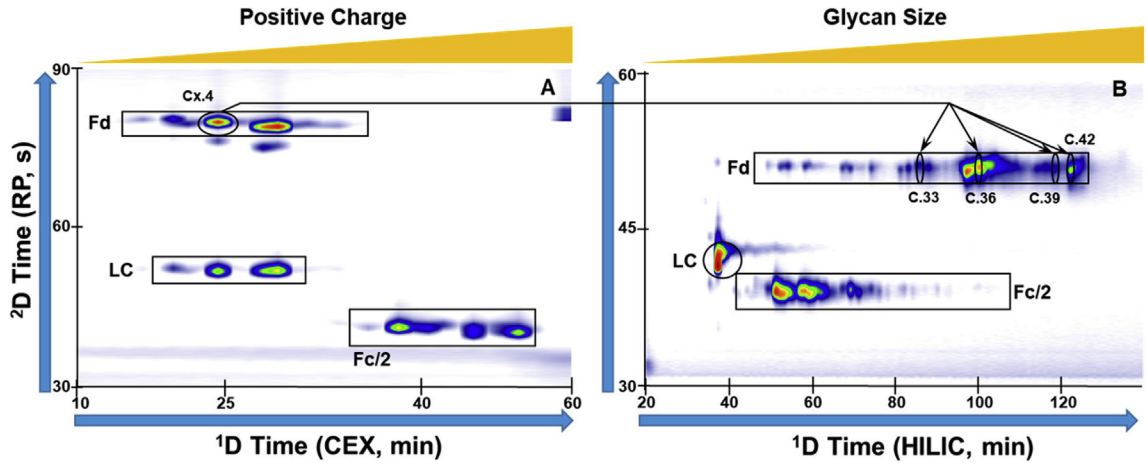

Fig. 4. Comparison of glycan analysis of cetuximab (*IdeS*-digested and reduced) using (**A**) CEX × RP and (**B**) HILIC × RP. It was demonstrated that using HILIC in the first dimension, instead of 1D-CEX, could resolve several co-eluting glycoforms (Cx.4) on the heavy chain portion of the antigen binding fraction (Fd) of cetuximab. Chromatograms are based on UV absorbance at 280 nm, peak identification was based on TOF-MS detection. For experimental conditions and peak annotations, the reader is referred to the original article. Reprinted with permission from Stoll, Harmes, Staples, Potter, Dammann, Guillarme, Beck. Development of Comprehensive Online Two-Dimensional Liquid Chromatography/Mass Spectrometry Using Hydrophilic Interaction and Reversed-Phase Separations for Rapid and Deep Profiling of Therapeutic Antibodies. *Anal. Chem.* 2018, 90, 5923–5929 [120]. Copyright 2018 American Chemical Society.

dimensions, post-ionization separation techniques have also gained increasing interest. In particular, the use of ion-mobility spectrometry – mass spectrometry ((IM)-MS) has gained increasing attention thanks to the introduction of significant technical advances. IM-MS allows separating ions based on their size, charge and shape, as they migrate through a buffer gas driven by an electric field. Due to their mobility differences, the arrival time distribution can be measured and used to calculate the collision cross section (CCS). The CCS is a physical property related to the shape of the ion, representing a 3D fingerprint that can be used during characterization [121]. In addition to providing structural information, IM-MS data can significantly reduce the complexity of mass spectra by group separation in complex (biological) samples. By plotting the CCS data against the *m/z* values, differences in drift times between, e.g., peptides, lipids and carbohydrates can be used to distinguish and exclude specific molecular classes (having overlapping masses) from the data [122,123]. Therefore, IM-MS offers a major improvement in throughput by using one single analytical platform to measure both glycans and peptides directly after release with PNGaseF. This makes elaborate derivatization and clean-up procedures redundant, while simultaneously minimizing sample loss during pre-treatment procedures [124,125].

The combination of structural information and potential for HTS, makes IM-MS a compelling technique for the field of glycomics. Indeed, due to the isomeric nature of many carbohydrate structures, the identification of chemically similar glycopeptides and glycoproteins requires comprehensive structural information.

The potential of IM-MS for analysis of isobaric structures was demonstrated by Hofmann et al. by showing the separation of carbohydrate linkage-isomers as well as stereoisomers without prior derivation [126]. Furthermore, in-depth analysis of site-specific glycosylation of peptides was demonstrated for sialic acid-linked isomers and N-acetyl neuraminic acid linked isomers (Fig. 5) [127,128]. In the above-mentioned methods, glycopeptides were fragmented and CCS values of smaller oligosaccharide structures were used to identify distinct glycan motifs. Since small structural differences have a negligible impact on the shape of large intact precursor ions, their CCS information provides limited resolving power, compared to smaller oligosaccharide structures. However, performing structural elucidation of intact glycans based on glycan fragments requires multidimensional databases,

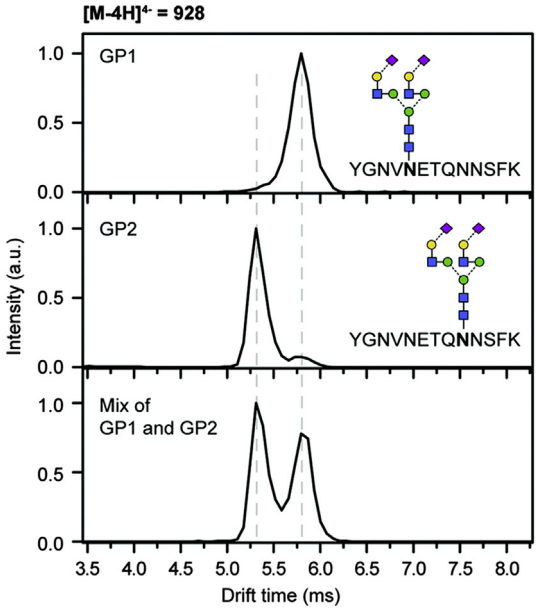

Fig. 5. Separation of isobaric glycopeptides GP1 and GP2 using IM-MS. Differences in obtained drift time allow to distinguish between two isobaric glycopeptides having identical peptide sequences but differ in site-specific glycosylation. For experimental conditions the reader is referred to the original article. Reprinted from Hinneburg, Hofmann, Struwe, Thader, Altmann, Varón Silva, Seeberger, Pagel, Kolarich. Distinguishing N-acetylneuraminic acid linkage isomers on glycopeptides by ion mobility-mass spectrometry. *Chem. Commun.* 2016, 52, 4381–4384 [127] - Published by The Royal Society of Chemistry.

including both CCS and masses for the precursor and fragment ions. Fortunately, CCS data is highly reproducible and databases have emerged, containing comprehensive data for many peptides, glycopeptides, glycans and glycan fragments for accurate ion identification [129,130].

The use of IM-MS for the analysis of originator mAb and biosimilar products has recently emerged as an interesting analytical technique for global conformational characterization. IM-MS allows to create a 3D fingerprint of the higher order structure of the protein and is easy to integrate in routine analysis, due to the

limited (manual buffer exchange) or no sample preparation (automated removal of salts) [117,131,132]. Structural information of mAbs regarding the disulfide bridge heterogeneity as well as the presence of monomer and dimer conformations can be rapidly obtained via IM-MS [133]. Beck et al. compared originator cetuximab and trastuzumab with their model biosimilar candidates to demonstrate the potency of the comparability exercise (Fig. 6) [131]. The use IM-MS revealed heterogeneities in the trastuzumab biosimilar product bearing 1 or 2 glycans on the Fc-region, while for all other mabs homogenous driftscope plots were obtained, indicating the absence of structural differences. Upton et al. applied IM-MS to analysis of lot-to-lot differences of trastuzumab and demonstrated conformation heterogeneity in 1 out of the 3 approved lots. Subsequently, they revealed how the variety in N-linked glycosylation influenced the protein conformation and advocated how the observed range of conformational heterogeneity could provide general acceptance criteria in the approval process for new protein therapeutics [134].

As result of the ms-timescale separations, IM-MS is an attractive technique to combine with orthogonal separation techniques, e.g., LC and CE, to further increase the resolving power. This has been demonstrated by coupling ion mobility to CE and LC separation for the analysis of intact glycans [135,136]. More interestingly is the coupling of IM-MS to 2D-LC techniques previously described, to further improve the separation of glycoforms and PTMs at subunit level [119,137,138]. A comparable approach was recently demonstrated by Ehkirch et al. that coupled SECxSEC-native IM-MS for the comprehensive analysis of mAb size variants [139]. The online 2D-LC setup consisting of SEC in both dimensions allowed the use non-volatile salts for optimal separation performance prior to native IM-MS [140]. It was shown that a comprehensive IM-MS approach is crucial for the unambiguous identification and quantification of all detected size variants, especially to emphasize the difference between different monomeric conformers in forced stressed material.

4.4. Site-specific glycan analysis for therapeutic proteins

Site-specific glycosylation is of vital importance for the folding, stability and efficacy of therapeutic proteins. Therefore, regulatory agencies demand comprehensive information on the site-specific profile as well as site occupancy, for approval of new mAb products or during the comparability exercise for biosimilar mAb products. Because of the complexity of glycan patterns and intrinsic heterogeneity between expression systems, there is an increasing demand for site-specific analytical techniques that can provide the information required by regulatory agencies.

Recently, Cao et al. developed a robust and versatile approach based on LC-MS/MS for the global analysis of site-specific N-glycosylation (intended for a HIV-envelope glycoprotein containing 75–90 glycans) that is potentially applicable to every glycoprotein

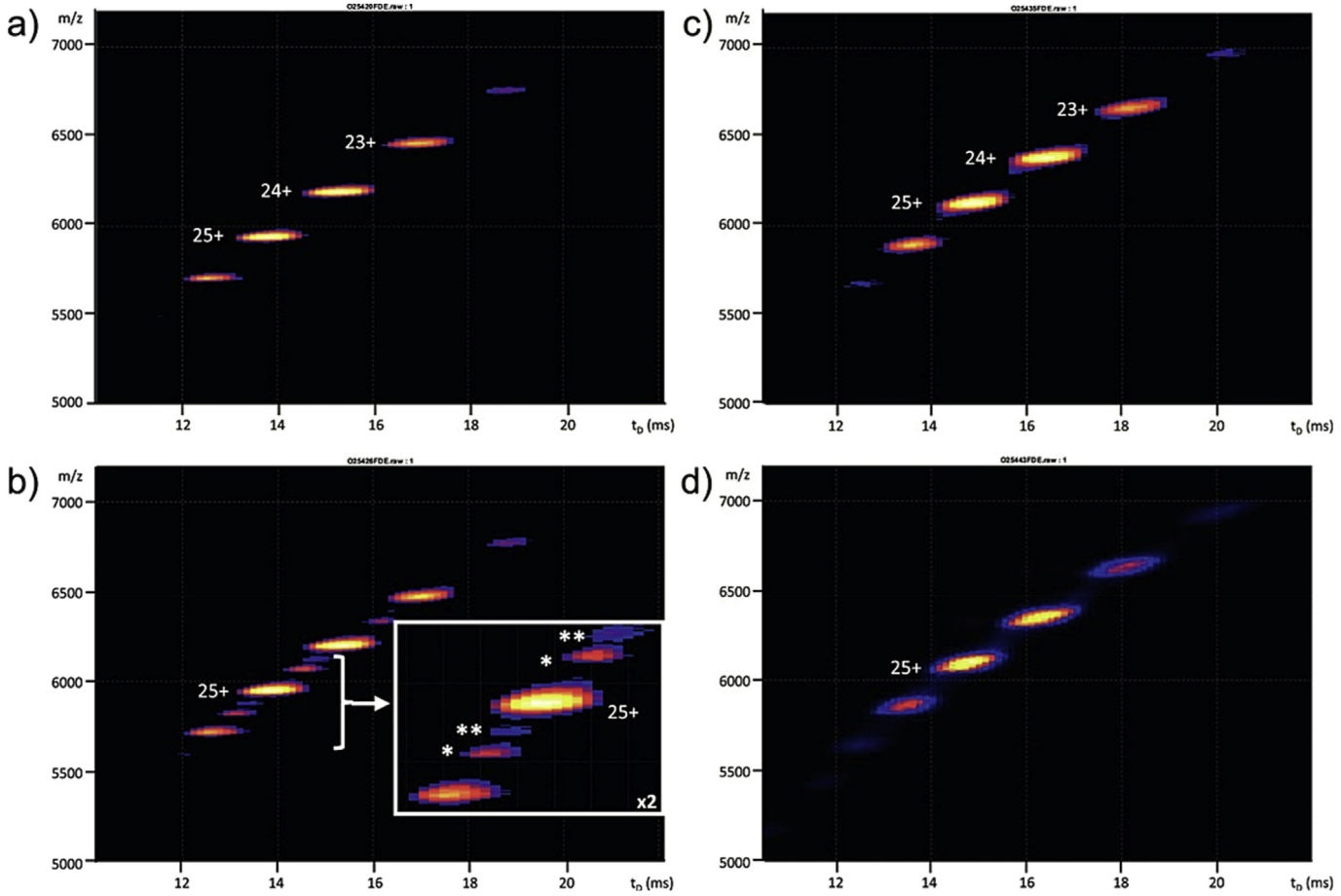

Fig. 6. Analysis of intact trastuzumab, cetuximab with their biosimilar candidates using intact native IM-MS. Comparison of driftscope plots of trastuzumab (a) and trastuzumab-B (b) demonstrated structural heterogeneity in the glycosylation profile of the biosimilar product bearing 0 (*) or 1(**) glycan per Fc. Homogeneous results were obtained in the comparison of cetuximab (c) and cetuximab-B (d). For experimental conditions, the reader is referred to the original article. Reprinted from *J. Mass Spectrom.*, Vol. 50, Beck, Debaene, Diemer, Wagner-Rousset, Colas, Van Dorsselaer, Cianférani. Cutting-edge mass spectrometry characterization of originator, biosimilar and biobetter antibodies, pp 285–297 [131]. Copyright 2015, with permission from John Wiley & Sons, Ltd.

[141]. The approach is based on the use of multiple endoglycosidase enzymes, to create unique mass signals (Fig. 7a) after cleavage of either the high-mannose (with Endoglycosidase H) or complex-type glycans (with PNGase F). The main benefit of this approach is that site-specific quantitative information is obtained based on peptides without the attached glycans. Therefore, the analyzed peptides have similar ionization efficiencies during the MS analysis and provide a more reliable quantification. A second key feature is the use of multiple proteases during digestion, which results in a significant increase of sequence coverage and allows the use of robust proteomics software for glycomics purposes. However, the total workflow consists of 7 days, and is therefore not applicable to routine analysis of originator and biosimilar mAb products. Moreover, by removing the glycans prior to the LC-MS/MS analysis, only quantitative information can be obtained on the presence of either high-mannose or complex-type glycan species. Therefore, the presented workflow is limited in its use for the comprehensive glycan analysis required during the comparability exercise.

Yang et al. demonstrated the use of multiple reaction monitoring (MRM) with UHPLC-MS/MS, to monitor site-specific glycosylation for multiple mAb products [75]. MRM allowed to monitor the site-specific glycan profiles based on the glycopeptides directly after digestion, which significantly reduced the analysis time, to approximately 10 min per sample. Subsequently, removal of the glycans using PNGase F allows to detect the asparagine to aspartic acid conversion (Fig. 7b) and quantitate the occupancy. It was shown that all six mAb products (IgG1 and IgG2) had similar glycosylation sites and an average occupancy of 97%. However, this represents the overall glycan occupancy and does not indicate the occupancy tailored to distinct glycoforms. In a similar approach with CZE-ESI-MS, site-specific glycan profiling was achieved within ~9 min with superior detection limits (i.e., 2 orders of magnitude) and a 200-fold smaller injection volume compared to nano-LC [142]. However, no information on site-occupancy was achieved, and only Fc glycosylation was studied.

4.5. Multiple attribute monitoring to enable quality by design manufacturing

Integration of QbD approaches for glycosylation on therapeutic proteins has been of main interest for many pharmaceutical companies, to create more robust and efficient manufacturing processes and develop mAbs with increased efficacy and safety [39]. The QbD approach aims to incorporate in-depth knowledge on the product, to design the desired quality rather than testing it [40]. Furthermore, the premises of QbD can simplify the development of new biosimilar products by better understanding of the CQAs that could hamper the desired clinical effect of the product and the process conditions that alter these given CQAs [143].

Essential for the implementation of QbD are multi-attribute monitoring (MAM) techniques with MS detection to obtain the required in-depth information on structure-function relationships as result of PTMs and the elucidation of the manufacturing attributes that affect the product characteristics [144,145]. However, implementation of MS systems in QC/GMP environments remains controversial in the biopharmaceutical industry, due to the expensive equipment and complicated data analysis [83].

To overcome the current hurdles and ensure the implementation of QbD in QC/GMP environments, Xu et al. demonstrated a MAM approach based on the use of a single quadrupole MS device [146]. The fully ICH-validated approach was applied to the selective characterization of pQA, e.g., deamidation, oxidation, glycosylation and disulfide bond heterogeneity and was verified by using forced degraded material. Therefore, the proposed QC-friendly platform has the potential to become a core module in QC labs and can pave the way towards the implementation of QbD strategies.

Another interesting approach is the use of CZE-ESI-MS for the relative quantitation of N-glycan species on a site-specific glycopeptide level. Similar to the previously mentioned LC-MS/MS approaches, the proposed CZE based method is not restricted to the analysis of glycopeptides but allows complete primary sequence

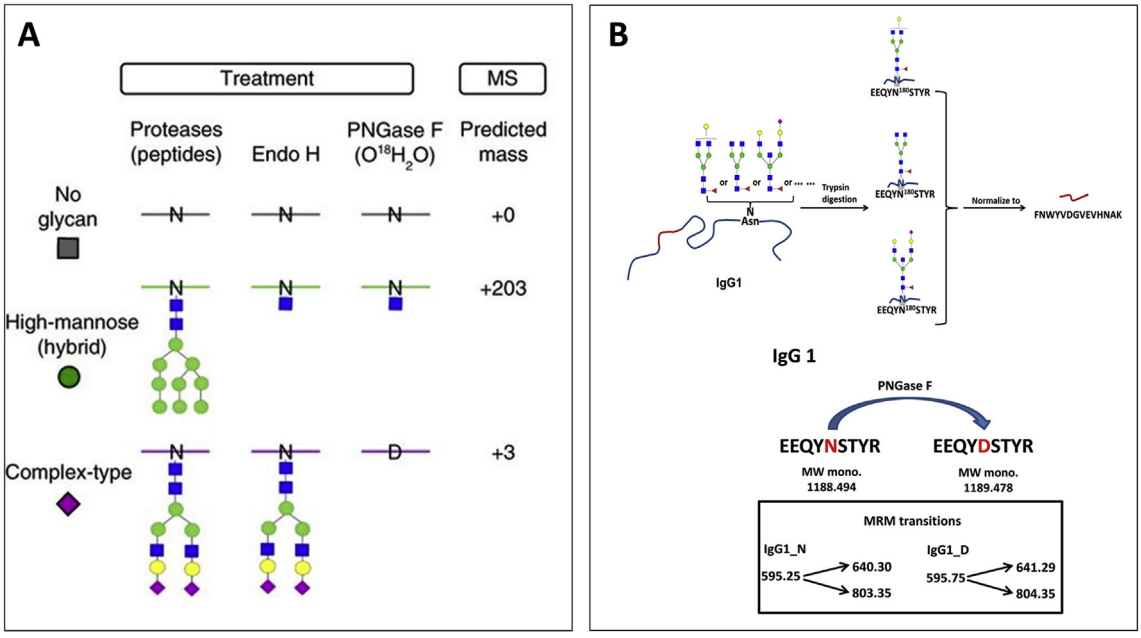

Fig. 7. Comparison of qualitative and quantitative methods for site-specific glycosylation. (**A**) By using two different endoglycosidases, unique mass signals are formed for high-mannose and complex-type glycans that can provide reliable site-specific quantitative information based on peptide level. (**B**) Site-specific glycan profiles are obtained directly after digestion and normalized by using a distinguishing parent peptide. Subsequently, the relative occupancy is determined based on the mass difference that is introduced by PNGase F during cleavage of the glycans. Readapted from Cao et al. [141] and Yang et al. [75].

and quantification of multiple PTMs in a single analytical workflow [142,147]. The use of CZE provides very high separation efficiencies, owing to the limited peak broadening effect in absence of a stationary phase. In addition, CZE is particularly well suited for the analysis of small peptides that elute within the column dead volume in LC and large peptides that adsorb on the stationary phase. Moreover, CZE is considered a miniaturized technique, which favors coupling to nano-ESI and improves ionization, while simultaneously reducing the sample consumption significantly [148,149]. In this context, Boley et al. showed that the peptide analysis throughput could be greatly enhanced by introducing multisegment injections in CZE [150].

In a recent study, Giorgetti et al. assessed and validated the potential of CZE-ESI-MS for N-glycosylation profiling, by analysing 10 different mAb drugs (including 2 biosimilar products of infliximab) [151]. The obtained glycosylation profiles were compared with profiles generated from the reference HILIC-2AB method. It was demonstrated that glycosylation profiles obtained with CZE-ESI-MS were highly similar to the ones obtained with the reference method, with accurate and precise levels of quantitation. However, the real attractiveness of the proposed platform was revealed by the comparability exercise for originator infliximab-Remicade and biosimilars Remsima and Inflectra, as discussed below.

5. Case study: clinical impact of structural heterogeneity in infliximab-biosimilars

The importance of comprehensive analytical characterization of biosimilar products was recently demonstrated by Giorgetti et al., who compared infliximab originator Remicade and biosimilars Remsima and Inflectra using a CZE-ESI-MS platform [151]. Significant differences between Remicade and both Remsima and Inflectra were observed in the relative abundance of eight selected N-glycan species (Fig. 8). Moreover, independent comparison of the peptide mapping results from both the LC-MS/MS (presented by Pisupati et al.) and CZE-ESI-MS workflow showed striking similarity in glycan profiling and determination of the relative abundance [152]. Combined, these results confirmed the significant differences in glycosylation profiles between Remicade, Remsima and Inflectra (Fig. 8). Additionally, by using an array of (bio-)analytical techniques (e.g., native MS, peptide mapping and bio-layer interferometry), multiple differences in quality attributes between the originator and the approved biosimilar product could be identified [152]. Observed differences in C-terminal truncation, glycation and soluble protein aggregates were considered negligible because of the limited clinical impact. More interestingly, a difference in afucosylated glycan levels between Remicade (19.7%) and Remsima (13.2%) was identified, which could be related to a two-fold reduction in FcγIIIa receptor binding for Remsima. These findings were confirmed by Lee et al., who showed even further differences in glycosylation profiles of infliximab's biosimilar products in terms of structure and biological activity among biosimilar products produced in different cell lines [153]. Therefore, it is of paramount importance to perform extensive analysis of glycosylation patterns, to ensure that all approved products are within the predefined quality limits [24].

In the case of infliximab biosimilars, significant differences were found in afucosylated glycan levels, which is considered as CQA for mAbs with Fc-mediated effector functions [34]. More specifically, afucosylation levels were correlated to significantly stronger ADCC effects, as a result of improved binding affinity to human FcγRIIIα expressed on, e.g., human natural killer (NK) cells or macrophages. Therefore, when the drug MoA is related to ADCC effects, afucosylation can potentially influence the clinical outcome (Fig. 9) [30].

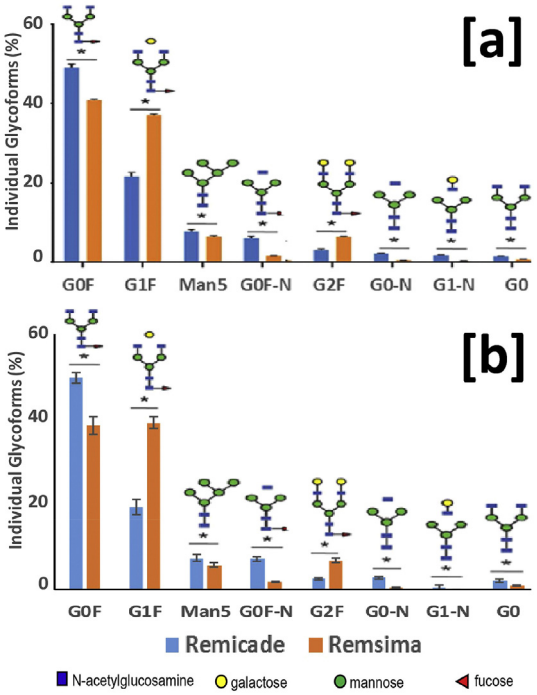

Fig. 8. Overview of glycan profile differences between originator Remicade and biosimilar Remsima. Independent studies using LC-MS/MS (A) and CE-ESI-MS (B) showed similar results in identification and quantitation of the N-glycosylation profile. Comparison of the results confirmed the significant differences in afucosylation and terminal-mannose levels between the biosimilar and reference product of infliximab. Reprinted from *Talanta*, Vol. 178, Giorgetti, D'Atri, Canonge, Lechner, Guillarme, Colas, Wagner-Rousset, Beck, Leize-Wagner, François. Monoclonal antibody N-glycosylation profiling using capillary electrophoresis – Mass spectrometry: Assessment and method validation, pp 530–537 [151]. Copyright 2018, with permission from Elsevier.

For anti-TNFα drugs, such as infliximab, the proposed MoA is related to neutralization of the soluble and transmembrane expressed TNF-α via Fab-mediated binding. In addition, Fc-mediated binding to FcγRIIIα expressed on NK cells facilitates ADCC of the target cells, which are bound by the Fab region [154]. Therefore, the clinical impact of glycan differences is strongly related to the drug MoA, which is dependent on the disease indication. In general, neutralization of TNF-α is solely accountable for the clinical outcome in rheumatic arthritis (RA), whereas in inflammatory bowel disease (IBD), the Fc-dependent ADCC is potentially involved in drug efficacy [155–157].

Health authorities were aware of the abovementioned differences in N-glycosylation, disease-MoA differences during the approval process of the infliximab biosimilar. Nonetheless, the biosimilar product was approved and extrapolated to all eight indications supported by the innovator product, based on only two clinical trials performed in RA and ankylosing spondylitis (AS) patients [158]. Therefore the approval of infliximab biosimilars for the treatment of IBD, based on clinical data obtained for RA, creates an important regulatory precedent. Regulators acknowledged the statistically significant differences in afucosylation, binding affinity and ADCC activity, but did not consider the level of afucosylation as a Tier 1 (highest clinical relevance) CQA [28]. Moreover, the extrapolation was based on clinical studies performed in RA patients where the Fc-mediated effector function of infliximab is not relevant [159]. Additionally, it is often mentioned that clinical studies are less sensitive than analytical studies [27]. Often the small 500–800 patient trials do not have enough power to detect meaningful differences in both safety and efficacy. If noteworthy differences in adverse events are detected, it is often difficult to

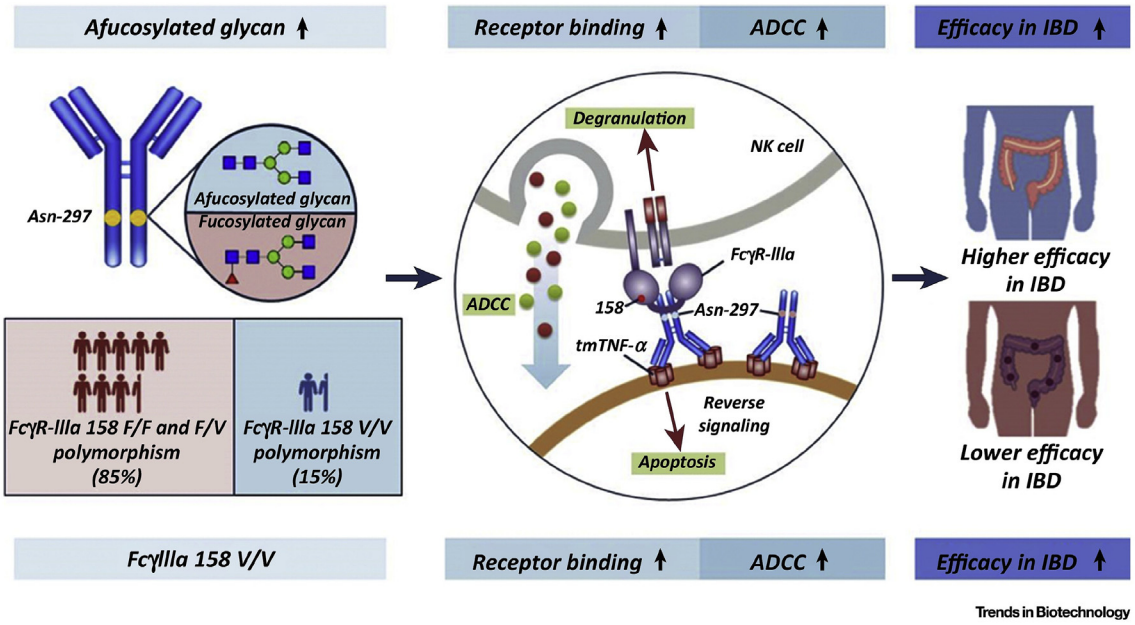

Fig. 9. Graphical representation of parameters influencing the clinical efficacy of infliximab in IBD patients. (**Top**) Increased levels of afucosylation improve the FcγRIIIα receptor binding and subsequently increase the ADCC activity, which results in a higher clinical efficacy in IBD patients. Therefore, afucosylation levels between the biosimilar and originator product should be considered as CQA. (**Below**) Patients with the FcγRIIIα 158 V/V polymorphisms have superior receptor binding and ADCC effects compared to F/V and F/F patients and have an increased therapy response to infliximab. Therefore, biosimilar products with increased levels of afucosylation would be preferable for patients with *F/F* and *F/V* polymorphisms. Reprinted from *Trends Biotechnol*, Vol. 36, Kang, Pisupati, Benet, Ruotolo, Schwendeman, Schwendeman. Infliximab Biosimilars in the Age of Personalized Medicine, pp 987–992 [159]. Copyright 2018, with permission from Elsevier.

correlate these effects to specific biosimilar drug effect, due to complexity of the studied disease indications and patient population [160,161].

The presented case study demonstrates the increasing importance of comprehensive analytical platforms for the characterization of biosimilar products. As previously described, glycosylation patterns can significantly alter the excretion rates, immunogenicity and clinical outcome of biological products, and therefore should be closely monitored during the manufacturing process. Moreover, multidimensional analytical platforms provide important knowledge on the relation between specific product attributes and their *in vivo* consequences [144]. At last, integrating the analytical similarity assessments in the design and analysis of Phase 3 efficacy studies could assist in lowering the residual uncertainty and support a demonstration of clinical similarity [162].

6. Conclusion and perspectives

The introduction of biosimilar products, following patent expiration of the originator products, may help to reduce the overall healthcare costs and improve the access to life-changing mAb treatments for all patients. Recent loss of patent protection for major blockbuster biologics has rapidly matured the biosimilar market to the current *status quo*, in which multiple biosimilars are available for a single originator drug. The intrinsic biological variability of biosimilars is a significant challenge for the current analytical platforms. As result, a plethora of new techniques has been developed to monitor various product characteristics and important PTMs, such as glycosylation, that can significantly affect the safety and efficacy of the biosimilar product.

Here, we reviewed multiple new analytical strategies that are of specific interest for glycan analysis during biosimilar development (Table 3). The use of HILIC-MS at protein subunit level has been discussed as interesting approach for glycan analysis in the development and approval process of biosimilar mAbs. Newly developed stationary phases have enabled to rapidly compare glycosylation moieties between originator and biosimilar mAbs without the need of complex sample preparation procedures. In order to further increase the resolving power at protein subunit level, new multidimensional chromatography approaches have been introduced. The benefits of 2D-LC for biosimilar analysis has been discussed in detail. However, introduction of 2D-LC approaches for routine analysis provides a significant challenge due to the complex data analysis and extensive technical requirements.

Recent introduction of IM-MS allows the fast comparison of the glycan heterogeneity and HOS of biosimilars at intact level. However, the current commercially available techniques have limited resolving power at intact protein level and therefore the application of IM-MS to analyze glycopeptides is more interesting for accurate glycoform determination. In addition, analysis performed at glycopeptide level allows obtaining important site-specific glycosylation information, which are not achieved with released glycans or intact protein level analysis. Several distinct sequential enzymatic procedures have been introduced to obtain additional site-specific occupancy information. However, we believe that broad application of these procedures in the development of biosimilar products will be limited due to the long sample preparation procedures.

Currently, one of the main challenges is the development of fast, cost-effective and sensitive platforms that allow the characterization of multiple product attributes in a simple, automated and robust workflow. The recently introduced MAM-platforms (e.g., CE-ESI-MS and LC-MS/MS) allow the characterization and comparison of multiple quality attributes, e.g., disulfide bond heterogeneity, oxidation and site-specific glycosylation in a single analysis. Therefore, we expect that the discussed MAM-platforms will rapidly progress from academic concepts to core modules in the biopharmaceutical industry.

In respect to biosimilar development, these new analytical platforms are crucial in correlating distinct product attributes with

observed *in vivo* effects. Understanding the relationship between the product attribute and clinical performance is of major importance for glycosylated protein therapeutics, due to the significant effects of glycans on the clinical efficacy, stability, excretion rate and immunogenicity. Moreover, a better understanding of the product characteristics that determine the therapeutic effect and insight into the manufacturing process parameters that influence these quality attributes is a crucial aspect for QbD strategies. Implementation of QbD strategies to control glycosylation will improve the manufacturing consistency and result in safe and efficacious products on the market. Moreover, combination of QbD strategies with glycan engineering strategies could open up the possibility of creating tailor-made mAb products with increased clinical efficacy. In this context, detailed analytical information on glycosylation patterns with specific physiological effects can be used to develop precision medicine applications in a foreseeable future. Since these products cannot be considered as biosimilars, a close collaboration between health authorities and the pharmaceutical industry is the key for a continuous progression in the field of protein therapeutics.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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