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Review

Glycan characterization of biopharmaceuticals: Updates and perspectives

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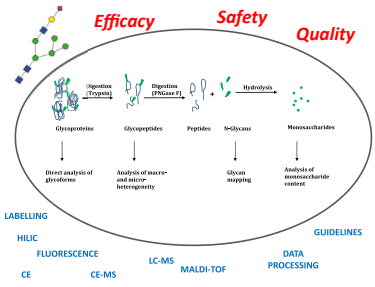
HIGHLIGHTS

- Biopharmaceuticals have emerged as the new class of blockbuster drugs in the pharmaceutical industry.
- More than 60% of the approved biopharmaceuticals are glycosylated.
- Glycosylation has an effect on the efficacy and the safety of therapeutic glycoproteins.
- N-glycosylation characterization of therapeutic glycoproteins is a regulatory requirement.
- Biosimilar releases are increasing and demonstration of comparability poses challenges for N-glycosylation characterization.

GRAPHICAL ABSTRACT



Abbreviations: 2-AA, anthranilic acid labeling; 2-AB, 2aminobenzamide labeling; ADCC, antibody-dependent cellular cytotoxicity; AEC, anion-exchange chromatography; APTS, 8-aminopyrene-1,3,6-trisulfonate labeling; Asn, amino acid residue; BHK, baby hamster kidney cell line; C18, octadecyl carbon chain bonded silica; CE, capillary electrophoresis; CHO, Chinese hamster ovary cell line; CIEF, capillary isoelectric focusing; CZE-ESI-MS, capillary zone electrophoresis-electrospray-mass spectrometry; DMT-MM, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride; EMA, European medicines agency; ESI, electrospray ionization; FDA, food and drug administration; Fuc, fucose; Gal, galactose; GalNAc, N-acetylgalactosamine; GC, gas chromatography; Glc, glucose; GlcNAc, N-acetylglucosamine; GN, N-acetylgalactosamine; HILIC, liquid chromatography using hydrophilic interaction chromatography; HPAEC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; HR-MSMS, high-resolution tandem mass spectrometry; ICH, International Conference on Harmonization; IEC, ion-exchange chromatography; IgGs, immunoglobulins G; LC, liquid chromatography; LIF, laser-induced fluorescence; mAbs, monoclonal antibodies; MALDI, matrix-assisted laser desorption ionization; Man, mannose; MS, mass spectrometry; neuNAc, N-acetylneuraminic acid; PGC, porous graphitized carbon; PNGase F, peptide-N-glycosidase F; RP, reverse phase; SP2/0, NS0, mouse myeloma cell line; TOF, time-of-flight; UHPLC, ultra-high performance liquid chromatography; VEGF, vascular endothelial growth factor; WHO, world health organization.

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ABSTRACT

Therapeutic proteins are rapidly becoming the most promising class of pharmaceuticals on the market due to their successful treatment of a vast array of serious diseases, such as cancers and immune disorders. Therapeutic proteins are produced using recombinant DNA technology. More than 60% of therapeutic proteins are posttranslationally modified following biosynthesis by the addition of N- or O-linked glycans. Glycosylation is the most common posttranslational modifications of proteins. However, it is also the most demanding and complex posttranslational modification from the analytical point of view. Moreover, research has shown that glycosylation significantly impacts stability, half-life, mechanism of action and safety of a therapeutic protein. Considering the exponential growth of biotherapeutics, this present review of the literature (2009–2015) focuses on the characterization of protein glycosylation, which has witnessed an improvement in methodology. Furthermore, it discusses current issues in the fields of production and characterization of therapeutic proteins. This review also highlights the problem of non-standard requirements for the approval of biosimilars with regard to their glycosylation and discusses recent developments and perspectives for improved glycan characterization.

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Contents

1. Introduction: importance of the glycan characterization of biopharmaceuticals

In 1972 Paul Berg, who won Nobel Prize in 1980, produced the first recombinant DNA facilitating the beginning of recombinant technology and heralding the birth of recombinant proteins as a new class of pharmaceuticals. Improved recombinant technology enabled production of the first human recombinant protein, recombinant human insulin, in 1982. Using human preparations instead of animal proteins led to great breakthrough in diabetes treatment [1]. Many other recombinant proteins were introduced soon after facilitating the treatment of severe deficiencies due to their ability to supplement the activity of the essential endogenous molecules, such as hormones, enzymes and clotting factors. Importantly, more than 60% of those recombinant proteins are glycosylated [2]. It has been highly demonstrated that changes in the glycosylation pattern of the expressed therapeutic has an effect on the efficacy, half-life, stability and safety of therapeutic glycoproteins. To obtain consistent glycosylation patterns, efficient manufacturing process and effective glycan characterization are required [3].

Glycosylation is the most common protein post-translational modification (PTM). This complex process occurs in endoplasmic reticulum and Golgi apparatus [2] (Fig. 1). In general, glycans are divided into two main groups, including N-glycans (oligosaccharides attached to asparagine amino acid residue through a nitrogen atom) and O-glycans (oligosaccharides attached to serine or threonine through an oxygen atom) [4]. In the current review we focus on N-linked glycosylation due to its importance in modulating the stability, function and structural integrity of biopharmaceuticals [5]. New approaches for O-glycosylation characterization were recently shown [6,7].

N-glycans are classified into three main groups, namely oligomannose glycans, complex glycans and hybrid glycans. Oligomannose glycans contain only mannose monosaccharide units (Fig. 1: M3, M5, M8). Complex glycans (Fig. 1: A3, A2B, FA2G2S2, FA2G2Ga1) are usually galactosylated, sialylated, and core-fucosylated. Hybrid glycans contain elements of both high mannose glycans and complex glycans due to the processing of α1-3 linked mannose residue of the trimannosyl chitobiose core structure (Fig. 1: FM5A1). N-glycans biosynthesis consists of removal and addition of sugars (N-acetylglucosamine (GlcNAc), glucose (Glc), galactose (Gal), N-acetylgalactosamine (GalNAc), fucose (Fuc), mannose (Man), and N-acetylneuraminic acid (NeuNAc)) depending on the presence of substrates and enzymes, namely glycosidases (glucosidases, mannosidases) and glycosyltranferases (GlcNAc transferases, galactosyltransferases, sialyltransferases and fucosyltransferases) (Fig. 1) [8,9].

In order to help scientists to describe glycans without the need to draw the full chemical structure of the oligosaccharide, different

nomenclatures have been introduced, including Oxford Glycobiology Institute (UOXF) notation, UOXF color notation, Consortium for Functional Glycomics (CFG) notation, CFG black and white notation and CFG with linkage placement notation [10].

Genetically engineered cells in culture such as bacteria, plants cells, insect cells, yeast, or mammalian cells are used for the production of glycoproteins. Current recombinant technology for therapeutic glycoprotein production is majorly based on mammalian cell lines, predominantly Chinese Hamster Ovarian (CHO) cells, baby hamster kidney cells (BHK), SP2/0 and NS0 cells (mouse myeloma cells). Exceptions do exist with recent reports outlining glyco-engineering strategies to produce certain glycosylated therapeutic proteins in bacteria and yeast [11]. Mammalian cell lines are more widely used for therapeutic protein expression due to their ability to perform human-like PTMs (including glycosylation) [3]. Of the mammalian cell lines used within the industry, CHO cell lines predominate as a commercial production host cell line due to an established regulatory track record, their ease of use, their ability to grow in suspension culture under chemically defined media conditions and their exemplary safety record to date. Nevertheless, a disadvantage of CHO cell line is that the protein glycosylation is not consistent due to the differential activity of hundreds of enzymes involved in the glycosylation process that vary in response to differences in the cellular environment during culture. This results in different glycosylation patterns and lower batch-to-batch repeatability [12]. Importantly, these molecular variations might lead to an alteration on the therapeutic effect of the expressed protein. Changes in glycosylation patterns of therapeutic proteins play a crucial role in terms of structure, stability, solubility, clearance, activity, and even immunogenicity [13–16]. For example, therapeutic antibodies which lose terminal sialic acid residues are thought to be removed faster from the blood circulation due to interaction of exposed galactose residues with the asialoglycoprotein receptor on hepatocytes. Moreover, the anti-inflammatory effect of the therapeutic antibodies which lose terminal sialic acid increases due to the improved ADCC and CDC effects [17–19]. A third example is therapeutic antibodies lacking core-fucosylation, which have been demonstrated to have higher ADCC activity than

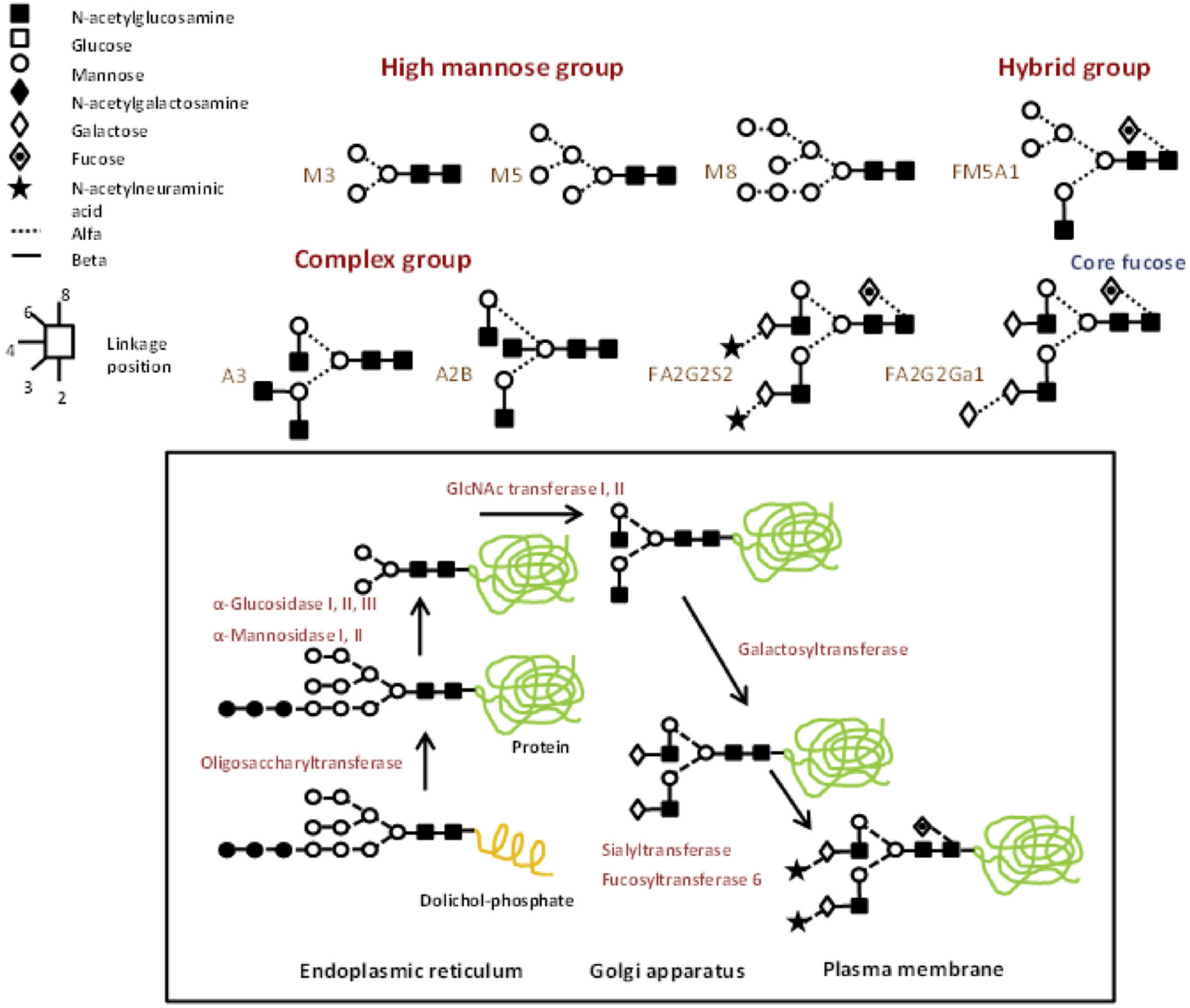


Fig. 1. Schematic picture of N-glycans and their biosynthesis using UOXF notation.

their fucosylated counterparts [20]. The changes in mannosylation can impact the half-life and cause off-target hepatic toxicities [21].

Modulation of N-glycosylation during the recombinant engineering in the pharmaceutical industry is challenging. The N-glycosylation profile of recombinant glycoprotein depends on several culture conditions [22,23], including nutrient levels, dissolved oxygen level, pH, temperature, high stress, use of serum, growth on micro carriers and media additives [24]. It has been shown that β1,4-galactosylation of the N-glycans could be controlled during the manufacturing process by adjusting the media concentrations of manganese, galactose and uridine, which are metabolic effectors of galactosylation [25]. Moreover, several important control points during manufacturing process of recombinant glycoproteins have been recognized, such as glycosylation enzymes (galactosyltransferase 4) and transport proteins [26,27].

Recent years have also witnessed considerable activity in the area of cellular glycoengineering using a variety of engineering strategies to produce specific or homogenous glycosylation variants. For example, establishment of FUT8 knockout CHO cell line enabled the production of completely a fucosylated therapeutic antibodies, which were demonstrated to have high-ADCC activity and fixed quality and efficacy [28]. Another interesting strategy has been lately introduced, called GlycoDelete, which shortens the Golgi N-glycosylation pathway with a view to reducing complexity of the N-glycans. Therefore, N-glycosylation is more consistent [29] but might conversely miss important more complex glycans. Even yeast cell lines can provide human-like glycosylation after humanization of the glycosylation machinery [30], illustrating that glycoengineering has a promising future.

The arrival of biosimilars has increased the market segment of biotherapeutics and has emphasized the requirement of strong analytical tools required to demonstrate physiochemical comparability during the evaluation stage. As many patents of currently marketed therapeutic proteins will expire in the coming decade [31] the biosimilar market is likely to rapidly increase. A biosimilar is a version of an already authorized therapeutic biological product with demonstrated similarity in physicochemical characteristics, efficacy and safety, based on a comprehensive comparability exercise [32]. Accordingly, the term “biosimilar” is a preferred expression than “generic product”, which is used for chemically synthetized active pharmaceutical ingredient (API) products.

According to the projected large reduction of healthcare cost, the arrival of biosimilars on the market could be a great improvement for patients and social health care departments. On one hand, cost studies projected that biosimilars could save the USA more than $ 250 billion between 2014 and 2024. In Europe and Asia, application of biosimilars instead of innovator therapeutic proteins could result in savings of up to 40%. However, subsequent studies showed that cost savings were not as substantial as initially thought due to the significant costs involved in the development and manufacture of biosimilars [32].

Authorities, such as European Medicines Agency (EMA) or United States Food and Drug Administration (FDA), control the market of medicines including therapeutic proteins and biosimilars in Europe and The USA, respectively. The first framework of guidance for approval of biosimilars was published in 2005 by EMA. Five years later, EMA published additional guidelines for biosimilar medicinal products (containing mAbs) highlighting the regulatory pathways needed for mAb biosimilar approval [33]. In the same year, FDA published new guidance documents for biosimilars to define recommendations for the American market [34]. Most recent guidelines were published by EMA in December 2014, entitled ‘Guideline on Similar Biological Medicinal Products containing Biotechnology Derived Proteins as Active Substance: Non-clinical and Clinical Issues’, which came into effect in July 2015 [35]. Concerning the glycobiological aspects of therapeutic proteins, the regulatory authorities highlight that this point is important but no specific guidelines are provided concerning their analysis. As an example of lack of details within the regulatory guidelines, the monograph for follitropin in the European Pharmacopeia only requires the control of sialylation rate (via the Z number calculation) but does not require any control of the possibility of immunogenic glycans [36].

The pharmaceutical industry must follow the most up-to-date guidelines for biosimilars published by EMA or FDA. For example, guidelines published by EMA define biosimilar as a product which should be highly similar to the reference medicinal product in physicochemical and biological terms. Any observed difference has to be duly justified with regard to their potential impact on safety and efficacy. In specific circumstances, e.g. for structurally more simple biological medicinal products, a comparative clinical efficacy study may not be necessary if similarity of physicochemical characteristics and biological activity/potency of the biosimilar and the reference product can be convincingly shown and similar efficacy and safety can clearly be deduced from these data and comparative pharmacokinetic data. Such an approach may have to be supported by additional data, for example in vitro and/or clinical pharmacodynamics data from a comprehensive comparative pharmacodynamics fingerprint approach [37]. Indeed, guidelines for biosimilars can become more uniform and clearer and therefore, will guide manufacturers to produce biosimilars that will truly benefit patients [38].

This review not only reports current analytical tools but also addresses issues in the characterization of glycans present on therapeutic glycoproteins. The necessity for powerful analytical tools is discussed focusing on appropriate characterization of attached N-glycans, to enable scientists to characterize and understand the role of glycans on the therapeutic glycoprotein and to understand the role of the process in the generation of the therapeutic glycoprotein.

2. Analytical methods for glycoproteins characterization

Glycomics is an emerging scientific discipline and guidelines, which define reports of glycoanalysis and associated databases, are still improving. The EMA guidelines (in Section Production and Quality Control of Monoclonal Antibodies) dictate that “the mAb should be characterized thoroughly”. Characterization of the mAb includes the determination of physicochemical and immunochemical properties, biological activity, purity, residual host cell impurities and quantity of the mAb, in line with International Conference on Harmonization (ICH) guidelines. With regard to N-glycosylation, EMA guidelines suggest that particular attention should be paid to their degree of mannosylation, galactosylation, fucosylation and sialylation and that distribution of the main glycan structures should be determined [39,40].

Determination of the main glycan structures is not enough. Content of all N-glycans structures should be reported. Even the presence of small amounts of N-glycans epitopes (such as α1-3-Gal-Gal, see Fig. 1) can be potentially dangerous (“drug allergens”) [41]. For example, the infliximab biosimilar Remsima®, which was released on European market in 2013, is produced in murine hybridoma cells as is the innovator (Remicade®). Infliximab produced in this cell line has been shown to contain low amounts (less than 5% of total amount of glycans) of glycans bearing N-glycolyl neuraminic acid residues and alpha-1,3 Gal-Gal epitopes. Recent studies revealed that there are differences between the innovator and its biosimilar particularly in the amount of sialic acid, stressing the importance of monitoring low abundance glycans, due to their potential immunogenicity [42].

Moreover, additional guidelines, which will help to standardize reporting limits for qualitative and quantitative glycoanalyzes, are essential. Reports should contain all necessary analytical information, including conditions and techniques that are used in glycans preparation, analysis that generate the primary data, and tools and parameters that are employed to process primary data to ensure reproducibility of the data at independent laboratory sites.

Analytical technologies must be accurate, precise, reproducible, robust, and scalable, must have the best possible resolution, produce minimal artifacts, and be based on separation parameters that are linked to the analyzed structure. Briefly, analyses of therapeutic proteins require state of the art techniques which typically include:

Amino acid analysis is in use in order to confirm correctness in the primary sequence and absence of mutations. Peptide mapping it is used as a confirmatory tool. Amino acid analysis is usually performed after total enzymatic or chemical hydrolysis of the therapeutic protein followed by reverse phase liquid chromatography coupled to UV or fluorescence detection. Classical post-column derivatization reagents for amino acid detection include ninhydrin, fluorescamine, dansylchloride, 4-fluoro-7-nitrobenzo-2,1,3 oxadiazole, phenylisocynate, and *o*-phthalaldehyde. For analysis of complex biological mixtures, such as samples taken during the production process of therapeutic proteins, pre-column derivatization with *o*-phthalaldehyde (OPA) and 9-fluoronylmethylchloroformate (FMOC) are used [43].

Peptide mapping enables to confirm the primary sequence and the absence of point mutations. Therapeutic proteins are usually digested by specific proteases (e.g. trypsin). Resulting peptide mixtures are separated by reverse phase liquid chromatography and detected by UV and/or high-resolution tandem mass spectrometry (HR-MS/MS). UV detection only enables detection of variation in combination with alterations in peptide retention time or selectivity due to the presence of a PTM. MS/MS enables the collection of higher quality information, including determination of the sequence of each peptide [44]. Furthermore, for glycan analysis, glycopeptide analysis can provide information regarding glycosylation site occupancy and heterogeneity. This is discussed below.

Analysis of heavy chains (Hc), light chains (Lc), Fab domains, and Fc domains (monoclonal antibody) provides information on the domain level for mAbs and is complementary to peptide mapping. Separation of the subunits of mAbs can be obtained by degradation of the mAbs using papain, endoproteinase Lys-C, pepsin and most recently immunoglobulin G-degrading enzyme (IdeS). Subunits of mAbs are usually characterized by HRMS analysis due to their larger molecular size (~25–50 kDa). HRMS analysis provides solid N-glycan profiling and micro variant identification [45].

Intact protein analysis and the detection of charge variants enables the determination of C-terminal lysine variation, asparagine-aspartic acid deamidation, sialic acid, and other variants. For the analysis of charge variants, intact proteins are commonly analyzed using exchange chromatography (IEC) coupled to UV detection. Lately, IEC has been largely improved through the implementation of different pH gradients rather than traditional salt gradient based elution, allowing for increased resolution of individual variants to be obtained [46]. Furthermore, analysis of the intact protein using reverse phase LC (RP-LC) coupled HRMS is now largely used. This analytical method enables rapid mass analysis of the proteins, which when compared to the theoretical expected mass values allows for the rapid identification of potential PTMs or point mutations in the primary sequence. If the protein is glycosylated, glycoform detection is also possible (see Section 2.1) which when performed in conjunction with the protein following deglycosylation provides information about charge variant like C-terminal lysine variation [47,48].

Detection of aggregates (dimers or multimers of the protein) is extremely important for formulation and stability assessments of the therapeutic proteins. Moreover, presence of certain aggregates can significantly change the characteristics of the therapeutic proteins, such as efficacy and immunogenicity. Aggregates are commonly detected by size-exclusion chromatography (SEC). SEC is definitely favourable method for its speed and reasonable reproducibility [49].

For a complete review on this topic, see the latest reviews Fekete et al. [50] and Reusch et al [51] and other reviews, as Sandra et al. [52] and Berkowitz et al. [53].

2.1. Characterization of glycosylation

Glycosylation characterization of therapeutic proteins has become regulatory requirement in the pharmaceutical analytics [53], due to the potential effects on activity and/or immunogenicity of the therapeutic proteins. Characterization of glycosylation includes the determination of the glycan sequences, linkage types, sites where glycans are attached to the protein, and macro- and micro-heterogeneity. Macro-heterogeneity is the heterogeneity of glycans which have variable percentage of occupancy of glycosylation site(s). While micro-heterogeneity is the heterogeneity of glycans that are attached to a specific glycosylation site [54].

In recent years, methods, instruments and reagents have rapidly progressed and current analytical strategies can provide highly detailed glycan composition. In the following section, state-of-the-art approaches for N-glycan analyses are described and discussed (Table 1). In Table 2, we suggest a choice of techniques for glycoprotein analyses. Fig. 2 illustrates analytical strategies of glycoproteins including glycan mapping and heterogeneity aspects.

2.1.1. Direct analysis of glycoforms

Direct analysis of glycoforms facilitates the analysis of glycosylation due to the minimal sample preparation. Development of such rapid methods is essential for the introduction of new glycoanalysis techniques to pharmaceutical industry. For this purpose, capillary electrophoresis (CE) and liquid chromatography (LC) both coupled to high resolution mass spectrometry (HR-MS) are largely used. CE offers excellent separation efficiency enabling the detection of individual intact glycoforms of glycoproteins, such as oxidized and acetylated variants of glycoforms. Accordingly, improvements in MS resolution facilitate the analysis of the intact molecules with higher accuracy [55,56]. Fig. 3 illustrates deconvulated mass spectra of four lots of reduced trastuzumab analyzed by RP-LC HR-MS. Only the heavy chain is shown where the major glycoforms are detected (FA2, FA2G1, FA2G2, A2G2, A2G1, A2, and M5). Furthermore, Table 3 describes the mass shift observed for the major described glycans. These mass shift values can be combined when several sites of glycosylation are present on a protein or a peptide. Intact glycoprotein analysis approach using capillary zone electrophoresis-electrospray-MS (CZE-ESI-MS) was described detecting minor glycan modifications of erythropoietin. Accordingly, excellent glycoform separation results, high dynamic range, and good sensitivity were achieved [57]. Another study describes analysis of intact recombinant human vascular endothelial growth factor (VEGF) using capillary isoelectric focusing (CIEF) coupled to the MALDI-TOF-MS [58]. Recently, it was shown that direct analysis of an intact mAb enabled quantitation of incomplete N-glycan occupancy based on HILIC separation of the glycoprotein [59]. However, analysis of intact protein for glycoprofiling may not enable the detection of low abundant glycans, therefore glycan mapping is also required for complimentary analysis of the total glycan pool present on a glycoprotein.

Table 1
Summary of glycosylation characterization analytical methods.

Glycan characterization	Separation/Detection techniques	Advantages	Disadvantages	References
Direct analysis of glycoforms	CE-HR-MS LC-HR-MS	- Intact mass profiling - PTM detection - Detection of different isoforms - Rapid method		[55–58,113,114]
Glycan mapping	CE/LC-fluorescence/ MS/ MALDI-TOF	- Detailed glycan composition - High-resolution separations - Consistent quality - Robust performance	- Time consuming (even if new rapid approaches are described)	[59,62,63,69,115]
Determination of N-glycan macro- and micro-heterogeneity	LC-MSMS (CID, ETD) LC-HRMS LC-fluorescence	- Determination of different occupying sites	- Time consuming - Requires high expertise	[65,66,113,116]
Analysis of monosaccharide content	AEC-PAD GC-MS CE-UVVIS/MS	- Determination of complex glycan structures - High sensitivity - Baseline separation - Avoidance of sugar derivatization	- Requires high expertise	[75,76]

Table 2
Proposed choice of analytical approaches for N-glycosylation characterization.

Glycan characterization	Sample preparation	Separation techniques	Detection techniques	References
Direct analysis of glycoforms	No sample preparation	CE (SI)	(HR)MS	[55,56]
Glycan mapping	Digestion by PNGase F 2-AB labeling	LC (HILIC)	Fluoresce-MS(/MS)	[115]
	NHS carbamate labeling and PNGase F (Done in only 30 min)	LC (HILIC)	Fluoresce-MS(/MS)	[59]
Determination of N-glycan macro- and micro-heterogeneity	Digested by trypsin/LysC/pepsin	LC (RP)	MS(/MS) (ETD)	[45]
	No sample preparation (If occupancy is more than 1%)	LC (RP/HILIC)	Fluoresce-MS	[117]
Analysis of monosaccharide content	Total hydrolysis by acetic acid or trifluoroacetic acid	HPAEC	PAD or MS	[75]

CE: Capillary electrophoresis; LC: Liquid chromatography; HILIC: Hydrophilic liquid chromatography; (HR)-MS: (High-resolution) mass spectrometry; RP: reverse phase; ETD: electron-transfer dissociation; HPAEC: high-performance anion-exchange chromatography; PAD: pulsed amperometric detection.

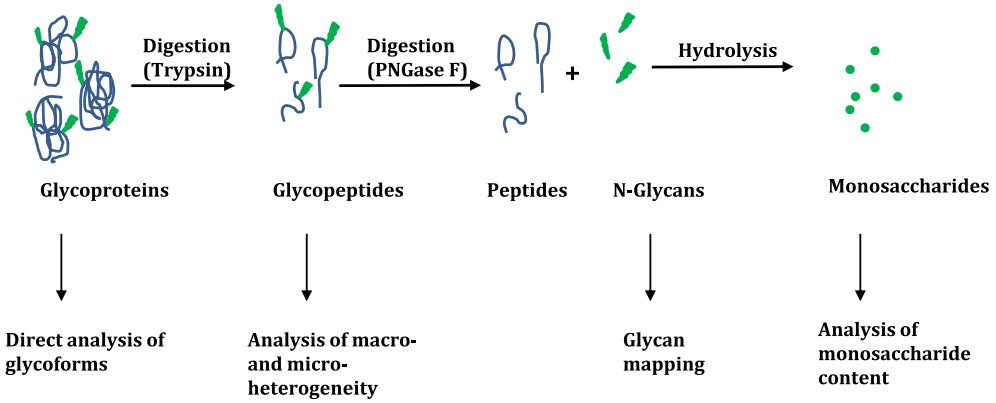


Fig. 2. Schematic picture of strategies for glycoprotein analyses.

2.1.2. Glycan mapping

Glycan mapping, or exact structural determination of glycans, is required for the release of batches of therapeutic protein. The most straightforward and reproducible way to release N-glycans is a digestion by peptide-N-glycosidase F (PNGase F) of unfolded protein. PNGase F catalyzes the cleavage between GlcNAc and asparagine amino acid residue. It releases almost all N-glycans from proteins except those bearing an α1-3 linked fucose residue attached to the reducing terminal GlcNAc residue [60,61]. In such cases, PNGase A can be used as an alternative. PNGase A shows lower efficiency in N-glycan release compared to PNGase F, but it releases N-glycans containing the α1-3 linked fucose on the reducing terminal GlcNAc residue, an epitope that is produced by insect and plant cell lines [60]. In certain cases, proteolytic digestion is recommended in order to improve the efficiency of the subsequent glycan release with PNGase F or PNGase A. However, PNGase F does not cleave glycans which are attached to the Asn residue in N- or C-terminal positions of a peptide [62]. After glycan release, glycans are separated either by liquid chromatography (LC) or by capillary electrophoresis (CE), which are coupled to appropriate detection instruments, including fluorescence and/or MS. Matrix-assisted laser desorption ionization time-of-flight MS (MALDI-TOF) analysis might also be used without any previous glycan separation and provides a mass based fingerprint of glycans

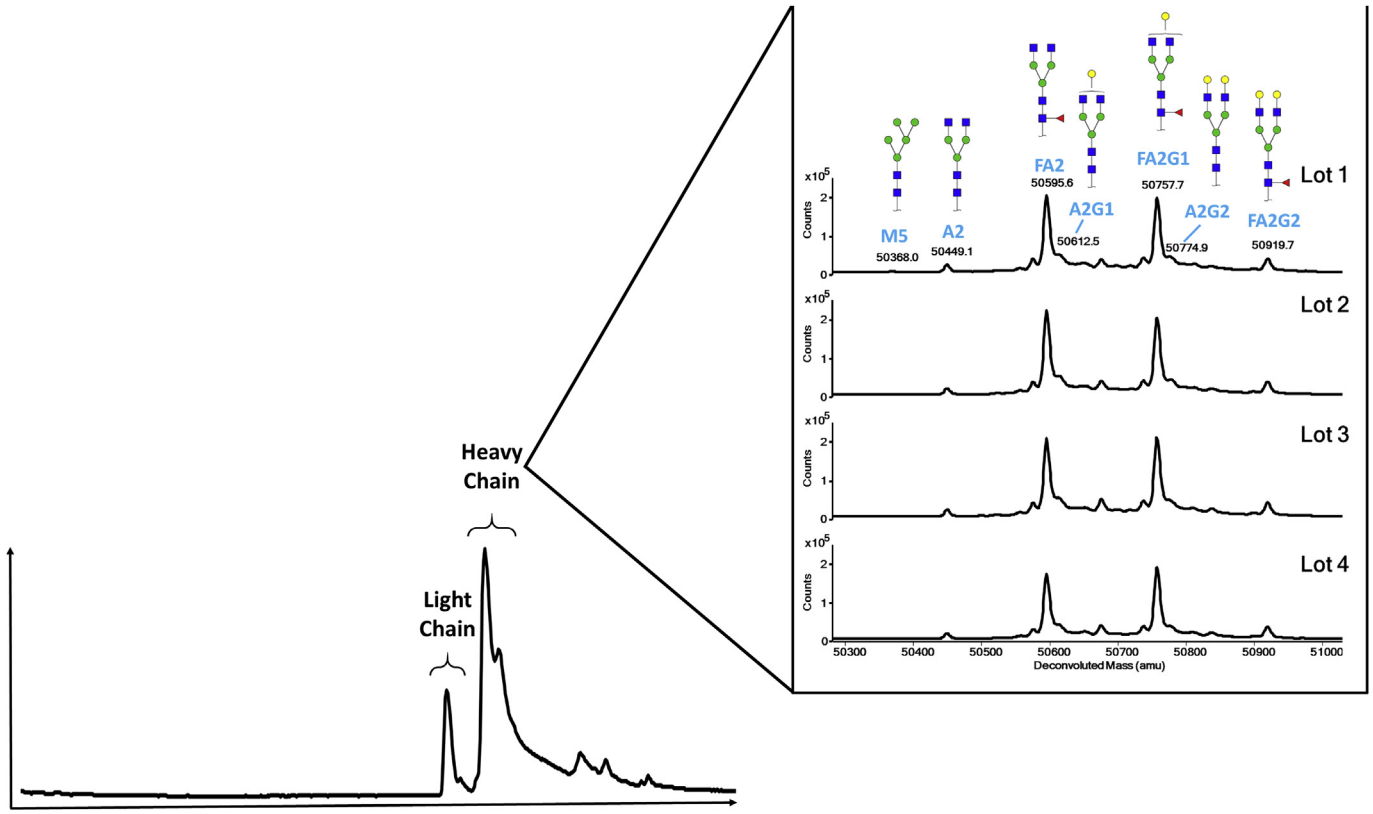


Fig. 3. Deconvulated mass spectra of the heavy chain of four lots of reduced trastuzumab analyzed by RP-LC HR-MS. The major glycoforms are detected (FA2, FA2G1, FA2G2, A2G2, A2G1, A2, M5).

Table 3
The most common N-glycans of therapeutic proteins and their formula and mass.

N-Glycan name	Formula	Delta monoisotopic Mass	Delta average Mass
A1	$C_{42}O_{30}N_3H_{69}$	1095.3965	1096.0120
A2	$C_{50}O_{35}N_4H_{82}$	1298.4760	1299.2142
A2G1	$C_{56}O_{40}N_4H_{92}$	1460.5288	1461.3574
A2G1S1	$C_{67}O_{48}N_5H_{109}$	1751.6241	1752.6241
A2G2 or A2G1Gal1	$C_{62}O_{45}N_4H_{102}$	1622.5816	1623.5007
A2G2S1	$C_{73}O_{53}N_5H_{119}$	1913.6769	1914.7497
A2G2S2	$C_{84}O_{61}N_6H_{136}$	2204.7723	2206.0076
FA1	$C_{48}O_{34}N_3H_{79}$	1241.4544	1241.1550
FA1G1	$C_{54}O_{39}N_3H_{89}$	1403.5073	1404.2974
FA2	$C_{56}O_{39}N_4H_{92}$	1444.5339	1445.3580
FA2G1	$C_{62}O_{44}N_4H_{102}$	1606.5867	1607.5013
FA2G1S1	$C_{73}O_{52}N_5H_{119}$	1897.6820	1898.7503
FA2G2	$C_{68}O_{49}N_4H_{112}$	1768.6395	1769.6445
FA2G2S1	$C_{79}O_{57}N_5H_{129}$	2059.7348	2060.8927
FA2G2S2	$C_{90}O_{65}N_6H_{146}$	2350.8302	2352.1506
FA2B or FA3	$C_{64}O_{44}N_5H_{105}$	1647.6131	1648.5450
M3	$C_{34}O_{25}N_2H_{56}$	892.3172	892.8171
M4	$C_{40}O_{30}N_2H_{66}$	1054.3699	1054.9500
M5	$C_{46}O_{35}N_2H_{76}$	1216.4228	1217.1019
M6	$C_{52}O_{40}N_2H_{86}$	1378.4756	1379.2443
M7	$C_{58}O_{45}N_2O_{45}$	1540.5284	1541.3867
M8	$C_{64}O_{50}N_2H_{106}$	1702.5812	1706.5291
FM5A1G1	$C_{66}O_{49}N_3H_{109}$	1727.6128	1728.5822

[63].

Glycan mapping is more challenging when isomeric structures are present (e.g. isobaric mass structures for MS detection and co-eluting structures in LC). Digestion of the glycans with exoglycosidase enzymes is highly beneficial in such cases, facilitating the determination of the monosaccharides present and their associated linkage information and annomericity. Stepwise digestion of the glycan pool using different combinations of specific exoglycosidases is performed, which remove terminal monosaccharides in a specific linkage orientation. Digested glycans are analyzed by LC-Fluorescence, LC-ESI-MS or MALDI-MS [64]. Linkage information is important for structural characterization of glycans. As an example, the type of sialic acid linkage can be determined using both specific sialidase NAN1 (releasing α-(2–3) linked non-

reducing terminal sialic acids) and nonspecific sialidase from *Athrobacter ureafaciens* (releasing both α(2–3) and α(2–6) linked non-reducing terminal sialic acids). Specific fucosidase, hexoaminidase, etc. are also available to identify different type of linkage [64].

Analysis limitation to the exoglycosidase digestion approach is the degree of user expertise required for successful data analysis, however, informatics tools such as GlycoBase assist with interpretation of the resulting experimental data.

2.1.3. Determination of N-glycan macro- and micro-heterogeneity

Analysis of glycosylation macro- and micro-heterogeneity usually requires digestion of intact glycoproteins to glycopeptides. Glycoproteins are digested using specific proteases (e.g. trypsin, Lys-C or pepsin etc.) [45]. Resulting peptide mixtures are commonly analyzed by LC-HR-MS/MS with N-glycopeptide fragmentation. Tandem MS analysis resulting from collision-induced dissociation (CID) of N-linked glycopeptides has recently been proven to be very effective in characterization of N-glycan microheterogeneity. Unfortunately, data processing, including MS/MS spectra of glycopeptides, is extremely time consuming and requires high expertise of the analyst even where improvements to databases and software tools for interpretation have been made [65–69]. As an alternative to CID, electron-transfer dissociation (ETD) tandem MS, which fragments the peptide backbone of glycopeptides leaving the modification intact enables easier identification of both the amino acid sequence of a glycopeptide and the unambiguous assignment of its glycosylation site [70]. For example, trastuzumab and erythropoietin have been successfully characterized using LC-ETD-QTOF-MS [71].

The application of ^{18}O based labeling for glycosylation site occupancy identification is also beneficial for macroheterogeneity analysis [72,73]. For example, occupancy changes of immunoglobulin G were detected by adding PNGase F to the immunoglobulin G, which were previously labeled with ^{18}O stable isotope labeling [74]. Occupancy analysis has also been demonstrated for intact mAbs analysis using HILIC chromatography and fluorescence detection [59].

2.1.4. Analysis of monosaccharide content

Monosaccharide analysis is the simplest form of glycan characterization, enabling a determination of the type and the relative percentage of individual monosaccharides present in the complex glycan chains of therapeutic glycoproteins. For example, if sialylation, synthesis of complex glycans or synthesis of high-mannose glycans was modulated, changes in relative presence of monosaccharides will be detected. To facilitate monosaccharide analysis, N-glycans are hydrolyzed under acidic conditions (acetic acid or trifluoroacetic acid 2–4 M at 80–100 °C for several hours, generally 2–4 h). Obtained monosaccharides are later detected by anion-exchange chromatography (AEC) coupled to pulsed amperometric detection (PAD) or MS. PAD is mainly used for the analysis of non-acidic sugars and offers numerous advantages such as high sensitivity, baseline separation and avoidance of sugar derivatization and associated sample clean up [75]. Monosaccharides can be also separated by capillary electrophoretic methods [76]. For a complete review of analytical methods for monosaccharide analysis, see Cataldi et al. [77] and Jandik et al. [78].

Another advantage of monosaccharide analysis is that it enables to study linkages. Indeed, after permethylation of glycans, acidic release of monosaccharides, deuteron-reduction and per-acetylation is performed. Using electron-impact-GC-MSMS analysis, specific monosaccharides are obtained. This method is based on known retention time of specific monosaccharides with a different acetyl tag. Described method enables to give more information about linkages [61].

2.2. Description of techniques used in the analysis of glycans

2.2.1. Glycan separation techniques

Glycans or glycopeptides are usually separated via LC or CE prior to detection. LC separations are based on analyte distribution between the stationary and the mobile phase, while CE separation is based on specific mobility of the analytes in an electrical field depending on charge, size and shape of the molecule [79–81]. LC and CE methods were compared for precision, accuracy and throughput, see Reusch et al [51], wherein it was shown that both methods have excellent precision and accuracy.

Different stationary phases are used for the liquid chromatographic separation of glycans [82] (Table 4), and were compared by Melmer et al. [83]. HILIC has become favourable method for separation of glycans mostly due to the higher solute diffusivity, higher retention, higher resolution, better sensitivity and better peak shapes compared to RP-LC [84]. Fig. 4 illustrates HILIC separation chromatogram of released N-glycans from human IgG. The only major downside of HILIC is that it demands high content of acetonitrile (up to 90%), which is harmful to the environment [85]. Porous graphitized carbon (PGC) columns are also successfully used for the separation of glycans and very polar sugar phosphates [86]. Reverse phase LC separations of anthranilic acid (2-AA) labeled N-glycans have also been described [87]. Weak anion exchange chromatography (WAX) is used for separation of charge variants of glycans, such as glycans with different number of sialic acid residues, sulphated glycans, and phosphorylated glycans [88]. In addition, nano-LC systems are used for glycans separation in case of characterization of small volume samples. Chip technology has also been used in combination with nano-LC systems [89] and a novel chip (polymeric microfluidic device with an on-chip enzyme reactor) has been developed facilitating the streamlined analysis of glycoproteins [90].

2.2.2. Glycan detection techniques

Glycans poorly absorb ultraviolet (UV) light. Therefore, detection of glycans is more demanding than detection of proteins or peptides. Glycans are usually detected by mass spectrometry or fluorescence detection after derivatization.

2.2.2.1. Derivatization/labeling and detection of glycans.. To increase the sensitivity of glycan detection, several structural modulations are described including permethylation (addition of methyl group to hydroxyl groups), modification of sialic acids (e.g. esterification of the carboxylate group or neutralization of the sialic acid [91]), reduction of the free reducing-end, modification of the reducing end by addition of a fluorescent-tag group (e.g. of the 2-AA (anthranilic acid), 2-AB (2aminobenzamide), aniline, or APTS (1-aminopyrene-3,6,8-trisulfonic acid) Table 5), and isotope labeling (e.g. addition of ^{13}C- and deuterium-labeled groups). Other labeling reagents are described in the literature and non-exhaustive list might be found in the review of D. Harvey [92].

The choice of glycan modification depends on the analytical platform used for glycan detection. Labeling might be important also in terms of separation of the glycans. Permethylation increases glycan hydrophobicity and for this reason reverse phase LC is preferred. However, due to the permethylation, the hydrophobicity can increase in such extent that glycans may not separate successfully [93]. Moreover, 2-AB labeling is the method of choice for HILIC separation due to the lack of the negative charge. APTS is highly negatively charged and is the tag of choice for CE separation, especially when laser induced fluorescence detection is used due to the compatibility of APTS with 488 nm lasers [92] (Table 5).

Table 4
Summary of the HILIC, PGC, C8–C18, and WAX characteristics.

	Mechanism	Advantages	Disadvantages
HILIC	Variation of reversed phase using a polar stationary phase, water in the mobile phase forms an aqueous-rich layer	High resolution	ACN consumption Lower resolution for highly sialylated glycans
PGC	Reverse phase, not fully determined	High resolution	Interactions with MS detector (when it is directly combined)
C8–C18	Reverse phase		Low resolution
WAX	Ion chromatography	Separation of charged glycans (sialic acid, sulphated glycans)	

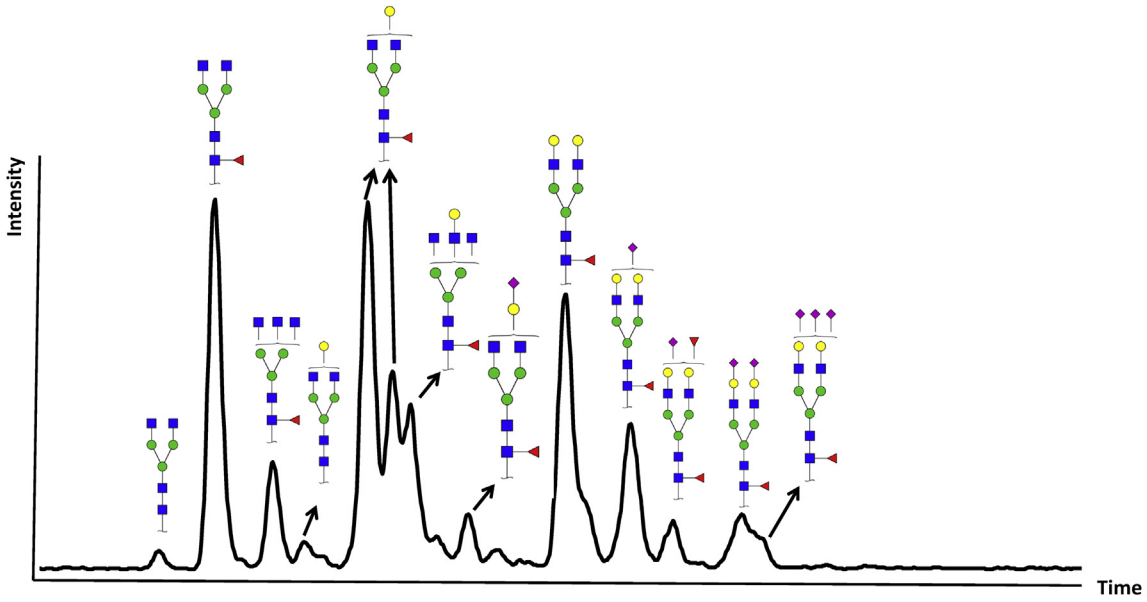


Fig. 4. Chromatogram of IgG released N-glycans using HILIC separation. Major peaks are illustrated with corresponding glycan structure.

Recently, labeling reagents have been improved and newer techniques have been introduced. N-glycans are labeled with an N-hydroxysuccinimide (NHS) carbamate rapid tagging group and a highly basic tertiary amine (for enhancing ionization). Rapid tagging has been directly integrated with PNGase F digestion prior to the LC-FLR-MS analysis. PNGase F releases N-glycans as glycosylamines which enables amine reactive NHS chemistry. Compared to other labeling processes, the aforementioned method is extremely rapid, making this labeling chemistry an excellent choice for routine analysis, such as batch-to-batch analysis. The downside of this novel labeling reagent is that it reacts with the terminal amino group of the released glycans, which is unstable and therefore the process has to be very rapid. Possible limitations of the technique include less effective labeling resulting from rapid hydrolysis of the reducing terminal glycosylamine or less effective glycan release with PNGase F due to short incubation times. This method is not inherently suitable for glycoprofiling of a new biological product, whereby N-glycans are not known in advance [59].

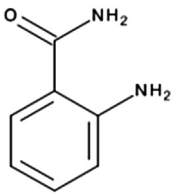
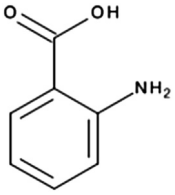
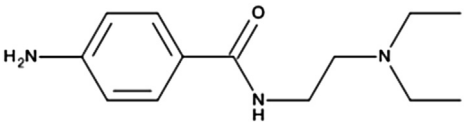
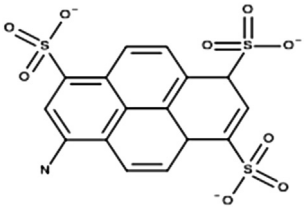
Reduction of the reducing-end of N-glycans is very important when performing LC separation. If N-glycans are not reduced, both closed ring and open chain anomeric forms of the same sugar (Fig. 5) might be separated by LC. This phenomenon leads to two chromatographic peaks for a single N-glycan. Reduction of the reducing terminal GlcNAc to its corresponding alditols avoids this anomeric interconversion but prevents further derivatization of the reducing terminal with reagents for optical or fluorescence detection [92].

Combined fluorescence and MS detection of labeled N-glycans enables informative glycan characterization due to the ability to generate simple fluorescence chromatograms but also to collect mass information for the glycan species present in each chromatographic peak. When fluorescence is followed by MS detection, several tags can be used. For example, 2-AA in negative mode MS detection is preferable to 2-AB, as 2-AA due to facilitated formation of the pseudomolecular anion of 2-AA labeled glycans in negative ion mode. In positive mode, procainamide has shown better ionization efficacy and better identification of minor glycans than 2-AB due to the presence of the quaternary amine functionality within the molecule (see Table 5 for preference ionization mode) [94].

2.2.2.2. Fluorescence detection. Fluorescence detection is generally more sensitive than MS detection for glycans. However, MS detection enables deeper structural determination of glycans. Fluorescence detection usually requires further experiments, e.g. exoglycosidase digestion or/and use of normalization and database searching for structural confirmation. LC coupled to the fluorescence detector has become standard technique for characterization of 2-AA and 2-AB labeled N-glycans [92]. Accordingly, several kits are available for N-glycan labeling.

Disadvantage of fluorescence detection compared to MS detection is that glycans have to be labeled before being detected. For this reason, sample preparation for fluorescence detection is time consuming compared to MS characterization [95]. Moreover, labeling reagents are usually present in large excess and later they have to be removed by additional cleaning steps using e.g. size-exclusion chromatography (SEC) and/or solid-phase extraction (SPE) [96,97]. For example, SPE is a commonly required method for the permethylated glycans purification, as permethylated glycans

Table 5
The most commonly used reducing end tags for glycan fluorescence detection [100].

Reducing-end tag name	Abbreviation	Structure	Separation/detection techniques
2-aminobenzamide	2-AB		LC (HILIC)-favourable Fluoresce MS (negative mode)
2-amino benzoic acid (Acide Anthranilic)	2-AA		LC (HILIC) Fluoresce MS (negative mode)- favourable
Procainamide			LC (HILIC) Fluoresce MS (positive mode)
8-aminopyrene-1,3,6-trisulfonic-acid	APTS		CE Fluoresce MS (negative mode)

are retained onto the solid reversed-phase of SPE where 2-AB or 2-AA labeled glycans can be clean-up using HILIC phase SPE. However, this purification step requires time and increases the potential for loss of material [98].

2.2.2.3. MS detection. Recently, a review paper focusing on MS for glycosylation analysis has been published, see Dotz et al. [99], which provides an extensive review of MS methods, including MALDI-MS, direct ESI-MS/(MS), LC-ESI-MS, CE-MS, and IM (ion mobility)-MS. Another review article has been published by Leymarie and Zaia [100], which focuses on tandem MS and describes dissociation mechanisms and product ions that arise there from. The majority of the methods available for glycans characterization are also extensively described in Alley et al. [101]. Hereinafter, we focused on these aspects that are not discussed in the mentioned review articles.

The major points to consider before starting analysis of glycans using MS detection are: the use positive or negative mode?; will MS suffice or is MS/MS required?; is high resolution MS analysis required?; what way will the data be collected?; which informatics tools will be used to analyze the resulting data? For example, to analyze MSMS fragmentation of human plasma IgG, negative mode high resolution MS/MS are useful as exact mass and arm specific fragments can be generated. Fig. 6 illustrates MS/MS spectra of FA2, FA2G1, and FA2G2S1 N-glycans.

Data collection using negative or positive mode is a major consideration. Positive mode is generally more sensitive but negative-ion formation provides more information than positive-ion formation about specific compositions, such as location of fucose. However, it is harder to achieve negative-ion formation of neutral N-glycans in certain cases, for example by using MALDI-MS. In order to provide better MS spectra using MALDI-MS in negative mode, special matrixes are in use, such as 6-aza-2-thiothymine and 2,4,6-trihydroxyacetophenone. Moreover, permethylation of N-glycans and characterization by RP-LC-MS provides a solution to detect neutral N-glycans and sialylated N-glycans simultaneously with normalized response factors.

In general, the stability of N-glycans is high in comparison to the stability of proteins. Recently N-glycans analysis in mAbs by a twoplex method using LC-fluorescence-MS detection, showed excellent stability even following exposure to forced degradation conditions [102].

However, it is known that N-glycans can be degraded or suffer from rearrangement during MS analysis. Terminal sialic acid and terminal fucose are indeed rather unstable during CID MS/MS. Several methods of derivatization are available to improve the stability of sialic acid and fucose. Rearrangements of fucose within the MS were observed in several experiments. Permethylation is certainly the most referenced technique and has been used for a long time in order to increase the stability of N-glycans in MS [61]. This derivatization technique also avoids rearrangements or loss of terminal sialic acid and terminal fucose during MS/MS.

For example, fucose rearrangement products can be detected after CID fragment-ion analysis in certain conditions, which makes the interpretation of the fragmentation spectrum more difficult. Fucose rearrangement products can be misleadingly interpreted as B-type ions of structural isomers. Indeed, when glycans are fragmented it has been demonstrated that rearrangements might occur generating potential fragments that cannot be synthetized *in vivo*. It is noteworthy that permethylation can solve the phenomenon of rearrangements [103,104].

Sialylated glycans have lower ionization efficiency than neutral glycans in positive mode and have been shown to lose terminal sialic acid during the MS analysis. Several methods of derivatization

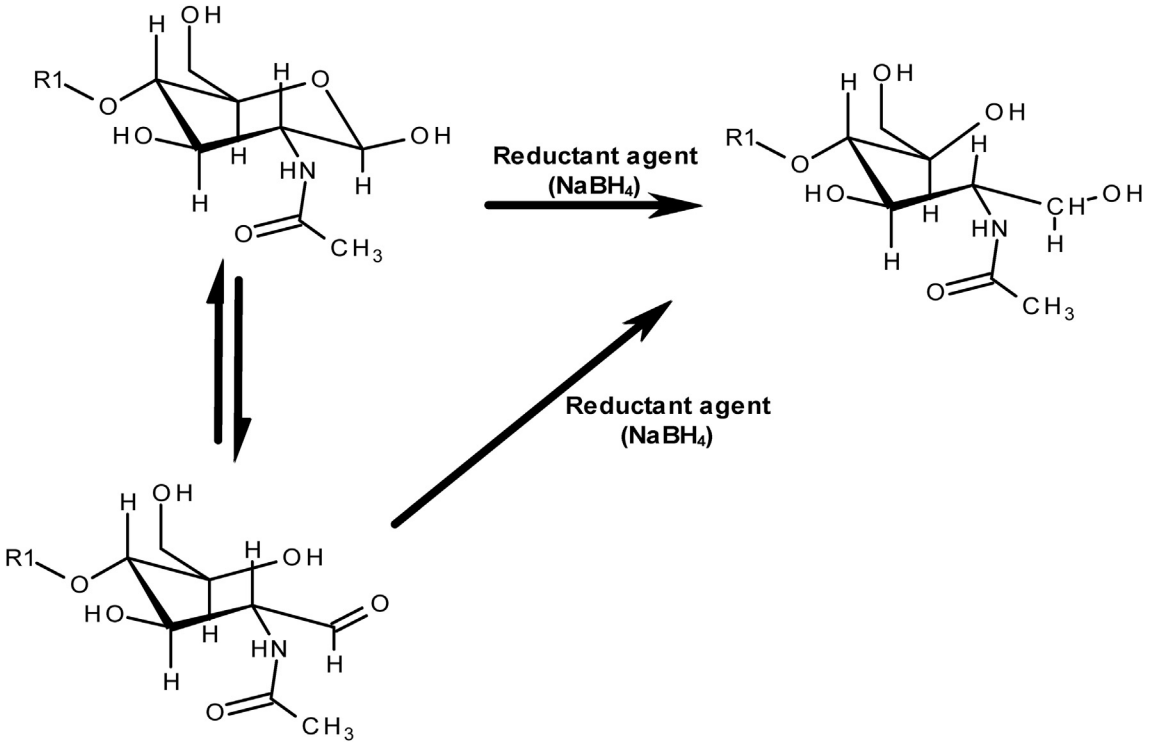


Fig. 5. Reduction of reducing-end of glycan.

are currently in use, which improve the stability of sialic acid residues by neutralizing their negative charge. Morelle and Michalski described several methods of derivatization, for example, methyl esterification of the carboxyl group of sialic acid residues, which allows simultaneous analysis of neutral and sialylated oligosaccharides in the positive ion mode by MALD-MS. Methylation is commonly used to stabilize the sialic acid residues [61]. Tousi et al. in their study combined linkage specific derivatization of sialic acids (reaction with DMT-MM) with nanoscale HILIC separation prior to HR-MS analysis. Furthermore, their derivatization also improves HILIC separation [105].

2.2.2.4. Detection of isomeric forms and linkage analysis.. Some glycans exist in multiple isomeric forms, with the same mass but different structure. With a view to distinguishing isomers, detailed structural characterization is required. Combination of specific exoglycosidase digestions and LC coupled to tandem MS (LC-MS/MS) or fluorescence is usually applied [106].

More recently, ion mobility MS (IM-MS) was shown as an interesting tool for isomeric glycan analysis. This analyzer brings one supplemental dimension to MS by introducing a separation based on the mobility of ions in a carrier gas. For more details about ion mobility approach see review of Yamaguchi et al. [107] and Harvey et al. [108].

2.3. Bioinformatics tools in analytics

Considering that glycomics is still in its infancy relative to the more mature omics such as genomics, transcriptomics and proteomics, accordingly, bioinformatics tools for the interpretation of glycomics data lag behind their other omics counterparts. Usually interpretations of glycomic data require high knowledge of glycobiology and considerable manual input. Advanced glycosylation characterization approaches reflect in creation of several new databases and bioinformatics tools. The purpose of databases is providing help to those who are dealing with glycosylation characterization [109]. There are several available databases containing N-glycan and O-glycan structures, for example Carbbank, the Consortium for Functional Glycomics, EUROCarbDB, GLYCOSCIENCES.de, KEGG, CFG's Glycan Structures Database, GlycoBase Database, etc. [110].

As an illustration, CFG's Glycan Structures Database provides detailed structural and chemical information about glycans, including both synthetic glycans and endogenous glycans. This database provides links to other external databases, which include additional information, such as binding sites and 3-D modeling feature.

Another example is GlycoBase Database which has been created in order to help researchers to determine glycan structures in samples. It is based on systematic analysis of released glycans from many different glycoproteins. Retention time for each glycan corresponds to the specific dextran ladder value, expressed as Glucose Units (GU values). Currently, GlycoBase 3+ contains GU values for more than 600 2-AB labeled N-glycans [111].

Another software, GlycoWorkBench (EUROCarbDB design study), was developed to help researchers to draw glycans, to determine glycan mass and to analyze MS and MSMS data. The software is even coupled to other databases [112]. Moreover, other software, such as SimGlycan® (Premier Biosoft International, Palo Alto, CA, USA), or online tools for glycan identifications, such as GlycoPeakfinder, were developed. However, there is still a lack of software required to make data processing really easier.

3. Perspectives and concluding remarks

Therapeutic glycoproteins are currently the most promising therapeutic group for the treatment of diseases, such as certain types of cancers and immune deficiencies. The exponential growth of the therapeutic glycoprotein market and the release of several biosimilars make the analytics of glycoproteins a major focus for

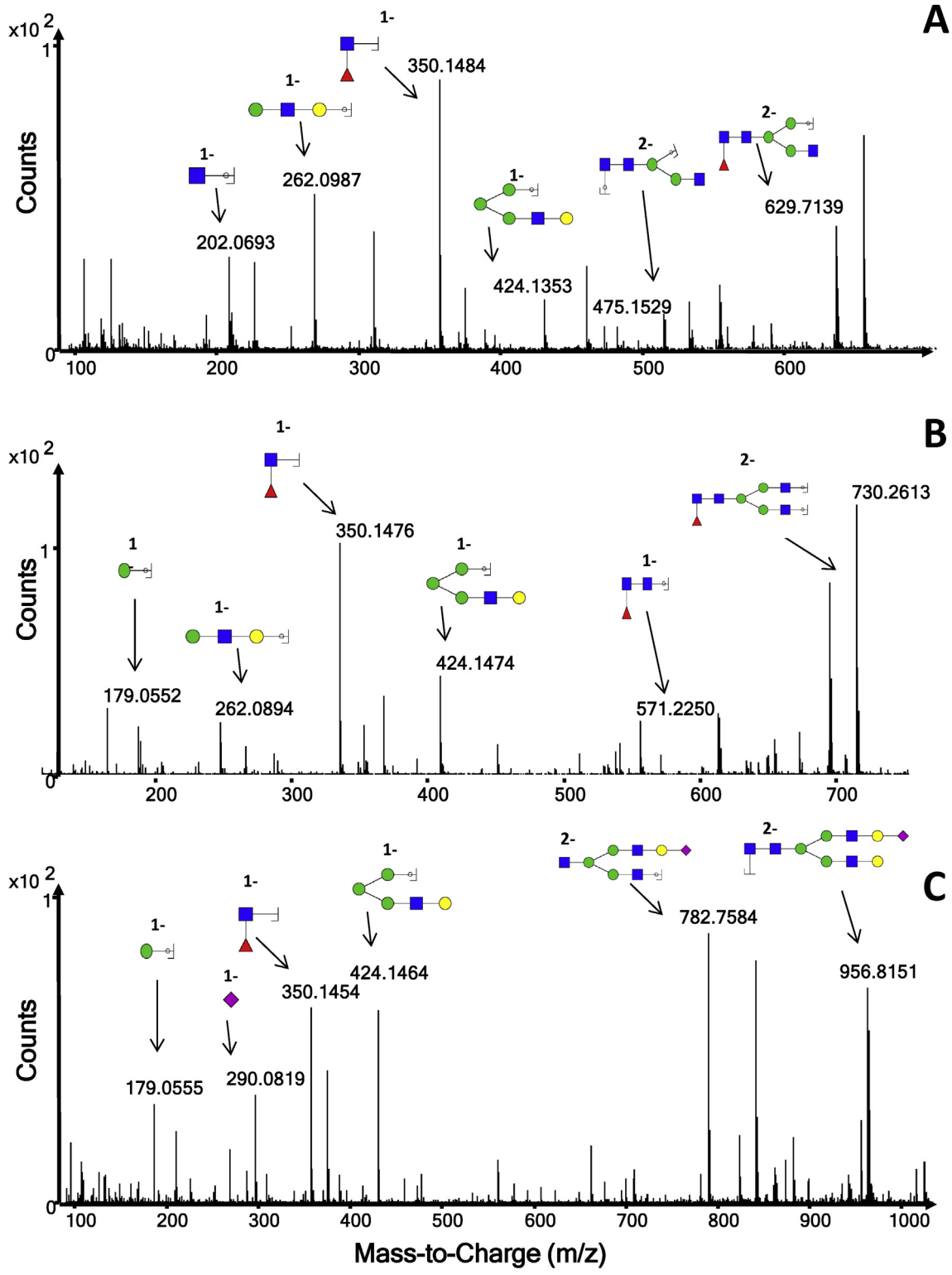


Fig. 6. MSMS spectra of (A) FA2 (B) FA2G1 and (C) FA2G2S1. The major peaks of the fragments are illustrated with the corresponding structure.

the future. Therapeutic glycoproteins have complex structures and consequently it is necessary to have access to advanced characterization methods in order to demonstrate safety and quality from the resulting physiochemical analytical data. Progressively, glycosylation characterization is becoming more and more important. Correct glycosylation patterns and batch-to-batch reproducibility of glycosylation are essential. Furthermore, in the near future, many patents of original therapeutic glycoproteins will expire. Therefore, the importance of biosimilar development and their characterization are also gaining significant importance. In this context, more efficient and faster analytical methods as well as better statistical approaches are required.

Indeed, there is a lack in the harmonization within regulatory guidelines, especially in the context of authorities' requirements.

The latter are indeed constantly being reviewed according to the new findings in the field. This will help to achieve more standardized reports for qualitative and quantitative glycoanalyzes. In the area of glycosylation characterization guidelines, only major glycans of certain therapeutic proteins are controlled. Although it is well known that specific glycan epitopes, as galactose-α-1,3-galactose (α-1,3-Gal) motive and terminal N-glycolylneuraminic acid (NeuNGc), can be potentially immunogenic even in very small quantities. Therefore, approaches which can detect very small changes in glycosylation profiles of the therapeutic glycoproteins, are of great importance.

With a view to introducing more detailed glycoanalysis to the industry, faster and improved methods are needed. In the last decade, many new approaches have been developed in terms of glycans and glycopeptides analysis, in the field of derivatization, separations and detection. Certainly, even better approaches for glycoanalysis are in progress. New sample preparation approaches might be promising, such as online systems, 2D-LC separations, rapid PNGase F digestion, etc. Those new approaches can shorten the protocols of glycoanalysis from one week to only a few hours, which is extremely promising. Furthermore, sensitivity of developed methods is constantly improving.

Although the analysis of released glycans is often preferred for glycobiological analysis, new instruments, columns, phase chemistry, and MS resolution also allow analysis of intact protein or glycopeptides. These methods are becoming increasingly popular and might become the standard quality control of therapeutic glycoproteins and might be implemented in the guidelines that will be released by authorities in the future.

Data processing also need to be improved. There is a need to obtain conclusions from MS and/or fluorescent data in a shorter timeframe. Nowadays, extraction of mass spectrometry data is still very time-consuming and appropriate statistical approaches should be applied in order to simplify data processing with the view to helping the manufacturers to assess the changes in glycosylation more easily and faster.

Author contributions

Planinc, A., Delporte C., Bones J. and Van Antwerpen P. designed the concept for the manuscript.

Planinc A. and Delporte C. wrote the manuscript.

Bones J., Van Antwerpen P. and Dejaegher B corrected the manuscript.

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