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GLOBAL AND SITE-SPECIFIC ANALYSIS OF PROTEIN GLYCOSYLATION IN COMPLEX BIOLOGICAL SYSTEMS WITH MASS SPECTROMETRY

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Abstract

Protein glycosylation is ubiquitous in biological systems and plays essential roles in many cellular events. Global and site-specific analysis of glycoproteins in complex biological samples can advance our understanding of glycoprotein functions and cellular activities. However, it is extraordinarily challenging because of the low abundance of many glycoproteins and the heterogeneity of glycan structures. The emergence of mass spectrometry (MS)-based proteomics has provided us an excellent opportunity to comprehensively study proteins and their modifications, including glycosylation. In this review, we first summarize major methods for glycopeptide/glycoprotein enrichment, followed by the chemical and enzymatic methods to generate a mass tag for glycosylation site identification. We next discuss the systematic and quantitative analysis of glycoprotein dynamics. Reversible protein glycosylation is dynamic, and systematic study of glycoprotein dynamics helps us gain insight into glycoprotein functions. The last part of this review focuses on the applications of MS-based proteomics to study glycoproteins in different biological systems, including yeasts, plants, mice, human cells, and clinical samples. Intact glycopeptide analysis is also included in this section. Because of the importance of glycoproteins in complex biological systems, the field of glycoproteomics will continue to grow in the next decade. Innovative and effective MS-based methods will exponentially advance glycoscience, and enable us to identify glycoproteins as effective biomarkers for disease detection and drug targets for disease treatment.

Keywords

mass spectrometry; protein glycosylation; glycoproteomics; chemical and enzymatic methods; complex biological samples

I. INTRODUCTION

Over the past two decades, mass spectrometry (MS)-based proteomics has become an increasingly powerful technique to study proteins and their modifications (Eng et al., 1994; An et al., 2003; Zhang et al., 2003; Cox and Mann, 2008; Wollscheid et al., 2009; West et

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al., 2010; Nwosu et al., 2011; Butterfield et al., 2014; Aebersold and Mann, 2016; Glaskin et al., 2017; Suttapitugsakul et al., 2017; Yu and Huang, 2018). Protein modifications are extremely important in biological systems because they regulate nearly every cellular activity including gene expression, signal transduction, and cellular response to environmental cues (Mahal et al., 1997; Spiro, 2002; Varki et al., 2008; Wolfert and Boons, 2013). Comprehensive and site-specific analysis of protein modifications is normally beyond the reach of conventional biochemistry methods. Modern MS technology provides a unique opportunity to globally and site-specifically characterize protein modifications (Ficarro et al., 2002; Peng et al., 2003; Trinidad et al., 2006; Siuti and Kelleher, 2007; Witze et al., 2007; Yates et al., 2009; Mertins et al., 2013; Nwosu et al., 2013; Huang et al., 2015a; Ludwig et al., 2015; Gu and Robinson, 2016; Lossl et al., 2016; Yue et al., 2016; Cao et al., 2017; Simithy et al., 2017; Hogrebe et al., 2018; Wu et al., 2018). However, it is still extraordinarily challenging to globally analyze them because of the low abundance of many modified proteins, sub-stoichiometry of protein modifications, and the complexity of biological samples. (Jensen, 2004; Reinders et al., 2004; Wu et al., 2011a, 2011b; Xiao et al., 2015a; Clerc et al., 2016; Banazadeh et al., 2017) In addition, no common method can be used to comprehensively analyze all types of protein modifications because every type of modification is different. Innovative and effective methods are crucial to achieve the global analysis of every type of protein modification.

Among hundreds of known modifications, glycosylation is one of the most common and essential modifications. It determines protein folding, trafficking, and stability, and regulates many cellular activities, especially extracellular ones (Mahal et al., 1997; Gabius et al., 2002; Spiro, 2002; Lau et al., 2007; Kudelka et al., 2015; Chandler and Costello, 2016; Neelamegham and Mahal, 2016; Wang et al., 2016a). Many glycoproteins are of extremely low abundance compared to nonmodified proteins; meanwhile, glycosylation is very complex due to heterogeneous glycan structures and various amino acid residues covalently bound to glycans. To overcome these challenges, many beautiful methods were developed to qualitatively and quantitatively study protein glycosylation events in different kinds of samples.

Here some major MS-based methods for global and site-specific analysis of protein glycosylation are discussed. In the first part, we summarize the chemical and enzymatic strategies for MS-based glycoproteomics, including different enrichment methods and the approaches to generate a common tag for global analysis of protein N- and O-glycosylation with MS. Second, MS-based methods for glycoprotein dynamic study are discussed. Reversible glycosylation makes glycoproteins highly dynamic, and the analysis of glycoprotein dynamics will provide insight into glycoprotein functions. In the last part, we include some applications of MS-based glycoproteomics in different complex biological systems, including yeast, plants, mice, human cells, and clinical samples. Due to the importance of protein glycosylation, many excellent reports on glycoprotein analysis were published in the literature. It is not possible to cover all of them in one Review, and we apologize to those whose beautiful works in this field are not included here.

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II. GLOBAL ANALYSIS OF GLYCOPROTEINS

A. Glycopeptide/Glycoprotein Enrichment

Glycopeptide/glycoprotein enrichment is imperative for global analysis of protein glycosylation with MS due to the low abundance of many glycoproteins, the dynamic nature of protein glycosylation, and the complexity of biological samples (Ramachandran et al., 2006; Wohlgemuth et al., 2009; Patwa et al., 2010; Wei et al., 2010; Novotny et al., 2013; Ahn et al., 2015; Lu et al., 2016; Dong et al., 2017; Zheng et al., 2017; Shao et al., 2018; You et al., 2018). Enrichment can allow us to minimize the interference from highly abundant non-glycoproteins on the analysis of protein glycosylation, and to reach low-abundance glycoproteins. In the literature, a variety of chemical/enzymatic methods were reported to enrich glycopeptides/glycoproteins, and each method has its own advantages and limitations.

1. Lectin—Lectin-based methods have been widely used to enrich glycopeptides/glycoproteins. Commercially available lectins are mostly originated from plants, with some also from bacteria and animals (Lotan et al., 1975; Weis and Drickamer, 1996). Each lectin can specifically bind one or several types of glycans, which limits lectins from enriching all glycopeptides with diverse glycans in complex biological samples. Several lectins, such as concanavalin A (Con A, mainly for internal and nonreducing terminal a-D-mannosyl and a-D-glucosyl groups), wheat germ agglutinin (WGA, for N-acetyl-D-glucosamine and sialic acids), and ricinus communis agglutinin I (RCA I/RCA 120, binding galactose, or N-acetylgalactosamine residues) were combined for glycopeptide/glycoprotein enrichment (Amari et al., 1994; Yang and Hancock, 2004; Qiu and Regnier, 2005; Larsen et al., 2006; Wuhrer et al., 2007; Scott et al., 2011; Cao et al., 2013a). Typically, lectins are immobilized onto a solid support and served for solid-phase extraction of glycopep-tides. The lectin-functionalized beads can also be packed into separation columns, allowing for enrichment of glycopeptides coupling with liquid chromatography (LC). Zielinska et al. (2010) combined lectin enrichment with filter-aided sample preparation (FASP) to map N-glycosylation sites in four mouse tissues and blood plasma, and they identified 6,367 sites on 2,352 glycoproteins. In addition to the well-known motif (NX[S/T], X stands for any amino acid residue other than proline) for protein N-glycosylation, they also found other rare motifs, such as the NXC motif. The same group further employed the FASP method coupled with lectins to profile N-glycosylation sites across seven evolutionarily distant species, and found the distant species have common characteristics including sequence recognition patterns, structural constraints, and subcellular localization although the N-glycoproteomes from those species are largely divergent (Zielinska et al., 2012).

Besides protein N-glycosylation, lectin-based enrichment methods were also applied for protein O-glycosylation analysis although the methods are not as mature as those of N-glycosylation analysis (Pan et al., 2011; Vakhrushev et al., 2013). For instance, Darula and Medzihradszky (2009) used the jacalin lectin (recognizing GalNAca1-O-extension of the core 1 structure) to enrich O-glycopeptides and identified O-glycosylation sites from bovine serum through MS analysis. The same lectin was also immobilized onto silica beads, which were further packed in an LC column for serial lectin affinity chromatography analysis of O-

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glycopeptides after N-glycopep-tides being removed with *Con* A (Durham and Regnier, 2006). Steentoft et al. (2011) performed O-glycosylation analysis combining vicia villosa agglutinin (VVA) lectin chromatography with the SimpleCell technology, which is discussed in more details below.

As an important and special type of O-glycosylation, O-GlcNAcylation has been extensively studied in the past three decades, and lectin-based methods have also been developed for comprehensive mapping of protein O-GlcNAcylation (Dehennaut et al., 2008; Skorobogatko et al., 2011; Nagel et al., 2013; Xu et al., 2017). In this context, WGA was exploited for the enrichment of O-GlcNAcylated proteins or peptides due to its substrate specificity. For example, by combining lectin weak-affinity chromatography (LWAC) with β-elimination/Michael addition with DTT (BEMAD) and electron-capture dissociation (ECD) mass spectrometry, Vosseller et al. (2004, 2006) analyzed 145 unique O-GlcNAcylated peptides from a postsynaptic density preparations. Overall, lectin-based strategies have been extensively employed for the enrichment of glycopeptides/glycoproteins, and in combination with MS-based proteomics, the experimental results provide valuable information about protein N- and O-glycosylation.

2. Hydrazide Chemistry—Hydrazide chemistry is another milestone method in the glycoproteomics field. In 2003, Zhang et al. (2003) developed an elegant strategy based on glycan oxidation and hydrazide chemistry for glycopeptide enrichment, followed by protein N-glycosylation analysis with MS (Fig. 1). Glycans were oxidized to generate the aldehyde groups, and then the oxidized glycopeptides were conjugated to a solid support using hydra-zide chemistry. After on-bead digestion, non-glycopeptides were removed, and the enriched glycopeptides were recovered from the beads by using Peptide-N-Glycosidase F (PNGase F) to cleave off N-glycans. The glycopeptides were then identified and quantified by LC-MS/MS. In this approach, the glycans are oxidized, and thus glycan structural information is lost. This method was further modified for better performance and has been extensively used to study protein glycosylation in a variety of samples, from cells to clinical samples, and from plant to animals (Liu et al., 2005; Wang et al., 2012; Huang et al., 2015b; Yu et al., 2016a).

Although initially this method was designed for protein N-glycosylation analysis as PNGase F can only release N-glycopeptides from the beads, it was further used for O-glycosylation analysis. Nilsson et al. (2009) combined hydra-zide chemistry with acid cleavage to analyze sialylated glyco-proteins and identified 36 N-linked and 44 O-linked glycosylation sites from human cerebrospinal fluid with glycan structural information. Later they applied a similar strategy to study human urinary glycoproteins, and 58 N- and 63 O-linked "intact" glycopeptides were identified from 53 glycoproteins (Halim et al., 2012). This method was further improved by pretreating the samples with PNGase F to remove N-glycans and using CID-MS^2/MS^3 and other fragmentation strategies including ECD or electron-transfer dissociation (ETD) to comprehensively analyze glycopeptides (Halim et al., 2013). Taga et al. (2012) combined hydrazide chemistry with the glycan oxidation with galactose oxidase and formic acid-induced cleavage of the hydrazone bond to study O-glycosylation on collagen. Klement et al. (2010) also applied hydrazide chemistry for O-GlcNAcylation studies where they elevated the concentration of sodium periodate and reaction temperature,

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and enriched the modified proteins through hydrazide chemistry. The O-GlcNAcylated peptides were released by hydroxylamine treatment and analyzed by tandem MS. A total of 12 OGlcNAcylated peptides from five proteins were identified. Hydrazide chemistry-based methods have been widely applied for comprehensive analysis of glycoproteins in different samples.

3. HILIC—Hydrophilic interaction liquid chromatography (HILIC) is a separation technique that has been extensively employed to separate/enrich glycopeptides (Calvano et al., 2008; Lam et al., 2010; Segu et al., 2010; Parker et al., 2013; Dedvisitsakul et al., 2014; Khatri et al., 2014; Qin et al., 2017; Lin et al., 2018). Contrary to reverse-phase chromatography, the stationary phase of HILIC is very hydro-philic, allowing binding of hydrophilic analytes to the column. The gradient of the mobile phase usually starts with a high percentage of relatively less polar organic solvent, and then the percentage of polar component (usually H_2O) is gradually increased through the gradient timeline, resulting in higher hydrophilicity of the solution and stronger elution power.

Glycans are highly hydrophilic, and therefore the retention times of glycopeptides on the HILIC column are generally longer than non-glycopeptides. In the literature, Hägglund et al. (2004) combined zwitterionic hydrophilic interaction chromatography (ZIC-HILIC) enrichment and partial deglycosylation to study protein N-glycosylation in human plasma, and identified 62 glycosylation sites from 37 glycoproteins. The same group later employed a similar strategy to study core fucosylated N- and O-glycosylation among human plasma proteins (Hagglund et al., 2007). Protein N-glycosylation in six breast and brain cancer cell lines was analyzed using HILIC and electrostatic repulsion liquid chromatography (ERLIC) enrichment and LC-MS/MS analysis. A total of 497 glycopeptides were characterized, of which 401 common glycopeptides were identified from both enrichment techniques (Zacharias et al., 2016).

HILIC enrichment was extensively applied for glycosylation analysis, including site mapping and intact glycopeptide analysis (Nettleship, 2011). However, if a non-glycopeptide contains multiple hydrophilic amino acid residues, its retention time may be comparable to those of glycopeptides, rendering the low specificity of HILIC. Nevertheless, HILIC can be coupled with other enrichment methods to perform two-dimensional separation and fractionation. There are some reports in the literature regarding the improvement of HILIC performance, such as introducing ion-pairing reagents in the mobile phase and further functionalizing the stationary phase (Ding et al., 2009; Mysling et al., 2010), which have considerably advanced the use of HILIC in glycoproteomics.

4. IsoTag—Recently, Woo et al. (2015) developed an innovative isotope-targeted glycoproteomic (IsoTaG) method, which combined metabolic labeling, isotopic recording, and MS-based proteomics to analyze intact N- and O-glycopeptides. They synthesized an isotopic affinity probe (shown in Fig. 2), which has four critical components: the azide for tagging glycans through copper-catalyzed azide-alkyne [3 [notdef] 2] cycloaddition (CuAAC), the biotin group for enriching the tagged glycopeptides through strong biotin-avidin interactions, the silane scaffold for being readily cleaved to release the glycopeptides after enrichment, the dibromide motif for detection of glycopeptides in the MS. Due to the

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natural abundance of the stable isotopes of Br (Br^{79}: Br^{81} = 1:1), glycopeptides tagged with the probe display a special pattern in MS analysis (termed IsoStamp) (Palaniappan et al., 2011).

The following steps are involved in the IsoTaG method (Fig. 3): (i) metabolically label glycans with a functional sugar analog (i.e., N-azidoacetylgalactosamine ($Ac_4GalNAz$)); (ii) tag the labeled glycoproteins with the probe, and then capture the glycoproteins on a solid phase through biotin-avidin interactions. The enriched glycoproteins were digested using trypsin, and the glycopeptides were released by cleaving the silane scaffold; (iii) analyze the glycopeptides by MS. Here they used a pattern-searching algorithm, which recognizes the 2:5:1 distribution of the dibromide motif in full MS to selectively sequence glycopeptides; and (iv) assign glycosylation sites and glycan structures using Byonic™ software. IsoTaG increases the selection and detection speed of glycopeptides due to the pattern-searching algorithm, and greatly aids in the analysis of intact glycopeptides, but metabolic labeling might limit its applications for clinical samples.

5. NGAG—Zhang and co-workers recently reported a beautiful chemoenzymatic method, named solid phase extraction of N-linked glycans and glycosite-containing peptides (NGAG), to comprehensively analyze N-glycoproteins and glycans in complex samples (Sun et al., 2016). This method utilizes various enzymatic and chemical reactions together with MS to analyze N-glycans and their parent deglycosylated peptides. The procedure is shown in Figure 4. Briefly, proteins were first digested, and the resulting peptides were guanidinized to block the ε-amino groups on the lysine side chains. Peptides were then conjugated to the solid phase via their N-termini, and the carboxyl groups of aspartic acid (D), glutamic acid (E), the peptide C termini, and sialic acids were reacted with aniline to facilitate the detection of glycopeptides with MS. N-glycans were then released from the solid support by PNGase F treatment and subjected to MS analysis while the asparagine residues on the glycopeptides were converted to aspartic acids. This provides the opportunity for Asp-N-induced cleavage of the peptides, and thus releasing them from the solid phase. However, the aspartic acid residues that were not generated from the PNGase F cleavage of N-glycans were not affected because they were modified by aniline in the prior step. The released deglycosylated peptides were also identified by MS.

Using this method they identified 2,044 unique N-glycopep-tides, and in parallel experiments comparing NGAG and hydrazide chemistry methods, a total of 3,083 unique N-glycosite-containing peptides were identified on 1,473 glycoproteins in OVCAR-3 cells. Quantitative analysis of glycopeptides based on NGAG was also performed, and they found that treating cells with tunicamycin mainly caused glycan occupancy reduction on the glycosylation sites. Further glycan dynamic experiments also showed differential alteration of glycans by the tunicamycin treatment. Recently they applied this method to analyze N-glycoproteins in serum (Sun et al., 2018). Overall, despite multiple steps, NGAG is a brilliant example of rational usage of chemical and enzymatic reactions to greatly advance MS-based glycoproteomics.

6. Boronic Acid-Based Methods—The covalent interactions between boronic acids (BA) and *cis*diols on glycans have been extensively studied and been applied for

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glycoprotein analysis (Dowlut and Hall, 2006; Jin et al., 2010; Zhang et al., 2012; Wang et al., 2013; Xu et al., 2013; Wang et al., 2014; Wu et al., 2014; Xiao et al., 2015b; Liu and He, 2017; Xiao et al., 2018a). Yang and co-workers designed a boronic acid-functionalized core-satellite-structured composite material to capture glycopeptides/proteins, and identified 194 unique glycosylation sites from 155 different glycoproteins (Zhang et al., 2009). Later the same group synthesized a boronic acid-functionalized detonation nanodiamond to enrich glycopep-tides for MS analysis, which resulted in the identification of 40 unique N-glycopeptides on 34 glycoproteins in mouse liver (Xu et al., 2013). Zeng et al. (2013) designed a surface patterned sample support with a hydrophobic outer layer and an internal boronic acid-modified gold microspot to selectively enrich glycopeptides, and the enriched glycopeptides were then directly subjected to matrix-assisted laser desorption ionization (MALDI) MS analysis. Metz and co-workers combined boronate affinity chromatography with ETD MS to analyze non-enzymatically glycated peptides (Zhang et al., 2007).

Chen et al. (2014a) conjugated boronic acids onto magnetic beads, and then used the beads to enrich glycopeptides digested from a yeast whole cell lysate for MS-based proteomic analysis (Fig. 5). Due to the nature of the pH-dependent reversible interactions between boronic acids and hydroxyl groups, the enrichment was performed under basic conditions to maximize the amount of glycopeptides captured on the beads. After the removal of non-glycopeptides, elution was performed in an acidic solution, where the reversible covalent bonds were broken. The enriched glycopeptides were then treated with PNGase F in heavy-oxygen water ($H_2{}^{18}O$) for only 3 hr to generate a common tag on N-glycosylation sites as well as to distinguish the glycosylation sites from spontaneous asparagine deamidation sites. They applied this method to study the yeast glycoproteome, and identified 816 N-glycosylation sites on 332 proteins.

Strong interactions between boronic acid and glycans are critical to catch glycopeptides/proteins with low abundance. In order to enhance the interactions between boronic acid and glycans, different boronic acid derivatives were examined (Xiao et al., 2018c). Among the derivatives tested, benzoboroxole was the most effective one, and it allowed us to identify the largest number of unique N-glycopeptides from human whole cell lysates, as shown in Figure 6. These results are consistent with the previous report that benzoboroxole can form strong interactions with sugars (Dowlut and Hall, 2006).

To further enhance the interactions between the boronic acid derivative and glycans, synergistic interactions were employed based on the common features of the vast majority of glycans containing multiple monosaccharides and every monosaccharide bearing several hydroxyl groups. A dendrimer was used as a platform because it can conjugate many benzoboroxole molecules and also provide structural flexibility to form strong interactions. Dendrimers were previously used for binding of saccharide molecules (James et al., 1996) and stereoselective recognition of monosaccharides (Smith and Diederich, 1998). Synergistic interactions based on a dendrimer was also employed for selective enrichment of trace glycoproteins (Wang et al., 2013). Dendrimer-conjugated Boronic Acid derivative (DBA, Fig. 7a) is effective for facilitating the synergistic interactions, and the molecular mechanism is shown in Figure 7b. We optimized the dendrimer size to maximize the glycopeptide enrichment (Fig. 7c). Comparing DBA to the same boronic acid magnetic

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beads without a dendrimer (cycle 0), many more unique N-glycopeptides were identified in human cells among parallel experiments. The enrichment can be accomplished rapidly, and the results from the incubation time of 10 min are similar to those from 2 to 3 hr. These also demonstrate that there is almost no glycopeptide degradation or other side reactions because prolonging the reaction time did not result in a lower number of glycopeptides (Fig. 7d).

The DBA method was employed for glycoprotein analysis in human cells (MCF7, HEK293T, and Jurkat), and 4,691 sites were identified on 1,906 N-glycoproteins. We also applied this method to analyze glycoproteins with a small glycan, that is, OGlcNAc. GlcNAc does not contain *cis*-diol, and thus it is almost impossible to be enriched with normal boronic acid-based methods. As shown in the comparison experiments, without the dendrimer, using the magnetic beads conjugated with the same boronic acid derivative, we were barely able to identify OGlcNAcylated proteins in human cells. With the synergistic interactions using DBA, more than 200 O-GlcNAcylated proteins were identified (Xiao et al., 2018c). Using the DBA method, global analysis of glycoproteins was also performed in yeast cells and mouse brain tissues. For protein N-glycosylation, we identified over 1,000 sites on 501 glycoproteins in yeast and 4,195 sites on 1,608 glycoproteins in the mouse brain tissues.

The reversible interactions between boronic acid and hydroxyl groups make boronic acid-based chemical enrichment universal for nearly all types of glycopeptides/glycoproteins. The reversible nature of this interaction and the mild reaction conditions ensures glycopeptide remaining intact after the enrichment, which allows us for site-specific analysis of glycopeptide with glycan structural information. This method can be further improved by strengthening the binding between boronic acid and glycans, allowing the capture of more glycopeptides and minimizing the glycopeptide loss during the washing step. Overall, the combination of boronic acid-based enrichment methods and MS-based proteomics have great potential to effectively analyze protein glycosylation on a large scale.

7. Click Chemistry-Based Methods—The combination of metabolic labeling and click chemistry has emerged as a powerful method to study proteins and protein modifications (Mahal et al., 1997; Hang et al., 2003; Bond and Kohler, 2007; Dieterich et al., 2007; Hanson et al., 2007; Hong et al., 2009; Smeekens et al., 2015; Chen et al., 2016; Cheng et al., 2016; Spiciarich et al., 2017; Xiao and Wu, 2017a; Xiao et al., 2018b). Metabolic labeling of glycans with unnatural sugar analogs is an excellent way to study glycans and protein glycosylation (Kayser et al., 1992; Saxon and Bertozzi, 2000; Feng et al., 2013; Aguilar et al., 2017a, 2017b; Park et al., 2018; Qin et al., 2018). In the recent two decades, the Bertozzi group has performed pioneering work on using unnatural sugar analogs to label glycoproteins (Mahal et al., 1997; Saxon and Bertozzi, 2000; Hang et al., 2003; Breidenbach et al., 2010). They first developed a cell surface engineering strategy by combining metabolic labeling with a modified Staudinger ligation reaction (Saxon and Bertozzi, 2000). Acetylated azide-containing sugar analogs were found to have much greater labeling efficiency than the non-acetylated versions. Azide-containing sugar analogs are commonly used because azide does not create an unacceptable steric hindrance, which allows glycotransferases to use the sugar analogs to modify glycoproteins, and is relatively stable and biologically inert.

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To date, a variety of sugar analogs have been used for metabolic labeling, such as GalNAz, GlcNAz, fucose analogs, and ManNAz (for labeling of sialic acid) (Yang et al., 2011; Chuh et al., 2014; Palaniappan and Bertozzi, 2016; Witzke et al., 2017; Woo et al., 2017). After incorporation of the azido sugar analogs, a click reaction is performed to introduce another chemical handle for affinity enrichment. The glycoproteins/glycopeptides can then be analyzed by a variety of methods including MS-based proteomics. Both identification and quantification of glycopeptides can be achieved using this strategy (Sprung et al., 2005; Hubbard et al., 2011; Yang et al., 2011; Chen et al., 2015; Xiao et al., 2016a; Konze et al., 2017).

8. Other Chemical and Enzymatic Enrichment Methods—Chemical and/or enzymatic methods facilitated the identification and quantification of protein glycosylation (Khidekel et al., 2007; Alfaro et al., 2012; Li et al., 2014; Sun et al., 2016; Yu et al., 2016b; Capicciotti et al., 2017; Wen et al., 2018), including O-GlcNAcylation. For instance, Khidekel et al. (2007) established a method, termed quantitative isotopic and chemoenzymatic tagging (QUIC-Tag), which enzymatically incorporates a ketone group onto O-GlcNAcylated proteins, and then tags them with biotin for affinity enrichment of OGlcNAcylated peptides/proteins for rapid and sensitive analysis. The ketone moiety generation and biotin tag incorporation are shown in Figure 8. Combining this method with quantitative isotopic dimethyl labeling, they studied protein O-GlcNAcylation dynamics in cultured neurons and rat brain samples.

Wang et al. (2010) developed a click chemistry-based method by combining chemical/ enzymatic tagging, photochemical cleavage, and ETD mass spectrometry to enrich O-GlcNAcylated peptides and map protein O-GlcNAcylation sites (Fig. 9). They used enzyme GalT1 to transfer an azide-containing sugar analog (UDP-GalNAz) onto the O-GlcNAc moieties of modified peptides, and then incorporated a biotin group on it through CuAAC for the enrichment. The enriched O GlcNAcylated peptides were then released by photochemical cleavage, followed by LC-MS analysis. They also investigated the cross-talk between O-GlcNAcylation and phosphorylation through proteomics-based methods. Their lab has made significant contributions in discovering protein O-GlcNAcylation and studying its biological functions (Wells et al., 2002; Vosseller et al., 2005; Alfaro et al., 2012; Ma et al., 2016).

Chemical and/or enzymatic strategies have significantly broadened the toolbox of MS-based proteomics, especially for studying protein PTMs. We envision that further development of chemoenzymatic methods will tremendously advance our understanding of protein glycosylation events.

B. Generating a Common Tag for MS Analysis

Glycans are highly heterogeneous, and the diverse structures contain a wealth of valuable information, which makes the analysis of glycoproteins and glycopeptides by MS extraordinarily challenging. Unlike other modified groups with a fixed structure, such as the phosphate group in phosphoproteins or the methyl group in methylated proteins with a constant mass shift (Wu et al., 2011b; Cao et al., 2013b), glycans on glycoproteins do not

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have a common mass tag for MS analysis. In order to globally identify glycosylation sites, generating a tag on glycosylation sites can provide convenience for glycopeptide identification. This section covers the methods generating a common mass tag for MS-based glycoproteomics analysis.

1. A Common Tag for Protein N-Glycosylation

Enzymatic methods.: The simplest strategy is to remove glycans while the residual mass can serve as a tag to localize the glycosylation site. To achieve this goal, enzymatic methods have been widely used to deglycosylate N-glycans. PNGase F is one of the most commonly used enzymes to remove protein N-glycans in order to assist glycoproteomic analysis (An et al., 2003; Liu et al., 2005; Alvarez-Manilla et al., 2006; Ramachandran et al., 2006; Kita et al., 2007; Zhao et al., 2007; Stavenhagen et al., 2013; Yin et al., 2013). It was initially isolated from *Flavobacterium meningoseptic* in 1984 (Plummer et al., 1984), and has been exploited to remove N-glycans for more than three decades. PNGase F was reported to have broad substrate specificity and can hydrolyze the glycosylamine linkage between N-glycans and asparagine, which generates a deglycosylated peptide and an intact oligosaccharide with the di-N-acetylchitobiose unit at the reducing end (Tarentino et al., 1985). In the process, the asparagine residue is converted to aspartic acid, introducing a mass shift of +0.9840 Dalton, which can serve as a universal mass tag for N-glycosylation site localization. The reaction is shown in Figure 10.

Although PNGase F has been widely used, there are still a few drawbacks. First, spontaneous deamidation of the asparagine residues occurs *in vitro* and *in vivo*, which generates the same mass shift as the PNGase F-induced deglycosylation (Hao et al., 2011). This makes it hard to control the false positive glycosylation site identification (Palmisano et al., 2012a). To solve this problem, heavy-oxygen water ($H_2{}^{18}O$) has been employed as the solvent to carry out the enzymatic removal of N-glycans by PNGase F (Kuster and Mann, 1999; Kaji et al., 2003). In this case, the mass tag (+0.9840 Da) of accumulated spontaneous deamidation is different from the one (+2.9883 Da) due to the authentic deglycosylation from glycopeptides with PNGase F. The reaction can be performed for a shorter period of time to limit the effect of spontaneous deamidation during the PNGase F treatment. In our previous experiment, for a 3-hr PNGase F treatment under neutral conditions, the spontaneous deamination effect was negligible (Xiao et al., 2016a). The other limit of this method is that although PNGase F is widely used, it is not able to remove N-glycans with fucose attached $\alpha 1 \rightarrow 3$ to the asparagine-linked N-acetylglucosamine residue (Tretter et al., 1991).

Aside from PNGase F, the endoglycosidases are a family of important deglycosylation enzymes reported in the literature (Stavenhagen et al., 2013; Cao et al., 2014; Yu et al., 2017a). Endoglycosidase H (Endo H) is one of the most widely used endoglycosidases. It cleaves within the chitobiose core of high mannose and some hybrid oligosaccharides from N-linked glycoproteins, but it does not work efficiently against complex glycans. Endo H was firstly isolated from *Streptomyces plicatus*, and its structure was described by Robbins *et al.* (1984). The residual mass after the Endo H cleavage is different from that due to the PNGase F treatment because the innermost GlcNAc residue remains on the peptide after the

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glycan cleavage. The innermost N-GlcNAc can serve as a common tag for glycosylation site identification.

Although the remaining GlcNAc can serve as the mass tag, it is larger than the one from the PNGase F treatment, which may affect the quality of tandem mass spectra for glycopeptide identifications, especially when collision-induced dissociation (CID) is used. In addition, Endo H has stricter substrate specificity compared to PNGase F, rendering the enzymatic glycan release less effective. However, the spontaneous deamidation of asparagine is not an issue while using Endo H. To improve the glycoproteome coverage, researchers have combined several endoglycosidases to remove glycans. For instance, H€agglund et al. (2004) combined Endo H and D to deglycosylate glycopeptides enriched by hydrophilic interaction LC, which led to the identification of 62 glycosylation sites on 37 glycoproteins from human plasma samples.

Chemical deglycosylation methods.: Enzymatic methods are commonly used especially for protein N-glycosylation analysis. However, each enzyme has its own specificity, and thus it is very difficult to universally remove all glycans using an enzyme. In addition, they are not cost-effective. Chemical methods were also developed to remove glycans for glycosylation mapping. More than three decades ago, Edge et al. (1981) removed glycans from fetuin using chemical deglycosylation, and found that it resulted in rapid cleavage of peripheral sugars, slow loss of serine- and threonine-linked GalNAc, and retention of N-linked GlcNAc. Other methods such as ammonium hydroxide/carbonate-based chemical deglycosylation were also reported in the literature (Triguero et al., 2010). Recently, based on the common core structure of N-glycans, Chen et al. (2014b) developed a method combining chemical deglycosylation and MS-based proteomics to perform large-scale analysis of protein N-glycosylation in complex biological samples. This method takes the advantage of the chemical property difference between the amide bond connecting the innermost GlcNAc with peptides and the glycosidic bond among the rest of monosaccharides in a glycan. A strong acid, trifluoromethanesulfonic acid (TFMS), was used to cleave the glycosidic bonds, but not the amide bond (Fig. 11a). Therefore, after the cleavage, the innermost GlcNAc remaining on the peptide can serve as a common tag for N-glycosylation site mapping. Although the tag is the same as that from Endo H treatment, this chemical method has the advantage of not being affected by the compositional and structural variation of N-glycans, which can lead to a much broader glycoproteome coverage. The experimental procedure is shown in Figure 11b.

Combining this chemical deglycosylation method with lectin enrichment of glycopeptides, the authors identified 555 N-glycosylation sites from 219 glycoproteins in yeast without further glycopeptide fractionation. They also compared this method with the Endo H method, which generates the same mass tag, and the results demonstrated that this chemical method outperformed the other. Based on the same principle, Ma et al. (2015) employed TFA to deglycosylate glycopeptides with the assistance of microwave heating, which shortened the treatment time to merely 10 min. Combining this method with ZIC-HILIC enrichment and higher-energy collisional dissociation (HCD) fragmentation, they identified a total of 257 N-glycosylation sites on 144 proteins from healthy human serum. Although chemical deglycosylation methods are generally more universal than enzymatic methods, the

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harsh deglycosylation conditions sometimes could damage the peptide backbone, which needs to be carefully considered.

2. A Common Tag for Protein O-Glycosylation

β-elimination methods.: Although it is well-known that protein O-glycosylation plays crucial roles in biological systems (Tian and Ten Hagen, 2009; Moremen et al., 2012), effective methods for global analysis of protein O-glycosylation are relatively under-represented compared to those for N-glycosylation. β-elimination has been used to deglycosylate O-glycans and generate a mass tag on peptides for MS analysis. Glycans on glycopeptides are removed under basic conditions, and simultaneously an alkene group on the glycosylation site is produced. The carbon–carbon double bond is reactive and susceptible to nucleophilic attack, and therefore, reduction was then performed on the alkene to stabilize the structure and create a mass tag for proteomic studies.

More than two decades ago, Greis et al. (1996) designed a β-elimination-based strategy to analyze O-GlcNAcylated peptides using MS. They demonstrated that β-elimination can create a mass shift for O-GlcNAcylated peptides, converting the previously glycosylated serine and threonine residues to alanine and 2-aminobutyric acid, respectively, which were served for site-specific identification of these glycopeptides. The same research group developed a method by coupling b-elimination with Michael addition of dithiothreitol, termed BEMAD (Wells et al., 2002). This method creates a 136.2 Dalton mass shift through the loss of glycans and the addition of DTT, serving as a common mass tag for O-glycosylation (especially O-GlcNAcylation) site analysis. In the literature, there are also many other reports on b-elimination-based methods for protein O-glycosylation analysis (Durham and Regnier, 2006; Vosseller et al., 2006; Hanisch et al., 2009; Overath et al., 2012; Hahne et al., 2013; Boysen et al., 2016). For instance, Rademaker et al. (1998) used NH_4OH to initiate b-elimination, and after completion, NH_3 was incorporated onto the amino acid residue from which the glycan was removed. This method yielded a unique mass tag for database searching and was effective for as low as 1 pmol of starting material. Despite that the β-elimination-based methods are often used to generate common mass tags for O-glycosylation sites, the major drawbacks are that they are not very effective, and the reaction conditions are relatively harsh, which induces the degradation of the peptide backbone, often rendering peptide sequencing difficult.

Enzymatic method.: Unlike N-glycans that can be removed by enzymes, such as PNGase F or Endo H, prior to LC-MS/MS analysis, it is more challenging to use enzymatic methods to release O-glycans because there are no universal enzymes available. However, attempts were still made on using enzymes to deglycosylate O-glycans for studying protein O-glycosylation. Hägglund et al. (2007) combined two enzymatic deglycosylation strategies to identify protein O-glycosylation sites from human plasma proteins. They carried out the N-glycan removal using PNGase F in $H_2{}^{18}O$ first. Then Endo D and Endo H along with several exoglycosidases (β-galactosidase, neuraminidase, and N-acetyl-glucosaminidase) were used to cleave the glycosidic bond between the two GlcNAc residues in N-glycans, leaving only one GlcNAc residue with the potential fucosyl side chain on peptides. Although initially this strategy was devised for N-glycosylation analysis, several O-glycosylated peptides were also

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identified with a single GalNAc attached to the modification site, which was attributed to partial de-O-glycosylation by the combination of endo- and exoglycosidases.

Simple Cell.: In addition to the enzymatic and chemical methods mentioned above, another very beautiful method was developed by Steentoft et al. (2011) in which zinc-finger nuclease (ZFN) was employed to genetically engineer cells, simplifying the glycan structures to create a common mass tag. They applied ZFN targeting to modify the O-glycan elongation pathway in human cells, and thus truncated O-glycans (Fig. 12). The modified cell lines with homogenous O-glycosylation were named as SimpleCell lines. These cell lines solely expressed GalNAc (Tn) or NeuAcα2–6GAlNAc (STn) O-glycans, allowing O-glycopeptides to be easily enriched by lectins. The glycopeptide sequencing process was also greatly simplified due to the common tags. A total of more than 350 O-glycosylation sites on over 100 proteins were identified by combining this method with LC-MS/MS with ETD fragmentation. This method has opened up a new avenue for O-glycoproteomic analysis.

Following their first publication, Steentoft et al. (2013) further optimized the experimental conditions and applied this method to site-specifically analyze protein O-glycosylation in 12 human cell lines. Nearly 3,000 glycosylation sites were identified in over 600 O-glycoproteins. These cells were from different organ origins, and the glycoproteomes were considerably different across these cell lines. Meanwhile, they also improved NetOGlyc4.0 as a tool for O-glycosylation prediction. Overall, even though O-glycan structure information is lost, SimpleCell is very powerful to globally and site-specifically analyze protein O-glycosylation in combination with MS-based proteomics. With the development of gene editing techniques in recent years, similar strategies should have broader applications in protein modification studies.

III. GLYCOPROTEIN DYNAMICS

Glycosylation is a type of reversible protein modification, and many enzymes are responsible for the addition and removal of glycans. Glycoproteins are highly dynamic in and outside of cells. Studying their dynamics will allow us to have a better understanding of glycoprotein functions and cellular activities. Although it is very challenging to systematically investigate glycoprotein dynamics, the rapid advancement in glycoproteomics and multiplexed proteomics has provided the exciting possibility. The following subsection reviews several studies on MS-based proteomic investigation of glycoprotein dynamics.

A. O-GlcNAcylated Protein Dynamics

Several methods have been reported to study glycoprotein dynamics, and most of these studies were conducted in recent years due to the technical advancements. For instance, Wang et al. (2016b) metabolically labeled O-GlcNAc by feeding cells with $^{13}C_6$-glucose. The isotopically labeled glucose was metabolically incorporated into ^{13}C-labeled UDP-GlcNAc through the hexosamine biosynthetic pathway, which was used to eventually label O-GlcNAcylated proteins. O-GlcNAcylated peptides were enriched using a boronic acid-based method for quantitative proteomics analysis. Through this strategy, protein O-GlcNAcylation turnover rates were determined. In total, they identified 105 O-GlcNAcylated peptides from 42 proteins and determined the turnover rates of 20 O-GlcNAcylated peptides

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from 14 proteins in the nuclei of HeLa cells (Wang et al., 2016b). They found that although the rates widely varied depending on both the protein and the site, O-GlcNAcylation turnover rates were generally slower than those published results for phosphorylation or acetylation. This methodology can be extensively applied to study turnovers/dynamics of protein O-GlcNAcylation in different samples, which will provide more valuable information about protein O-GlcNAcylation functions.

B. N-Glycoprotein Dynamics

Recently, Xiao and Wu (2017b) developed a method that integrated isotopic labeling, chemical enrichment, and multiplexed proteomics to simultaneously determine glycoprotein degradation and synthesis rates. Cells were cultured in a medium containing heavy lysine and arginine, and then chased in a medium with all light amino acids for different durations before being harvested. This pulse-chase labeling allowed for quantification of protein abundance changes. After cell lysis, proteins were extracted and digested, and the resulting peptides were subjected to boronic acid-based glycopeptide enrichment. The enriched glycopeptides from cells harvested at different time points were labeled with tandem mass tags (TMT) reagents, and then analyzed by LC-MS/MS. After chasing cells with the light medium, the abundance of existing proteins labeled with heave lysine and arginine (heavy proteins) decreases while proteins with light lysine and arginine (light proteins) increase. Heavy glyco-proteins were used for the determination of the degradation rates and light glycoproteins for the synthesis rates of glycoproteins. The synthesis rates of 847 N-glycoproteins and the degradation rates of 704 N-glycoproteins were determined. More glycoproteins have higher synthesis rate than degradation rate, which is consistent with the fact that the cells were growing.

We also globally quantified cell surface N-glycoprotein dynamics and measured their half-lives by combining metabolic labeling, click chemistry, and multiplexed proteomics (Xiao and Wu, 2017c). Surface glycoproteins with catalytic activities were more stable than those with binding and receptor activities. Glycosylation sites located outside of any domain had a notably longer median half-life than those within domains, which strongly suggests that glycans within domains regulate protein interactions with other molecules while those outside of domains are mainly responsible for protecting protein from degradation. Systematic investigation of glycoprotein dynamics allows us to have a better understanding of reversible protein glycosylation.

IV. APPLICATIONS OF MS-BASED GLYCOPROTEOMICS

Protein glycosylation is one of the most complex modifications in all types of organisms. Characterizing glycosylation events in various samples will certainly lead to a deeper understanding of many cellular processes and better solutions to biomedical problems. The strategies developed in the field of glycoproteomics have been widely applied to investigate a variety of samples, including plants and clinical tissue samples. In this last section, we review the applications of MS-based glycoproteomics in different biosystems.

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A. Yeast

Saccharomyces cerevisiae is one of the most commonly used model systems in laboratories. Although it has much fewer genes than mammalian cells, the global analysis of glycoproteins in yeast is still challenging (Breidenbach et al., 2010; Bailey and Schulz, 2013; Halim et al., 2015). Unlike glycans in human cells, the yeast glycans are mostly the high-mannose type (Kukuruzinska et al., 1987), and the molecular weight of N-glycans can sometimes be very large. Furthermore, yeast has cell walls, and many cell wall proteins are heavily mannosylated. Investigating protein glycosylation in yeast has long been intriguing to researchers.

In 2009, Schulz and Aebi (2009) designed a novel method to quantify glycosylation site occupancy in yeast. They enriched glycoproteins bound to the yeast polysaccharide cell wall and released glycans using Endo H, which also created a mass tag on the glycoproteins simultaneously. Peptides and glycopeptides were analyzed by LC-MS/MS. Their experimental results revealed that the paralogues Ost3p and Ost6p had crucial roles in efficient glycosylation of distinctly defined glycosylation sites. Bailey and Schulz (2013) later demonstrated that adding a protein deglycosylation step prior to enzymatic protein digestion can systematically improve N-glycoprotein identification in yeast lacking Alg3p. By treating proteins with PNGase F before protein digestion with Asp-N or trypsin, the coverage of the yeast cell wall proteome was improved.

As discussed above, Chen et al. (2014a) developed a chemical deglycosylation method to study the yeast N-glycoproteome, and 555 protein N-glycosylation sites were identified on 250 glycoproteins in yeast cells. They later devised a boronic acid-based enrichment strategy to universally analyze glycoproteins, and identified 816 N-glycosylation sites from 332 glycoproteins in yeast. In a recent report, with more effective enrichment of glycopeptides, over 1,000 protein N-glycosylation sites were identified on over 500 proteins (Xiao et al., 2018c).

Xiao et al. (2016b) quantified the proteome and glycoproteome changes in yeast cells treated with tunicamycin compared with untreated cells. Among a total of 4,259 quantified proteins, over 5% of them were found to be down-regulated by at least twofold, and many N-glycopeptides were down-regulated as expected because tunicamycin is a potent inhibitor for protein N-glycosylation. Smeekens et al. (2017) identified and quantified secreted yeast proteins and glycoproteins from tunicamycin-treated cells. The secreted yeast glycoproteins were separated from cells through mild washing and centrifugation that avoided cell death, limiting the impact of intracellular proteins on the secretome analysis. Among 27 secreted glyco-proteins, 26 of them were down-regulated with the tunicamycin treatment, testifying that the secretion of these glycoproteins is regulated by N-glycosylation.

Compared to protein N-glycosylation, global analysis of protein O-glycosylation in yeast has been relatively under-studied. In baker's yeast, O-glycans consist of only mannose, but the number of mannose per glycan varies. Taking advantage of an O-glycan elongation deficient yeast strain to simplify the sample complexity, for the first time, Neubert et al. (2016) globally analyzed protein O-mannosylation in yeast, and identified over 500 O-glycoproteins. More than 2,300 O-mannosylation sites were mapped by ETD-based MS/MS.

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They found that O-mannosylation is a prominent modification of not only cell wall and plasma membrane proteins, but also a large number of proteins from the secretory pathway with crucial functions in protein glycosylation, folding, quality control, and trafficking (Neubert et al., 2016).

In the DBA method discussed above, enriched glycopeptides remain intact, which also enables us to globally analyze O-glycoproteins. In order to increase the confidence of intact O-glycopeptide identification, HCD was employed for glycopeptide fragmentation, and the tandem mass spectra were recorded in the Orbitrap cell with high resolution and high mass accuracy. Byonic™ was used to search the raw files for the identification of protein O-mannosylation. In biologically duplicate experiments, we identified 987 unique O-glycopeptides on 206 proteins, and 971 unique O-glycopeptides on 196 proteins, respectively. In total, 234 O-glycoproteins were identified, and 168 proteins were found in both experiments. The distribution of the number of mannose per glycan was also studied. The number of unique glycopeptides with one mannose is the highest, and the second are those with four mannoses. For glycopeptides with glycans containing more than four mannoses, the number decreases with the increasing number of mannoses. O-glycoproteins located on the cell wall ($P = 4.25E\text{-}32$) are the most highly enriched. Based on reversible covalent interactions between DBA and glycans, protein O-glycosylation can be confidently identified, including valuable information about O-glycosylation sites and glycan structures (Xiao et al., 2018c).

B. Plant

Glycosylation is of great importance in plant cells. Similar to mammalian cells, many proteins in the extracellular and endomembrane systems are glycosylated by N-linked oligosaccharides in plants. Protein N-glycosylation impacts not only their physicochemical properties but also their biological functions (Rayon et al., 1998). Despite the fact that protein glycosylation is relatively conserved across all eukaryotic species, plant glycosylation does have its uniqueness compared to mammalian cells. However, unlike glycosylation in mammalian cells which has been extensively studied, plant glycosylation is still relatively not well-studied.

A plant proteomics experiment typically includes the following steps: cell/tissue preparation, protein extraction and digestion, peptide separation, and MS analysis (Jorrin-Novo, 2014). For protein modification studies, usually there is an additional enrichment step. The protein extraction procedure for plant can be very different from mammalian protein extraction because: (i) plants have a great variety of tissues and the extraction procedure varies for different tissues; (ii) protein concentrations in plant samples are usually low and yet plant tissues have high amount of proteases; and (iii) many plant-originated compounds, such as polysaccharides in the cell wall, lipids, pigments, and metabolites, may interfere with several steps of the typical proteomics workflow.

Glycoproteomic studies in plant samples have been reported in the literature (Bardor et al., 1999; Ytterberg and Jensen, 2010; Song et al., 2011; Zhang et al., 2011; Ruiz-May et al., 2012). Andon et al. (2003) performed a proteomic study on the mannose-binding proteins in rice (*Oryza sativa*). Instead of directly analyzing glycoproteins, they studied proteins

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involved in rice sugar metabolism, including several rice lectins. The method they used to enrich glycoproteins is column affinity chromatography, and a-D-mannose was employed as the ligand to pack the column for binding desired glycoproteins. Saravanan and Rose (2004) evaluated several extraction techniques to analyze proteins in recalcitrant plant tissues, and found that compared to acetone-based protein precipitation methods, phenol-based methods allowed them to identify higher numbers of proteins and glycoproteins.

Wimmer et al. (2009) reported a nice method to isolate "membrane-associated, boronic acid-interacting proteins," such as glycoproteins and glycosylphosphatidylinositol (GPI)-anchored proteins, using boronate affinity chromatography. Resin-immobilized phenylboronic acid was employed to capture glycoproteins from root microsomal preparations of arabidopsis (*Arabidopsis thaliana*) and maize (*Zea mays*). These proteins were then analyzed by 2D-gel electrophoresis and MALDI-TOF MS.

Albenne et al. (2009) systematically studied plant cell wall proteins including glycoproteins. They established a workflow to prepare cell wall peptides for MS analysis and developed a bioinformatics tool to interpret the data. In that study, N-glycosylation was found on peroxidases, such as PER32. Their MS data also provided insights into N-glycan structures and facilitated protein glycosylation prediction.

For those reports published in recent years, lectin-based enrichment methods were the most widely used to enrich plant glycoproteins. Typically, one or several types of lectins are immobilized on the resin to pack a column, and then lectin affinity chromatography is performed to separate and purify glycoproteins in plant samples. Through the enrichment, researchers were able to analyze both N- and O-glycosylation on plant proteins. For instance, Rose and co-workers carried out a comparative study to analyze N-glycoproteins in tomato fruit. In one experiment, by combining three lectins, namely concanavalin A, snowdrop lectin, and lentil lectin, to enrich glycoproteins, they identified 448 putative N-glycoproteins. In the other parallel experiment using lectin affinity chromatography plus hydrophilic interaction chromatography as the enrichment method, 318 putative N-glycosylation sites on 230 N-glycoproteins were identified. Of note, 17 N-glycan structures were also studied (Ruiz-May et al., 2014). Thannhauseret al. studied glycoproteins in tomato fruit by combining lectin affinity chromatography and LC-MALDI-MS/MS, and the glycoproteins identified are involved in multiple biological processes, such as carbohydrate metabolism, proteolytic activity, oxidative catabolism, phosphatase activity, nucleic acid catabolism/transcriptional regulation (Catala et al., 2011).

C. Mouse

Mouse is an excellent model to study the biology in complex and dynamic physiological systems, and is frequently used in biomedical research. Many clinically-relevant mouse models were generated to mimic the cellular processes of human diseases (Saxena and Christofori, 2013). Through these models, the molecular mechanisms of disease and clinical responses of disease treatment were investigated, and the results of these studies contribute immensely to the development of new therapies for disease treatments. Due to the biomedical importance of mouse models, analyzing mouse glycoproteins has been the focus

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of many reports in the literature (Kurogochi et al., 2010; Tian et al., 2010; Lee et al., 2011; Danzer et al., 2012; Noro et al., 2015).

Besides the mouse N-glycoproteome studied by Mann et al. that we described in the first section of this review (Zielinska et al., 2010), there are also many other impactful glycosylation studies on mouse samples. Cima et al. (2011) used a glycoproteomics approach to discover serum biomarkers for prostate cancer. Hydrazide chemistry coupling with solid phase extraction was employed for the enrichment, and they identified 775 N-glycoproteins from sera and prostate tissues of wild-type and *Pten*-null mice. Label-free quantification was then performed, and the results demonstrated that *PTEN* deletion led to changes of the glycoproteomes in the prostate tissue and serum samples. Based on these results, further targeted-proteomics and bioinformatics studies were carried out to identify potential biomarkers. This study is an excellent example regarding a rational design of proteomic analysis in mouse models for the discovery of biomarkers.

Goldberg et al. (2007) developed a program for automated N-glycopeptide identification, which can identify glycopeptides and also annotate glycan composition. To validate this strategy, they isolated proteins from mouse ovaries and analyzed N-glycoproteins. Zhang et al. (2010) optimized a protocol for the enrichment of both glycopeptides and phosphopeptides through electrostatic repulsion hydrophilic interaction chromatography. They identified 922 glycosylation sites on 544 glycoproteins, and 915 phosphorylation sites on 383 phospho-proteins from the mouse brain membrane.

O-glycosylation studies in many mouse samples were also reported in the literature. Alfaro et al. (2012) devised a strategy combining metabolic labeling, chemical/enzymatic enrichment, and photochemical cleavage to study mouse brain O-GlcNAcylated proteins as discussed above. From 100 mg tryptic peptides, they were able to identify 458 O-GlcNAcylation sites and 195 glycoproteins. Palmisano et al. (2012b) combined titanium dioxide enrichment with HILIC to investigate protein modifications during mouse brain development, and were able to identify 3,246 unique sialoglycopeptides. More than 10% of these peptides were found to be differentially regulated in the development process. Although O-glycosylation studies in mouse are still challenging, with the development in enrichment and MS fragmentation methods, we expect more applications of O-glycosylation analyses in mouse models will be performed, which will expand our knowledge of O-glycoprotein functions in cells with healthy or diseased states.

D. Human Cell Lines and Intact Glycopeptide Analyses

Glycoproteins in human cell lines and stem cells have been extensively studied (Alvarez-Manilla et al., 2010; Dai et al., 2011; Boheler et al., 2014; Bausch-Fluck et al., 2015; Konze et al., 2017; Li et al., 2017a; Stadlmann et al., 2017), which provide much valuable information. Glycoproteomic analysis has advanced from glycoprotein identification-centric studies to site-specific investigation, and glycan structural characterization of intact glycopeptides and glycoproteins. Although both bottom-up and top-down glycoproteomics have developed rapidly (Tran et al., 2011; Yang et al., 2016; Lakbub et al., 2017), glycopeptide-based analysis holds the advantages for global analysis of glycosylation sites and glycan structures. In addition, stoichiometry and glycan occupancy information can also

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be obtained from this working route (Schulz and Aebi, 2009; Pan et al., 2012; Li et al., 2017b; Yang et al., 2017a). To study intact glycopeptides in human cells, different fragmentation methods have been employed and each method has its strengths and weaknesses. CID can generate abundant b- and yions that are useful to identify the glycan composition and substructure. However, CID cannot produce adequate and reliable fragments for peptide identification and glycosylation site localization. Beam-type HCD usually can generate enough b- and y- ions for peptide identification, but the frequent detachment of glycans renders the site localization less effective. ECD and ETD fragmentation methods yield c- and z- ions to determine the peptide backbone sequence and glycosylation site, but there is not enough information for glycan structure elucidation.

Due to the limitations of each method, attempts were made to perform MS^3 experiments and/or combine different fragmentation techniques to analyze intact glycopeptides (Wu et al., 2013; Zeng et al., 2016). These attempts can improve the peptide backbone fragmentation efficiency, or allow pre-selection of glycopeptides, thus filtering out non-glycopeptides and allocating more analysis time for glycopeptides. Parker et al. (2013) combined glycomics and glycoproteomics to study the N-glycoproteome. They analyzed the glycosylation sites and glycan structures separately. The glycopeptides were deglycosylated with PNGase F and identified by LC-MS/MS to accurately localize N-glycosylation sites. The glycan-containing form of peptides were analyzed to identify the glycan composition, and eventually the data collected from both analyses were combined to reconstruct intact glycopeptides. With both the glycome and glycoproteome information, a total of 863 unique N-glycopep-tides from 161 glycoproteins were identified.

Other attempts were focused on developing computational algorithms for site-specific assignments of intact glycopeptides. Zhang et al. developed software called GPQuest to analyze intact glycopeptides. Using this software, they first generated a spectral library of glycosite-containing peptides from MS analysis using HCD as the fragmentation method. Intact glycopeptides were then selected based on the oxonium ions, and the spectra were compared with the library generated from glycosite-containing peptides. During this step, MS/MS spectra of intact glycopeptides were assigned to specific glycosite-containing peptides. The glycans were then determined through calculating the mass shift between the precursor ions of intact glycopeptides and the glycosite-containing peptides (Eshghi et al., 2015; Hu et al., 2017a).

Frese et al. (2012) developed a novel method that combined HCD with ETD (termed EThcD) to improve peptide backbone fragmentation. After the initial ETD, all ions were fragmented by CID. Therefore, in the end, b-, y-, c-, and z-ions were observed in the same spectrum. They later applied this method for protein phosphorylation analysis (Frese et al., 2013). Recently, glycopeptides were analyzed by EThcD MS after lectin and HILIC enrichment (Yu et al., 2017b). Since both the glycosidic and amide bonds were cleaved, the information about glycan structures and peptide sequences were obtained. The authors also compared the number of glycoforms identified from EThcD or HCD alone and a greater number of glycoforms were observed using EThcD.

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E. Clinical Samples

Due to the importance of protein glycosylation in biological systems, glycoproteomics has been extensively applied to disease-related research (Whelan et al., 2009; Hwang et al., 2010; Song et al., 2014; Reiding et al., 2017; Yang et al., 2017b; Kailemia et al., 2018), such as hepatitis, cancer (Dai et al., 2011; Liu et al., 2014; Kailemia et al., 2017; Miyamoto et al., 2018; Veillon et al., 2018), and infectious diseases (Vigerust, 2011; Go et al., 2017). Glycosylation can change chemical and physical properties of proteins, and regulate their binding and interactions with ligands or extracellular matrix. Aberrant glycosylation patterns reflect abnormal cellular processes, which can be used to understand the disease mechanisms and to monitor disease statuses (Varki, 2017). For instance, an increase in sialylation is a common feature of cancer cells (Vajaria et al., 2016). Furthermore, as described in the Warburg effect, cancer cells predominantly produce energy through glycolysis instead of mitochondrial oxidative phosphorylation (Heiden et al., 2009). This leads to a great increase in glucose uptake, which elevates the level of UDP-GlcNAc, the end product of the hexosamine biosynthetic pathway. With a high concentration of UDP-GlcNAc, as expected, protein O-GlcNAcylation was elevated in cancer cells (Ma and Vosseller, 2014).

Many secreted proteins are glycosylated, and secreted glycoproteins in body fluids may serve as excellent noninvasive biomarkers. One of the most important glycoproteomic applications in clinical samples is to discover effective disease biomarkers (Abd Hamid et al., 2008; Kaji et al., 2013; Benicky et al., 2014; Mayampurath et al., 2014; Pompach et al., 2014; Zhang et al., 2014; Song and Mechref, 2015; Cotton et al., 2017; Song et al., 2018). Unlike traditional strategies that screen a single or a small number of potential biomarker(s), monitoring a larger group of candidates using glycoproteomic approaches can often result in higher accuracy and specificity. For example, Ahn et al. (2014) attempted to screen biomarkers for small cell lung cancer through a glycoproteomic approach. They enriched glycoproteins using a lectin column, and then analyzed the glycoproteome changes using both label-free quantification and multiplexed proteomics. The results showed that the expression and glycosylation changes in fucosylated proteins, such as paraxonase 1, might serve as serological markers for small cell lung cancer. Qiu et al. (2008) profiled plasma glycoproteins in order to find biomarkers for colorectal cancer. They combined lectin glyco-array and LC-MS to determine the glycan patterns from the plasma samples of nine normal, five adenoma, and six colorectal cancer patients, and found several proteins with elevated sialylation and fucosylation as potential biomarkers of colorectal cancer. These markers were then validated by lectin blotting of plasma samples from 30 patients.

Halim et al. (2012) applied MS-based glycoproteomics to analyze glycoproteins with glycan structural information in clinical samples. In 2012, they performed a study to analyze N-and O-linked glycoproteins in urine samples. After eliminating the interferants by dialysis, they performed the enrichment with hydrazide chemistry, and characterized intact glycopeptides by CID and ECD. Later they combined PNGase F pretreatment and automated CID-MS^2/MS^3 fragmentation for glycopeptide identification, and ECD/ETD for glycosylation site localization, to analyze intact O-glycopeptides digested from glycoproteins in human cerebrospinal fluid samples (Halim et al., 2012). In 2014, by using synthetic glycopeptides,

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and glycopeptides from human cerebrospinal fluid samples, they found that oxonium fragmentation patterns were able to differentiate O-GlcNAc from O-GalNAc, which is an interesting discovery for intact O-glycopeptide analysis (Halim et al., 2014).

The applications of glycoproteomics in clinical samples are far more than what we described in this section, there are many comprehensive reviews covering the importance of glycoproteomic technologies in biomedical research (Kim and Varki, 1997; Tian and Zhang, 2010; Plomp et al., 2016; Ruhaak et al., 2018; Zhang et al., 2018). Due to the irreplaceable roles of glycoproteins playing in various diseases, high-throughput glycoproteomic technologies will continue to be developed to enable large-scale profiling of glycopeptides and glycan structures. Foreseeably, glycoproteomics will help shape the directions of future biomedical research.

V. CONCLUSIONS AND PERSPECTIVES

The tremendous development in chemical biology and MS technologies results in rapid advancements in the field of glycoproteomics. Although MS-based glycoproteomic analysis is challenging due to the low abundance of many glycoproteins and the heterogeneity of glycan structures (An et al., 2003; An et al., 2009), effective methods for glycopeptide/ glycoprotein enrichment, mass tag generation, and glycoprotein quantification have dramatically contributed to protein glycosylation studies. We believe that advances in methodologies will continue to revolutionize this field and accelerate our exploration in the field of glycoscience.

Here we discussed MS-based methods to globally analyze protein glycosylation. We first reviewed the methods for the enrichment of glycopeptides/glycoproteins, including lectin enrichment, hydrazide chemistry, HILIC, click chemistry-based, and boronic acid-based methods. Following this, we included several enzymatic and chemical methods for the generation of a common mass tag for MS analysis. With the methods described above and multiplexed proteomics, large-scale investigation of glycoprotein dynamics has come to the realization. Therefore, in the following section, we reviewed the methods to systematically study glycoprotein dynamics. In the last section, we discussed many applications of MS-based glycoproteomics for different samples, from yeast, plant, mouse models, to clinical samples, and also included MS analysis of intact glycopeptides.

With these methods and further developments, MS-based glycoproteomics provides a wealth of valuable information, from glycosylation sites to glycan structures. Analysis of intact glycopeptides allows us to obtain glycosylation site and glycan structure information. In addition, global analysis of protein glycosylation stoichiometry also becomes achievable (Sun and Zhang, 2015). These studies will have profound impacts on biochemical and biomedical research. In addition, as entering an era with immense advances in computation and MS instrumentation, we expect to see further developments in MS-based glycoproteomics, which will enable more sensitive peptide sequencing, higher-speed analysis, and more suitable fragmentation techniques.

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Higher computational power will also lead to the development of novel bioinformatics tools for glycoproteomics. New and innovative software can allow us to quickly and accurately identify glycopeptides, especially intact glycopeptides, and to obtain more valuable information regarding protein glycosylation sites and glycan structures (Hu et al., 2017b; Lakbub et al., 2018). Due to the importance and complexity of protein glycosylation, it is urgent to further develop effective chemical and enzymatic methods for global analysis of glycoproteins. It is expected that the field of glycoproteomics will grow exponentially in the next decade, which will further advance glycoscience and biomedical research.

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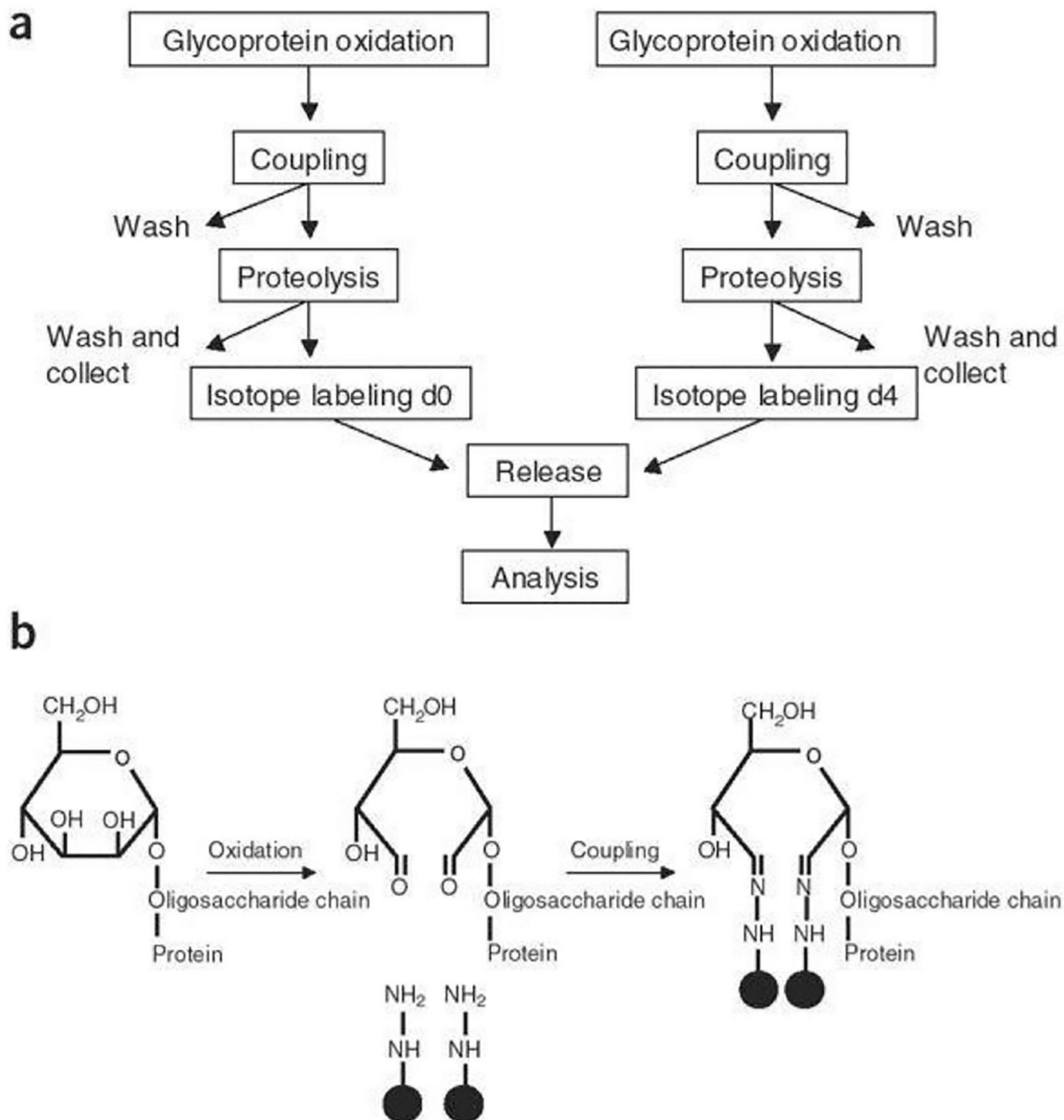

FIGURE 1.
(a) Experimental procedure for quantitative analysis of glycopeptides from two biological samples through hydrazide chemistry. (b) The core concept of hydrazide chemistry-based enrichment. Reproduced with permission from [Nature Biotechnology. 2003; 21:660–666]. Copyright © 2003 Nature Publishing Group.

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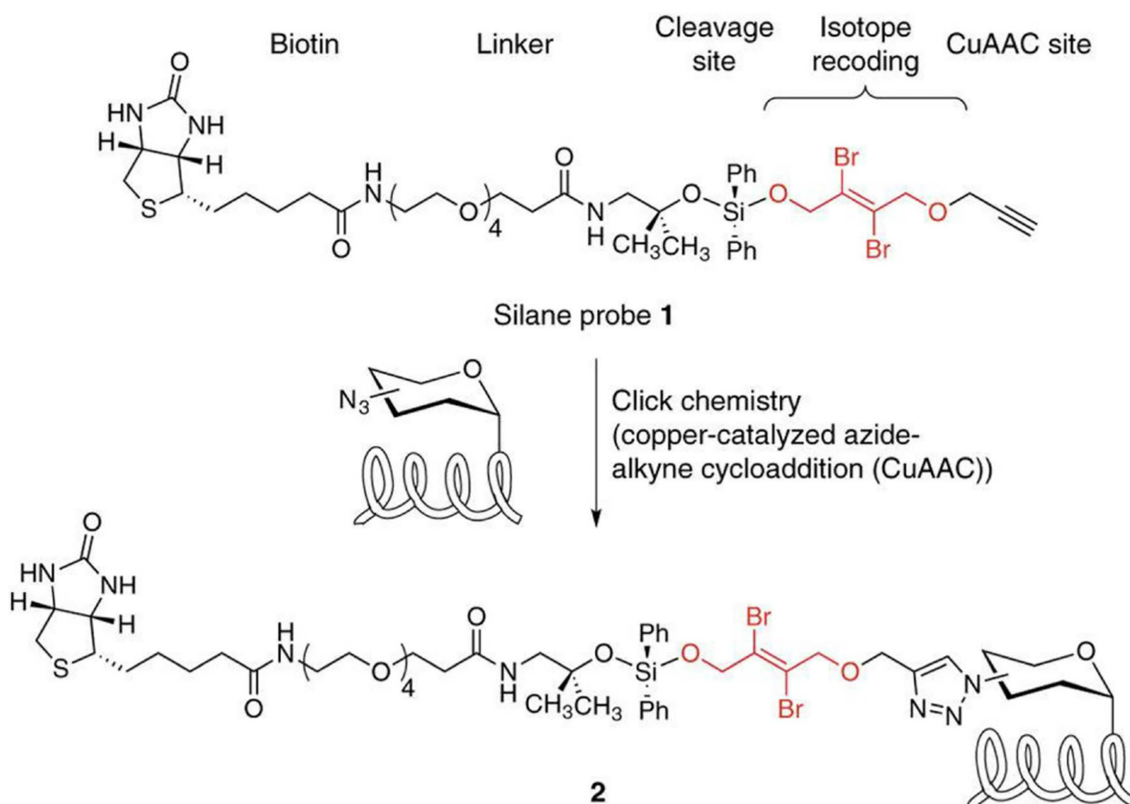

FIGURE 2.
Structure of the isotopic affinity cleavable probe 1 and glycoproteins/glycopeptides tagged by the probe *via* click chemistry 2. Reproduced with permission from [Nature Methods. 2015; 12:561–567]. Copyright © 2015 Nature Publishing Group.

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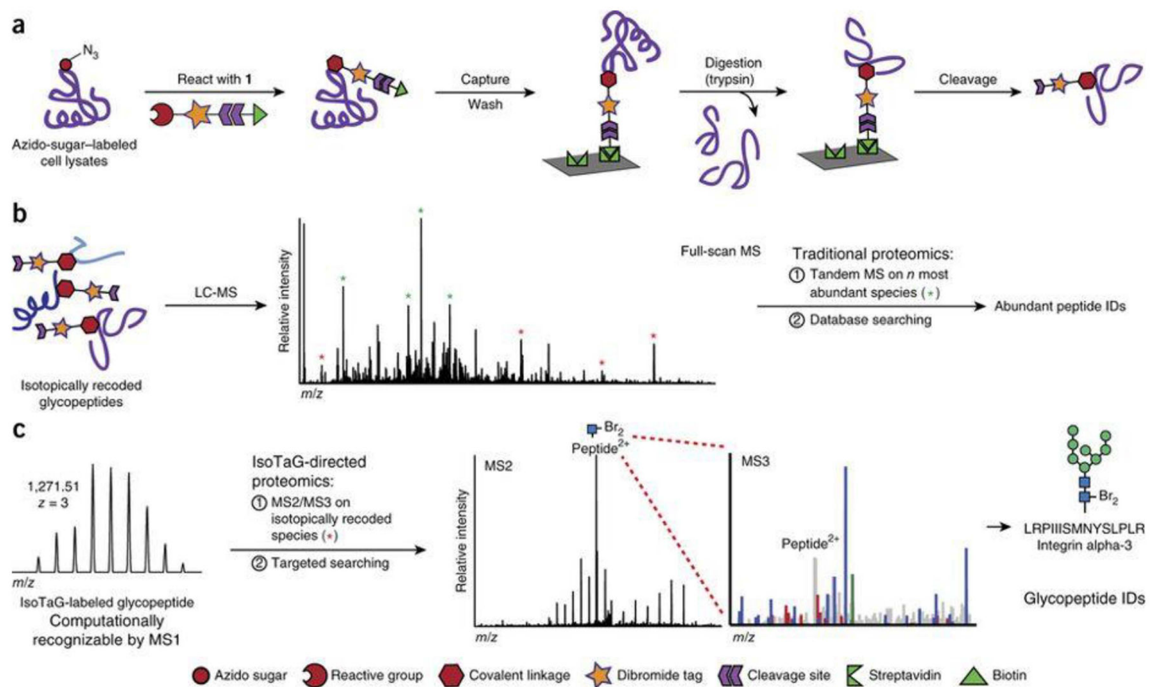

FIGURE 3.

The experimental procedures of IsoTaG-based identification of intact glycopeptides. (a) Metabolically labeled glycoproteins are tagged by dibrominated silane (probe 1), and then enriched with streptavidin. After protein digestion and the removal of non-glcopeptides, glycopeptides are eluted. (b) LC-MS analysis to characterize the isotope-coded glycopeptides. (c) High-confidence glycopeptide identification is enabled by the pattern-searching algorithm. Reproduced with permission from [Nature Methods. 2015; 12:561–567]. Copyright © 2015 Nature Publishing Group.

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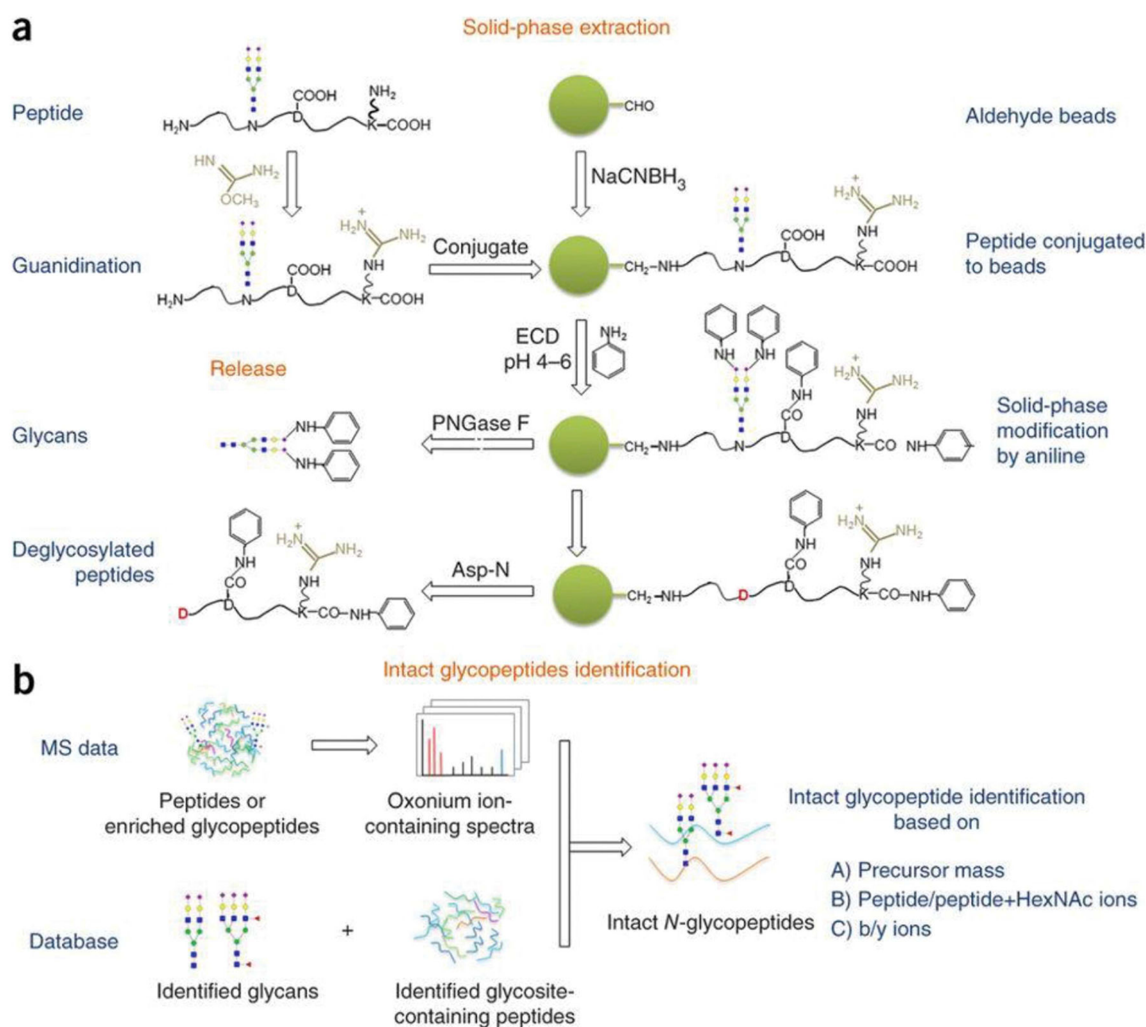

FIGURE 4.
(a) Workflow of the NGAG method and chemical/enzymatic reactions involved in the enrichment of N-glycans and their parent glycopeptides. (b) Identification of N-glycans and N-glycopeptides. Reproduced with permission from [Nature Biotechnology. 2016; 34:84–88]. Copyright © 2016 Nature Publishing Group.

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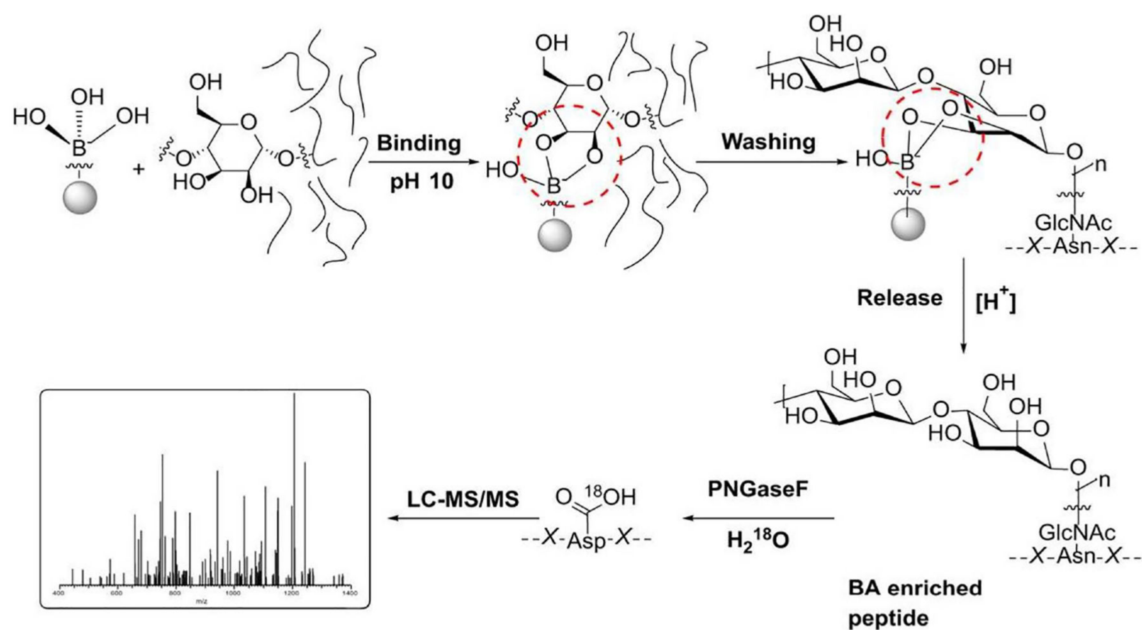

FIGURE 5.
Workflow of the boronic acid-based enrichment method for comprehensive analysis of protein N-glycosylation sites. Reproduced with permission from [Molecular & Cellular Proteomics. 2014; 13:1563–1572]. Copyright © 2014 American Society for Biochemistry and Molecular Biology, Inc.

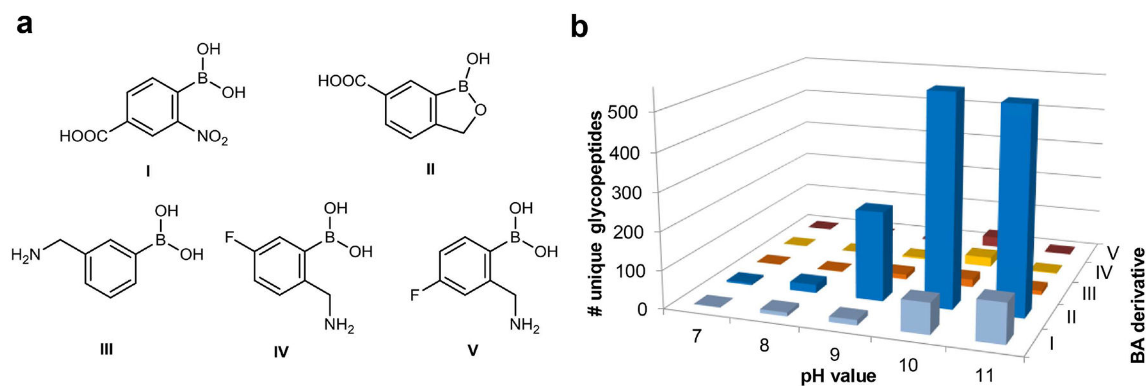

FIGURE 6.

(a) Structures of boronic acid derivatives tested for glycoproteomic analysis. (b) The number of unique N-glycopeptides identified with each boronic acid derivative from the parallel experiments. The same amount of starting materials (peptides digested from the whole cell lysate of HEK 293T cells) was used in each experiment. Reproduced with permission from [Nature Communications. 2018; 9:1692]. Copyright retained by the authors under a CC BY license.

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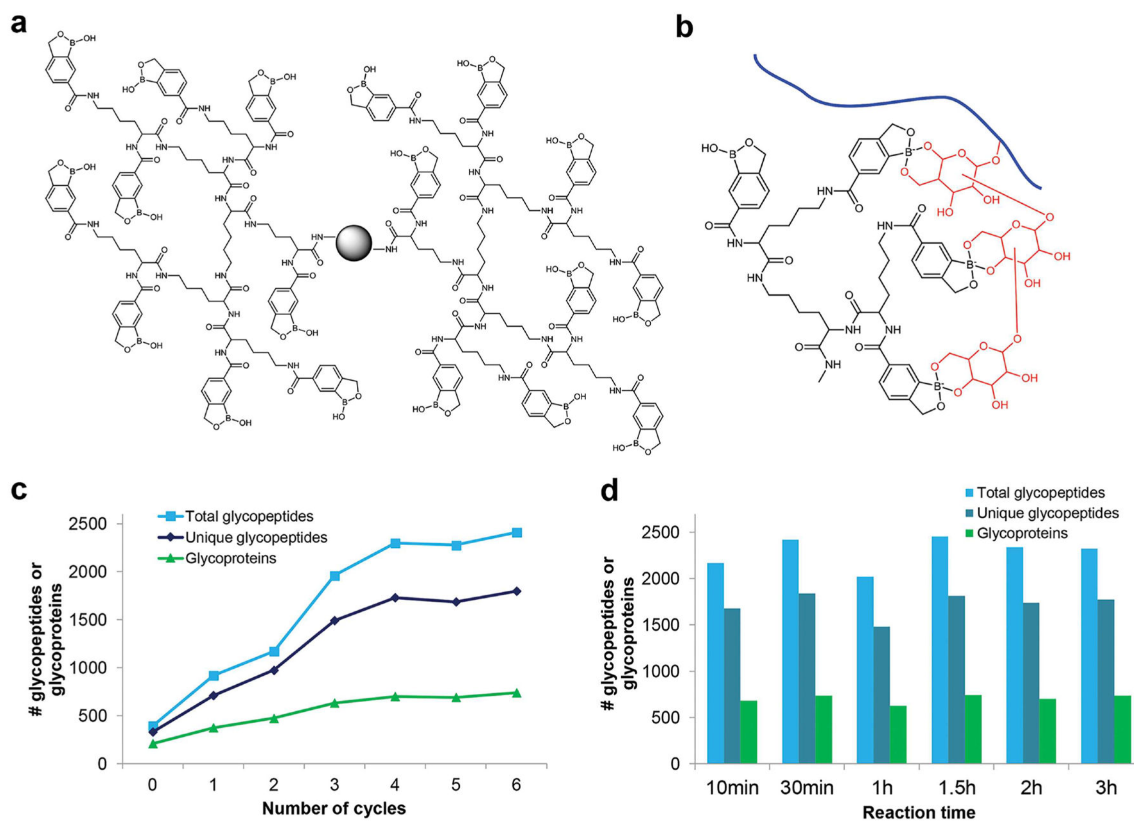

FIGURE 7.

(a) The structure of the dendrimer conjugated with benzoboroxole. (b) An example of the synergistic interactions between multiple benzoboroxole molecules in a dendrimer and several sugars within one glycan. (c) The effect of synthesis cycles and corresponding dendrimer size on the enrichment of glycopeptides. (d) The effect of reaction time on the N-glycopeptide identification. Reproduced with permission from [Nature Communications. 2018; 9:1692]. Copyright retained by the authors under a CC BY license.

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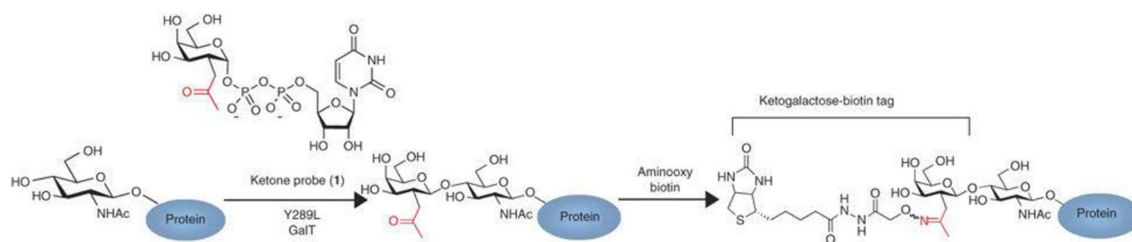

FIGURE 8.

Procedure for incorporating a ketone group onto the GlcNAc and tagging it with biotin. Reproduced with permission from [Nature Chemical Biology. 2007; 3:339–348]. Copyright © 2007 Nature Publishing Group.

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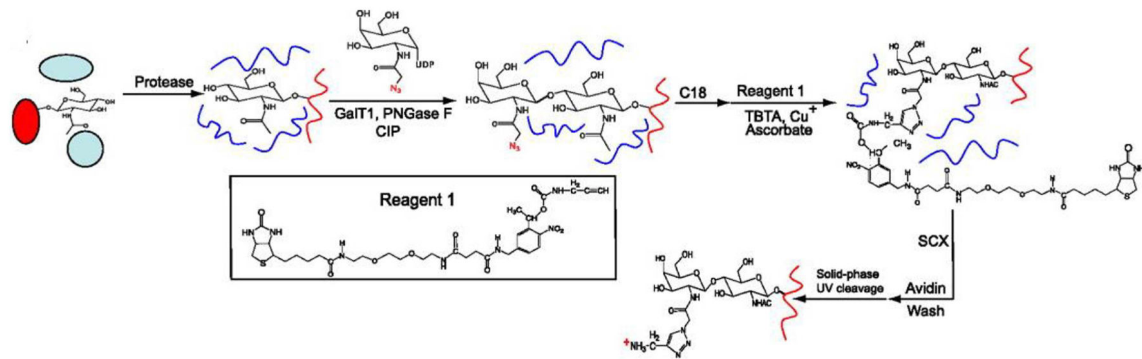

FIGURE 9.

Workflow of enrichment of O-GlcNAc-modified peptides by combining enzymatic labeling with UDP-GalNAz and chemical derivatization with PC-PEG-biotin-alkyne (Reagent 1). Reproduced with permission from [Molecular & Cellular Proteomics. 2010; 9:153–160]. Copyright © 2010 American Society for Biochemistry and Molecular Biology, Inc.

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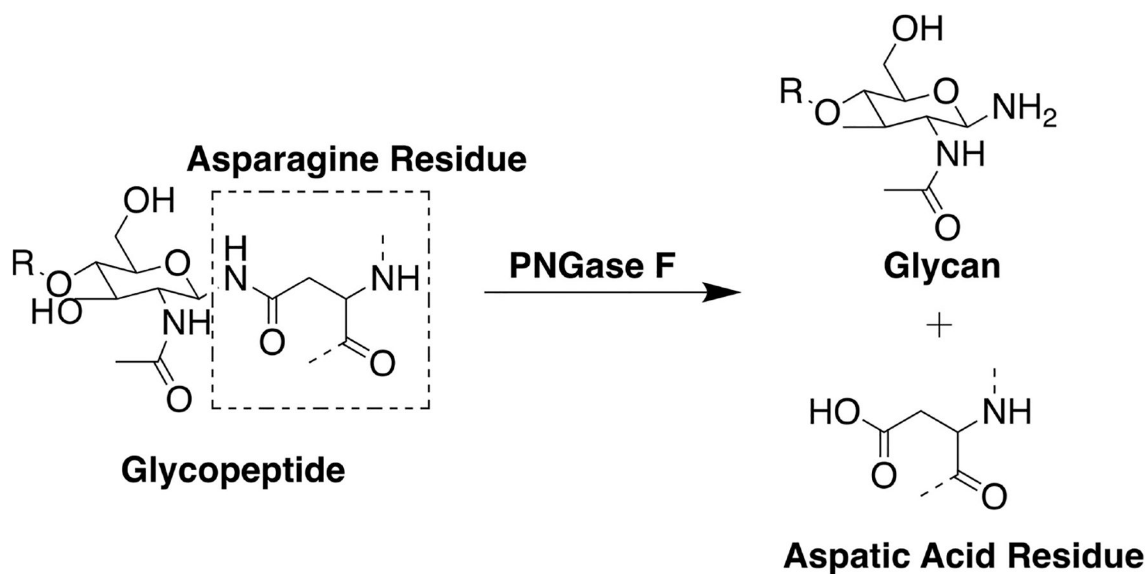

FIGURE 10.

Deglycosylation using PNGase F. After glycoprotein/glycopeptide enrichment, glycans are removed with PNGase F, converting Asn to Asp at the same time and causing a mass shift of +0.9840 Da.

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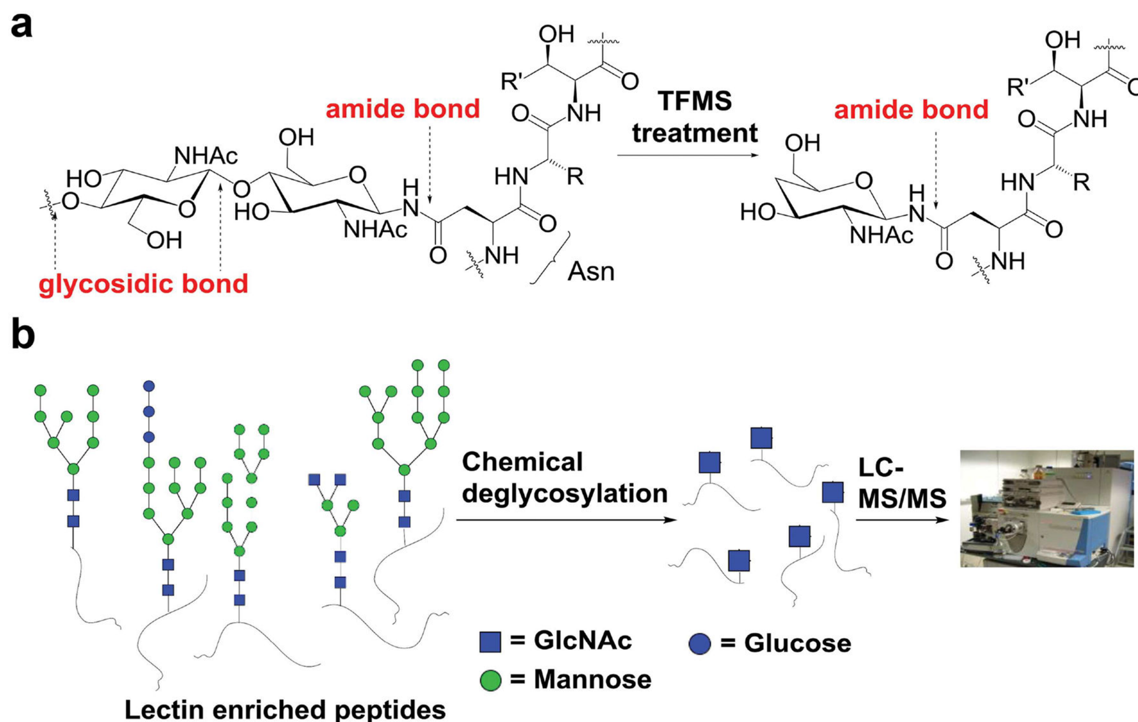

FIGURE 11.
(a) The difference between the glycosidic bond and the amide bond in response to TFMS treatment;(b) The experimental procedure of large-scale N-glycosylation analysis by combining chemical deglycosylation and MS-based proteomics. Reproduced with permission from [Journal of Proteome Research. 2014; 13:1466–1473]. Copyright © 2014 2014 American Chemical Society.

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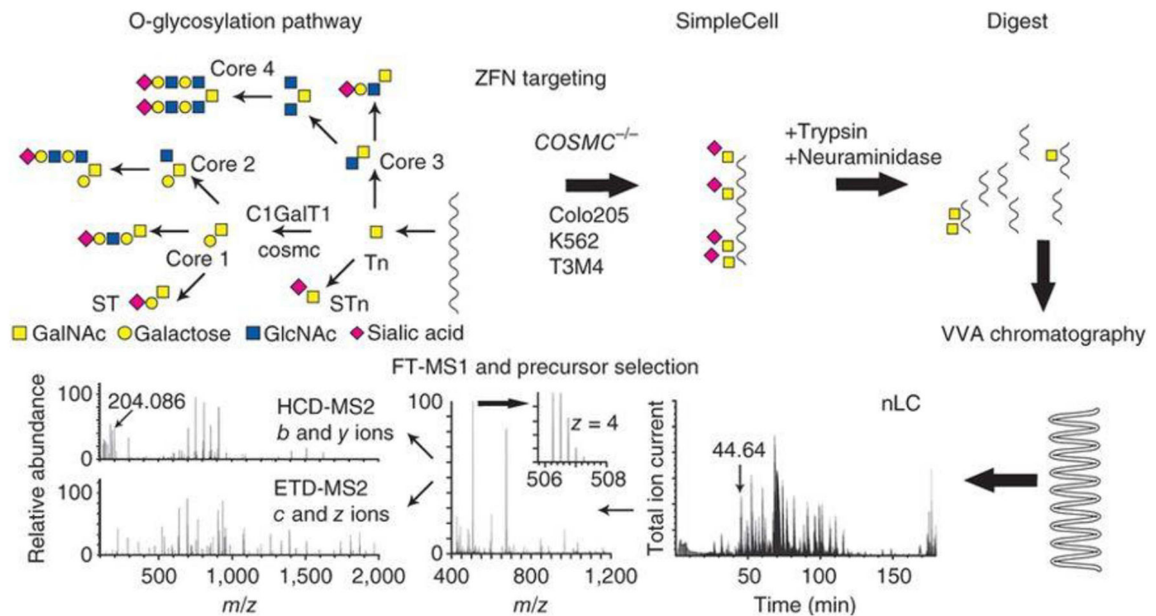

FIGURE 12.

Mapping the human O-glycoproteome using the SimpleCell strategy. The cells were first targeted by ZNF to knockout the gene that codes for COSMC, which is the chaperone for T-synthase. Without COSMC, the glycan structures were simplified to containing only GalNAc or NeuAc-GalNAc. The sialic moiety of the latter can be removed by neuraminidase, and O-GalNAc on the glycopeptides serves as a common tag for O-glycosylation site identification with MS. Reproduced with permission from [Nature Methods 2011; 8:977–982]. Copyright # 2011 Nature America, Inc.