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Recent advances in mass spectrometry (MS)-based glycoproteomics in complex biological samples

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Abstract

Protein glycosylation plays a key role in various biological processes and disease-related pathological progression. Mass spectrometry (MS)-based glycoproteomics is a powerful approach that provides a system-wide profiling of the glycoproteome in a high-throughput manner. There have been numerous significant technological advances in this field, including improved glycopeptide enrichment, hybrid fragmentation techniques, emerging specialized software packages, and effective quantitation strategies, as well as more dedicated workflows. With increasingly sophisticated glycoproteomics tools on hand, researchers have extensively adapted this approach to explore different biological systems both in terms of in-depth glycoproteome profiling and comparative glycoproteome analysis. Quantitative glycoproteomics enables researchers to discover novel glycosylation-based biomarkers in various diseases with potential to offer better sensitivity and specificity for disease diagnosis. In this review, we present recent methodological developments in MS-based glycoproteomics and highlight its utility and applications in answering various questions in complex biological systems.

Keywords

Mass spectrometry; Glycoproteomics; N-Glycosylation; O-Glycosylation; Biological samples; Biomarker; Disease

1. Introduction

As one of the most common protein post-translational modi-fications (PTMs), protein glycosylation plays an important role in protein stability, intra- and intercellular signaling, fertilization, embryogenesis, organ development, hormone activity, and immunological regulation [1]. It is estimated that half of the proteins expressed in cells are glycoproteins. There are many types of protein glycosylation, and the most widely studied types are N-linked (amide nitrogen of asparagine residue) and O-linked (hydroxyl oxygen of serine or threonine residue). There is a consensus amino acid sequence (Asn-X-Thr/Ser (X is any

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amino acid except proline)) that contains the glycosylation site of N-glycoproteins, while no consensus sequence has been found for O-linked glycoproteins yet.

Numerous studies have shown that altered glycosylation played a key role in the pathological process during disease progression. Mass spectrometry (MS)-based glycoproteomics is a powerful, high-throughput approach that enables system-wide screening of glycosylation-based biomarkers. In fact, many current disease biomarkers are glycoproteins, such as CEA for colorectal cancer, CA-125 for ovarian cancer, and AFP for hepatocellular carcinoma etc., and the glycans attached to them have been shown to be altered during oncogenesis [2–4]. With glycosylation being particularly sensitive to malignant transformation, glycosylation-based bio-markers hold great promise to improve the sensitivity and specificity of current protein-based biomarkers and may eventually contribute to disease early diagnosis and better treatment [5]. Therefore, comprehensive profiling of protein glycosylation is prerequisite to better understand its role in these pathological and physiological processes.

Over the past decade, substantial progress has been made to obtain detailed information of protein glycosylation, including glycan structures, glycosylation site and its occupancy, and protein sequence. Traditionally, there are two different approaches to study protein glycosylation: (1) the ‘glycomics’ approach, which focuses on the glycan structures after glycan release from proteins and other sugar-containing moieties and (2) the ‘glycoproteomics’ approach, which examines the localization of glycosylation site and structural elucidation of glycans, as well as protein sequence. In this review, we will focus on MS-based glycoproteomics approach. Substantial advances have been made in this field, and here we highlight recent methodological developments and their applications toward comprehensive understanding of the function of protein glycosylation.

2. Glycopeptide enrichment

Comprehensive profiling of the glycoproteome from a complex biological sample is still challenging due to the wide dynamic range of proteins and the micro- and macro-heterogeneity of glycosylation [6,7]. Isolating glycopeptides from complex samples by an appropriate enrichment method is the most efficient way to reduce the sample complexity and achieve an in-depth glycoproteome analysis.

2.1. Hydrazide chemistry enrichment

Hydrazide chemistry (HC) enrichment is based on the formation of covalent bonds between the $NaIO_4$ oxidized cis-diol on N-and O-linked glycans and the hydrazide groups on the hydrazide beads. The advantage of HC method is its high enrichment spec-ificity, as normally 90% of the enriched peptides are glycopeptides [8]. In the original workflow, the glycopeptides captured on hydrazide beads were deglycosylated and released by the treatment of PNGase F for glycosylation site analysis [9,10]. Recently, the capture and release steps were modified to allow glycopeptides to be released without losing the glycan. Specifically, samples were treated with moderate $NaIO_4$ to selectively oxidize the terminal sialic acid of the glycan to generate an aldehyde while leaving the other parts intact. Next, the sialylated glycopeptides were captured by the hydrazide beads and released through acid

hydrolysis of the glycosidic bond of sialic acid by trifluoroacetic acid (TFA) [11,12]. This method enabled sialylated glycopeptides to be analyzed, but, unfortunately, the degree of sialylation information was lost [13]. To preserve the sialylation information, Nishimura et al. employed ice-cold 1M hydrochloride to cleave hydrazone bond between the sialic acid and hydrazide beads, allowing the sialic acid to remain on the glycan [14].

2.2. Lectin affinity chromatography

Lectin affinity chromatography (LAC) is another popular enrichment method for protein glycosylation analysis and has been approved by FDA for cancer glycoprotein biomarker detection [15]. Several well-characterized lectins had been used for selective enrichment of specific type of N- or O-linked glycopeptides, which is based on the affinity of lectins to glycans with specific structure motif. For example, Concanavalin A (Con A) binds to mannose, wheat germ agglutinin (WGA) binds to sialic acid and N-acetyl-glucosamine, Vicia villosa (VVA) binds to N-acetyl-galactosamine, Aleuria aurantia lectin (AAL) binds to fucose, and Ricinus communis Agglutinin (RCA120) captures terminal β-galactose [16–19]. Multiple lectins can be combined to improve the glycoproteome coverage [20–22], and including additional enrichment methods sequentially after LAC enrichment would further improve the enrichment specificity [23]. Moreover, lectin enrichment has also been incorporated in serial online reactors to allow simultaneous online proteolysis and glycopeptide enrichment, which is useful for the glycopeptide analysis where sample amount is very limited [24].

2.3. Hydrophilic interaction chromatography

Hydrophilic interaction chromatography (HILIC) as a universal glycopeptide enrichment method is based upon glycopeptides being more hydrophilic than non-glycopeptides due to the attached N- or O-linked glycans [25,26]. To increase the hydrophilicity difference between glycopeptides and non-glycopeptides, ion pairing reagents like TFA can be used [27]. Compared with the HC and LAC method, the HILIC method is more versatile and thus can provide a more comprehensive glycoproteome profile [28]. The disadvantage of HILIC is its poor enrichment specificity, which calls for the development of new HILIC materials with stronger hydrophilic functional group to improve the specificity [29–33]. The enrichment specificity can also be further improved by combining the HILIC with other enrichment methods such as LAC [34,35]. New devices, which integrate the HILIC materials in micro-column or tip, have been developed to minimize the sample loss during enrichment procedure and facilitate the detection of low abundance glycopeptides [33,36–39].

Other than the three enrichment methods summarized above, methods using boronic acid materials [40–43], titanium dioxide [44,45], responsive smart polymers [46–49], porous graphitized carbon (PGC) [50], acetone precipitation [51,52], size exclusion chromatography [53], and molecular weight cutoff filter [54] for the enrichment of glycopeptides are also in the ascendant. It is worth mentioning that most of current glycopeptide enrichment methods are suitable for both the N- and O-linked glycopeptides; however, due to the relatively high abundance of N-linked glycopeptides, to efficiently analyze O-linked glycopeptides,

deglycosylation of N-linked glycopeptides or deep fractionation of sample should be performed.

3. Characterization and quantitation of glycopeptides

Qualitative characterization of glycopeptides includes two aspects: glycosylation site profiling and site-specific intact glyco-peptide analysis. A typical MS-based glycoproteomics workflow is shown in Fig. 1.

3.1. Glycosylation site profiling

3.1.1. N-glycosylation site profiling—Since N-glycosylation site profiling was originally performed by deglycosylation with peptide-N-glycosidase F (PNGase F) or endo-b-N-acetylglucosaminidase F&H (endo F&H), several studies focused on improving the deglycosylation efficiency. Huang et al. found that when glycosylation site profiling was performed by HC method, glycopeptides with an N-terminal serine/threonine can be oxidized on both the N-termini and glycans; thus, this type of glycopeptides cannot be released by PNGase F treatment due to being covalently coupling to the hydrazide beads through the N-termini. To overcome this problem, they utilized a peptide N-terminal protection strategy to block the primary amine groups on peptides, which avoided the adjacent amino alcohols on peptide N-termini being oxidized. The results showed that this strategy successfully prevented the oxidation of peptide N-termini and significantly improved the coverage of glycoproteome [55]. Recently, the same group found that releasing the glycopeptides captured on hydrazide beads by PNGase F deglycosylation was inefficient due to steric hindrance in the heterogeneous condition. Thus, they developed a hydroxylamine-assisted PNGase F deglycosylation method which used the hydroxylamine to efficiently cleave hydrazone bonds by transamination and release intact glycopeptides. As deglycosylation of the released glycopeptides was performed under homogeneous condition, the recovery rate of deglycosylated peptides was improved significantly [56]. Another study by Weng et al. reported that N-terminal glycosylated peptides are difficult to be deglycosylated due to the limitation of PNGase F enzymatic specificity, which cannot cleave N-glycans attached to N- or C-termini and require the presence of an extra amino acid at the termini. To overcome this drawback, they developed an N-terminal site-selective succinylation strategy by incorporating an amide bond to mimic an amino acid at the peptide N-termini, which greatly improved N-glycosylation site coverage [57].

Other studies involved combining different sample preparation techniques, enrichment methods, and fractionation strategies to improve the glycoproteome coverage. Mann and coworkers developed an N-glyco-FASP sample preparation approach, where the lectin column in conventional method was replaced with ultrafiltration units, to decrease the glycopeptide loss. In this method, the glycopeptides were enriched by binding to lectins on top of a filter, which greatly reduced the sample loss and improved the glycosite coverage. The robustness of this approach was successfully demonstrated in the large-scale glycosylation site profiling in mouse plasma and four different tissues where 6367 N-glycosylation sites were identified. Combining different enrichment methods is also an effective approach to increase the glycosite coverage [58]. Recently, Qian group combined

two widely used glycopeptide enrichment methods, HC and HILIC, for N-glycosylation site analysis of the secretome of two human hepatocellular carcinoma (HCC) cell lines. A total of 1212 unique N-glycosylation sites from 611 N-glycoproteins were confidently identified. Overall, the overlap of N-glycosylation sites determined by the two methods was only 28.4% [59]. Zou group performed a similar strategy which combined the click maltose-HILIC and the HC method to comprehensively map the N-glycosylation sites of human liver tissue. Altogether,14,480 N-glycopeptides, corresponding to 2210 N-glycoproteins and 4783 N-glycosylation sites, were identified [60]. As another example, Yang group combined seven protease treatments (trypsin, trypsin coupled with Lys-C (Try + Lys), trypsin coupled with Glu-C (Try + Glu), Lys-C, Glu-C,chymotrypsin and pepsin), four different enrichment techniques (HILIC, ZIC-HILIC, HC, and TiO_2 chromatography), and two different fractionation strategies (SCX and high-pH RP), which aided in identifying a total of 13,492 N-glycopeptides, corresponding to 8386 N-glycosylation sites on 3982 proteins in the mouse brain. Considering the efficiency and simplicity, a workflow combining the use of trypsin, Try + Lys and Try + Glu for protein digestion, HILIC and ZIC-HILIC for glycopeptide enrichment, and 1D-RPLCMS/MS for N-glycopeptide detection can also produce a comparable glycosite coverage [61].

3.1.2. O-glycosylation site profiling—Mapping of O-glycosylation is also an active area of research. Among the different types of O-glycosylation, the O-GlcNAcylation and O-GalNAcylation are the most widely studied [62,63]. Profiling of O-glycosylation sites is even more difficult compared to N-glycosylation due to a lack of a consensus sequon and the lack of an enzyme that can effectively deglycosylate the O-linked glycans.

To date, the most successful approach for profiling of OGlcNAcylation has been metabolic and enzymatic labeling, which incorporates an azide-containing group to the O-GlcNAc moiety [64]. Then, the derivatized O-GlcNAc is enriched by an alkynyl biotin or photo-cleavable tag containing alkynyl beads to be analyzed by LC-MS/MS. By using this highly specific strategy, tens to hundreds of O-GlcNAcylation sites can be mapped [65–68]. The drawback of this approach is relatively low labeling efficiency, leading to the limited coverage of O-GlcNAcylation sites [69]. Besides the enzymatic and metabolic labeling methods, Burlingame and coworkers developed a lectin weak affinity chromatography (LWAC) strategy to enrich O-GlcNAc peptides with wheat germ agglutinin (WGA) lectin [70]. The same group recently optimized this LWAC strategy and identified over 1750 sites of O-GlcNAcylation from murine synaptosomes [71]. Due to the particularly low abundance, low hydrophilicity of the O-GlcNAcylation peptides, and severe interference from other N/O-glycopeptides, isolating O-glycopeptides from a complex sample by HILIC enrichment was originally thought to be ineffective. However, after combining PNGase F, sialidase and O-glycosidase to selectively cleave and remove most of the N/O-linked glycans in glycoproteins, Shen et al. were able to eliminate the interference of other N/O-glycopeptides while still preserving the O-GlcNAcylation modified peptides. Benefiting from the improved enrichment specificity of the OGlcNAc peptides, a total of 474 O-GlcNAc peptides from 457 proteins were identified from a human urinary sample. In comparison, performing HILIC enrichment without the deglycosylation step only identified

107 O-GlcNAc proteins, and an immunoprecipitation (IP) approach using an anti-O-GlcNAc antibody only profiled 31 O-GlcNAc proteins [72].

For O-GalNAcylation, the glycan structures are of higher diversity than O-GlcNAcylation. To facilitate the MS identification of these glycopeptide sequences and their attached sites, Medzihradszky et al. utilized exoglycosidase digestion to partially deglycosylate O-GalNAcylation peptides and reduce the complexity of glycan structures and was able to identify 124 O-GalNAcylation sites in 51 O-GalNAcylated proteins from human serum samples [73]. Besides this *in vitro* approach, Clausen and coworkers developed an alternative method called SimpleCell strategy *in vivo*, which utilized a zinc-finger nuclease gene targeting to block the O-GalNAcylation elongation pathway to generate short glycan homogenous O-GalNAcylation. This strategy allowed directly enrichment by the LWAC method followed by MS/MS detection of OGalNAcylation peptides from different cell lines [74]. Recently, they extended this approach to characterize samples from 12 human cell lines and profiled almost 3000 O-GalNAcylation sites in over 600 OGalNAcylation glycoproteins, which represented the first map of the human O-glycoproteome [75].

3.2. Site-specific characterization of intact glycopeptides

Along with rapid development of glycosylation site profiling, two major breakthroughs have significantly facilitated the site-specific characterization of intact glycopeptides. These breakthroughs are the advancement of MS/MS dissociation methods towards acquiring both the glycan and peptide backbone fragments and the development of new search engines to decipher the MS/MS spectra of intact glycopeptides.

3.2.1. Comparison of dissociation methods for intact glycopeptides—The dissociation modes for peptide analysis mainly include collision-induced dissociation (CID), beam-type CID (occurs in triple quadrupole (QQQ) and quadrupole time-of-flight (Q-TOF) instruments, and the so-called high-energy collisional dissociation (HCD) in Thermo-Fisher™ instruments), and electron-induced dissociation (ExD, such as electron transfer/capture dissociation, ETD/ECD). Each of these methods alone cannot provide a full picture of the glycopeptide structure [76]. CID prefers to break glycosidic bonds, and it generates strong characteristic ions of peptides bearing different numbers of glycans after the stepwise release of peripheral monosaccharides (Y ions) (Fig. 2). It provides abundant information for deciphering glycan structures but limited information for peptide backbone identification. As for HCD dissociation, in low collision energy, a series of Y ions are preferentially generated, which is similar to CID; while in high collision energy, the peptide backbone fragmentation yielded a decreased intensity of Y ions. ExD mode mainly fragments the peptide backbone while leaving the glycan intact (Fig. 2), which is suitable for the localization of glycosylation sites with a wealth of peptide fragments [77].

3.2.2. Intact glycopeptide analysis by combining different dissociation methods—As no single dissociation method is available to produce a complete picture of intact glycopeptides, combining the complementary fragment information from multiple dissociation modes is an effective strategy to decipher the intact glycopeptides. Larson and coworkers combined CID and ExD to analyze desialylated glycopeptides, where CID-MS^2

spectra of glycopeptides were used for the glycan characterization and the subsequent CID-MS^3 spectra of selected CID-MS^2 fragment ions for peptide sequence identification. Moreover, ExD as a complementary peptide fragmentation mode was used for the characterization of O-glycosylation sites, where 58 N- and 63 O-glycopeptides from 53 glycoproteins were identified and 40 of the 57 putative O-glycosylation sites were accurately localized [78]. However, the requirement of prior knowledge of the targeted peptides to be selected for MS^3 and the longer duty cycle due to ExD reaction time in ExD-MS^2 limit its capacity compared to HCD- and CID-MS^2, and currently only tens to hundreds of intact glycopeptides can be profiled from complex biological samples using this strategy [79].

Sun et al. developed an integrated method that enabled comprehensive characterization of N-linked glycans and glycosite-containing peptides of glycoproteins and generated the glycan and glycosylation site database for spectral interpretation of intact glycopeptides acquired from another enrichment. This strategy allows simultaneous profiling and monitoring of N-linked glycans, glycosites, glycoproteins and site-specific glycosylation in a single experiment [80]. Chen et al. developed an alternative complementary method that enabled analysis of intact glycopeptides by sequentially interrogating the deglycosylated peptides and intact glycopeptides using CID and HCD, respectively. A total of 811 N-glycosylation sites from 567 glycoproteins were identified from HEK293T membrane proteins, and 177 intact N-glycopeptides were also identified by manually integrating the CID and HCD spectra. The number of identified intact glycopeptides was much smaller than the number of identified N-glycosites, which can be attributed to the low ionization efficiency of intact glycopeptides and manual interpretation of the complicated MS/MS spectra [81]. Recently, the same group developed a fully-automated software platform for high-throughput characterization of intact N-glycopeptides. They used the strong correlation of retention time to effectively remove the random matches and were able to control the probability of random matches within 1%. In total, 2249 intact glycopeptides, representing 1769 site-specific N-glycans on 453 glycosylation sites, were identified [82]. Liu et al. developed a similar strategy which profiled 1145 non-redundant glycopeptides from 225 core peptides and 95 glycoproteins from human serum samples [83].

3.2.3. Intact glycopeptide analysis by integrated dissociation methods—

Rather than implementing two dissociation methods to obtain the complementary structure information in two separate LC-MS/MS runs, it would be beneficial if hybrid fragmentation spectra were acquired in a single run. To this end, the evolution of several new dissociation approaches offered an effective solution to this problem. Among them, the stepped collision energy HCD (step-HCD), beam-type CID with high energy, and electron-transfer/higher-energy collision dissociation (EThcD) show great potential.

As different HCD collision energies could generate complementary fragments, performing step-HCD (e.g. 30 ± 10%) will give a more complete intact glycopeptide structure information in a single spectrum. Qian and coworkers first applied the step-HCD to analyze partially deglycosylated core-fucosylated glycopeptides in mouse liver tissue and HeLa cell samples and found that the overall performance increased by 7-fold [84]. Recently, this method has been widely used in intact glycopeptide analysis with impressive results being reported [85,86]. Current MS instruments can only provide a three-step collision energy in

one spectrum, and more flexible collision energy settings could definitely improve intact glycopeptide analysis.

Under typical beam-type CID conditions, ions produced from dissociation of the peptide backbone are in low abundance. Zaia and coworkers reported that abundant peptide backbone fragments could be generated by increasing the collision energy, along with oxonium ions and intact peptide ions with varying numbers of saccharide units attached. They successfully used this approach for intact glycopeptide analysis from several standard N-glycoproteins [87]. Recently, Sung et al. utilized the same strategy in complex sample analysis and profiled 36 intact glycopeptides of 26 glycoproteins in a HeLa cell sample [88]. Ye & Zou and coworkers continuously explored this strategy in intact O-GalNAcylation peptide analysis and established an automated workflow for O-GalNAcylation peptide MS^2 spectral interpretation, enabling identification of 407 intact O-GalNAcylation peptides from 93 glycoproteins in human serum sample [89].

Integrating the HCD and ETD in one spectrum (Fig. 2), EThcD also enables information of both glycan and peptide fragments to be acquired [35,90,91]. Li and coworkers first optimized the parameters of EThcD for intact glycopeptide analysis, and determined that the efficiency of dissociation was greatly improved by using charge-dependent optimized ETD reaction times. Large-scale experiments in rat carotids collected over the course of restenosis progression resulted in over 2000 N-glycopeptide identifications [92]. Qian and coworkers found that EThcD provided more complete fragmentation information on O-GalNAcylation peptides and a more confident site localization of O-GalNAcylation than HCD method. By combining multiple enzyme digestions and multidimensional separation, they identified 173 O-glycosylation sites, 499 non-redundant intact O-glycopeptides, and 6 glycan compositions originating from 49 O-glycoprotein groups from normal human serum [93].

3.2.4. Database search for intact glycopeptide analysis—One of the biggest challenges for intact glycopeptide charac terization is the accurate interpretation of the resulting spectra. Based on different strategies, several new search engines have been developed for intact glycopeptide identification, such as Glyco-Master DB [94], GPQuest [95], I-GPA [96], Byonic [97], SweetNET [98], pGlyco [99], pGlyco 2.0 [85], etc. Particularly, pGlyco 2.0 conducted a comprehensive false discovery rates (FDR) evaluation at all three levels of glycans, peptides and glycopeptides, greatly improving the accuracy of intact glycopeptide identification (Fig. 3). Moreover, a quantitative analysis method utilizing $^{15}N/^{13}C$ meta bolically labeled glycoproteome samples to validate glycopeptide identification was specifically designed [85]. By taking advantage of the optimized step-HCD collision for fragmentation of the HILIC enriched intact glycopeptides and sophisticated algorithm of pGlyco 2.0, the researchers were able to identify 10,009 distinct glycopeptides in five mouse tissues, in site-specific manner, corresponding to 1988 glycosylation and 955 glycoproteins. Some other analytical tools, like MAGIC and SugarQb, which translate the intact glycopeptide spectra and enable them to be analyzed using current peptide search engines, have also been developed [86,88,100]. More details about the development of search engines can be found in several recent reviews [101,102]. Due to space limitations and lack of standard glycopeptide spectral datasets, a fair comparison between different software has not been performed [103]. Comprehensive

evaluation of the current software regarding the coverage of the glycoproteome and quality control of the identification results would provide valuable insights for future software design [104]. Large-scale glycoproteomics research would especially benefit greatly from the improvement of automated glycopeptide identification, due to the large volumes of data being generated.

3.3. Quantitation of glycopeptides

Quantitation of protein glycosylation can be performed at the glycan, glycopeptide or glycoprotein level based on the target molecule, and at relative or absolute quantitation levels based on the strategies used. Absolute quantitation is often conducted by employing targeted MS approach. As the theme of the current review is non-targeted bottom-up glycoproteomics, the quantitation strategy discussed here will focus on quantitation at the glycopeptide level.

3.3.1. Label-free quantitation—The label-free approach has been regularly used in proteomics studies to measure protein abundance changes, and it offers the advantage of a simple workflow, low cost and high proteome coverage [105]. Normalization is needed to overcome the MS response variations in different samples and reliable quantitation results could be obtained by normalizing the data to the total ion abundance [106,107]. However, it could be problematic for glycopeptide analysis due to the low ionization efficiency of glycopeptides, which means small changes of nonglycosylated interferences could lead to large variability in quantitative assays. To overcome this problem, Desaire and coworkers developed a new normalization strategy based on the intensity of all glycopeptides and a twotiered quantitative analysis to discriminate between glycosylation changes of a given protein and glycoprotein's concentration changes [108]. Additionally, the large volumes of data produced by label-free experiments need rigorous statistical assessment for accurate data processing and interpretation, which requires effective algorithm models and software tools to be developed [109]. Mayampurath et al. developed a novel ANOVA-based mixed effects model for label-free glycopeptide quantitation and demonstrated its effectiveness by applying this method to biomarker discovery in human serum [110]. To facilitate simultaneous identification and label-free quantitation of glycopeptides, Park et al. developed an automated Integrated GlycoProteome Analyzer (I-GPA) platform and successfully quantified 598 N-glycopeptides from human plasma sample [96].

3.3.2. Label-based quantitation—Compared with label-free methods, the greatest advantage of stable isotope labeling is that different samples are mixed together and analyzed simultaneously, which largely reduces instrument time and run-to-run variations. In general, stable isotope labeling can be classified into three major categories: metabolic labeling, chemical labeling and enzymatic labeling.

The most commonly used metabolic labeling in quantitative proteomics is the stable isotope labeling by amino acids in cell culture (SILAC), which incorporates stable isotope-encoded essential amino acids into living cells [111]. The main advantage of SILAC is that it allows different samples to be combined at the intact cell level, minimizing the possible quantitation error introduced by the sample preparation process [112]. By incorporating a

glycopeptide enrichment step, the regular SILAC workflow can be easily modified for quantitative glycoproteomics studies. After treatment with PNGase F, the total glycosylation expression changes at each site can be quantified through comparison of light and heavy-labeled deglycopeptides, and a number of studies have successfully utilized this approach to quantify glycosylation changes on hundreds of N-glycosites [113–117]. Furthermore, Parker et al. utilized the SILAC approach to quantify the intact glycopeptides without PNGase F treatment, which enabled the changes in N-glycosylation micro-heterogeneity to be revealed [118]. By combining glycopeptide enrichment using hydrazide chemistry with SILAC, Taga et al. conducted a quantitative analysis of O-glycosylation and showed increased glycosylation of collagen in Osteogenesis Imperfecta [119].

However, the disadvantage of metabolic labeling is that some biological systems are not suited to efficient metabolic labeling and the cost is relatively high [120]. To this end, chemical labeling approaches have been developed to label proteins or peptides extracted from tissues/cells or biofluids with stable isotope-incorporated tags. In early 2003, Zhang et al. used stable isotope labeling by succinic anhydride after glycoprotein capture by hydrazide beads for identification and quantitation of N-glycopep-tides [9]. However, succinic anhydride labeling method requires repeated labeling to achieve reaction completeness and side reactions may happen during the process [121]. To overcome this problem, Sun et al. developed an approach that enables sequential glycopeptide enrichment and dimethyl labeling on hydrazide beads, which showed high quantitation accuracy over two orders of magnitude in dynamic range [122]. However, both succinic anhydride and dimethyl labeling have limited capability for quantitative analysis across different samples; hence, isobaric tags have been developed to allow for multiplexing capability, such as 10-plex tandem mass tag (TMT) [123,124], 8-plex isobaric tags for relative and absolute quantitation (iTRAQ) [125,126], and 12-plex N,N-dimethyl leucine (DiLeu) isobaric tags [127,128] etc. Employing a 6-plex TMT labeling strategy, Kroksveen et al. conducted a quantitative glycoproteomics analysis between 21 subjects in relapsingremitting multiple sclerosis group and 21 subjects in neurological control group, and successfully quantified 1700 deglycopeptides with 235 deglycopeptides showing significant differences between disease group and control group [129]. Notably, Melo-Braga et al. conducted a global comparative proteomic study, as well as changes in N-glycosylation, phosphorylation, and Lys-acetylation with 4-plex iTRAQ tagging scheme [130]. The reason that multiple quantitative PTMs analysis could be conducted in parallel is because both proteome and PTMs analysis shared the same upper stream steps and samples could be split into aliquots and subject to different PTMs-targeted enrichment methods after isobaric labeling. Such capacity allows multiple PTMs to be analyzed from a limited amount of sample and largely facilitates the study of crosstalks between different PTMs. Besides chemical labeling, enzymatic labeling was also developed by incorporating ^{18}O into the peptides during the enzyme-catalyzed digestion process [131]. Later, Liu et al. developed a tandem ^{18}O stable isotope labeling strategy for quantitation of N-Glycoproteome by combining ^{18}O labeling in the C-terminal carboxylic acid during proteolytic process and another ^{18}O labeling in the asparagine residue during deglycosylation process by PNGase F hydrolysis [132].

4. Application of MS-based glycoproteomics in complex biological samples

4.1. In-depth glycoproteome profiling in complex biological samples

4.1.1. Human serum and other human tissues—In-depth glycoproteome profiling has been extensively conducted in different biological systems, including body fluids, cells and tissues, etc. Serving as an indicator of physiological and pathological states alteration in the body system, serum/plasma is the most common clinical specimen for disease diagnosis. The majority of serum proteins are glycosylated as many proteins are secreted in glycosylated form, with an estimated 50% after removing high abundance proteins [133]. To facilitate the detection of low abundance glycoproteins, Sparbier et al. utilized magnetic lectins (ConA, LCA, WGA) beads and boronic acid beads for the enrichment at both protein and peptide levels, resulting in 95 N-glycosylation sites from 193 N-glycoproteins [134]. Nevertheless, the coverage is still not desirable mainly due to the extreme complexity of serum and the wide dynamic range of proteins with concentrations spanning over 10 orders of magnitude [135]. To further decrease sample complexity, various approaches have been applied, including immunoaffinity depletion of high-abundance serum proteins (albumin, IGG etc.), sequential enrichment strategies (lectins, HILIC etc.), off-line fractionation (HpH, SCX) and 2D-LC, which yielded more than 600 N-glycosylation sites from over 300 N-glycoproteins [136,137]. Faced with the challenges of sample complexity brought by various glycoforms, several studies focused on a subset of total glycopeptides such as core-fucosylated peptides which could be enriched by highly specific binding afforded by lectin LcH [138–140]. Park et al. developed a novel automated Integrated GlycoProteome Analyzer (I-GPA) with FDR control for fast and confident intact N-glycopeptide identifications, and successfully identified 619 intact N-glycopeptides with an FDR below 1% from human serum [96]. Compared to N-glycosylation, O-glycosylation in serum is less studied mainly due to its lack of consensus motif and diversity of core structures. Recently, Zhang et al. developed a systematic strategy that combined multiple enzyme digestion, multidimensional separation and EThcD fragmentation, and identified 499 non-redundant intact O-glycopeptides in serum, covering singly, doubly and triply O-glycosylated peptides [93]. Besides serum, in-depth glycoproteome profiling has also been conducted in other human body fluids/tissues such as urine [141–143], liver [60,144] and so on.

4.1.2. Cell culture—In addition to human serum, the glycoproteome of different cell types have also been extensively explored. Cell culture has helped us gain valuable insights into various biological processes and disease-related pathological alterations, and has contributed enormously in drug discovery and development [145]. Adding glycoproteome data to the cellular models would help us gain a better understanding of the inherent complexity in biological systems [146]. Notably, the Clausen group developed a robust SimpleCell approach for an O-GalNAc study [75], and successfully mapped human O-GalNAc glycoproteome with almost 3000 glycosites from over 600 O-glycoproteins in 12 human cell lines from different organs [147]. Although SimpleCell approach has shown extraordinary performance in terms of O-glycosite mapping, it falls short in intact glycopeptide analysis due to glycan truncation during the process. To this end, Bertozzi group developed an IsoTaG strategy for intact glycopeptide characterization [148], and 1375

intact N-glycopeptides and 2159 intact O-glycopeptides were successfully identified from 15 human tissue-derived cell lines [149]. Later, this approach was also applied for human T-cells O-GlcNA-cylation analysis, with over 2000 O-GlcNAcylation peptides identified [150]. Some other studies focused on the glycoproteome on the cell surface, which are crucial for the understanding of cell-cell communication and cell-environment interaction [151,152]. In order to selectively capture surface glycoproteins, in 2009, Wollscheid et al. developed a powerful unbiased cell surface-capturing (CSC) technology through covalently labeling cell surface N-glycan moieties [153]. Since then, this approach has been widely used for cell surface N-glycoproteome profiling including embryonic stem cells [154], induced pluripotent stem cells (iPSCs) [155], gastric adenocarcinoma cells [156], hepatocellular carcinoma cells [157], and hundreds of surface glycoproteins have been identified. Another rich source of glycoproteins come from the secreted proteins, or secretome, as many proteins undergo glycosylation prior to secretion [158]. For secreted glycoprotein analysis, conditioned media from serum-free cell culture is usually collected, followed by extraction of the secreted proteins, and then is subject to a typical glycoproteomics workflow. Li et al. have extensively conducted glycoproteome profiling of hepatocellular carcinoma cell lines [59] and have mapped 1213 unique N-glycosites from 611 N-glycoproteins [159]. Cell component analysis revealed that these N-glyco-proteins were primarily localized to the extracellular space and plasma membrane, indicating important role of N-glycosylation in the secretory pathway. The study of secreted glycoproteome of other commonly used cell lines such as human embryonic kidney (HEK) cells [160], Chinese hamster ovary cells (CHO) and endothelial cells [161], and some microorganisms such as green algae [162] and filamentous fungi [163] have also been conducted, which provide valuable insights into the secretory pathway and their responses to the environmental stimuli.

4.1.3. Animal tissues and plants—Due to their easy accessibility, the glycoproteome of animal tissues and plants have also been comprehensively profiled. Among them, mouse or rat brain is perhaps among the most extensively studied tissue. By employing different enrichment strategies, including lectin, HILIC, hydrazide chemistry and TiO_2, Zhang et al. have successfully mapped 3446 unique glycosylation sites from 1597 N-glycoproteins in mouse brain, and 65% of the identified N-glycoproteins are membrane or extracellular proteins [28]. To take a step further, Fang et al. further increased the coverage by optimizing protease treatments and fractionation strategies and identified 8386 glycosylation sites on 3982 N-glycoproteins, representing the largest N-glycosylation site dataset in mouse brain ever reported [61]. Site-specific N-glycoproteome study in rat brain has also been conducted by utilizing a combined glycomics and glycoproteomics approach, resulting in the identifications of 863 unique intact N-glycopeptides [164]. The N-glycosylation site mapping studies in other mouse/rat tissues such as liver, kidney, heart, plasma, stomach, ovary etc. revealed a tissue-specific expression pattern of N-glycosylation, indicating the close relation between glycosylation and the specialized function of different organs/tissues [58,165,166]. Compared with the large number of glycoproteome studies in mammalian, the glycoproteome studies in plants are quite limited, despite an increased interest in deciphering the plants glycoproteome [167–169]. So far, hundreds of N-glycosites have been mapped in rice [170], cereal crop *Brachypodium distachyon L.* [136], tomato[22], flowering

plant Arabidopsis [171] etc., providing valuable insights into the biological role of this ubiquitous protein modi-fication in different plant species.

4.1.4. Microorganisms—As one of the most popular models for basic biological research, yeast has also attracted substantial interest in the glycoscience field. Breidenbach et al. started out mapping the N-glycosites in yeast, yielding a total of 133 N-glycosites spanning 58 glycoproteins, which were mainly distributed in the yeast ER, plasma membrane, vacuole, and cell wall [172]. It has been a puzzle for researchers that O-GlcNAcylation was found in all eukaryotic cells except yeast until Halim et al. discovered and mapped nucleocytoplasmic O-mannose glycoproteome in yeast in 2015, which opened new avenues for the investigation of O-glycosylation based biological events in yeast [173]. Later, Neubert et al. successfully mapped 2300 O-mannosylation sites in 500 O-glycoproteins from whole yeast cell lysates, and one interesting finding was that these O-mannosylation sites were in the proximity of N-glycosylation sites, indicating their potential interplay [174]. The glycoproteome of some common bacteria [175–177] and viruses [178,179] have also been mapped, providing a molecular foundation for further understanding of glycosylation-assisted physiological processes.

4.2. Comparative MS-based glycoproteomics in complex samples

4.2.1. Disease biomarker discovery—Previously, glycosylation-based biomarker studies relied on lectin staining or 2D gel electrophoresis approaches to measure the total glycosylation changes or the total glycoprotein changes, which suffer from low-throughput, limited sensitivity, and limited site-specific glycosylation information [180,181]. With the advancement of glycoproteomics methodologies, glycosylation level changes can be pinpointed on a specific site and further micro-heterogeneity differences can be revealed through intact glycopeptide analysis in a high-throughput manner.

In a quantitative proteome and glycoproteome study of relapsing-remitting multiple sclerosis and neurological controls, Kroksveen et al. identified 96 altered deglycopeptides where their associated protein abundance was not affected, indicating the alterations were due to glycosylation occupancy changes instead of changes at the protein level [129]. Similarly, Zhang and coworkers utilized an integrated proteomics and glycoproteomics approach to explore the mechanism of castration resistance for androgen-deprivation therapy in prostate cancer [182]. This integrated omics approach not only allowed the detection of changes in glycosylation occupancy and microheterogeneity, but also identi-fied associated altered fucosyltransferase and fucosidase expression. To take one step further, after the initial finding of the increased terminal galactosylation and up-regulation of B4GalT5 galactosyltransferases upon TNF-Alpha-Induced insulin resistance in adipocytes through an integrated proteomics and glycoproteomics approach, Parker et al. showed that the knockdown of B4GalT5 down-regulated the terminal galactosylation, confirming the involvement of B4GalT5 in the TNF-alpha-regulated N-glycome [118]. Instead of analyzing the whole glycoproteome, some other studies focused on a specific type of glycopeptides (fucosylated, sialylated etc.) to improve the coverage depth. Tan et al. employed an LCA enrichment approach to selectively enrich core-fucosylated glycopeptides, and were able to identify 613 core-fucosylated peptides and 8 of them exhibited a significant difference

between pancreatic cancer and controls [183]. Due to close crosstalks between cells and the extracellular space, the secreted glycoproteins in extracellular space is another rich source for biomarker discovery. Li et al. conducted a glycoproteomics study in the secretome of human hepatocellular carcinoma metastatic (HCC) cell lines, and two glycoproteins FAT1 or DKK3 were proposed as novel prognostic biomarkers of HCC after validation with Western blot and tissue array immunohistochemistry (IHC) [159]. Specifically, extracellular vesicles (EVs) in the secretory system have been exploited as an attractive source for biomarker discovery. Very recently, Chen et al. identified 1453 unique deglycosylated glycopeptides from 556 glycoproteins in plasma-derived EVs, among which 20 were veri-fied to be significantly higher in breast cancer patients [184]. Additionally, 5 of these glycoprotein candidates were later successfully validated in patients and healthy individuals through a novel polymer-based reverse phase glycoprotein array (polyGPA) platform.

4.2.2. Biological process exploration—With glycosylation playing a key role in many biological processes, comparative glycoproteomics could reveal the dynamic changes and further shed light upon its functions along these processes. In a recent study, Kang et al. employed quantitative glycoproteomics approach to explore the molecular mechanism underlying the increased insulin secretion of normal pancreatic islet b-cells (PBCs) in response to elevated blood glucose levels [185]. Their results showed that altered sialylation of surface glycoproteins, such as integrins, integrin ligands, semaphorins and plexins was involved in the process of glucose-stimulated insulin secretion (GSIS). In order to uncover the glyco-markers in the neuronal differentiation process, Tyleckova et al. have successfully quantified hundreds of N-glycoproteins at onset and upon neuronal differentiation, as well as in mature hNT neurons using the cell surface capture (CSC) technology, and validated the glycosylation alterations of several cell adhesion glycoproteins using selected reaction monitoring (SRM) strategy [186]. Glycosylation has been known to affect the development of central nervous system (CNS) and defective glycosylation has also been shown to impair development and neurological function [187]. To this end, Palmisano et al. conducted a glycoproteomics study to monitor the glycosylation changes associated with cell signaling during mouse brain development using the postnatal mice from day 0 until maturity at day 80 [188]. Their results confirmed the role of sialylation in organ development and provided the first extensive global view of dynamic changes in N-glycosylation during mouse brain development. A comprehensive N-glycoproteomics analysis was also conducted to investigate the role of N-glycosylation during the deetiolation process, which is one of the most dramatic developmental processes known in plants [189]. The study has shown 186 N-glycosylation sites from 162 N-glycoproteins were significantly regulated over the course of the 12-h de-etiolation period, indicating the important role of N-glycosylation during deetiolation process. Besides the biological process without disturbance, the biological processes that are the result of environmental stimuli, such as infection, have also been investigated. Melo-Braga et al. explored the modulation of N-glycosylation in grape by *Lobesia botrana* pathogen infection and demonstrated the importance of N-glycosylation in plant response to biotic stimulus through the glycosylation changes of disease-resistance response glycoprotein DDR206 [130]. In another study into regulation of protein N-glycosylation in human macrophages and their secreted microparticles (MPs) upon *Mycobacterium tuberculosis* infection, Hare et al. showed an increased complex-type

glycosylation and concomitant down-regulation of paucimannosylation of macrophages upon infection [190].

5. Concluding remarks

With rapid advancements in various methodologies, including improved enrichment methods, novel MS/MS fragmentation techniques, powerful workflows, and advanced bioinformatics, MS-based glycoproteomics is gaining more attention and has been increasingly applied to studies of various biological systems. Unprecedented glycoproteome depth has been achieved in different complex samples, providing valuable molecular basis for further structure-function studies of glycosylation. Due to the advances in hybrid fragmentation methods and maturing search engines for intact glycopeptide analysis, site-specific glycoproteomics have become increasingly feasible and will eventually become a routine and practical approach for large-scale glycosylation analysis, which could help decipher the long-time puzzle of glycosylation microheterogeneity. Although great advancements have been made, limitations still exist in the following aspects. The current O-glycopeptide enrichment efficiency is not ideal, and intact O-glycan structure information was often lost in many existing O-glycopeptide enrichment methods. Because of these remaining challenges, the O-glycoproteome information from different complex samples is still quite sparse, with most of the studies focusing on O-glycosite mapping or O-glycoproteome profiling with truncated O-glycans information. More efficient O-glycopeptide enrichment methods that preserve native O-glycan structures are highly desirable to advance the O-glycoproteomics forward. Furthermore, integrated workflows that enable both N-glycosylation and O-glycosylation to be analyzed simultaneously are in great demand. Additionally, the current glycoproteomics methodologies only allow glycan composition and partial glycan structure information to be revealed; although isomer differentiation has been advanced by utilizing PGC columns [191], more diverse tools are needed to improve the resolution for isomer differentiation. Future direction includes improving glycan structural analysis by incorporating isomer differentiation tools such as infrared spectroscopy (IR) or ion mobility (IM) into the workflow.

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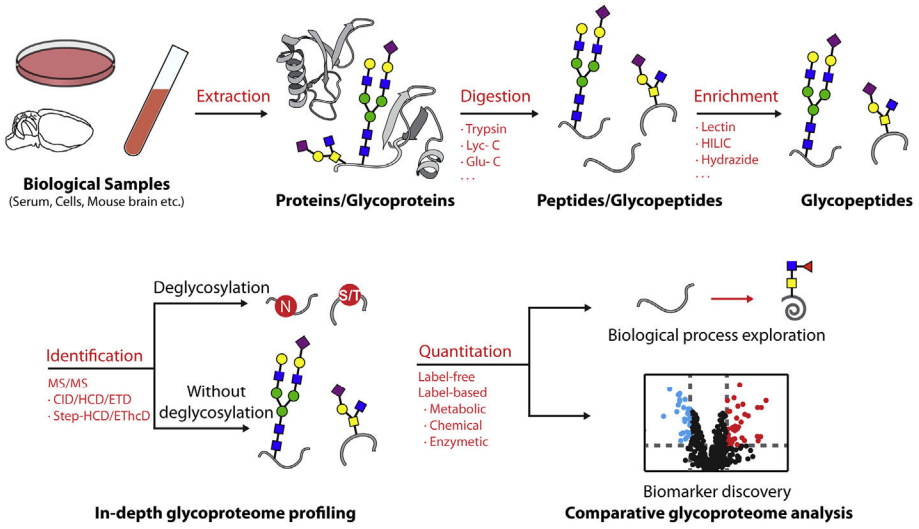

Fig. 1.

A typical workflow for MS-based glycoproteomics in different complex biological samples.

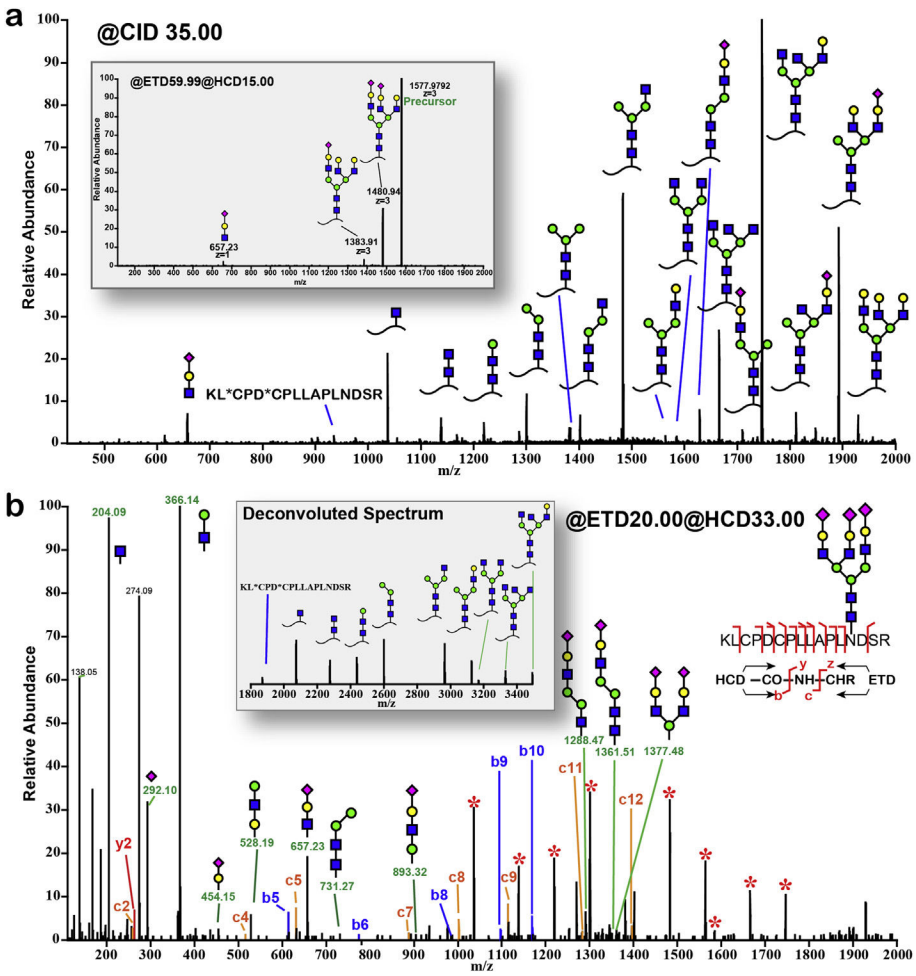

Fig. 2.

MS/MS of 3 + charge state precursor ion at *m/z* 1577.9 of bovine fetuin triantennary N-glycopeptide KLCPDCPLLAPLNDSR (AA 126–141). Alternating between CID/ETD/EThcD resulted in different sets of ions. (a) CID and ETD spectra (inset). Asterisk (*) in the peptide sequence indicates carbamidomethylation. (b) EThcD spectrum. Starred peaks (*) in the spectra were deconvoluted and annotated in the inset. Adapted from Ref. [68] with permission.

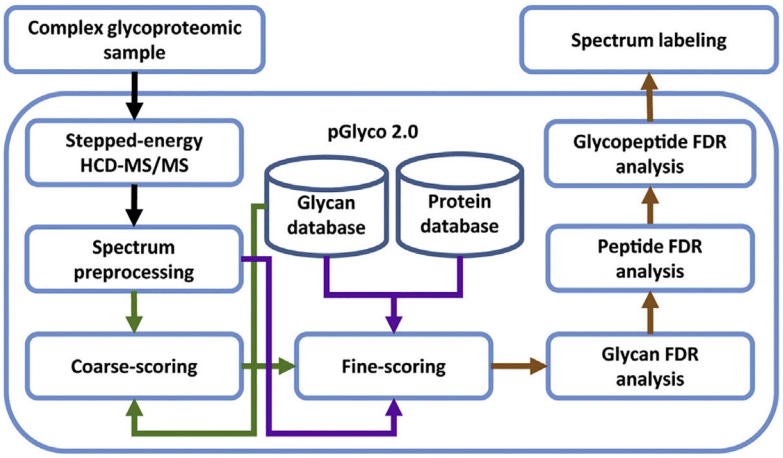

Fig. 3.

Design of a dedicated software pGlyco 2.0 for intact glycopeptide interpretation. Adapted from Ref. [60] with permission.