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REVIEW

Glycan analysis of therapeutic glycoproteins

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ABSTRACT

Therapeutic monoclonal antibodies (mAbs) are glycoproteins produced by living cell systems. The glycan moieties attached to the proteins can directly affect protein stability, bioactivity, and immunogenicity. Therefore, glycan variants of a glycoprotein product must be adequately analyzed and controlled to ensure product quality. However, the inherent complexity of protein glycosylation poses a daunting analytical challenge. This review provides an update of recent advances in glycan analysis, including the potential utility of lectin-based microarray for high throughput glycan profiling. Emphasis is placed on comparison of the major types of analytics for use in determining unique glycan features such as glycosylation site, glycan structure, and content.

Abbreviations: AE, anion-exchange; CE, capillary electrophoresis; cIEF, capillary isoelectric focusing; ESI, electrospray ionization; HILIC, hydrophilic interaction liquid chromatography; HPAEC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; HPLC, high-performance liquid chromatography; ICH, International Conference on Harmonization; IEF, isoelectric focusing; IEX, ion-exchange; IM, ion-mobility; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PAGE, polyacrylamide gel electrophoresis; PGC, porous graphitic carbon; RP, reversed-phase; SEC, size-exclusion chromatography

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Introduction

Many biopharmaceuticals are glycosylated proteins (also named glycoproteins) produced by living cell systems. These include monoclonal antibodies (mAbs) and other recombinant protein products (e.g., fusion proteins, growth factors, cytokines, therapeutic enzymes, and hormones), which are approved (*see* examples in Fig. 1A–B and supplement I) or under development as treatments for cancer, autoimmune diseases, and other life-threatening conditions. Appropriate glycosylation of a therapeutic protein is critical for product solubility, stability, pharmacokinetics and pharmacodynamics (PK/PD), bioactivity, and safety (e.g., immunogenicity).[1] It is well documented that protein glycosylation not only increases protein stability in vitro, but also protects proteins from proteolytic degradation in vivo.[2–5] In this regard, non-glycosylated erythropoietin is known to be more susceptible to denaturation or degradation induced by chemicals, pH changes or heating conditions compared to the fully glycosylated form.[6]

Protein glycosylation can also influence PK/PD properties of therapeutic glycoproteins.[2,7] There is evidence that partially glycosylated proteins, which usually contain terminal galactose, have much shorter circulatory lifetimes compared to fully glycosylated proteins with terminal sialic acid. This is mainly due to the binding of galactose with hepatic asialoglycoprotein receptors expressed on hepatocytes, which promotes hepatic clearance of the partially glycosylated protein.[8] Other lectin-like receptors with binding specificity to terminal mannose, N-acetyl-glucosamine (GlcNAc) or fucose also contribute to clearance of glycoproteins containing these glycans.[2] Additionally, IgG Fc glycosylation is critical to many antibody effector functions through modulating Fc-FcγR interactions.[9–12] Human FcγR family includes activating (FcγRIa, FcγRIIa, and FcγRIIIa) and inhibitory (FcγRIIb) receptors. Fc glycosylation plays important roles in modulating antibody binding affinities with FcγRs or C1q on effector cells, and thus affects immune effector functions such as antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and complement-dependent cytotoxicity (CDC) (Table 1).[13] Optimization of Fc glycosylation has been explored to enhance the ADCC activity of therapeutic mAbs.[14]

Protein glycosylation is a complex post-translational modification (PTM) involving attachment of glycans at specific sites on a protein, most commonly at Asn (*N*-linked) or Ser/Thr (*O*-linked) residues. The *N*-linked glycosylation occurs at the consensus sequence of Asn-X-Ser/Thr (where X is any amino acid except proline). By contrast, *O*-linked glycans are usually attached to serine (Ser) or threonine (Thr) residues via an α-*O*-glycosidic bond formed between *N*-acetylgalactosamine (GalNAc) and the hydroxyl group (-OH) of Ser/Thr (murine α-GalNAc *O*-glycans).[15] Protein *N*-glycosylation starts in the endoplasmic reticulum (ER), where a glycan chain ($Glc_3Man_9GlcNAc_2$) is added to Asn of the Asn-X-Ser/Thr sequon. The glycan chain is then trimmed by various glycosidases and the resultant glycoprotein is transported to the Golgi

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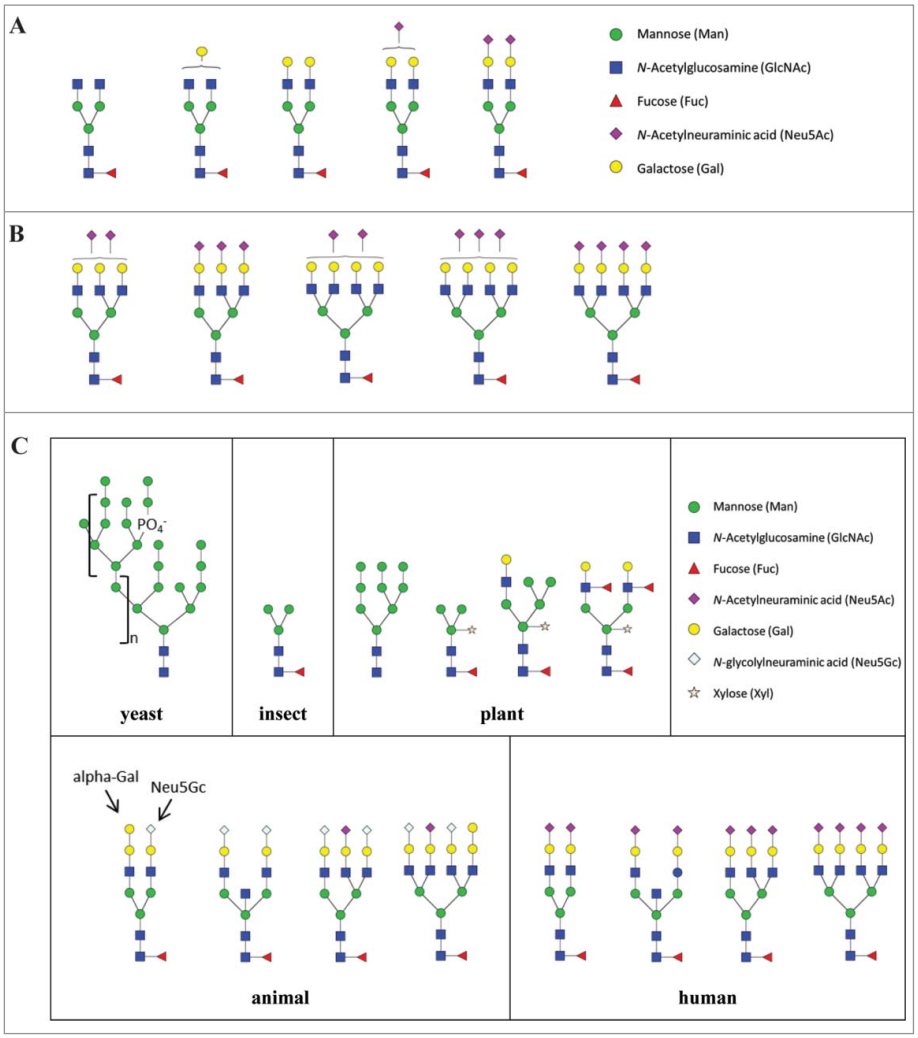

Figure 1. Examples of *N*-glycans found in recombinant glycoproteins. Shown are typical glycan structures for therapeutic humanized IgG1 mAb (A), recombinant human erythropoietin (B), and *N*-glycans produced in the commonly used expression systems (C) (derived from Ghaderi D et al. 2012).[115]

apparatus for further modifications. Within the Golgi, protein glycans are subjected to a multi-step trimming and modification process, which is catalyzed by various glycan-processing enzymes, resulting in diverse *N*-glycan structures such as high-mannose, complex, and hybrid glycans.[16] Protein *O*-glycan biosynthesis is initiated in the Golgi by directly transferring GalNAc from UDP-GalNAc to Ser/Thr residues.[17] The *O*-GalNAc precursor undergoes further derivations, thereby producing up to 8 *O*-GalNAc glycan core structures with 4 (core 1, 2, 3 and 4) of them commonly seen in mammals.[18]

Table 1. Potential impacts of Fc glycosylation on therapeutic mAbs.[13]

Glycans/glycosylation	Impacts
α1–3-galactose; *N*-glycolylneuraminic acid	Immunogenicity
Terminal sialylation	↓ binding to FcγRIIIa, ↓ ADCC; ↑ PK/PD
Afucosylation	↑ binding to FcγRIIIa, ↑ ADCC, ↑ ADCP
Galactosylation	↑ binding to C1q, ↑ CDC, moderate effect on ADCC
High-mannose	↓ PK/PD; ↑ binding to FcγRIIIa, ↑ ADCC; ↓ binding to C1q, ↓ CDC

↑ positive impact; ↓ negative impact.

To add complexity, protein glycosylation is influenced by the type of host cells and fluctuations in fermentation conditions (e.g., media, pH, temperature, agitation).[19] Therapeutic glycoproteins can be produced by mammalian cells, yeast strains, plant cells, or genetically modified animals in which each host system has its own unique glycosylation machinery that produces proteins with distinct glycan patterns (Fig. 1C and Supplement I). For instance, mammalian cells (e.g., Chinese hamster ovary cells) are widely used for production of therapeutic glycoproteins containing human-like glycans. However, mammalian cell-expressed proteins may also contain minor non-human glycans such as *N*-glycolylneuraminic acid (Neu5Gc) and terminal α1-3-Galactose (α-Gal) modification (Fig. 1C). By contrast, yeast strains usually express proteins with high content of mannose (up to 100 units). Other platforms for expressing therapeutic glycoproteins include engineered plant cells and genetically modified animals.[20,21] However, these systems may produce proteins with non-human glycan variants such as xylose, Neu5Gc or terminal α-Gal, which are known to be immunogenic.[21] Due to the complexity of the glycosylation process, glycoproteins produced by living cell systems usually contain macro- and micro-heterogeneity in terms of glycosylation patterns. Macro-heterogeneity

refers to the variability of glycosylation sites and glycan numbers while micro-heterogeneity concerns glycan structural variations at a specific site. Both macro- and micro-heterogeneities contribute to diversity of glycoforms, which share identical amino acid backbones, but may be different in glycosylation sites, glycan contents or structures.[22]

Analytics for therapeutic protein glycosylation

The multiple levels of glycan heterogeneity pose a daunting analytical challenge. In the development of therapeutic glycoproteins, especially biosimilar products,[23] glycan analysis usually involves the use of complementary methods for assessing specific glycosylation attributes, including glycosylation site, glycan structure and abundance.[24–26] The analytical methods, which include high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), mass spectrometry (MS), isoelectric focusing (IEF), and lectin-based microarray, are grouped and discussed according to their utility in analyzing intact glycoproteins, glycopeptides, released glycans, and monosaccharides (Fig. 2).

Glycan profiling of intact glycoproteins

Intact glycoproteins can be directly analyzed for general glycan patterns and glycan heterogeneity. In this regard, electrospray ionization - time of flight (ESI-TOF) MS coupled with reversed-phase (RP) HPLC or size-exclusion chromatography (SEC) have been commonly used.[27] RPLC-MS provides better chromatographic separation of protein variants[27] and has shown ability in the detection of intact mAb with 10 ppm accuracy (approx. 150,000 ± 1.5 Da), but the procedures require high column temperatures (60–80°C).[22,28,29] SEC-MS using either a non-denaturing or denaturing mobile phase is operated at room temperature and produces good quality of mass spectra, but SEC resolution is relatively lower than RP.[30-32] The LC-ESI-MS approach has been applied to glycoform profiling of more complex therapeutic proteins such as erythropoietin, which contains one *O*- and 3 *N*-glycosylation sites.[33] While traditional ESI-TOF-MS analysis of even more heterogeneous glycoproteins such as darbepoetin alfa (known to contain 5 *N*-glycosylation sites) under denaturing conditions remains challenging, the recently developed Orbitrap Exactive™ Plus high-resolution mass spectrometer enables accurate determination of highly complex glycan profiles at the native intact protein level. This technology takes advantage of the substantially fewer charges on a given protein when ionized in an aqueous ammonium acetate buffer, where the protein retains more folded "native" structure.[34,35] In addition to ESI-MS, matrix-assisted laser desorption ionization (MALDI) MS can be used for fast glycan analysis of small intact proteins, but with a lower mass accuracy.[36]

To increase the sensitivity of MS analysis of mAbs, one approach has been to reduce a mAb into individual heavy and light chains.[28,37] For this purpose, commonly used reducing reagents include dithiothreitol (DTT), tris(2-carboxyethyl)phosphine (TCEP), β-mercaptoethanol (β-ME) and mercaptoethylamine (MEA).[38] Alternatively, a mAb can be cleaved into smaller fragments using selective proteases such as papain, pepsin, IdeZ, and IdeS.[39,40] Papain cleaves IgG above the hinge region into 3 fragments, 2 Fab and one Fc, while pepsin cleaves IgG below the hinge region to produce $F(ab')_2$ fragment and a degraded Fc fragment. IdeZ and IdeS both cleave IgG to yield a $F(ab')_2$ fragment and an intact Fc fragment.

The heterogeneity of glycoforms can also be analyzed by CE-MS.[41–43] Additionally, protein sialylation heterogeneity can be analyzed at the intact glycoprotein level using charge-based electrophoresis such as IEF,[41,44] CE,[45] capillary isoelectric focusing (cIEF),[41,46] or ion-exchange (IEX) chromatography.[43,47] These approaches are commonly used for quality control testing;[48] however, although IEF and IEX are traditional methods, they are incompatible with MS and time-consuming. CE and cIEF are emerging technologies with attractive features such as high speed, high resolution, and compatibility with MS, but their drawback is that the capillaries tend to adsorb intact proteins.[49]

Analysis of glycopeptides

Characterization of protein glycosylation site(s) and occupancy is usually achieved at the glycopeptide level using ESI-MS or MALDI-MS.[50,51] This approach is also important for characterization of *O*-glycans because it has been difficult in achieving quantitative *O*-glycan release (US. Pharmacopoeial Convention (USP) general chapter <1084 >).[52,53] Other PTMs can also be detected through peptide analysis. The analytical procedures start with digestion of glycoprotein into suitably sized glycopeptides by a highly specific protease such as trypsin, Lys-C, or Glu-C based on the sequence of the protein as well as the glycan modifications close to the peptide backbone. A combination of multiple proteases may be necessary in specific cases like erythropoietin.[54]

Following digestion and prior to MS analysis, enrichment of glycopeptides by HPLC is often required in order to overcome ion suppression of glycopeptide signals by regular peptide signals.[55] This can be achieved by coupling HPLC with ESI-MS in an online manner, or with MALDI-MS in an off-line manner, where the LC-ESI-MS with online separation is a more efficient approach, and thus is most widely used. As for traditional RPLC-MS analysis using C18 columns, detection of low-abundant glycopeptides may be challenging due to the ion suppression of co-eluting glycopeptides with high abundance. Hydrophilic interaction liquid chromatography (HILIC) provides better separation of hydrophilic glycopeptides as well as regular peptides.[56] However, glycopeptide isomers with the same mass cannot be distinguished by this approach. A novel nano-LC-MS method was recently introduced to differentiate glycopeptide isomers by using microfluidic chip-based capillaries packed with porous graphitic carbon (PGC) stationary phase, which can resolve isomeric glycopeptides containing very short peptide moieties (as short as 3 residues) produced by unique and nonspecific digestion enzyme pronase E.[57] On the other hand, a high-throughput work flow for monitoring Fc-glycosylation during fermentation was tested where the IgGs were purified by protein A immobilized on 96-well plates, and the tryptic glycopeptides were extracted from the plates by HILIC beads followed by ESI-MS analysis without HPLC separation of the extracted glycopeptides.[58]

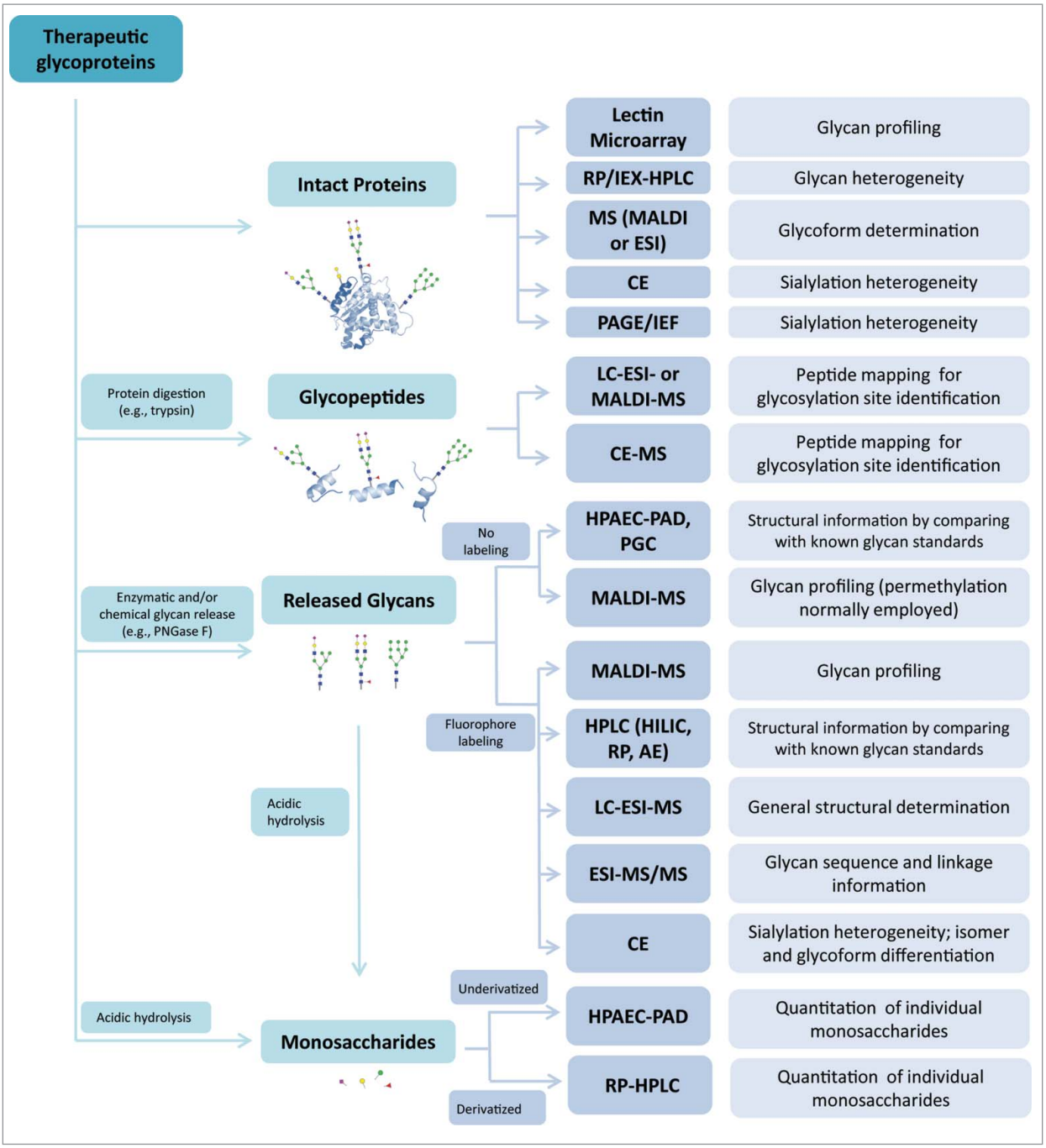

Figure 2. Overview of analytics for assessing glycans. The commonly used methods for glycan analysis are divided into 4 main groups per their applications in analyzing intact proteins, glycopeptides, released glycans or monosaccharides (*see* detail in the text). These methods are usually used in combination to determine glycan profiles, glycan structures and heterogeneity, glycosylation site(s) and the content of specific glycans.

Compared to ESI, MALDI is more suitable for rapid glycopeptide profiling because of the relatively simple mass spectra resulting from the singly charged ions, but its drawback is related to degradation of the underivatized sialylated glycopeptides due to in-source and post-source decay,[59] which could be avoided by sialic acid derivatization prior to MALDI-MS analysis.[60]

Tandem MS analysis of glycopeptides allows determination of glycosylation site(s) and glycan composition present on a glycopeptide. The recently developed in-source electron-transfer dissociation (ETD), which predominately cleaves the peptide bonds, can be used to identify the glycosylation site(s) of glycoproteins.[61] Classical collision-induced dissociation (CID) method, on the other hand, mainly cleaves the glycan moiety attached to a glycopeptide, which generates information about carbohydrate composition and sequence.[50,54] The combination of these methods in one LC-MS experiment could provide an efficient sequencing of glycopeptides.[61,62]

CE-MS is also a useful tool in determining site-specific glycan micro-heterogeneity of glycoproteins at the glycopeptide level.[63] In traditional RPLC-MS, highly hydrophilic peptides may have insufficient interaction with the RP chromatography matrix, and thus get lost during the loading and desalting step. In this case, CE-MS can be a good alternative to detect these

small hydrophilic peptides and achieve complete sequence coverage.[63,64]

Analysis of released glycans

N-linked glycans on a glycoprotein can be released by an amidase such as peptide-*N*-glycosidase F (PNGase F). *O*-linked glycans are commonly released through reductive alkaline β-elimination.[65] However, it is difficult to achieve complete glycan release and the unreleased glycans are left undetermined. Released glycans can be rapidly profiled by MALDI-TOF MS where glycan permethylation is usually employed to improve ionization efficiency and to stabilize sialylated glycans during simultaneous analysis of neutral and sialylated glycans in the positive-ion mode.[27,66,67] Moreover, with linkage-specific sialic acid esterification, not only the sialic acids can be stabilized, but different sialic acid linkages (α2–3 and α2–6) can also be distinguished by MALDI-MS.[68]

To facilitate HPLC- and MS-based glycan analysis, released glycans are usually fluorescently labeled through reductive amination. The two most commonly used fluorescent tags are 2-aminobenzamide (2-AB) and 2-aminobenzoic acid (2-AA).[69] 2-AB lacks negative charges and is used as a gold standard for HPLC approaches with extensive database established, but shows low ESI ionization efficiency. 2-AA carries one negative charge, and thus is suitable for CE and MALDI analysis.[69] A recent study indicated that procainamide [4-amino-N-(2-diethylaminoethyl) benzamide]-labeled *N*-glycans show enhanced ESI ionization efficiency and fluorescence intensity comparing to 2-AB-labeled glycans, and thus improve identification of low abundance *N*-glycans.[70] Prior to HPLC and MS analysis, purification steps are usually required to remove excess tags and salts, which include HILIC, RP, PGC, or gel filtration approaches based on the labeling method.[69]

The labeled glycans can be separated using chromatographic approaches such as HILIC, RP-HPLC, or anion exchange HPLC (AE-HPLC). HILIC has shown better resolution in separating glycans over other HPLC methods.[71–73] The elution profile of 2-AB-labeled glycans is compared against a 2-AB-labeled dextran ladder (glucose homopolymer), yielding an estimation of the number of glucose units of each species.[67,74] This information is then used to calculate the abundance of individual monosaccharides, which allows prediction of glycan structure.[75] On the other hand, *N*-glycans without fluorescence-labeling can be separated by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) or PGC chromatography,[74,76] but with limited robustness and reproducibility.[73]

Released glycans, upon fluorescence labeling, can also be determined using CE at high sensitivity and resolution. The fluorescent tag 8-aminopyrene-1,3,6-trisulfonate (APTS) is widely used for CE analysis of oligosaccharides;[77,78] sometimes other labeling reagents such as 2-AA are also used.[79] This approach offers a unique ability to differentiate isomeric glycan structures.[77–81] However, only a limited number of glycan standards is available for use in making the assignment of a glycan structure to a corresponding CE peak signal. In this case, sequential exoglycosidase digestion of oligosaccharide followed by CE analysis has the potential to provide detailed carbohydrate sequence information.[81,82] When hyphenated with MS, CE-MS proves to be useful in peptide mapping and glycoform characterization;[42,64,83–85] however, its application in oligosaccharide analysis is somewhat limited.[86,87] A recent study compared 7 orthogonal methods (e.g., HILIC, CE, and HPAEC-PAD) in analyzing released Fc glycans, which showed similar results for glycan patterns and content.[88]

Glycan structure assessment is challenging due to the isomeric and branched nature of oligosaccharides. The traditional approach involves the use of exoglycosidases to selectively and sequentially release terminal monosaccharides, which produces trimmed glycans to be analyzed by HILIC, CE or MALDI-MS. The structure of the originating glycans can be postulated according to respective shifts in glucose units, migration time and mass following each enzymatic digestion.[89] These approaches are valuable for new glycan structure analysis, but they involve a lengthy procedure and require highly pure enzymes to achieve cleavage at the desired sites. Recently, LC-ESI-CID-MS/MS was applied to glycan sequencing, which showed improved speed and sensitivity.[90] To determine the linkages in a glycan by MS/MS, cleavage across the glycosidic ring (cross-ring fragmentation) is required, which can be done by ESI-QTOF MS in a negative-ion mode under low energy CID conditions.[91–93] However, MS generally cannot resolve the structural isomers, or determine the 3-dimensional structure of the glycans. To fill this gap, nuclear magnetic resonance (NMR) and X-ray crystallography are employed.[94,95] In fact, NMR remains the only technique that can determine anomericity and linkage information in a new glycan structure,[67] but it requires relatively large amounts of purified glycans. A newly developed ion-mobility (IM) MS technique, which separates ions in gas phase based on size, shape, and charge, was shown to effectively detect glycan isomers.[96]

Analysis of monosaccharides

Quantification of released monosaccharide provides information on the content of a specific-terminal monosaccharide such as sialic acid, mannose-6-phosphate (M6P), GlcNAc, or *O*-linked monosaccharides.[97–99] Sialic acid residues can be selectively released by mild acid hydrolysis or by an enzyme neuraminidase (USP<1084>). Released sialic acids are normally quantitated by HPAEC-PAD without derivatization.[100] The absolute amount of sialic acids can be determined by HPAEC-PAD using commercially available sialic acid standards,[25] e.g., 3-deoxy-D-glycero-D-galacto-nonulosonic acid (USP<1084>). Alternatively, released sialic acids can be labeled with a fluorescent tag 1,2-diamino-4,5-methylenedioxybenzene followed by RP-HPLC analysis.[101]

To analyze the composition of monosaccharides other than sialic acids, these monosaccharides are commonly released by strong acid hydrolysis and separated by HPLC.[102] Specifically, trifluoracetic acid (TFA) is widely used for hydrolysis of glycans after selective release of sialic acids, and released amino monosaccharides are normally re-*N*-acetylated.[67,103] Common problems associated with acid hydrolysis are incomplete glycan cleavage and instability of the released monosaccharides under the acidic conditions.[102] Identification and quantification of non-sialic acid monosaccharides are normally conducted by HPAEC-PAD without derivatization, and 2-deoxy-glucose can

be used as standard for these neutral monosaccharides (USP<1084>).[104]

Lectin array-based glycan analysis

Lectins, which are a group of glycan binding proteins (GBPs) that are naturally occurring in plants and many other species, are known to selectively interact with glycan molecules either released or attached to a peptide backbone. This unique property has been explored in the development of analytics for glycan determination. Several relevant platforms have been reported in which lectin-based microarray has demonstrated a utility in capturing glycan profiles of therapeutic glycoproteins.[105–109]

In the design of a lectin microarray, specified lectins are immobilized onto a solid phase (e.g., glass chips) that is activated either chemically (e.g., epoxy, NHS ester, gold or amino group) or biochemically (e.g., streptavidin).[105,107] After lectin coating, residual active groups on the solid surface are blocked by appropriate agents (e.g., Tris buffer or bovine serum albumin). When a glycoprotein is applied onto the lectin chip, the binding events detected at specific lectin spots provide a picture of glycan variants that are likely present in the sample. In conventional microarray analysis, washing procedures are required to remove unbound glycoproteins from the lectin chips. Such operation procedures will disrupt the steady-state interactions between lectins and glycans because lectin-glycan binding is generally weak ($K_d \sim 10^{-4}$–10^{-7} M) compared to high binding affinity of antibody-antigen and biotin-streptavidin complexes. The recently developed evanescent-field activated fluorescence system allows real-time detection of lectin-glycan interactions at equilibrium without the need of washing procedures.[106,110,111] Such a platform has been used in assessing glycan variants of purified glycoproteins, crude extracted membrane proteins,[112] and live cell plasma membrane proteins.[113] A recent study compared 3 chromatographic methods (HPAEC-PAD, 2-AA HILIC, 2-AB HILIC) and a lectin microarray method for glycan profiling of a therapeutic mAb.[114] The lectin-based assays identified "glycan structural classes" in the 4 mAb lots, which were generally consistent with those detected by other classical methods.

Our laboratory has recently tested over a dozen Food and Drug Administration-approved therapeutic glycoproteins (including 9 mAbs) using commercial lectin chips that contain 45 distinct lectins (GlycoTechnica Ltd.). The derived glycan profiles (e.g., glycan variants and relative content) are generally consistent with the known glycosylation patterns of each product (data not shown). For example, a recombinant human glycoprotein shows strong binding signal at phytohemagglutinin-L (PHAL)-coated spots, confirming the presence of tri-/tetra-antennary glycan structures in the sample (Fig. 3A). No binding signal was detected for a recombinant IgG1 monoclonal antibody that is known to be deficient of such glycan structure. In another case, we found that the lectin-based assay was able to distinguish terminal sialylation variants (e.g., α2–3- vs. α2–6-sialylation) (Fig. 3B). Our data show promise for lectin microarrays in profiling glycan variants that are commonly present in therapeutic proteins. Compared to MS-based methods, which usually involve multiple sample processing steps, a lectin microarray directly works on intact glycoproteins with only minimal alteration to a testing sample (e.g., fluorescent labeling). The lectin microarray, when coupled with a sophisticated detection system, appears to provide a high throughput platform for rapid screening of glycan profiles of therapeutic proteins. However, the lectin-based microarray still faces major challenges before being adopted as a tool for characterization of therapeutic glycoproteins. In this regard, most commercial lectin microarrays utilize lectins from natural sources (e.g., plants) that are

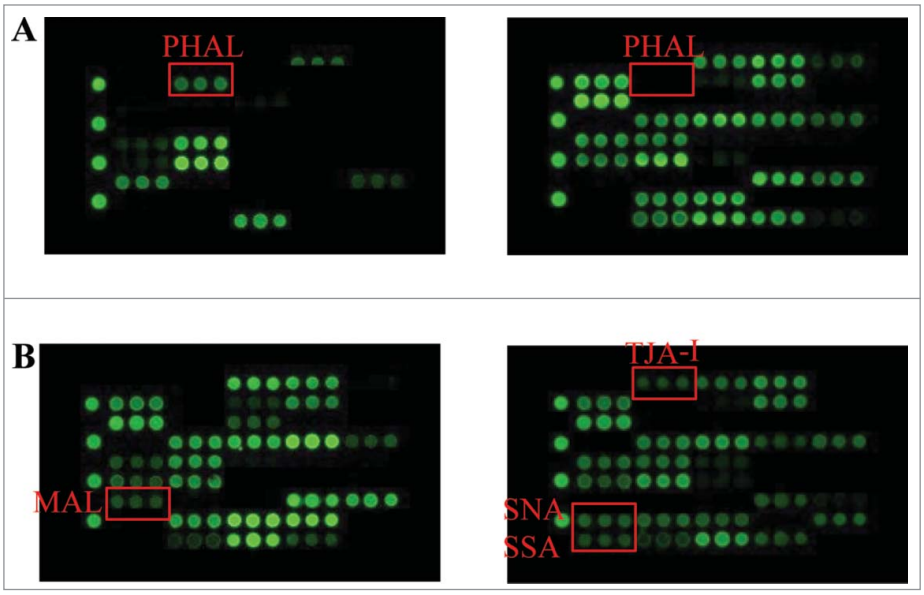

Figure 3. Lectin microarray analysis of glycans. (A). A recombinant human glycoprotein showed strong binding signal at PHAL coated spots (*left panel*), confirming the presence of tri- and/or tetra-antennary glycan structures in the testing sample. By contrast, no binding signal was detected for another mAb lacking such glycans (*right panel*); (B) Two therapeutic mAbs, which were known to contain α2–3- or α2–6-sialylation, respectively produced signals at α2–3-sialic acid binding lectin MAL (*left panel*) and α2–6-sialic acid binding lectins (SNA, SSA, TJA-I)(*right panel*).

Table 2. Comparison of analytical methods for glycoprotein characterization.

	Sample type				Advantages						Disadvantages		
Method	Intact protein	Glycopeptides	Released glycan	Monosac-charides	Lot release testing	Quick glycan profiling	Glycosylation site identification	Glycan sequencing	Glycan isomer differentiation	*O*-glycosylation analysis	Incomplete deglycosylation when releasing glycan	Semi-quantitative	Others
IEF	√				Charge variants on intact protein							√	Time consuming
IEX	IEX-UV		Fluorescence	HPAEC-PAD		Monosaccharides content							Not for LC-MS
CE	UV	UV	Fluorescence						√		√		Protein adsorption
HPLC-fluorescence			RP, HILIC	RP	Oligosaccharide map	Sialic acid content					√		
HPLC-ESI-MS	RP, SEC	RP, HILIC	√				ETD MS/MS of glycopeptide	CID MS/MS of glycopeptide or released glycan	√*	√	√	√	Complex data sets
HPLC with off-line MALDI-MS		RP, HILIC	√							√	√	√	Require sialic acid derivatization
MALDI-MS	√		Permythylation			Released glycan							Low accuracy
Lectin microarray	√					GLYCAN structural epitopes			√	√		√	Lectin availability

*PGC-chip based nano-LC-MS of short glycopeptides produced by pronase-E digestion, or ion-mobility MS of released glycan.

expected to display weak binding affinities to a spectrum of glycans. This phenomenon complicates interpretation of the detected binding events with a specific lectin molecule. Nonetheless, the lectin microarray platform appears to allow high throughput screening of the presence or absence of specific glycan variants in glycoprotein samples.

Perspectives

The complexity of protein glycosylation poses a daunting analytical challenge in the development of therapeutic glycoproteins (e.g., mAbs). Orthogonal methods are required to characterize specific features: 1) the carbohydrate content (neutral sugars, amino sugars, and sialic acids); 2) the structure of the carbohydrate chains, the oligosaccharide pattern (antennary profile), and 3) the glycosylation site(s) (ICH guidance Q6B). The selection of appropriate methods for a specific glycoprotein will depend on the purpose of testing and the ability of individual method (Fig. 2 and Table 2). IEF, IEX, or CE alone or in combination is commonly used to monitor heterogeneity in sialic acids on intact glycoproteins. HPLC is widely used to quantify the amounts of released oligosaccharides. MS coupled with HPLC remains a powerful tool in the characterization of glycosylation site(s) occupancy and carbohydrate structures. However, those oligosaccharide-based methods involve release of glycans from a glycoprotein. When conducting these assays, cautions must be taken to ensure that glycans are effectively released and recovered using appropriate procedures. Ideally, glycan analysis should involve procedures that only minimally alter the test samples. In this regard, lectin microarray appears to directly measure glycan variations in an intact protein without the need of clipping glycans from the protein backbone. Such a platform could be used as a complementary tool for characterization of protein glycosylation.

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