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Review Article

Identification and Quantification of Protein Glycosylation

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Glycosylation is one of the most abundant posttranslation modifications of proteins, and accumulating evidence indicate that the vast majority of proteins in eukaryotes are glycosylated. Glycosylation plays a role in protein folding, interaction, stability, and mobility, as well as in signal transduction. Thus, by regulating protein activity, glycosylation is involved in the normal functioning of the cell and in the development of diseases. Indeed, in the past few decades there has been a growing realization of the importance of protein glycosylation, as aberrant glycosylation has been implicated in metabolic, neurodegenerative, and neoplastic diseases. Thus, the identification and quantification of protein-borne oligosaccharides have become increasingly important both in the basic sciences of biochemistry and glycobiology and in the applicative sciences, particularly biomedicine and biotechnology. Here, we review the state-of-the-art methodologies for the identification and quantification of oligosaccharides, specifically *N*- and *O*-glycosylated proteins.

1. Protein Glycosylation

Carbohydrates are essential for cell metabolism and energy production and are building blocks of the extracellular matrix. Carbohydrates also play a role in a variety of biological processes, such as intra- and intercellular signaling and mediation of cell-cell interactions. In plants and animals alike, they may be found in various forms and structures—monosaccharides, oligosaccharides, or long and complex polysaccharides. Saccharides may be present in their free forms or they may be linked to other molecules, such as proteins and lipids. In proteoglycans the saccharide is the dominant component, whereas in glycoproteins, the saccharide constitutes only a small portion of the entire molecule.

Protein glycosylation, that is, the attachment of a saccharide moiety to a protein, is a modification that occurs either cotranslationally or posttranslationally. The two major types of glycosylation, *N*-linked and *O*-linked, are both involved in the maintenance of protein conformation and activity, in protein protection from proteolytic degradation, and in protein intracellular trafficking and secretion [1]. *N*-glycan moieties also play a key role in the folding, processing, and secretion of proteins from the endoplasmic reticulum (ER) and the Golgi apparatus [1]. In the past few decades, growing attention has been directed to protein glycosylation in the biomedical field, since aberrant glycosylations have been associated with various congenital [2], metabolic, neurodegenerative [3], and immune diseases [4] and with cancer [5]. Thus, the study of glycans and their localization and structural pattern will shed light on their role in protein regulation and function, both under normal conditions and in pathologies. In biotechnology, too, there is a need for an indepth understanding of glycosylation, since the efficient production of recombinant proteins, including glycoproteins [6], is becoming increasingly important. Thus, there is a great deal of interest in the use and development of tools for glycobiology research. This paper is focused on protein glycosylation and on state-of-the-art methods for the identification and quantification of glycoproteins.

1.1. N-Glycans. In proteins, the *N*-linked oligosaccharide is attached to the amide group of an asparagine residue within the consensus peptide sequence NXS/T, where X is any amino acid except proline. *N*-glycosylation occurs solely on proteins that shuttle via the secretory pathway [7]. The assembly of the *N*-glycan oligosaccharide begins in the cytosolic side of the ER and then it flips to the lumen side, where the assembly is completed, and attaches to the translated protein. The

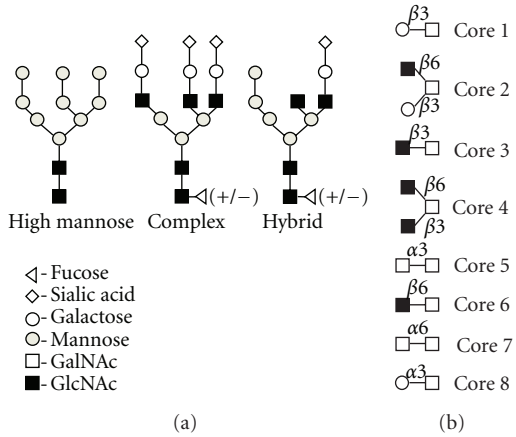

Figure 1: Basic core structures of *N*-glycans (a) and *O*-glycans (b).

attached oligosaccharide-protein complex has the form of $Glc_3Man_9GlcNAc_2$ in the ER, but following the correct folding of the protein, it is transferred to the Golgi apparatus as $Man_8GlcNAc_2$ [8]. In the Golgi apparatus, the glycan moiety can remain in the high-mannose form or it can be processed further—with some glycans being removed and a variety of other glycans added—to form a hybrid and complex structure. This glycan "tree" may be bi-, tri- or tetraantennary, and it may also be bisected by a GlcNAc attachment to the core mannose (Figure 1(a)).

1.2. O-Glycans. The most common *O*-glycosylations are *O*-acetylgalactosamine (*O*-GalNAc) and *O*-acetylglucosamine (*O*-GlcNAc). *O*-GalNAc is attached to the hydroxyl group of the protein serine or threonine residues through an α-linkage, while *O*-GlcNAc is attached through a β-linkage. Unlike *N*-linked glycosylation, no consensus sequence has been found for *O*-linked glycosylation [9], and it could occur at various intracellular sites. Structurally, *O*-glycosylation may involve monosaccharides and/or oligosaccharide complexes of several different monosaccharides [9]. *O*-GalNAcylation, which occurs in the Golgi and hence on secreted proteins, is a common *O*-glycosylation, with over 10% of human proteins being *O*-GalNAcylated. There are eight types of core *O*-GalNAc structures (Figure 1(b)), which can be further elongated. This is often a complex process, since the same protein can carry different *O*-glycan structures. The most abundant *O*-GalNAcylated proteins are mucins, the heavily glycosylated mucus proteins that are secreted mainly from the epithelium of the gastrointestinal and respiratory tracts and also from the salivary glands [9]. A number of congenital, developmental, and immune diseases have been attributed to mucin type *O*-glycan alterations [10].

Unlike *N*- and *O*-α-glycosylation, β-*O*-GlcNAcylation, which occurs on cytosolic and nuclear proteins, is a dynamic modification resembling protein phosphorylation [11]. Moreover, there is a dynamic crosstalk between *O*-GlcNAcylation and phosphorylation: the two modifications can reciprocally occur on the same Ser/Thr residue or occupy adjacent residues [12–14]. Since the discovery of *O*-GlcNAc [15], a myriad of *O*-GlcNAcylated cytosolic and nuclear proteins have been found in eukaryotes, including transcription and translation factors, metabolic and signaling components, cytoskeletal proteins, oncogenic products, and tumor suppressor proteins [12, 16–21]. Protein *O*-GlcNAcylation is known to be vital for cell life and to be linked to regulation of the cell cycle and cell growth, proliferation, and apoptosis.

2. Analysis of Protein Glycosylation

The analysis of protein glycans is complicated by their vast variety and the large number of potential glycosylation combinations: even a single protein can undergo a number of *N*- and *O*-glycosylations. Moreover, the same glycosylation site can be occupied by different glycans in different copies of a protein, a phenomenon termed microheterogeneity. Thus, reliable, detailed characterization of glycans often requires the use of several methods, as described below.

2.1. Glycoprotein Detection. For detection of glycosylated proteins, two types of methods are generally used: staining and affinity-based methods.

2.1.1. Staining Procedures. A basic, simple method to determine whether a protein is glycosylated is to resolve it on SDS-PAGE and to stain the gel for glycoproteins. Most gel-staining procedures are based on the periodic acid-Schiff (PAS) reaction, in which periodic acid oxidizes two vicinal diol groups to form an aldehyde, which reacts with the Schiff reagent to give a magenta color [22]. This chromogenic gel staining is most commonly performed with acid fuchsin [23], which can be detected fluorescently at 535 nm and is thus two- to fourfold more sensitive than the visible staining method [24]. Some commercially available fluorescent stains utilize periodate oxidation to attach a fluorescent hydrazide, with no further reduction being required. These fluorescent dyes are highly sensitive, detecting <18 ng of glycoproteins [25]. However, these periodate oxidation methods may be problematic, since nonspecific reactions can occur with proteins that possess endogenous oxidizable moieties, for example,

aldehydes or ketones. In addition, the staining intensity is lower for proteins with fewer glycosylation sites and "poorer" glycan trees than for heavily glycosylated proteins.

Two other gel stains that should be mentioned are alcian blue and Stains-All [26, 27], which are used for detecting proteoglycans, glycosaminoglycans, and negatively charged glycoproteins. However, one should bear in mind that for these stains, too, non-specific reactions with phosphoproteins and other negatively charged proteins are also possible.

2.1.2. Affinity-Based Procedures. The following methods are more specific and facilitate the determination of the glycosylation type.

(a) Saccharide-Binding Proteins. Lectins are proteins (found in animals, plants, and microorganisms) that specifically bind mono- or oligosaccharides [28]. Over the years, numerous lectin-based applications for glycoprotein detection and characterization have been developed (Table 1). In the widely used lectin blot, a protein sample is resolved on SDS-PAGE and transferred onto a nitrocellulose or polyvinylidene fluoride (PVDF) membrane. The membrane is then incubated with a specific lectin and labeled with a group, such as digoxigenin (DIG) or biotin that will further bind to a secondary antibody or to avidin, respectively [29, 30]. Several lectins are commercially available from companies such as Roche Applied Science and Sigma. The antibody or avidin is conjugated to an enzyme that catalyzes a color-producing reaction (alkaline-phosphatase) or a more sensitive luminescence-producing reaction (horseradish peroxidase).

(b) Enzyme-Based Methods. Click chemistry, utilizing specific enzymes, may be used to label and isolate glycosylated proteins. For example, a protein that is *O*-β-GlcNAcylated or that carries *O*-α- and/or *N*-glycans with a terminal GlcNAc (either purified or in a protein mixture) is incubated with GALT (β-1,4-galactosyltransferase), which specifically adds an azidogalactose to GlcNAc. The modified *O*-GlcNAc is then allowed to react with a fluorescent alkyne, the sample is resolved on SDS-PAGE, and the modified protein is visualized by UV, using a gel imager [24]. Similarly, *O*-GlcNAcylated peptides can be labeled and further analyzed by mass spectrometry (MS).

(c) Antibody-Based Methods. *O*-β-GlcNAc may be analyzed in a method that is specific to this unique glycan moiety, since primary antibodies, for example, commercially available CTD 110.1 and RL2, have been developed against it. The cross-reaction of these antibodies with secondary antibodies conjugated to enzymes that catalyze a color- or luminescence-producing reaction can be exploited in procedures such as blotting to detect *O*-GlcNAcylated proteins [31–33].

2.2. Glycan Structure Analysis. After protein glycosylation has been confirmed, the glycan moiety structure can be elucidated by chromatography and mass spectrometry. The glycan could be analyzed when attached to the protein or following its release. We will focus on the latter approach, since it is more reliable.

2.2.1. Glycan Release from Proteins. Depending on its nature, the glycan moiety can be removed from the protein by enzymatic or chemical means.

(a) Enzymatic Cleavage of Glycans. Most of the commercially available glycan-cleaving enzymes are specific for *N*-glycans, with fewer being available for *O*-glycans. The large variety of *O*-glycans dictates that several enzymes might be required for the analysis of a single sample, and their utility is thus limited. Thus, chemical removal is more applicable for *O*-glycans.

The most efficient cleaving enzyme for *N*-glycan moiety would be a glycoamidase, particularly glycoamidase F (PNGase F), which cleaves the bond between GlcNAc and an Asn residue, thereby converting Asn to Asp. PNGase F cleaves most types of *N*-linked oligosaccharides, including complex structures (e.g., high mannose, hybrid, and multisialylated structure), up to tetraantennary trees and oligosaccharides containing sulfate-substituted residues [34]. However, for native proteins high concentrations of PNGase F may be required. If the oligosaccharide contains α-1,3 core fucosylation or an individual *N*-acetylglucosamine attached to asparagine, PNGase F will not cleave the *N*-glycan moiety, and in that case another glycoamidase, PNGase A, should be used [35]. However, the use of PNGase A may be problematic for glycan cleavage from intact protein or large peptides, probably due to its high molecular weight (80 kDa) that limits its accessibility to the cleavage site [34]. In addition, PNGase F and A activity is reduced when the *N*-glycan is close to the protein N- or C-terminus. Thus, for native glycoproteins or known glycan moieties, endoglycosidases that cleave the *N*-glycan tree between the two core GlcNAcs (GlcNAc1-4GlcNAc) are preferred. It should be remembered that endoglycosidases are rather specific: endo-H cleaves most hybrid-type oligosaccharides and high-mannose, core-fucose, and bisecting glycans; endo F_1 cleaves high-mannose and hybrid-type glycans [36]; endo F_2 cleaves high-mannose and biantennary glycans; endo F_3 cleaves bi- and tri-antennary glycans [34].

(b) Chemical Removal of Glycans. The two main chemical methods for removing *O*- and *N*-linked oligosaccharides are β-elimination and hydrazinolysis. In β-elimination, exposure of a glycoprotein to an alkaline environment (e.g., of sodium hydroxide) results in glycan release. The free glycan moiety can be further degraded into monosaccharides by additional β-elimination reactions, until complete degradation—termed a "peeling reaction." Yet, glycan degradation can be prevented by performing the reaction in the presence of sodium borohydride, which reduces the remaining aldehydes [37]. Although efficient, this method has a major disadvantage in that oligosaccharides possess only one group available for labeling, the reduced end aldehyde. This group is eliminated during the reaction, thus labeling following glycan release is not possible.

Table 1: Lectins and their specific affinity to glycans.

Lectin	Origin	Specific affinity
Mannose binding lectins		
Con A	*Canavalia ensiformis*	Branched α-mannosidic structures; high-mannose type, hybrid type and biantennary complex type *N*-glycans
GNA	*Galanthus nivalis*	α-1–3 and α-1–6-linked high-mannose structures
LCH	*Lens culinaris*	Fucosylated core region of bi- and triantennary complex type *N*-glycans
Galactose/*N*-acetylgalactosamine binding lectins		
RCA	*Ricinus communis*	Galβ1-4GlcNAcβ1-R
ECL	*Erythrina cristagalli lectin*	Galβ1-4GlcNAcβ1-R
PNA	*Arachis hypogaea*	Galβ1-3GalNAcα1-Ser/Thr (T-Antigen)
AIL	*Artocarpus integrifolia*	(Sia)Galβ1-3GalNAcα1-Ser/Thr (T-Antigen)
VVL	*Vicia villosa*	GalNAcα-Ser/Thr (Tn-Antigen)
Sialic acid/*N*-acetylglucosamine binding lectins		
WGA	*Triticum vulgaris*	GlcNAcβ1-4GlcNAcβ1-4GlcNAc, Neu5Ac (sialic acid)
PHA	*Phaseolus vulgaris*	*N*-Acetylglucosamine
SNA	*Sambucus nigra*	Neu5Acα2-6Gal(NAc)-R
MAL	*Maackia amurensis*	Neu5Ac/Gcα2-3Galβ1-4GlcNAcβ1-R
Fucose binding lectins		
UEA	*Ulex europaeus*	Fucα1-2Gal-R
AAL	*Aleuria aurantia*	Fucα1-2Galβ1-4(Fucα1-3/4)Galβ1-4GlcNAc; R_2-GlcNAcβ1-4(Fucα1-6)GlcNAc-R_1

In hydrazinolysis, anhydrous hydrazine is added to a salt-free, lyophilized glycoprotein to start the hydrolysis reaction. This method may be used for both *N*- and *O*-linked carbohydrates, which can be even released separately when mixed together, by altering the reaction conditions, specifically the temperature; *O*-linked oligosaccharides are released at 60°C, while *N*-linked oligosaccharides are released at 95°C [38]. The released glycan moiety remains intact, whereas the protein may be degraded.

2.2.2. Glycan Desalting and Purification. Following their release from the glycoprotein, free glycans are usually found in a solution that contains salts, detergents, proteins, peptides, amino acids, and so forth. Before further analysis, these contaminants must be removed. There are several methods for glycan desalting and purification. The graphitized carbon desalting method is based on the ability of oligosaccharides to bind to carbon beads, while unbound simple monosaccharides, salts, and detergents can be washed away with water [39]. The glycan moiety is eluted from the beads with acetonitrile (ACN), while strongly bound proteins and peptides remain attached. Polar oligosaccharides are usually eluted by 25% ACN, while charged oligosaccharides require the addition of 0.05% trifluoroacetic acid (TFA) [39]. In another desalting method, a drop of glycan sample is loaded onto a cation-exchange Nafion membrane, which is floated on water. Proteins, peptides, and salts within the sample will bind to the Nafion, and the purified glycan sample can be retrieved [40]. Similarly to the method with the Nafion membrane, the cation-exchange AG-50 resin can be used. Salts and some detergents may also be removed by dialysis [41].

2.2.3. Glycan Structure Analysis. Following purification, two approaches may be taken for glycan structure analysis: chromatography and MS.

(a) Glycan Analysis by Chromatography. In chromatography, fluorescent labeling of the glycan moiety usually improves the detection. The fluorescent molecule is conjugated to the glycan reducing end via reductive amination [42]. The most commonly used fluorophores are 2-aminobenzoic acid (2-AA), 2-aminobenzamide (2-AB), and 2-aminopyridine (2-AP). However, aminopyridine-based fluorophores can hinder specific glycan characterization, since their conjugation may cause desialylation [42], whereas the other two fluorophores, 2-AA and 2-AB, may cause only mild desialylation. The negative charge of 2-AA makes it less appropriate for chromatography and MS, but more suitable for electrophoresis [42]. The 2-AB fluorophore labels glycans nonselectively in a 1 : 1 ratio, allowing subpicomolar detection, while maintaining the right molar proportions [43].

The most widely used chromatographic methods for glycan separation are weak anion exchange (WAX) [44], gel filtration (on Bio-Gel P4) (Ashford,1987), high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) [45], and normal-phase high-performance liquid chromatography (NP-HPLC) [43]. Although all methods are efficient, one should carefully choose the appropriate method in the light of the specific requirements and the specific properties of the particular glycans being analyzed. WAX and gel filtration chromatography require large amounts of oligosaccharides. HPAEC-PAD requires high-pH and high-salt buffers (which must be removed before further analysis of the separated glycans,

such as in the case of exoglycosidase sequencing). NP-HPLC, having high resolution and reproducibility, is the most efficient method for glycan analysis. In this method, a partially hydrolyzed 2-AB-labeled dextran is used as an external standard, and the retention time of the unknown glycan (relative to the standard) is converted to glucose units (GU) [43]. This GU value is compared to a database of experimental values to obtain a preliminary structure assignment for the glycan [46, 47]. Yet, the NP-HPLC resolution may be too low for some glycans; thus, a complementary method, such as reverse-phase-HPLC (RP-HPLC), is required [48]. Since the dextran standard does not resolve well on RP-HPLC, the alternate standard is arabinose [48].

Further validation of the glycan sequence can be performed with an array of exoglycosidases, that is, the sequential application of specific exoglycosidases to cleave terminal monosaccharides from the nonreducing end of the glycoprotein [49]. This chromatographic method also allows relative glycan quantification: by calculating the peak area one can assess the percentage of a specific type of oligosaccharide out of the total glycan repertoire. Furthermore, relative quantification of the same oligosaccharide in several samples can be achieved [43].

(b) Glycan Analysis by MS. MS analysis is based on glycan ionization, fragmentation, and mass identification of the fragments. The two main MS methods for oligosaccharide analysis are electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). Before analysis, the glycan sample must be desalted to prevent ionization masking in ESI and MALDI analysis. In MALDI analysis, the analyte is ionized through the matrix, usually to a single sodiated ion, $[M+Na]^+$. Several matrices are applicable for glycans, with 2,5-dihydroxybenzoic acid (DHB) being the most widely used. Yet, in the light of the variety of glycans and their different polarities, 2,4,6-trihydroxyacetophenone (THAP) is a more suitable matrix [50]. In ESI and nanospray, the analyte in solution is converted to an aerosol by the electrospray. Here the ions are usually multiply charged, which may complicate the glycan analysis [51].

In MS, the ionization polarity mode should be considered. Although most proteomics and glycomics analyses are performed in a positive-ion mode, the glycan moieties composition is diverse, and if the particular moiety contains *N*-acetylation and acidic residues, such as sialic acid, ionization may be prevented. In that case, a negative-ion mode could be employed, but further MS/MS and fragmentation analysis (performed in a positive mode) is usually not applicable. The above notwithstanding, positive ionization can be improved by adopting one of the following two strategies. The glycan moiety can be desialylated by sialidase that cleaves the terminal sialic acid [50]. Although desialylation enables better ionization, some information is lost: in a pool of released oligosaccharides it is practically impossible to identify which oligosaccharide possessed the sialic acid. Alternatively, permethylation, in which all the glycan-free OH groups are methylated [52, 53], will stabilize oligosaccharides containing sialic acid residues. Moreover, permethylation masks highly polar groups and confers a slight hydrophobicity; thus, the oligosaccharides are more easily separated from contaminants (e.g., salts) that may interfere with the analysis, and they also become more uniformly and efficiently ionized [54]. Indeed, fragment ions obtained from permethylated derivatives are easily assignable to glycan sequences [55].

Glycan fragmentation is achieved by cleaving either the glycosidic bonds between monosaccharides or the bonds within a monosaccharide ring (termed cross-ring cleavage). The ions obtained from the reducing side are designated X in cross-ring cleavage and Y and Z in glycosidic cleavage. The nonreducing side ions are designated A in cross-ring cleavage and B and C in glycosidic cleavage (Figure 2) [41]. In collision-induced dissociation (CID) fragmentation, ions enter a collision cell filled with a gas (usually argon) and are subjected to high- or low-energy collisions. When performed with high-collision energy, CID results mostly in cross-ring cleavages, providing data on the monosaccharide linkages, while low-collision energy yields glycosidic cleavages, providing data on the monosaccharide sequence and branching [56]. Recently, high-energy C-trap dissociation (HCD) fragmentation was applied for glycan and glycoprotein analysis [57]. The resolution of this method in the low-mass spectrum is higher than that of CID, enabling the identification of the monosaccharide masses. Thus, all the fragment ions, obtained by a combination of techniques, are assigned, providing the full glycan sequence [57, 58]. For detailed structural characterization, multiple ion isolation and fragmentation cycles are needed. For that ion-trap analysis with tandem MS fragmentation is the method of choice as described by Prien et al. [59].

Although MALDI and ESI spectra can be used for the elucidation of glycan composition and structure, full glycan characterization by MS is rather complicated: glycan quantity limitations are common, and more importantly, the isobaric structure of many monosaccharides (i.e., having the same molecular weights) hinders their specific identification based solely on the number of carbon atoms. While glycan fragmentation may contribute some information on the glycan sequence, branching, and linkage, the combination of several methods is usually necessary to produce a highly reliable picture. For example, data acquired by MS and exoglycosidase array will give a near-complete characterization of the glycan sequence, branching, and linkage [60]. Similarly, oligosaccharide hydrolysis after permethylation, followed by gas-chromatography mass-spectrometry (GC-MS) analysis would identify unmethylated carbons of the monosaccharide. Moreover, structural characterization and branching can be best resolved by NMR analysis if enough material in sufficient purity is available. Thus, information on branching and inner oligosaccharide linkages could be obtained [53].

2.3. Glycan Site Mapping. Identification of a glycosylation site is important, since such knowledge could provide an indication of the function of that glycan. Moreover, assignment of a certain structure to specific site can also shed light on the protein's glycosylation profile and microheterogeneity and hence, plausibly, its activity. As mentioned above, removal of an entire *N*-glycan moiety by PNGase F or A results in

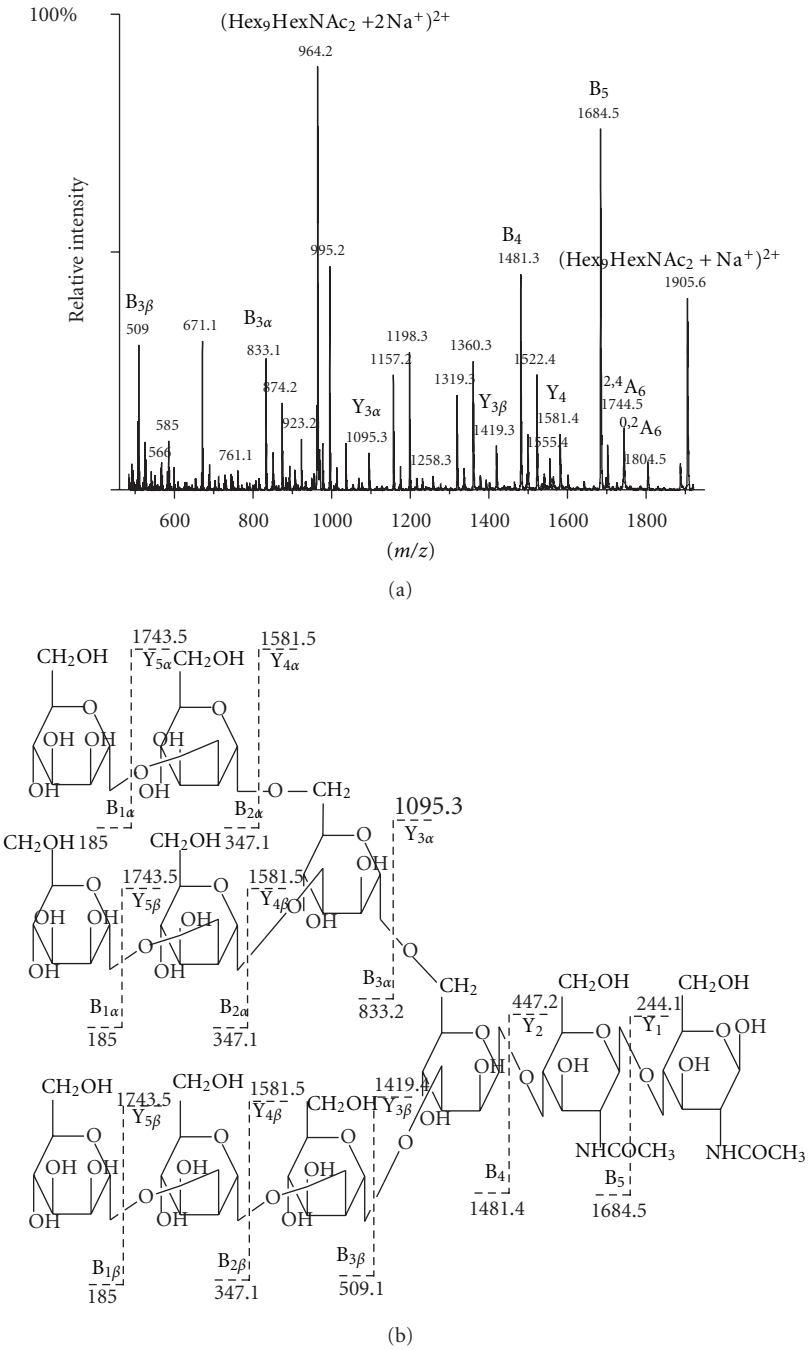

Figure 2: ESI-MS/MS for *N*-glycan structural analysis. MS/MS spectrum of the $Hex_9HexNAc_2$ *N*-glycan (a). Numbers above the parent ions peaks represent $[M+Na]^+$, while other peaks represent $[M+H]^+$ of the fragments. Expected fragment type and mass are indicated in the oligosaccharide structure (b).

the conversion of Asn to Asp and a shift of one mass unit for each *N*-glycosylation site. Thus, when a deglycosylated protein is further digested with trypsin, the peptides that are bound to the glycan moiety will be 1 Da heavier than the expected theoretical mass. By subjecting these peptides to MS/MS, each peptide that possesses Asp (instead of Asn) is identified as formerly attached to the glycan moiety [61]. A similar approach involves glycan removal with PNGase F in the presence of $H_2{}^{18}O$: the deglycosylated Asn would be labeled with ^{18}O, thus its mass, altered by 3 Da (1 Da for the Asn-Asp conversion and 2 Da contributed by ^{18}O), can be followed [62].

Glycan site mapping is not always straightforward, and some challenges may be encountered. One challenge is to identify a glycopeptide in a large pool with an absolute majority of nonglycosylated peptides. Moreover, the glycan

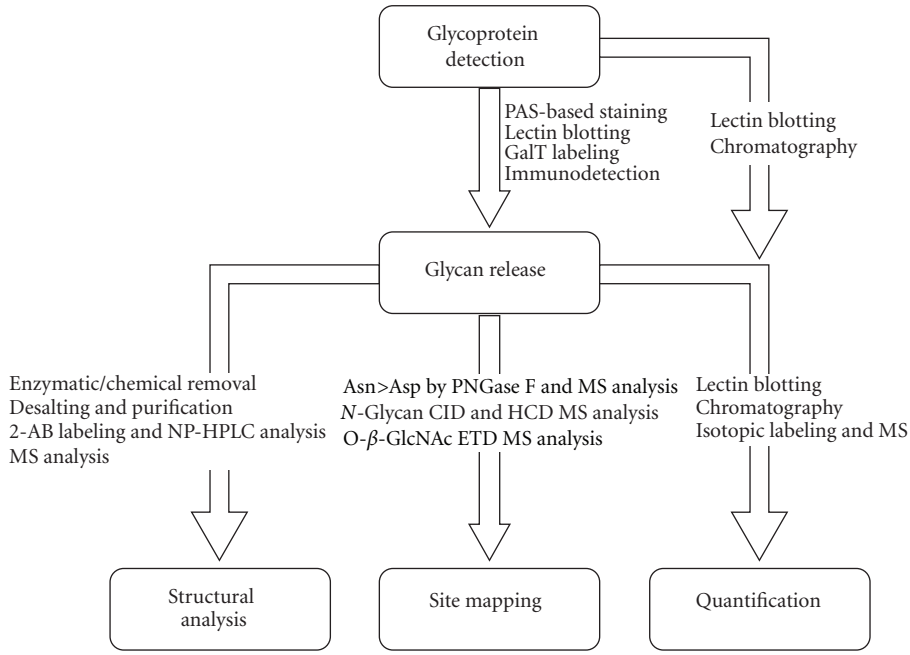

Figure 3: Schematic representation of glycoprotein identification, glycan structural elucidation, and protein glycosylation site mapping and quantification.

moiety tends to suppress ionization compared to unmodified peptides. Therefore, in such a case glycopeptide enrichment should be employed. A vast variety of commercial resin-bound lectins are available for column chromatography [63]. Another challenge involves the analysis of modified peptides, specifically glycopeptides; in this case, the commonly used CID and HCD fragmentation methods result in intensive fragmentation of the glycan moiety, while the peptide remains intact. Two recently developed fragmentation methods, electron capture dissociation (ECD) and electron transfer dissociation (ETD), may solve this problem. Both these methods, based on electronic excitation energy, result in peptide fragmentation that leaves the modification intact and attached to the amino acid. Thus, enriched glycopeptides can be subjected to CID-ETD fragmentation, in which the CID fragments the glycan moiety and the ETD sequences the glycopeptide with the attached glycan moiety [58].

A study on *N*-glycan site mapping by HCD was recently published [57]: a linear ion-trap orbitrap hybrid mass spectrometer (LTQ-orbitrap) was used to sequence a glycopeptide through repeated fragmentation (MS^n). In the first round of fragmentation (MS^2), CID was used for analyzing glycan sequencing and branching, and HCD (analyzed in the orbitrap) was used—due to its ability to form glycan oxonium ions—to detect glycopeptides. HCD yields a unique ion, designated Y1, which represents the mass of the peptide with Asn-GlcNAc. The Y1 intensity in the HCD spectra is high, and the ion can thus be subjected to further MS^3 for peptide sequencing [57]. The newly released GlypID 2.0 software, utilizing the accurate precursor mass and both the CID and HCD spectra, enables the glycosylation site characterization [64].

In contrast, localization of the dynamic mono-glycosylation *O*-β-GlcNAc is elusive. Due to its extreme labile nature in the gas phase and due to ion suppression, *O*-β-GlcNAc detection and site mapping of an intact *O*-β-GlcNAcylated peptide are virtually impossible by standard MS methods relying on CID; ETD is the only applicable type of fragmentation. ETD-produced C and Z fragment ions that contain Ser or Thr residues would carry an additional mass of 203 Da, indicating the attachment of *O*-β-GlcNAc [65]. Alternatively, *O*-β-GlcNAcylated peptides may be identified following *O*-GlcNAc removal. Use of conventional β-elimination to remove the *O*-β-GlcNAc results in the conversion of glycosylserine to 2-aminopropenoic acid (2-ap) and glycothreonine to 2-amino-2-butenoic acid (2-ab) [66], which decreases the glycopeptide mass by *m/z* 222. The obtained CID fragment ion represents a loss of *m/z* 69 (2-ap) instead of *m/z* 87 for serine, while the mass of threonine and its derivative 2-ab remains *m/z* 101. This method enables rapid identification of *O*-GlcNAc-modified peptides in a complex mixture, as well as its site mapping at the low picomole level.

2.4. Glycoprotein Quantification. The substoichiometric relation between glycosylation and the protein constitutes a further challenge in glycoprotein quantification. Yet, lectin purification, followed by a labeling procedure, can be used for relative quantification of a specific glycoprotein. The simplest method is lectin blotting (see the previous), followed by densitometry of the lectin cross-reactive band. In addition, lectin chromatography can be used for comparative quantification of a specific glycoprotein in several samples; the relative amounts of a particular glycoprotein, purified by a

specific lectin column, in different samples, are determined by densitometry of SDS-PAGE protein bands, stained with either Coomassie blue or another specific stain.

Chromatography by NP-HPLC has previously been used for relative quantification of 2-AB-labeled oligosaccharides in one or more samples [43]; calculation of the area under the chromatographic peaks indicates the amount of the specific glycan. In addition, chromatography and MS are often coupled to provide a reproducible and reliable glycan characterization and quantification. MS is also applicable for the relative quantitation of glycopeptides by state-of-the-art methods based on stable isotope labeling, for example, isotope-coded affinity tagging (ICAT) [67] or isobaric tags for relative and absolute quantitation (iTRAQ) [68]. In these methods, lectin-purified glycoproteins from different samples are each labeled with either light or heavy isotopes during trypsin digestion, mixed together, and analyzed by MS. The relative abundance of the light- and heavy-isotopic peaks of the same peptide from the two different samples indicates their relative quantities. In case of microheterogeneity, that is, the existence of several glycoforms (Figure 1), quantification by a modified permethylation procedure, using isotopic labeling, is applicable [54]. Following glycan release, each free glycan sample is labeled with either ^{12}C- or ^{13}C-methyl iodide; the two samples are mixed in equal proportions, and the labeled glycans are analyzed by MS. This method has a high-dynamic range, adequate linearity, and high reproducibility [54].

3. Concluding Remarks

Glycosylation is fundamental for protein function and for cell physiology. Analysis of the structure and localization of protein glycans is necessary in life sciences and biotechnology. The most reliable analytical tools currently available are chromatography and mass spectrometry. To begin with, characterization of glycans by lectin chromatography is the primary method to be used. For structure elucidation, exoglycosidase array followed by a combination of NP-HPLC and MS is the method of choice. MS analysis, by either ETD or isotopic labeling, is suitable for glycosylation site mapping. Isotopic labeling is further appropriate for quantification of glycans (Figure 3).

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