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Protein-Centric N-Glycoproteomics Analysis of Membrane and Plasma Membrane Proteins

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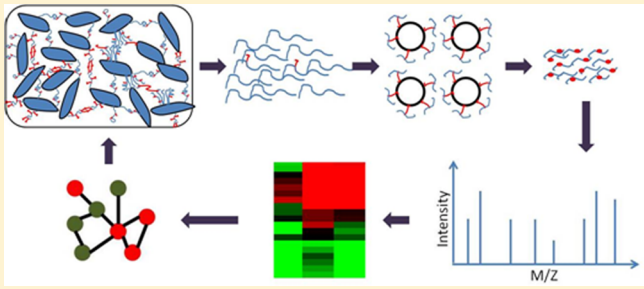
ABSTRACT: The advent of proteomics technology has transformed our understanding of biological membranes. The challenges for studying membrane proteins have inspired the development of many analytical and bioanalytical tools, and the techniques of glycoproteomics have emerged as an effective means to enrich and characterize membrane and plasma-membrane proteomes. This Review summarizes the development of various glycoproteomics techniques to overcome the hurdles formed by the unique structures and behaviors of membrane proteins with a focus on N-glycoproteomics. Example contributions of N-glycoproteomics to the understanding of membrane biology are provided, and the areas that require future technical breakthroughs are discussed.



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MEMBRANE AND PLASMA MEMBRANE PROTEINS AND THEIR ANALYSIS TECHNIQUES

The plasma membrane segregates a mammalian cell body from its environment.[1] Proteins inserted in the plasma membrane help connect cells to their immediate environment by transporting nutrients and wastes, protecting cell integrity and facilitating cell motility and adhesion, and by sensing the environmental cues and eliciting appropriate responses. Inevitably, surface proteins play crucial roles in cell survival and fitness. Often membrane proteins can be proteolytically cleaved from the cell surface and shed into body fluids. As a result, circulating membrane proteins can be biomarkers for disease diagnosis and prognosis, and plasma membrane proteins are frequently targeted by drugs.

Functioning importantly at the interface, the plasma membrane is physically and chemically close connected to the endomembrane system and the extracellular environment.[1,2] For example, cell surface proteins are synthesized and recycled through the secretory and endocytic pathways including the endoplasmic reticulum (ER) and Golgi apparatus complex as well as the endosomes and lysosomes. In the meantime, cells also modify their immediate environments and send various signals by secreting extracellular matrix components, small molecules, peptides, as well as microparticles and exosomes, as shown in Figure 1. Therefore, cellular membrane system in general, and plasma membrane in particular, are closely related, and this review will start from the techniques developed to analyze plasma membrane proteins and ends with a discussion about the intimacy between membrane and plasma membrane proteins, which pose significant challenges and opportunities for N-glycoproteomics.

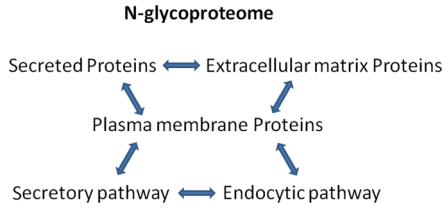

Figure 1. Composition and relationship of proteins in the N-glycoproteome.

Membrane proteins represent a diverse and poorly characterized protein category in the cell. Possibly due to the biological and physiological importance, current studies suggest that in many genomes including the human genome[3,4] around one-third to half of the annotated genes[5−7] encode membrane proteins. However, <1% of known protein structures in the Protein Data Bank (PDB) (http://www.rcsb.org/pdb/home/home.do, 2013 statistics) are derived from membrane proteins. Albeit large-scale proteomics efforts employing multidimensional separations that have been carried out to characterize cellular states and behaviors,[8,9] membrane proteins are often under-represented.[10] Moreover, a large number of surface receptors such as many G-protein-coupled receptors are expressed at extremely low level (<100 copies/cell[11,12]) that survey-based global proteomics studies often miss.[13]

The difficulty in studying plasma membrane proteins arises mainly from three factors. (1) Structural complexity. This complexity includes the heavy modifications on both the ecto- and endodomains of membrane proteins. In addition,

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membrane proteins tend to form homo- or heterodimers and oligomers that further increase the dimensionality of their structural complexity. (2) High diversity and low abundance. As mentioned above, membrane proteins occupy a larger percentage of the genome than other protein classes such as enzymes, cytoskeletal proteins, and proteins regulating translation and transcription. Nevertheless, membrane proteins only occupy less than 1% of the overall cellular protein mass. (3) Low solubility. Membrane proteins especially the integral membrane proteins have high hydrophobic transmembrane spans and hydrophilic domains and loops that heavily rely on interactions with lipid bilayers for appropriate folding. The removal of the lipids causes these proteins to collapse and forms aggregates that are difficult to dissolve in aqueous solutions without the help of detergents.

Because of these challenges, many analytical and bioanalytical techniques have been developed to assist proteomic characterization of plasma membrane proteins.[14−19] Because most detection techniques are concentration-dependent, significant efforts have been focused on the enrichment of plasma-membrane proteins through solubilization and separation of the enriched proteins and their derived peptides.[15,17−22] For enrichment and isolation, early efforts have involved using two-phase partition[23,24] as well as continuous density-gradient technique.[25,26] Concerns regarding the substantial organelle contaminations of plasma membrane proteins have been raised in the later applications of these methods.[27,28] Ever since, more selective techniques to analyze cell-surface proteins have been developed using biotinylation[29−31]or colloidal-particle affinity binding of functional groups or antigen epitopes on the intact-cell surface.[28,32−35] Enzymatic shedding (proteolytic cleavage) of cell-surface proteins is another clever method to specifically isolate protein domains on the external-surface of the plasma membrane.[36−39] These methods still more or less suffer from the cytosolic protein contaminations arising from the natural cell death inherent to in vitro cell cultures. Additional separations of the enriched proteins and the proteolytic products by 2D-gel,[40,41] SDS-PAGE/LC, or gel-free liquid chromatography[17,42,43] and capillary electrophoresis[44,45] have all been pursued. Most methods rely on peptide MS for protein identification and quantification. Because of the lack of proteolytic sites, different enzymes beyond trypsin have been explored to improve the digestion efficiency, including proteinase K[46] and LysC,[47] prior to MS detection. In the meantime, solubilization and denaturation conditions have also been extensively studied to strip lipids off the membrane proteins and aid the enzymatic digestion.[19,20,48] Several excellent reviews have summarized the technical efforts in membrane proteomics.[17,20,22,42]

This review will, however, focus on a different strategy to enrich membrane and plasma-membrane proteins from the above-mentioned techniques, that is, the N-glycoproteomics. We will highlight the current trend and some future directions of this technology in its development and applications.

GLYCOSYLATION OF PLASMA MEMBRANE PROTEINS

Most plasma membrane proteins are modified by two main types of glycosylation:[49] N- and O-GalNAc glycosylation. In N-glycosylation, oligosaccharides are attached to the β-amide of the asparagine (Asn) in an Asn-Xxx-Ser/Thr consensus sequence (where Xxx can be any amino acid but not proline),[50,51] whereas in O-GalNAc glycosylation, oligosaccharides are attached to the hydroxyl group of the Ser or Thr side chains. Both N- and O-GalNAc glycosylations modify selectively extracellular regions of the plasma membrane proteins. Specifically, the addition of N-glycans takes place cotranslationally in the ER[52] to the nascent polypeptide chain, and the further processing and maturation of N-glycans are finished in the Golgi, while the O-GalNAc addition occurs in the Golgi.[53] Most proteins in the ER−Golgi complex are glycosylated, and this modification has broad biological implications.[54−57] They are known to assist protein folding, to stabilize protein structure, and to guide the sorting and trafficking of membrane proteins.[58−61] Glycan moieties also protect membrane proteins from undesired enzymatic attacks and participate in signaling cascades, immune responses, and cell adhesion to mention just a few.[54,62−64] Defects in glycosylation cause congenital disorders of glycosylation,[65−67] and the complete abolishment of this modification is embryonically lethal.[62,68]

The prevalent glycosylation on cell surface offers a unique opportunity to enrich low-abundance surface proteins. Several techniques have been developed to capture N- or O-GalNAc glycoproteins, and these methods can be divided into three main categories based on the interaction forces. Group 1: Covalent bonding that can be further separated into reversible and irreversible covalent bonding. The reversible bonding can be formed through the boronic acid,[69] which reacts with vicinal diols in native saccharides to generate cyclic boronate,[70−74] whereas the irreversible bonding can be formed via the hydrazide chemistry with the aldehydes or ketones introduced in saccharides by oxidizing cis-diols using periodate[75−79] or by metabolic oligosaccharide engineering and bioorthogonal chemistry.[80−83] The metabolic incorporated bioorthogonal groups are more frequently used for imaging[84] than glycoproteomics analysis. Ketones and aldehydes can also be introduced into saccharides through enzymatic engineering, which has been achieved to study GlcNAc proteome.[85,86] The two later studies are not included in this review. Group 2: Affinity binding. Affinity forces can be generated by lectin−carbohydrate and antibody−antigen interactions as well as manually introduced affinity tags, for example, biotin and histags. Lectins are naturally existing carbohydrate binding proteins[87,88] that have been frequently used to enrich specific types of glycoproteins,[89,90] but they lack the comprehensiveness to bind all glycoproteins. Universal affinity binding can be achieved by introducing biotin or small-peptide tags, such as FLAG or histag, into sugar moieties through chemical reactions of hydrazide[75] or bioorthogonal chemistry,[84] as previously described. Group 3: Hydrophilic interactions or size exclusion separation.[91,92] This group of methods separate glycosylated proteins and peptides from their nonglycosylated counterparts by chromatography based on the hydrophilic properties or the bulky structure of oligosaccharides. The representative column-filling materials for these types of separations include amine-derivatized resin (i.e., HILIC)[93] or monolithic columns,[94,95] TiO_2 columns for sialioglycopeptides,[94−97] graphitic carbon,[98−101] as well as porous size exclusion resins[102] for small glycopeptides, (such as those generated from pronase digestion).[98−100] Other new techniques also include ion pairing−normal phase liquid chromatography (IP-NPLC) enrichment,[103] and the use of hexapeptide to normalize glycoproteins.[104] Most methods have been reported for both protein and peptide analyses.

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The enriched glycoproteins or glycopeptides are not readily identified by MS compared with their unglycosylated cytosolic counterparts. The sugar attachment possesses high complexity and heterogeneity.[105] The heterogeneity can arise not only from the glycan structure itself but also from the sites attaching to proteins. The same protein can be glycosylated at different sites that is usually termed macroheterogeneity, and each glycosite can carry different glycoforms, which is termed microheterogeneity. As a result, in SDS-PAGE, glycoproteins often form a smear rather than a single band; in MS analysis, complex spectra with low signal intensity are a characteristic of glycopeptides.[106,107] The negative charges from abundant sialylation on terminal glycans can further reduce the MS signal in positive ion mode.

To facilitate the MS identification and quantification, we frequently removed oligosaccharide moieties from glycoproteins and glycopeptides after the enrichment. For N-glycosylation, the β-aspartylglycosylamine linkage can be selectively cleaved using peptide-N-glycosidase F (PNGase F),[108] except for those moieties in which the root β-1,4-acetylglucosamine has been fucosylated. The sites of N-glycosylation are readily identified by the deamidation of sugar-modified asparagine in the triamino-acid consensus sequence. The reaction converts the asparagine to aspartic acid with a close to 1 Da mass shift[108] (i.e., 0.9840 Da). Identification and quantification of the deglycosylated glycopeptides are then handled similarly as standard proteomics.[109] Because no single enzyme can cleave O-GalNAc protein linkage, N-glycoproteomics is relatively easy and straightforward to pursue in comparison with O-glycoproteomics.

To completely remove O-GalNAc glycosylation, both a cocktail of glycosidases and chemical deglycosylation methods have been broadly employed individually or in combination.[110−114] With the development of special MS fragmentation techniques such as IRMPD, ETD, and ECD,[105,106,115,116] intact or partially deglycosylated peptide products are now possibly characterized. A recent bioengineering effort has remodeled in vivo O-GalNAc glycosylation pathway to yield "SimpleCell",[117] in which only one GalNAc is attached to the hydroxyl group of Ser/Thr side chains in the O-GalNAc proteins. This drastically simplifies the analyses of O-GalNAc glycoproteome.

The rest of this Review will detail the hydrazide-based N-glycoproteomics, with an emphasis on the methodology development for membrane and plasma-membrane protein characterization. In this context, we will discuss some critical and inherent issues, such as the comprehensiveness and selectivity of membrane and plasma-membrane proteins and the sensitivity and accuracy of the identification and quantification of proteins, their sites of modification, and their interaction with other molecules.

HYDRAZIDE-BASED N-GLYCOPROTEOMICS

Among all protein-centric proteomics strategies for N-glycosylation, the hydrazide chemistry appears to achieve high comprehensiveness and specificity on glycoproteins.[109,118] The high selectivity of this method is originated from the robust covalent bond, which can tolerate stringent wash conditions. The better removal of other macromolecule contamination through stringent washes helps the deep and comprehensive membrane proteomics analyses.

The selectivity of this method is based on the periodate oxidation of the cis-diols that are ubiquitous in carbohydrates, a reaction first described in 1950s.[76] The newly formed aldehydes and ketones can then react with hydrazide under mild acidic conditions to form chemically stable hydrazones.[75,77,79] The method gained popularity in 1980s as a means for labeling carbohydrates,[79] and the first application of this method to immobilize proteins was carried out with antibodies.[77] In 2003, Zhang et al. pioneered its use in proteomics analysis,[109] in which glycoproteins were first immobilized on hydrazide-derived resins and then were digested into peptides. After the removal of the unbound ones, the N-glycopeptides were selectively released from the resin by PNGase F prior to LC−MS proteomics analyses. The protocol of this method has been documented.[119]

Later, this method was optimized into a one-tube reaction,[120] in which the glycoproteins were first digested into peptides and then the oxidation, capture, and releasing steps can be carried out in one tube by using quencher to deactivate the excessive oxidants prior to the capture. A video protocol of this new method has been made available recently.[2] The digestion of membrane proteins into peptides greatly improved the efficiency of bringing membrane proteins into aqueous solutions, and the one-tube procedure makes the analysis available to capture N-glycoproteins of low abundance. These improvements were reflected by the high selectivity (>90%) of both the N-glycoproteins and N-glycopeptides in the final result, which yielded the highest percentage of glyco-enrichment in membrane−protein applications. In addition to quenching, a desalting column has been used[121] to remove excessive periodate after the initial protein digestion. Because the desalting step is more difficult to be integrated in a one-tube scheme, the method is more suitable for studies of a relatively large quantity of samples. The high glyco-selectivity (or specificity) in peptide-capture method has been verified later in many studies,[12,122−124] and the optimized protocol can achieve a selectivity as high as 97−99%[122] for soluble blood proteins and 95% for membrane proteins.[2] The application of the one-tube peptide capture has helped to identify cancer cell-surface biomarkers,[120] the N-glycoproteome of mouse embryonic stem cells,[12] and so on. Ramachandran et al.[125] applied both the one-tube peptide method and the previous protein method to the saliva samples and suggested that the number of identified proteins from both methods were similar; however, the false positives were greatly reduced in the one-tube peptide method. Because most saliva proteins are soluble proteins, this study indicated that even though the one-tube peptide capture was designed for membrane proteins, it can be applied to study soluble and secreted glycoproteomes with demonstrated benefits.

Several other improvements were also carried out to the hydrazide N-glycoproteomics. Enzymes, such as pepsin and thermolysin, have been utilized in combination of trypsin to effectively digest membrane proteins.[126] For the enlarged capture area and the ease of operation, the agarose resins used in the original protein/peptide capture have been replaced by paramagnetic nanoparticles.[122,127,128] The capture step itself has been performed in the regular microcentrifuge vials as well as in spin-filter columns[122] and the recent microspin-filter columns, such as stage-tips.[129] To improve the accuracy in mapping the N-glycosites, the PNGase F deglycosylation has been performed in ^{18}O-labeled water, as introduced initially in 1992 by Gonzalez et al. to free glycoproteins[130] and later by Kuster and Mann in 1999 to gel-separated glycoproteins.[131] In this step, the ^{18}O in water is incorporated in the deglycosylated peptides, causing a mass shift of +2.9840 Da as opposed to the

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0.9840 Da shift formed in regular ^{16}O water. Kaji et al. implemented this deglycosylation strategy in their lectin-based N-glycoproteomics in 2003,[132] and this labeling has been integrated with hydrazide N-glycoproteomics by various researchers.[133,134] Besides ^{18}O-labeled water, endo-β-*N*-acetylglucosaminidases (EC 3.2.1.96) were also used in the none-hydrazide N-glycoproteomics[135] to cleave the glycosidic bond between the two proximal N-acetylglucosamines (GlcNAc) in the pentasaccharide core and to resolve peptides carrying a large mass shift of 203.08 Da (or 349.14 Da for the fucosylated saccharide)[136137] that can be easily distinguished from nonglycosylated peptides by MS.

INTEGRATED N-GLYCOPROTEOMICS METHODS

The low abundance of membrane proteins demands the most sensitive analytical techniques to improve their characterization efficiency. Integrated and online analyses will without doubt benefit the studies of low abundance proteins. The hydrazide-based capture often involves several steps, and some of them can be combined into a one-tube protocol as previously addressed, yet it is still challenging to conduct the whole procedure in a single vessel or online prior to MS analysis. In this case, other N-glycoproteomics studies have been more advanced. For instance, the lectin-coupled filter-aided sample preparation (N-glycol-FASP) method developed in Mann's group can achieve one-tube N-glycoproteomics and has been implemented to study many different biological species.[138] Besides lectin, Ti-IMAC-based FASP has also been reported for the integrated N-glycoproteomics.[139] A flexible online proteomics reactor was developed in Figeys' lab[140] and was recently demonstrated to have the capacity to combine all steps of the lectin enrichment, ^{18}O-isotopic-labeling, deglycosylation, and trypsin digestion online with the multidimensional liquid chromatography separation and MS for N-glycoproteomics analysis. Automatic online separation integrating HILIC or graphitic carbon for glycopeptides enrichment has also been developed for the N-glycoproteomics.[141,142]

N-GLYCOPROTEOMICS FOR PLASMA MEMBRANE PROTEINS

The plasma membrane is intimately related to the endomembrane system as well as the extracellular matrix and fluids. A majority of these membranes have most of their resident proteins N-glycosylated. Whole-cell hydrazide N-glycoproteomics does not distinguish plasma-membrane N-glycoproteins from those localized in other membranes. Hydrazide-based cell surface capture (CSC)[118]was developed and applied to the intact cells to achieve the surface selectivity. Because of the cytotoxicity raised by periodate and the endocytosis of intact cells, the labeling of glycoproteins in the secretory pathways had been observed in the CSC method. Mild periodate oxidization of surface sialic acids was subsequently developed and performed at a lower temperature to minimize endocytosis and to achieve the high selection of plasma membrane proteins.[83] Studies have also compared the surface proteome obtained by hydrazide chemistry with those from the biotinylation of all surface membrane proteins.[143,144] The results suggest a complementarity between the two methods, yet the N-glycocapture is able to identify post-translational modifications but not the biotinylation method.[144]

COMPARISON OF DIFFERENT METHODS

With the rapid advancement of glycoprotein analyses, numerous biological applications have been published. There is an urgent need to compare the results obtained among different methods for their pros and cons, a piece of information that is important to guide researchers in biological fields to choose and design an appropriate strategy for their specific research interests. All studies thus far have reported the total number of the identified proteins, but it is difficult to compare only these numbers due to various differences in the biological samples, the parameters used in analysis, and the applied methods themselves. We attempted to assess the GO (gene ontology)-enrichment *p* value instead of just the number of protein identification among several published results to reveal their similarities and differences in our recent study of the N-glycoproteome of mouse embryonic stem (mES) cells.[12] In this study, we compared our N-glycoproteome with the published global proteome and transcriptome of mES cells, as well as with other membrane proteomes obtained by differential centrifugation, surface−protein biotinylation, and the CSC method. GO enrichment analysis has been frequently used to identify the biological nature of the sample, and the biological difference among samples; however, it is rarely used for the comparison of different methods. The heatmap visualization we used in the study of mES-cell N-glycoproteome helped discover the patterns formed across different analysis methods, and the use of the same set of GO terms unified the comparison criteria. This analysis allowed us to reveal technical concerns raised by the original authors as well as the known biological difference in the used samples. This exercise demonstrated that it is possible to disclose pros and cons from different studies using bioinformatics, an analysis that not only saves the time and cost of experimentally reproducing the published work but also can maximize the usage of existing data.

DYNAMICS OF PLASMA MEMBRANE PROTEINS

Plasma membrane proteins are highly dynamic. Both their composition and their post-translational modifications are constantly being remodeled by the extracellular environment as well as the intracellular molecular machinery. Using N-glycoproteomics, researchers have gained fundamental insights into these dynamics. For example, using the whole N-glycoproteomics and CSC, the desensitization after stimulation has been characterized by the internalization of cell surface receptors.[145] Glycosylation can also be used to monitor the onset and progression of diseases. Metastatic cancer tissues are known to carry distinct glycan moieties than those from normal population,[146,147] and N-glycoproteomics has contributed greatly to the characterization of these changes and to the discovery of biomarkers in disease diagnosis, prognosis, and in patient responses to treatments. Details can be found in several excellent reviews.[148−150] During evolution, such as speciation, and biological development processes, such as embryogenesis and organogenesis, profound changes in surface−protein glycosylation have been known, and N-glycoproteomics is actively pursued to systematically characterize the similarity and differences among species, organs, tissues, and cell types.[118,138,151,152] An evolutionarily conserved complementarity between transmembrane domain and N-glycosylation has also been discovered recently in a comparison of five different

animal species including human, mouse, fish, fly, and worm using N-glycoproteomics.[12]

LANDSCAPE OF PLASMA MEMBRANE PROTEINS

From genomic information, we know the cell surface is rich in various glycoproteins, such as receptors, adhesion molecules, transporters, ion channels, and enzymes. However, we know little about their overall abundance in terms of both the absolute quantity and the quantity relative to each other. Our recent survey of the N-glycoproteome of mouse embryonic stem cells offers unique quantitative information at the molecular resolution on the protein landscape of cell surface. Using the mutually exclusive cataloguing scheme defined by Almen et al.,[153] our study summarized membrane proteins into four categories, that is, receptor, transporter, enzyme, and miscellaneous,[12] in which the transporters had the highest expression levels, whereas the receptors exhibited the largest diversity. On the basis of the results, the composition of surface proteins can be shown in Figure 2, where the receptors are twice as diverse as the transporters, yet the transporters express at two times higher quantity than any other protein category on the membrane. This global experimental information helps materialize our conceptual understanding of the cell surface proteins and also helps elucidate the related cellular physiology and biology. High-throughput proteomics encompasses enormous data such that studies aiming to mine and to analyze these data from different perspectives should be greatly encouraged. These computational efforts will also maximize the scientific value of the obtained results.

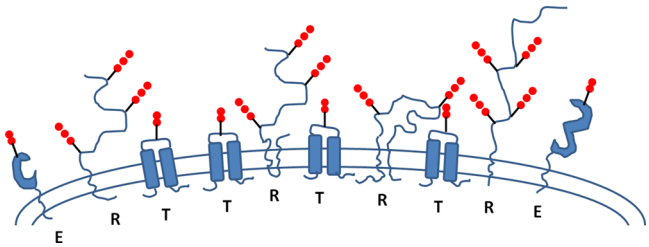

Figure 2. Illustration of major membrane glycoproteins, in which the red dots represent glycans: E, enzyme; R, receptor; T, transporter.

The structure and function of membrane proteins are heavily influenced by their interaction partners including those from the extracellular matrix and cytosolic scaffolds, for example. These interactions have been recently characterized systematically in yeast.[154] In this study, Babu et al. have researched four types of detergents to obtain membrane protein−protein interactions with various strengths.[154] A daunting total of 1726 membrane protein−protein interactions were individually studied, and the acquired information is an invaluable addition to our understanding of membrane proteins.

LINKING N-GLYCOPROTEOME BACK TO THE PROTEOME

Membrane proteins are biologically, physically, and chemically important to study, yet it is necessary and important to view their dynamics and functions together with the rest of the proteome. Previously, the membrane proteome has been a critical part in the whole proteome analysis. Nevertheless, most N-glycoproteomics efforts reviewed here have been primarily focused on the N-glycoproteome itself. New studies have emerged to examine the overall proteome together with the N-glycoproteome.[123,155] Within such a strategy, the capture approach has been used as a separation procedure instead of merely an enrichment step. An application of such an strategy termed: glycocapture-assisted global quantitative proteomics (gagQP), to the blood toxicoproteome has successfully identified more than 20 organ-specific biomarkers, indicating the toxic response of drug in eight organs.[123]

REMARKS

Biological membranes possess unique and critical functions in a cell; the proteins in the membrane have been an indispensable component of membrane structure and function, and they play crucial roles in modern medicine. Proteomics have been an effective tool to directly interrogate membrane proteins. The low abundance and the special localization of membrane proteins will constantly challenge the sensitivity and accuracy of any proteomics approach. In the past 10 years, the instrumentation of capillary nanoflow high-performance liquid chromatography and mass spectrometry has been quickly advanced. These efforts significantly improved our capacity to detect low-abundance membrane proteins and advanced the penetration of the membrane proteomes. Interestingly, one logical drawback arising from these instrumental improvements is an increased identification of the undesired contamination in the results, which motivates the development of more selective strategies.

Different methods introduced here will coexist due to their unique characteristics in sensitivity, selectivity, coverage, and ease of use. It is important to design and choose an appropriate strategy or a combination of a set of techniques based on the defined biological questions. Among all N-glycoproteomics strategies for membrane proteins, the chemical covalent bonding by the hydrazide chemistry potentially possesses the best selectivity through its tolerance to stringent washes. The nonhydrazide methods are readily employed for one-vessel or online N-glycoproteomics that offers better sensitivity than off-line approaches with multiple isolated steps. The increased precision to selecting a particular cell component, such as the plasma membrane, will eliminate the membrane proteins in the secretary and endocytic pathways that are trafficking toward or away from this site. This elimination will decrease the coverage of plasma membrane proteome, particularly the traveling low-abundance membrane proteins, yet this fundamental conflict is similar to the "uncertain theory" in quantum physics that may never be completely resolved.

The static examinations of membrane and plasma membrane proteins are likely replaced in the near future by the spatially and temporally resolved dynamic studies of membrane protein and their post-translational modifications. The mapping of the N-glycosylation sites has been a relative straightforward task compared with that of the O-glycosylation partially due to the available endoglycosidases. However, a recent report on the potential chemical deamidation[156] has confounded the enzymatic deglycosylation pursued in both ^{18}O- and ^{16}O-labeled water. Therefore, the use of additional endohydrolases, such as endo-β-N-acetylglucosaminidases, will be a useful alternative. The dynamics of N-glycoproteome including both the N-glycoproteins and their sites of N-glycosylation will be better understood by the rapid development of quantitative proteomics.[157−162] All major quantitative proteomics strategies including isotope labeling and label-free analyses and the targeted multiple-reaction monitoring have been coupled to the N-glycoproteomics.[109,123,145,163]

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More hybrid analyses combining different methods in sequel or in parallel have emerged to improve their effectiveness to address biological questions.[164] Computer informatics will be increasingly important to mine vast proteomics data sets and recognize the hidden patterns. The new problems arising from these endeavors should propel the technological innovations, and the pioneering analytical and bioanalytical tools will constantly push the frontiers of membrane glycoprotein research and provide fundamental molecular insights for human health and wellbeing.

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Notes

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