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A Comprehensive Review of Recent Advances in the Enrichment and Mass Spectrometric Analysis of Glycoproteins and Glycopeptides in Complex Biological Matrices

Mohamed A. Gab-Allah[1,2] and Jeongkwon Kim[1]*

[1]*Department of Chemistry, Chungnam National University, Daejeon, 34134, Republic of Korea*
[2]*Organic and protein analysis laboratory, National Institute of Standards, Tersa St., Al-Haram, P. O. Box 136 Giza 12211 Egypt*

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Abstract : Protein glycosylation, a highly significant and ubiquitous post-translational modification (PTM) in eukaryotic cells, has attracted considerable research interest due to its pivotal role in a wide array of essential biological processes. Conducting a comprehensive analysis of glycoproteins is imperative for understanding glycoprotein bio-functions and identifying glycosylated biomarkers. However, the complexity and heterogeneity of glycan structures, coupled with the low abundance and poor ionization efficiencies of glycopeptides have all contributed to making the analysis and subsequent identification of glycans and glycopeptides much more challenging than any other biopolymers. Nevertheless, the significant advancements in enrichment techniques, chromatographic separation, and mass spectrometric methodologies represent promising avenues for mitigating these challenges. Numerous substrates and multifunctional materials are being designed for glycopeptide enrichment, proving valuable in glycomics and glycoproteomics. Mass spectrometry (MS) is pivotal for probing protein glycosylation, offering sensitivity and structural insight into glycopeptides and glycans. Additionally, enhanced MS-based glycopeptide characterization employs various separation techniques like liquid chromatography, capillary electrophoresis, and ion mobility. In this review, we highlight recent advances in enrichment methods and MS-based separation techniques for analyzing different types of protein glycosylation. This review also discusses various approaches employed for glycan release that facilitate the investigation of the glycosylation sites of the identified glycoproteins. Furthermore, numerous bioinformatics tools aiding in accurately characterizing glycan and glycopeptides are covered.

Keywords : glycomics, glycoproteomics, enrichment strategies, nanomaterials, separation, mass spectrometry, bioinformatics, biological matrices

Introduction

Glycosylation is a fundamental process for cells, being one of the most prevalent post-translational modifications of proteins.[1-3] The study of glycoproteins has long been a fast-growing field of scientific endeavor and is still attracting significant research interest, which is not surprising since it is estimated that more than 50% of mammalian proteins are attached to oligosaccharide chains ("glycans").[4]

*Reprint requests to Jeongkwon Kim
https://orcid.org/0000-0002-0087-1151
E-mail: jkkim48105@cnu.ac.kr

The numerous studies exploring genetic and cellular aspects of glycosylation processes have demonstrated the fundamental significance of glycoproteins, thus leading glycosciences into the spotlight of modern biomedical research.[5,6] As a result of their high abundance, it is clear that glycoproteins play an important role in numerous biological functions, including immunological responses, cell adhesion, intra and intercellular signaling, cell−matrix interactions, tumor progression and metastasis, and protein stability.[7-10] Moreover, alteration in protein glycosylation has been associated with the development of many diseases, including carcinogenesis,[11-14] Alzheimer's disease,[15-17] congenital disorders,[18,19] diabetes,[20,21] inflammation,[22,23] bacterial/viral infections,[24,25] and immune deficiencies.[26]

There are two major forms of glycosylation for proteins, N-linked glycosylation and O-linked glycosylation. The primary distinction between the two types of glycosylation lies in the location of the sugar molecule and its attachment to an amino acid. N-linked glycosylation refers to the attachment of sugar to the amide of asparagine (Asn) residues in proteins within the consensus amino acid sequence

of Asn-X-Ser/Thr, where X can be any amino acid except proline.[27] This unique amino acid motif includes the glycosylation site for N-glycoproteins. On the other hand, O-linked glycosylation refers to the attachment of sugar to the hydroxyl of serine (Ser) or threonine (Thr) residues in proteins and does not necessitate a particular amino acid motif for attachment.[28]

Achieving a comprehensive analysis of protein glycosylation with enhanced sensitivity is an important step for better understanding various glycoprotein bio-functions, early diagnosis of diseases, and assessment of therapeutic efficiency for disease treatment.[27] However, the structural characterization process may be hampered due to various factors inherent to glycan and glycopeptides, such as micro-heterogeneity and extensive structure diversity since glycan biosynthesis is a non-templated driven process.[29-31] Diverse glycan compositions that are linked to a single glycosylation site and the polarity difference among individual saccharide residues induce additional complexities in glycomics and glycoproteomics research.[27] Hence, there has been an ongoing interest in establishing sophisticated bioanalytical methods and instrumentation for glycomic and glycoproteomic analyses for their application in biomedical studies. Indeed, research into protein glycosylation has gained more popularity due to advancements in modern instrumentation and computational approaches.

Mass spectrometry (MS) is an excellent technique for investigating protein glycosylation, especially with electrospray ionization (ESI)-MS[32-34] and matrix-assisted laser desorption/ionization (MALDI)-MS.[35-38] This is attributed to their high sensitivity and capacity to reveal structural information of glycopeptides and glycans. Nonetheless, the most efficient strategies have relied on MS-based separation techniques, such as liquid chromatography-tandem mass spectrometry (LC-MS/MS),[32-34] ion mobility (IM)-MS,[39-41] and capillary electrophoresis (CE)-MS[42-44] since these approaches can enhance glycopeptide sensitivity and selectivity by decreasing the number of species that undergo ionization simultaneously. However, establishing efficient strategies for glycopeptide enrichment and isolation from complex biological samples before MS analysis is a prerequisite to better enhance the detectability and sensitivity of MS-based glycoproteomics. This review summarizes and highlights the recent developments in enrichment techniques and separation hyphenated MS methods for the analysis of different types of protein glycosylation. Various glycan release methods facilitating the study of the glycosylation sites are discussed. Moreover, several software tools aiding in the rapid and accurate identification of glycan and glycopeptide isomers are overviewed.

Enrichment methods for glycopeptides

Although there have been significant advancements in MS-based approaches for large-scale glycoproteomic studies, direct analysis of glycopeptides derived from biological matrices continues to present considerable challenges.[27, 45] This is due to several factors, including low glycopeptide

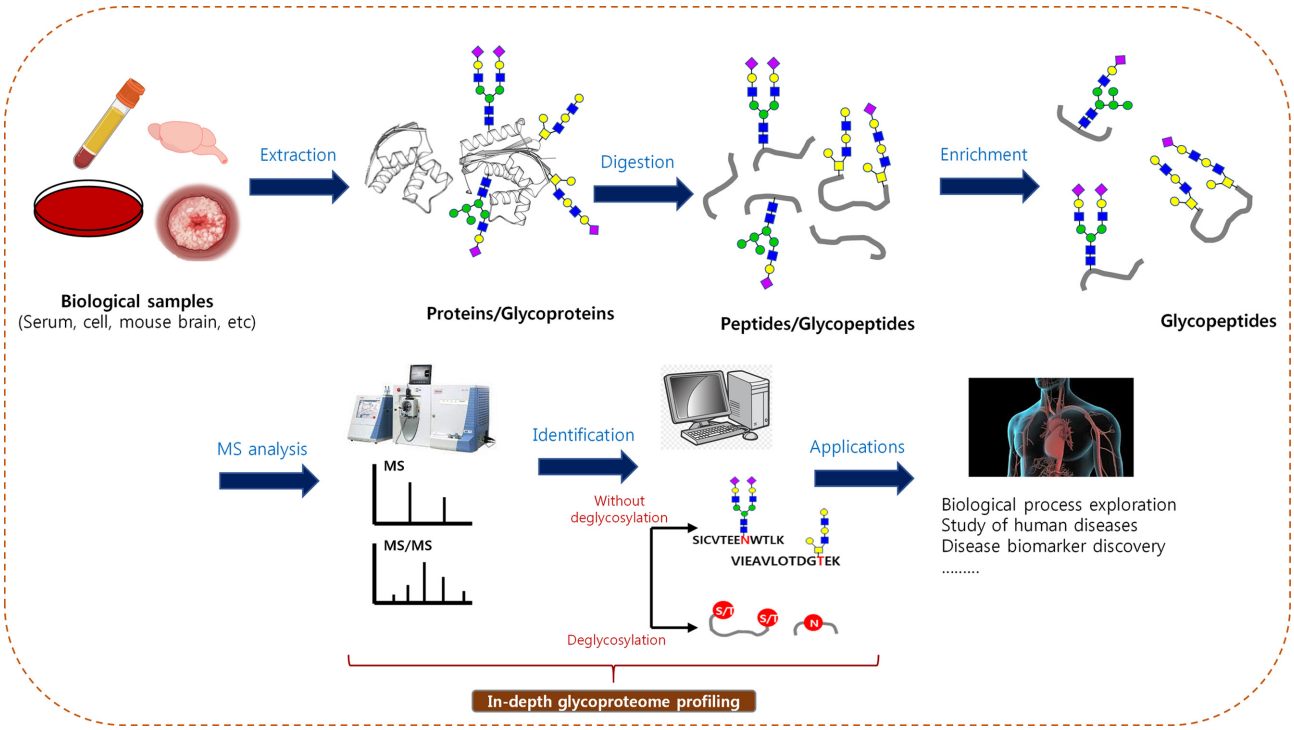

Figure 1. General protocol for MS-based glycoproteomic analysis in different biological samples.

abundance compared to native peptides in protein digests, complexity in glycan structures, heterogeneity of each glycosylation site, and poor ionization efficiency due to ion suppression of native glycopeptides.[27,46] Therefore, developing efficient strategies for selective glycopeptide enrichment and isolation from complex samples is required to enhance the detectability and sensitivity of MS-based glycoproteomics. Accordingly, diverse enrichment methods for glycoproteins and glycopeptides have been introduced based on boronate affinity materials, hydrazide chemistry, hydrophilic interaction liquid chromatography (HILIC), lectin affinity chromatography, polymeric materials, and other novel materials (i.e., metal-organic frameworks and functional composite nanomaterials).

The general protocol for MS-based glycoproteomic analysis from various complex biological samples is outlined in Figure 1. Glycoproteins extracted from biological samples are subjected to proteolysis, resulting in mixtures containing both peptides and glycopeptides. Subsequently, an enrichment step is implemented to isolate glycopeptides before they undergo MS analysis. Specialized software programs are then utilized to analyze the MS data for glycopeptide identification.

Boronate affinity materials

Boronic acid is one of the most commonly utilized ligands for separating glycopeptides and glycoproteins from different biofluids, such as saliva, serum, and plasma. So far, the most extensively investigated boronate monomers have been amine, thiol, carboxyl, formyl, acrylamide, or vinyl terminal phenylboronic acids.[8] The ability of phenylboronic acids to establish reversible interactions with diol-containing substances, including carbohydrates, has prompted various advancements with potential applications in sensor technology, drug delivery, and affinity chromatography.[47-50] During the enrichment process, materials based on boronic acid interact with diol groups present in glycan to capture the glycopeptides, thereby offering an enhanced specificity. Typically, the interaction between glycans and boronate necessitates alkaline pH conditions. Additional secondary interactions, including hydrogen bonding, hydrophobic coordination, and electrostatic are recognized to be involved in the process.[50] Xu et al.[1] synthesized boronic acid-modified mesoporous silica, termed as GA or GLYMO-APB, for glycopeptides enrichment from biological samples using a two-step post-graft method, which involved reacting 3-aminophenylboronic acid monohydrate (APB) with 3-glycidyloxypropyltrimethoxysilane (GLYMO) to synthesize boronic acid-bonded GLYMO, and then acquiring mesoporous silica modified with boronic acid through reaction with the GA solution. The newly developed material displayed a high surface area, a large accessible porosity, and precisely defined pore structures and entrances, thereby providing considerable benefits for glyco-specific enrichment, such as increasing binding rate and reducing the entire loading time (15 min),

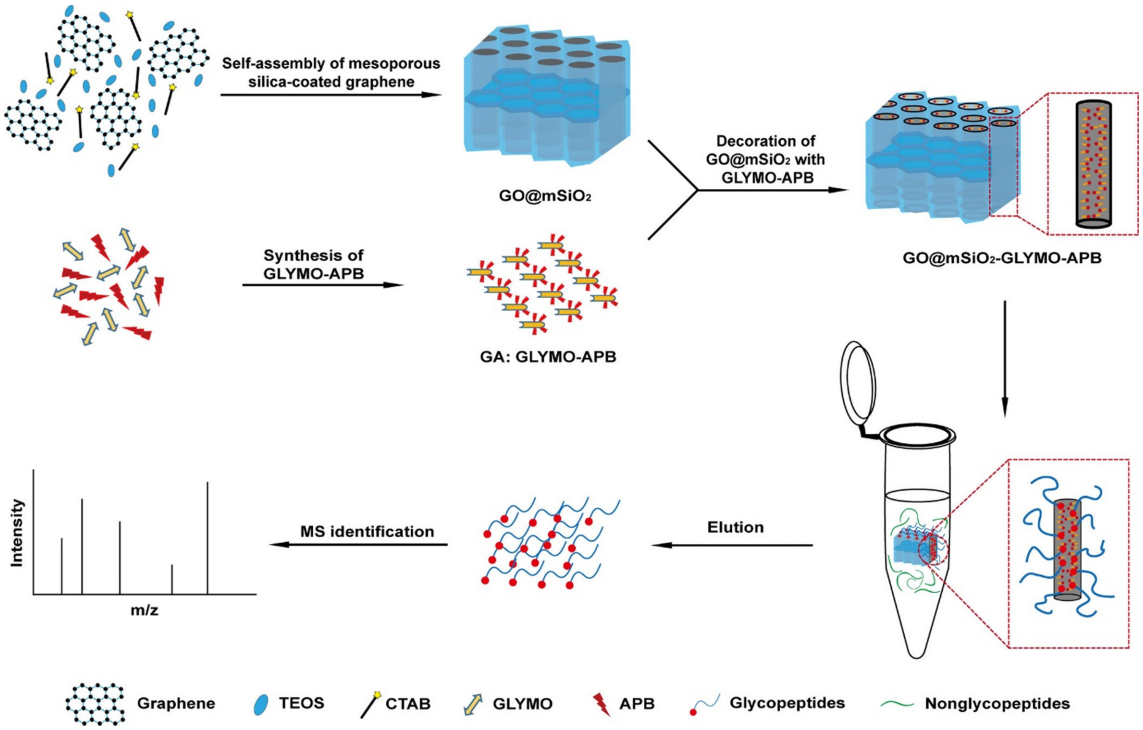

Figure 2. Graphical representation for the preparation of GO@mSiO$_2$–GLYMO–APB along with the procedure for glycopeptide enrichment from biological matrices. Reproduced with permission from Kong et al.[2] (2021).

compared with other conventional materials. Due to the formation of surface-rich boronic acid, the material showed significant selectivity for binding glycopeptides even in the presence of prominent tryptic BSA. The detection limit of glycopeptides was significantly enhanced, and the recovery rate was as high as 83.5%.

Recent advancement in enhancing boronic acid efficacy involves its grafting onto substrates with significantly larger specific surface areas and their hydrophilic properties, such as graphene oxide. Among diverse materials recently developed, the boronic acid-modified mesoporous composite of graphene and silica (denoted as GO@mSiO_2−GLYMO−APB), capable of enriching intact glycopeptides in complicated biological matrices, garners our interest.[2] The synthesis of the material (see Figure 2) involved the reaction between mesoporous silica-coated graphene (GO@mSiO_2) and GLYMO-APB, thereby benefiting from the hydrophilic nature of silica, the unique structure of graphene, and the reversible covalent binding capacity of BA. Combining the aforementioned benefits and their synergistic interactions, a highly sensitive and selective enrichment strategy utilizing GO@mSiO_2−GLYMO−APB was introduced for efficient glycoproteomics analysis in human serum. The approach facilitated a comprehensive analysis of both N-glycosylation and O-glycosylation, thereby enhancing the protein glycosylation profiling and providing a deeper comprehension of glycoprotein functionalities.

More recently, Li et al. fabricated a novel dual-functional boronic acid-modified metal-organic framework (MOF) nanomaterial (denoted as Fe_3O_4@SiO_2@UiO-PBA) for specific enrichment and purification of glycopeptides.[51] The proposed material exhibited two characteristics, including boronic acid affinity and hydrophobicity due to boronic acid grafting and the presence of abundant active amino groups on the material surface. Under physiological conditions (pH 7.4), the nanoparticles demonstrated significant binding capacities towards glycoproteins, such as horseradish peroxidase (530.79 mg/g), transferrin (241.17 mg/g), and ovalbumin (327.28 mg/g). Moreover, Fe_3O_4@SiO_2@UiO-PBA maintained its outstanding enrichment capability even after undergoing six consecutive uses. The applicability of the material was successfully confirmed through the separation and enrichment of OVA from egg white real samples under a physiological state. Thus, these observations demonstrated that the material exhibits a notable capability in extracting glycoproteins from intricate biological specimens.

Hydrazide chemistry enrichment

Glycopeptide enrichment using hydrazide functional beads relies on the formation of a covalent bond between hydrazide functional groups and N, O-linked glycan moieties in a pH-reversible reaction following the oxidation of their cis-diol groups with periodate to form aldehyde.[52] One of the most important features of this strategy is its increased specificity for capturing glycopeptides, accounting for approximately 90% of the enriched. By utilizing PNGase F treatment for N-glycans removal from hydrazide beads, these techniques have demonstrated significant capacity in mapping N-glycosylation sites.[53] The process of capturing and releasing glycopeptides was modified to avoid glycan loss. Specifically, samples were subjected to moderate $NaIO_4$ treatment to oxidize the terminal sialic acid residue of the glycan, while preserving the integrity of the other components. This approach facilitated the analysis of sialic acid-containing glycopeptides; nevertheless, it resulted in the loss of sialylation information.[54] To maintain the sialylation information, ice-cold 1 M HCl was used by Nishimura et al. to break the hydrazone link between the hydrazide beads and sialic acid. This process enabled the sialic acid to remain attached to the glycan.[55] Yen et al. employed the periodate oxidation/hydrazide-modified magnetic bead approach to identify a total of 486 glycoproteins from 14 breast cancer cell lines.[56] Based on the glycoprotein profiles from the 14 breast cell lines, hierarchical cluster analysis of glycoproteins was utilized to distinguish various subtypes of breast cancer, thereby serving as molecular markers for the classification of breast cancer subtypes and the differentiation between normal and benign breast cells from breast cancer cells. Moreover, extracellular matrix components including proteins were differentially expressed across various cell types, which makes them potentially valuable as biomarkers. In another interesting study, Sun et al.[57] presented a novel approach for analyzing the glycoproteome in human normal serum and liver cancer serum. They employed a solid phase-based strategy that integrates glycopeptide enrichment and stable isotope labeling on hydrazide beads. This innovative method allowed for the differential analysis of glycoproteins, providing valuable insights into the glycoproteome alterations associated with liver cancer. The method demonstrated a satisfactory linearity range for glycopeptide quantification with two orders of magnitude. This technique provided a greater enrichment recovery (10%-330%) and excellent detection sensitivity, allowing for the quantification of 42% of annotated glycosites (compared to 26%) with only 10 μg of glycoprotein mixtures and 400 μg of BSA interference as an initial sample.

The majority of the reported methods for enriching glycopeptide use a solid-liquid heterogeneous reaction that employs an insoluble or solid matrix. However, reaction rate and enrichment efficiency are severely constrained by the drawbacks of this system, including its nonlinear kinetic behavior and interfacial mass transfer resistance, in addition to the substantial steric hindrance of the matrix materials. In recent times, a stimuli-responsive soluble polymer system was developed to obtain a homogeneous reaction-based enrichment using hydrazide chemistry.[30,58] This approach is anticipated to yield higher enrichment efficiency in comparison to other conventional methods. Poly-

meric platforms, such as poly(N-isopropylacrylamide) (PNIPAM) or poly(acrylic acid) (PAA), known for their thermal and pH responsiveness respectively, were utilized to attach the hydrazide functionalities.[30,58] This allowed for the enrichment polymeric matrix to dissolve and self-assemble in an aqueous solution under specific reaction conditions. Consequently, this facilitated a homogeneous reaction between the hydrazide groups and oxidized N-glycoproteins/glycopeptides. Additionally, increased collision possibilities and binding capabilities are particularly advantageous for enriching low-abundant biomolecules from an exceedingly intricate biological sample. By controlling the temperature or pH, the polymer target molecule conjugates can be easily precipitated and recovered, which permits homogeneous reaction-based glycopeptide enrichment. By utilizing this approach, a total of 329 N-glycosylation sites were successfully identified in plasma exosomes,[30] and 1317 N-glycopeptides from 458 N-glycoproteins were enriched from mouse brain tissues.[58]

Lectin affinity chromatography

Lectin affinity chromatography is a well-established enrichment technique that is commonly utilized in the analysis of protein glycosylation.[59] It has received official recognition from the FDA for the identification of glycoprotein biomarkers linked to cancer.[60] Lectins are carbohydrate-binding proteins that are present in animals, plants, and microorganisms.[8] Lectin materials bind selectively to complex carbohydrate structures and can distinguish the slightly varied glycan forms since each lectin exhibits a distinct affinity for particular carbohydrates and contains carbohydrate recognition domains within its structure.[60,61] Van der Waals, hydrophobic, and interactions have facilitated the binding interaction between lectins and carbohydrates.[62,63] The application of lectins for the enrichment and detection of glycoproteins has been thoroughly reviewed.[64,65] Various well-characterized lectins have been utilized for the enrichment of glycoproteins and N- or O-glycopeptides. Concanavalin A (Con A) specifically recognizes α-linked mannose, Vicia villosa preferentially binds to N acetyl-galactosamine, wheat germ agglutinin (WGA) and *Sambucus nigra* lectin (SNA) has a strong preference for N-acetyl-glucosamine and sialylated complex glycans, Aleuria aurantia lectin (AAL) binds to α-linked fucose, and Ricinus communis Agglutinin (RCA120) offers high affinity toward terminal b-galactose.[28,61,66] Among these, WGA and Con A are the most commonly used lectins for enriching N-glycopeptides since they recognize terminal N-acetyl-glucosamine and α-linked mannose residues, both of which are common glycostructures present in many glycoproteins.[28,61,67,68]

A mixture of diverse lectins, which is normally described as multi-lectin affinity chromatography (M-LAC), is applied to enhance glycoproteome coverage in complex samples within one analysis.[69-71] For instance, Hancock, Hincapie, and co-workers explored the application of WGA, Jacalin, and ConA for the identification of N-linked, O-linked, and sialylated glycoproteins, and have recognized this technique for discovering cancer biomarkers in plasma or serum within the concentration level of 10–100 ng/mL.[12,72,73] In another study, Hirao and co-workers reported a multi-lectin affinity chromatography strategy using AAL, ConA, and Hippeastrum hybrid lectin (HHL) to identify glycoproteins and N-linked glycosylation sites in non-small cell lung carcinoma (NSCLC).[74] The report indicated the identification of 948 HHL/ConA-bound glycoproteins and 1092 AAL-bound glycoproteins from NSCLC tissue samples. However, the findings do not provide information regarding the glycosylation sites of the identified glycoproteins. Typically, lectins are l attached to solid surfaces like agarose or magnetic nanoparticles (MNPs) to immobilize them efficiently. Particularly, MNPs have garnered considerable interest due to their large area-to-volume ratio, enhanced lectin density on their surfaces, easy preparation and separation, and good biocompatibility.[14,75-77] Waniwan et al. presented a novel approach for analyzing the site-specific glycosylation of drug-resistant nonsmall cell lung cancer cells.[14] They utilized a lectin−magnetic nanoprobe (MNP@lectin) conjugated to SNA, AAL, and ConA, which allowed for comprehensive glycotope-specific enrichment. This technique enabled a detailed analysis of the glycosylation patterns in these cancer cells, providing valuable insights for further research and potential therapeutic strategies. The workflow scheme of the nanoprobe integrated into mass spectrometry analysis for specific glycopeptide enrichment is displayed in Figure 3. In total, 2767 and 2290 non-redundant glycopeptides were certainly identified in EGFR-TKI- resistant PC9-IR and -sensitive PC9, respectively with Byonic score ≥100.

Sequential integration of lectin enrichment with additional chemical immobilization methods, such as solid phase extraction separation techniques can further improve the enrichment efficiency by permitting specific isolation and identification of glycopeptides.[78,79] Li et al. developed a novel strategy combining lectin enrichment followed by solid-phase extraction of glycosite-containing peptides (SPEG) that was used for rapid identification and quantification of glycopeptides with specific glycan motifs. from serum with high specificity and sensitivity.[78] Zhou et al. reported that the identification of fucosylated glycopeptides was significantly improved by sequential application of lectin enrichment and Oasis MAX solid-phase extraction cartridges.[79] They utilized this tandem enrichment approach for the identification and quantification of 973 intact fucosylated glycopeptides derived from 252 fuc proteins in both nonaggressive and aggressive prostate cancer cell lines. On the other hand, lectin enrichment has been integrated into online reactors to enable fully automated high-speed and simultaneous proteolysis and glycopeptide enrichment, which is particularly valuable for analyzing glycopeptides

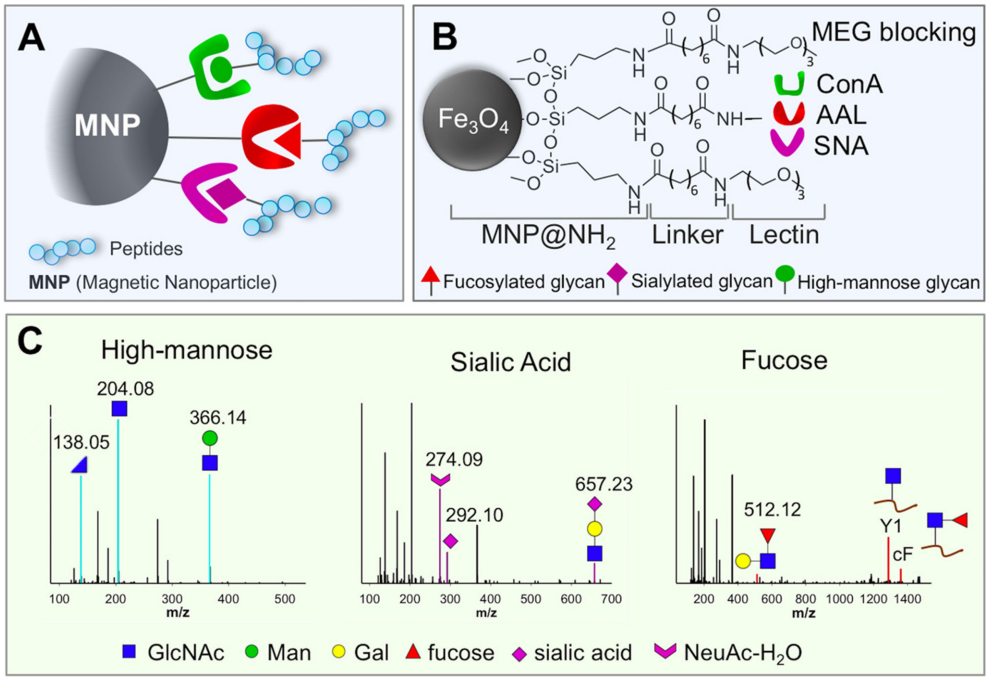

Figure 3. Workflow scheme illustrating the integration of lectin-magnetic nanoprobe (MNP@lectin) with HCD-CID-MS/MS for comprehensive enrichment of glycopeptides and analysis of site-specific glycosylation. (A) Glycotope-specific enrichment of high-mannose, fucosylated, and sialylated glycopeptides employing MNP@ConA, MNP@AAL, and MNP@SNA, respectively. (B) Synthesis of MEG-protected MNP@lectin. (C) Glycan-specific oxonium ions for identification of glycan motif. Reproduced with permission from Waniwan et al.[14] (2018).

when sample quantity is very limited. Based on this approach, a total of 262 N-glycopeptides were identified from 155 glycoproteins in 1.5 μL of human plasma [80].

Hydrophilic interaction liquid chromatography

Hydrophilic interaction-based enrichment, which mainly depends on the difference in hydrophilicity between glycopeptides and nonglycopeptides due to the attached N-linked or O-linked glycans, has demonstrated broad applications and superior performance in glycoproteomic analysis.[81-83] The interactions occurring between the analyte and HILIC materials comprise hydrogen bonding, dipole-dipole interactions, and electrostatic forces.[84,85] Ion-pairing reagents can be utilized to improve the hydrophilic properties between glycopeptides and non-glycopeptides, thereby enhancing their interaction.[86] In contrast to the hydrazide chemistry and lectin affinity chromatography methods, the HILIC method exhibits greater versatility, thereby providing a more comprehensive profile of the glycoproteome.[81,87] Liu et al. established an automated method employing a HILIC column for glycopeptide enrichment from serum, which allowed for the analysis of site-specific N-glycoproteome.[88] Based on this method, a total of 2081 unique N-glycopeptides and 324 N-glycosites from 190 glycoproteins were identified with a false discovery rate (FDR) of glycopeptide spectrum match (GPSM) of < 1%. The method displayed great potential for clinical utilities since it can identify significant changes in monofucosylated and nonsialylated oligosaccharides and the site-specific glycoforms on IgG1 site 180 between pancreatic cancer patients and healthy controls.

Although the HILIC approach enables unbiased recognition ability of glycopeptides along with great reproducibility and MS compatibility, the poor enrichment specificity as a result of the coelution of hydrophilic nonglycopeptides is one of its limitations.[89] Thus, recent advances in HILIC enrichment focus on designing novel HILIC materials featuring enhanced hydrophilic functional groups to increase the enrichment specificity. Zwitterionic stationary phase-based HILIC (ZIC HILIC), characterized by surface properties carrying both positive and negative charges, exhibited superior enrichment specificity in comparison to conventional HILIC methods. Cao et al. functionalized poly(amidoamine) dendrimer (PAMAM) with zwitterionic groups (ZICF-PAMAM) for glycopeptide enrichment from human serum.[90] Due to good solubility and multiple branched structure of the material, adequate interaction with glycopeptides occurred which resulted in remarkable glycopeptides enrichment capacity with high recovery of > 90.01%, and minimum detection concentration in the femtomolar level. By utilizing this method, a comprehensive analysis revealed a total of 48 glycosylation sites from 28 glycopro-

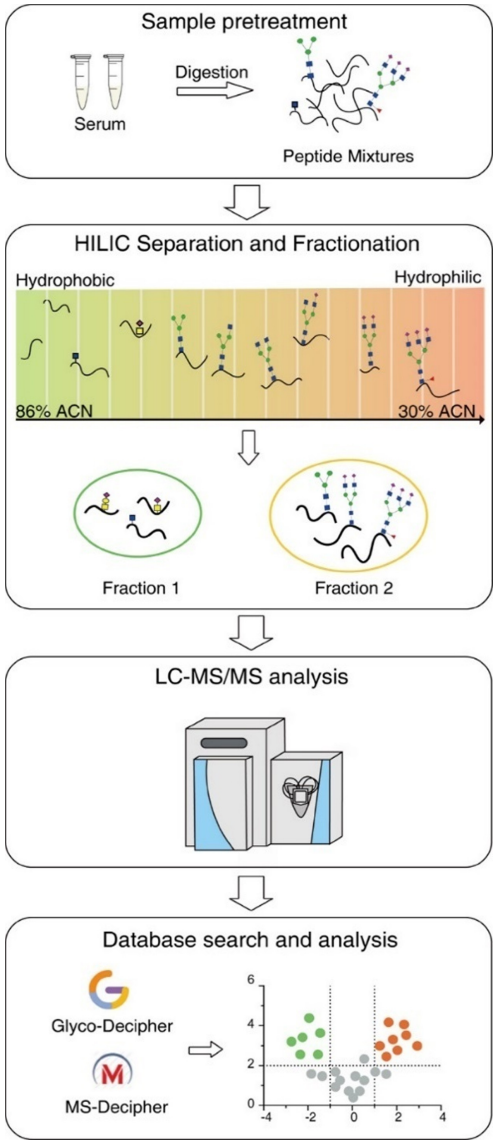

Figure 4. Schematic representation of the integrated platform used for the simultaneous identification of N- and O-glycopeptides. Reproduced with permission from Wang et al.[93] (2023).

teins, all from just 100 nL of human serum. In another research, a novel magnetic bead-based zwitterionic HILIC material with high specificity and high recovery yield (95-100%) was developed for glycopeptide enrichment from a standard glycoprotein (fetuin).[91] Using this approach, 85 N-glycosites in 53 glycoproteins were identified from urine samples. Moreover, the authors employed a two-step HILIC enrichment for a large-scale analysis of glycoproteins and succeeded in identifying, for the first time, two glycosylation sites on N^{513} of uromodulin and N^{470} of lysosomal alpha-glucosidase. Recently, Huang et al. designed a dual-functional Ti(IV)-IMAC material for capturing N-glycopeptides, phosphopeptides, and mannose-6-phosphate (M6P) glycopeptides from mouse lung protein based on both hydrophilic and electrostatic interaction.[92] Different sequential elution profiles were employed to allow the separation of mono-, multiphosphopeptides, and neutral and sialyl glycopeptides which resulted in the identification of 3896 N-glycopeptides from the mouse lung. The number of glycopeptides identified, by this approach was 1.9 greater than the number identified by using the traditional method relying only on single HILIC enrichment. More recently, Wang and co-worker investigated the use of the HILIC column (click maltose, 5 μm, 100 Å) to simultaneously enrich and characterize N- and O-linked intact glycopeptides from biological samples.[93] The schematic illustration of the integrated platform utilized in this study is depicted in Figure 4. Due to the distinct hydrophilicity of N- and O-glycopeptides, this integrated platform allowed their selective separation into two fractions. The first fraction predominantly contained 85.1% O-glycopeptides (85.1%), while the second fraction mainly contained 93.4% N-glycopeptides (93.4%); making this method very useful for simultaneously analyzing site-specific N- and O-linked glycosylation.

The combination of HILIC with other enrichment techniques such as LAC has been reported to further enhance the enrichment specificity.[94,95] Additionally, recent advancements have introduced new technologies that involve the incorporation of HILIC materials into a micro-column or tip aiming at minimizing the loss of sample during the enrichment stage while also improving the identification of low abundance glycopeptides at the same time.[96-98]

Metal-organic frameworks

Metal-organic frameworks (MOFs) are a special type of polymer-coordinated compounds with three-dimensional (3D) pores constituted by the self-assembly of positively charged metal ions and repeating units of organic linker molecules.[99] Due to their unique characteristics, including ultrahigh specific surface area, uniform structure, rich porosity, strong hydrophilic surface, ordered nano-channels, and abundant binding sites, MOFs have emerged as outstanding candidates to fulfill the requirements of glycopeptide enrichment, compared to other conventional porous materials.[7,46,100] Recently, various novel techniques have been established for synthesizing MOFs, thereby enabling the investigation of their potential applications. Depending on the synthesis technique, MOFs can be classified into three categories, namely pristine MOFs, chemically functionalized MOFs, and MOF-derived composites.[99,101] Ali et al. reported the fabrication of a highly hydrophilic and stable titanium-based metal organic framework (NH_2-MIL-125(Ti)) by employing an amino-functionalized organic linker for N-linked glycopeptide enrichment.[102] The newly prepared material possesses good hydrophilicity, excellent porous structure, extraordinary stability, high selectivity, sensitivity, and reusability. NH_2-MIL-125(Ti) was packed

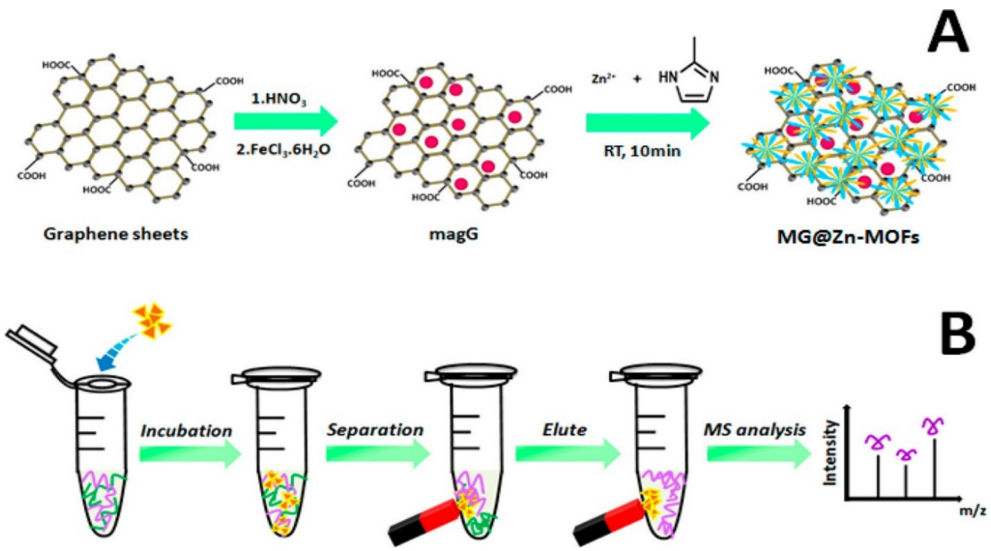

Figure 5. Graphical representation for MG@Zn-MOFs biocomposite synthesis (A) and the procedure for glycopeptides enrichment by MG@Zn-MOFs (B). Reproduced with permission from Wang et al.[46] (2016).

into a pipette tip to enable rapid, simple, and reliable analysis with a selectivity of up to 1:1000 (HRP digest to BSA digest) and a limit of detection of 1 fmol/μL. Following the application of NH_2-MIL-125(Ti) to a healthy saliva sample, a total of 64 unique endogenous glycopeptides were identified.

To further improve the functionality, selectivity, and hydrophilicity of synthesized MOFs, chemical modification has been achieved that enables the grafting of desirable functional moieties onto MOFs via stable chemical bonds.[100,103] In 2020, Pu et al. significantly enhanced the performance of Cerium-based MOFs (Ce-MOF) through synergistic etching and surface modification using phytic acid (PA).[100] Due to outstanding hydrophilicity and enlarged pore channels, Ce-MOF@PA exhibited excellent enrichment selectivity; effectively capturing a total of 422 N-linked glycopeptides corresponding to 155 glycoproteins in 2 μL of human serum. Following five successive reuses, Ce-MOF@PA displayed consistent signal intensity and retained the same number of identified N-linked glycopeptides as observed during its initial use, indicating its high recyclability and chemical stability. These findings indicated that the incorporation of PA elevated the hydrophilicity of Ce-MOF while also improving its stability through robust chelation with rich coordination-unsaturated metal sites within the MOF structure.[100] In another research, the amino-functionalized MOF (MIL-101(Cr)-NH_2) allowed for the enrichment of 116 N-linked glycopeptides from 42 glycoproteins in 10 μL of human serum,[104] while its maltose functionalized structure (MIL-101(Cr)-maltose) captured 111 glycopeptides corresponding to 65 glycoproteins in 5 μL of human serum.[105]

Among MOF-derived composites, magnetic materials are frequently utilized, thanks to their magnetic properties which allow for effortless separation and reusability.[46,106-109] Li et al. synthesized magnetic Mg-based MOF biocomposite (Fe_2O_3@Mg-MOF-74) via conjugating Mg-MOF-74 onto the surface of magnetic Fe_2O_3 using the facile epitaxial growth method.[109] The graphical illustration for MG@Zn-MOFs biocomposite synthesis and the procedure for glycopeptides enrichment is shown in Figure 5. Due to their inherent hydrophilic surface characteristics and size-exclusive effects, the fabricated MOFs exhibited notable selectivity in enriching N-linked glycopeptides, resulting in the identification of 441 N-glycosylation sites within 418 glycopeptides from 125 glycoproteins in only 1 mL sample of human serum. In another pioneering research, Wang et al. fabricated a novel magnetic graphene Zn-based MOFs composite, denoted as MG@Zn-MOFs through grafting Zn-MOF crystals on the surface of magnetic graphene for capturing glycopeptides from human serum.[46] The newly functionalized MOF composite owned a high specific surface area, strong magnetic responsiveness, unique size-exclusion properties, and excellent biocompatibility. Its practical utility was demonstrated by the analysis of human serum (1 μL), characterizing 517 N-glycopeptides corresponding to 151 glycoproteins with outstanding selectivity and good recyclability.

Polymeric materials

A range of polymeric materials has been developed for the enrichment of glycoproteins and glycopeptides. Particularly, flexible polymeric materials offer several advantages for capturing glycopeptide from different biological matrices. By incorporating different functional monomers into the polymeric matrix to improve their properties, enrichment performance toward glycopeptides is increasingly enhanced. Moreover, hydrophilic polymeric materials, such

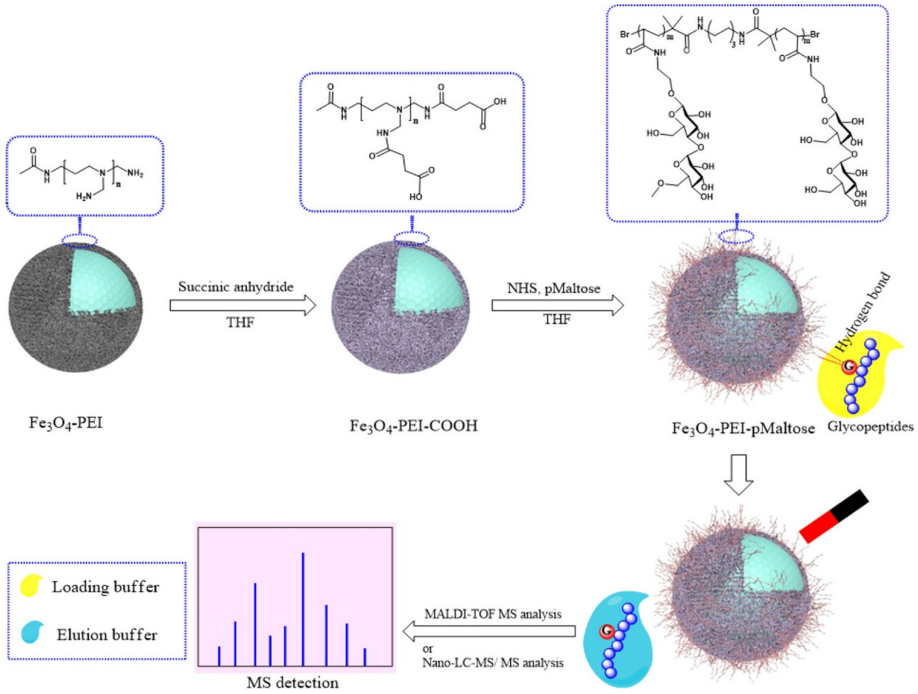

Figure 6. Synthesis flow chart of Fe_3O_4–PEI–pMaltose NPs and the selective enrichment for the N-Linked glycopeptides. Reproduced with permission from Bi et al.[112] (2016).

as polydopamine (PDA), and polyethyleneimine (PEI) provide unique adhesion properties, a great degree of functionality, and a promising spatial conformation for the target glycan, thereby facilitating glycopeptides binding. More recently, Ali et al. introduced a flexible and hierarchical polymeric-based material namely MF@PDA@UiO-66-NH_2 as a composite-based affinity tip which provided good and tunable permeability for the selective enrichment of glycopeptides and phosphopeptides from human saliva samples.[110] Melamine foam was modified with a monolayer of polydopamine to form PDA-modified melamine, and UiO-66-NH_2 crystals were grown on its surface. Thanks to the specific functionality, material flexibility, excellent size exclusion effect, and enhanced mechanical strength, the novel material showed a great performance for enriching glycopeptide and phosphopeptides with high selectivity of 1:200 (β-Casein digest to BSA digest) for phosphopeptides, 1:1000 (HRP digest to BSA digest) for glycopeptides and very low detection limit of up to 0.5 fmol/μL from tryptic digests of horseradish peroxidase and β-Casein.[110]

More recently, Zhang and co-workers introduced a novel hydrophilic polymer-modified magnetic nanospheres functionalized with 2-aminopurine (Fe_3O_4@poly(MBA/EH)@2AP) for selective enrichment of glycopeptides and glycosylated exosomes.[111] The presence of a polymer layer on the surface of the magnetic sphere introduces numerous polar groups, thereby markedly enhancing the material's hydrophilicity and increasing the grafting sites. From the serum of uremic patients, the newly synthesized material identified 290 glycopeptides and 184 glycosylation sites from 185 glycoproteins in the serum of uremic patients with high selectivity (BSA:HRP=1000:1), and good loading capability (125 μg/mg). Moreover, Zhang's group conjugated polymaltose polymer brushes, as a source of hydrophilic functional groups, on PEI-coated Fe_3O_4 via a facile two-step modification to introduce a super hydrophilic nanoparticle (denoted as Fe_3O_4–PEI–pMaltose).[112] Due to its outstanding features, including good selectivity, high binding capacity, high enrichment recovery, and low detection limit, the as-prepared magnetic adsorbent successfully identified 449 N-glycopeptides and 476 glycosylation sites, representing 323 glycoproteins. The excellent enrichment performance of the material was attributed to the attachment of a maltose polymer brush and the efficient assembly technique. These findings reinforce the perspective that polymers often provide increased binding sites for bonding functional groups, consequently enhancing the enrichment capability of glycoproteins and glycopeptides.[112]

Hydrogels are three-dimensional polymeric networks characterized by their hydrophilic nature and diverse hydrophilic groups distributed along the polymer chains.[113] These features afford hydrogels exceptional properties such as reversibility, excellent functionality, mechanical stability, and biocompatibility.[114,115] Cerrato et al. reported the preparation of a new hydrogel polymer employing 2-acrylamido-2-methyl-1-propanesulfonic acid as the monomer and ethylene glycol dimethacrylate as the cross-linker.[115] The

acquired material enriched up to 762 N-linked glycopeptides with 77% selectivity, thanks to its outstanding hydrophilicity, porosity, and enhanced swelling properties. In addition, magnetic molecularly imprinted materials have garnered significant interest for their notable specificity towards glycoproteins.[116,117] Sun et al. modified the surface of porous TiO_2-coated Fe_3O_4 microspheres with a molecularly imprinted polymer containing phenylboronic acid groups to fabricate a new magnetic molecularly imprinted polymer based on boronate affinity.[116] The authors demonstrated that the use of porous TiO_2 instead of smooth SiO_2 would provide more binding sites due to improved rough surface. Notably, the high electron-withdrawing properties of Ti(IV) reduced the pKa values of boronic acid, thereby facilitating the identification of glycoproteins from moderate acidic samples. Based on this approach, horseradish peroxidase (HRP) was selectively extracted and quantified in spiked fetal bovine serum (FBS) with a satisfactory adsorption capacity of 69.4 mg/g.

Functional composite nanomaterials

Recent developments in the synthesis technology of composites have enabled the effective use of an increasing range of composite nanomaterials for enriching glycopeptides. These include graphene composites [118,119], magnetic nanoparticles[120,121], porous graphitized carbon (PGC)[122,123], and functional nanosheets.[9,124] These composite nanomaterials offer the benefits of various compositions, high chemical stability, large specific surface area, and rich functional groups, thereby significantly enhancing the selectivity and efficiency of glycopeptide enrichment. Chen group was able to fabricate a hydrophilic chitosan-functionalized magnetic nanocomposite termed Fe_3O_4-GO@PDA-Chitosan by using a straightforward two-step synthesis approach, involving dopamine polymerization and Michael addition.[118] The inclusion of graphene oxide with a large specific surface area resulted in increased modification sites for chitosan immobilization, thereby enhancing the efficiency of glycopeptide enrichment. Subsequently, this composite was utilized to selectively enrich 393 N-glycopeptides from tryptic digests of human renal mesangial cells (HRMC), corresponding to 195 glycoproteins and 458 glycosylation sites. In another recent research, Zhang et al. introduced a single-step assembly method to develop maltose-modified oligopeptides functionalized Fe_3O_4@SiO_2, exhibiting superior performance in glycopeptide enrichment.[120] The method displayed high selectivity (with mass ratios of HRP and BSA digests reaching up to 1:150), remarkable detection sensitivity (0.001 ng/mL HRP) and favorable enrichment recovery (exceeding 86.3%). As a result, the material enriched 24 glycopeptides from HRP, 31 glycopeptides from IgG and 282 glycopeptides from human serum digests. Zhan and co-workers employed a sequential deposition technique for surface modification of a negatively charged magnetic graphene oxide and dendritic mesoporous silica nanoparticles (DMSNs) surfaces with polyethyleneimine (PEI) and hyaluronic acid (HA) through electrostatic interactions, resulting in the formation of hydrophilic magnetic materials.[119] The efficacy of this magnetic adsorbent in glycopeptide enrichment was validated by its application in capturing glycopeptides from a 2 μL serum sample. Consequently, 419 N-glycopeptides derived from 105 glycoproteins were identified using DMSNs@PEI@HA, and 376 N-glycopeptides derived from 102 glycoproteins were identified MagG@PEI@HA. This study offers a viable approach for designing magnetic nanocomposite affinity materials utilized for glycopeptide enrichment.

Additionally, Chu et al. were able to identify 438 glycopeptides from HeLa cell digests (100 μg) by utilizing a magnetic binary metal oxide (Fe_3O_4@ZrO_2/TiO_2) modified with glutathione.[121] More recently Lin group modified the surface of 2D covalent organic framework (COFs) nanosheets with hydrophilic monomers to be utilized for efficient glycopeptides enrichment from complex biological samples.[9] The as-prepared 2D COF nanosheets (designated as NUS-9) exhibited an exceptionally high density of sulfonic groups grafted onto their surface and demonstrated remarkable super-hydrophilic properties. Thanks to its large specific surface area, abundant accessibility sites, high chemical stability, and low steric hindrance, the NUS-9 was applied to human saliva and identified up to 631 endogenous glycopeptides with high sensitivity (0.01 fmol/μL), satisfactory recovery (92.2 ± 2.0%), and excellent selectivity.

Advancements in MS-based glycoproteomics

The fundamental principle of mass spectrometry (MS) involves the ionization of molecules in the gas phase, followed by separation and detection of these ions based on their mass-to-charge ratio.[125-127] MS has emerged as the preferred analytical method for investigating protein glycosylation due to its high sensitivity and capacity to reveal structural information of glycopeptides and glycans. Soft ionization techniques such as electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) are frequently employed for MS analysis of glycopeptides in complex biological samples.[2,71,82,115,121,128] Due to the complications in MS analysis of glycopeptides induced by their heterogeneous structure and glycan complexity, the most efficient strategies have relied on MS-based separation techniques since this approach can enhance glycopeptide sensitivity by decreasing the number of species that undergo ionization simultaneously.[45,129] Various separation techniques have been developed to enhance the MS-based characterization of glycopeptides, but the most commonly employed technique is LC-MS/MS. Due to the availability of various stationary phases, this method is often considered the preferred option for researchers in the field.[130-133] Alternative methods have replaced LC with CE systems. IM-MS, another method relying on gas phase separation,

has attracted significant attention in the field of glycoproteomics analysis. On the other hand, MALDI–MS has been commonly used as a high-throughput approach for glycopeptide analysis. Owing to the limited availability of convenient analytical standards in glycoproteomics, the identification process in MS relies heavily on the accurate interpretation of the generated MS spectra using robust bioinformatic tools. Consequently, exploring appropriate fragmentation techniques is critical for reliable glycopeptide identification.[4,134]

Characterization of glycopeptides using LC-MS/MS

LC analysis of glycopeptides is frequently employed using nano-LC-MS/MS systems, whereby eluted glycopeptides from the LC column are subjected to ionization using ESI and fragmented by a variety of tandem MS sophisticated fragmentation methods, such as collision, electron, or photo-induced fragmentations.[31,89,135,136] Due to the availability of various stationary phases, such as reversed-phase liquid chromatography (RP-LC), HILIC, porous graphitized carbon (PGC), and ion-pairing chromatography, this technique has emerged as a prevailing tool in glycoproteomics. RP-LC applies to both N- and O-glycopeptides; however, more polar stationary phases like PGC and HILIC are frequently utilized, particularly for their enhanced selectivity in separating N-glycopeptide isomers.[137,138] On the other hand, the selection of appropriate LC and MS/MS analytical parameters can play a critical role in glycoproteomic experiments and ultimately have a significant influence on the quality of data generated and the subsequent interpretation strategy.[137] In the LC-ESI-MS/MS technique, the flow rate, which is closely associated with the column's inner diameter, significantly influences the ionization efficiency of glycopeptides. Sensitivity gains rise exponentially when transitioning from analytical flow rates (0.1–0.4 mL/min for 2.1 mm ID column) to capillary flow rates (1–15 μL/min for 0.1–0.3 mm ID column) to nanoflow rates (300 nL/min for 75 μm ID column).[139] Lower flow rates also contribute to enhancing the MS signal of neutral glycans lacking sialic acid.

Reverse-phase liquid chromatography

The majority of glycoproteomic techniques employ reverse-phase liquid chromatography (RP-LC) at low pH (pH <2) to separate glycopeptides before MS/MS analysis, which proved to be advantageous due to its simplicity and high capacity.[33,34,140] This separation typically utilizes an octadecylsilane (C18) column under nano-flow rates typically ranging from tens to hundreds of nanoliters per minute. The interactions between C18 columns and glycopeptides are mainly influenced by the hydrophobic nature of the peptide backbone in the high aqueous phase; however, the hydrophilic behavior of monosaccharide content within peptide-attached glycans also contributes to the retention mechanism.[32,84,141,142] For instance, glycopeptides of lengthy peptide chains exhibit increased affinity for the C18 column, while glycopeptides that possess microheterogeneity at identical glycosylation sites elute in clusters depending on the sialic acid content.[29,33] Moreover, within a given cluster, glycopeptides of a greater number of glycan units tend to elute sooner. In an interesting work, the retention characteristics of several glycopeptides using a BEH^{TM} C18 nano column were investigated by Kozlik and coworkers.[142] The findings revealed that the retention time of glycopeptides decreases with an increase in the number of neutral sugar units. Conversely, the retention time exhibits a gradual rise as sialic acid units present in the glycopeptide increase. Additionally, it was noted that the presence of fucose residues did not impact the glycopeptide's retention time in the case tri- and tetra-antennary glycans are linked to the peptide sequences. While RP-LC remains the predominant method in the analysis of glycopeptides by LC-MS/MS, this technique still has some limitations. One such drawback is the simultaneous elution of isomeric glycoforms because of their similar peptide backbone.[134,140] In a recent study, Ji et al. demonstrated that high-temperature RPLC can improve the retention of sialylated N- and O-glycopeptides of different glycan isoforms.[34] However, it is worth noting that although this approach may facilitate the separation of some isomeric glycopeptide species, it may not consistently achieve complete separation of all isomers present in the samples.[137] C18 columns demonstrate superior stability compared to HILIC and PGC columns, particularly in nano-flow scale applications. Furthermore, peaks tend to exhibit narrower widths on C18 columns in comparison to PGC and HILIC columns. Nonetheless, HILIC and PGC columns have more capability to separate glycopeptides that elute in clusters on reversed-phase C18 columns.[33,123,139,143,144]

Hydrophilic interaction liquid chromatography

HILIC columns have gained popularity in glycopeptide analysis, where the interaction between the HILIC phase and glycopeptides is based on the hydrophilicity imparted by glycan moieties, and the presence of polar amino acids in the peptide backbone.[32,33,144] Therefore, glycoproteins and intact glycopeptide isomers that vary in glycan linkage position and branching can be effectively separated by HILIC columns. In other words, glycopeptides with shorter peptide chains exhibit greater affinity for HILIC columns, while those with microheterogeneity at a single glycosylation site are notably better separated compared to C18 columns. The combination of HILIC LC with multiple reaction monitoring (MRM) was explored to characterize isomeric α2,3- and α2,6-sialoglycopeptides derived from human prostate-specific antigen,[32] the latter isomer exhibited an increased LC retention on HILIC column in another research.[143] HILIC LC was also utilized for the separation of topological N-glycopeptide isomers of human hemopexin, aiming to determine the fucose residue position on

N-glycans (core versus outer arm) and to identify the position of α2,6-sialic acid residues on the glycan arm.[144] HILIC offers the advantage of its capability to analyze unmodified glycans or glycopeptides.[145] Nevertheless, to enhance the sensitivity of detection or facilitate quantification, derivatization procedures are sometimes carried out before HILIC analyses.[146,147] Several HILIC columns with different materials for stationary phases are now available for the analysis of isomeric glycans and glycopeptides, including amide columns equipped with primary amine groups, ZIC-HILIC column containing zwitterionic functional groups (e.g., tetramethyl ammonium group and sulphonic acid group or phosphoryl group), and HALO HILIC column incorporating multiple hydroxyl groups.[45,139] However, a recent report comparing various HILIC stationary phases indicated that HALO penta-HILIC demonstrates superior separation capability for isomeric forms of N-glycopeptides compared to the commonly utilized ZIC-HILIC and amide columns.[148] So far, a significant drawback of HILIC separation is that certain glycopeptides may exhibit poor solubility in high organic phase conditions.

PGC liquid chromatography

Another stationary phase alternative to RP-LC utilizes PGC which retains polar compounds using solvents compatible with MS and offers significant benefits in separating released glycans.[149,150] It serves as a complementary stationary phase alongside the C18 column, particularly useful in separating polar substances and almost similar analytes.[149] Despite its common use in glycomics, PGC LC remains underutilized in glycoproteomics. Glycopeptides of a high sialic acid content that are produced from frequently utilized proteases such as trypsin, chymotrypsin, or glutamyl endopeptidase (GluC) are challenging to elute from the column; thus, nonspecific proteolysis is typically required to generate shorter glycopeptides.[151,152] In this separation process, both charge and hydrophobicity play a role in analyte retention, which makes the separation of large glycopeptides somewhat complicated.[153,154] Research has demonstrated that PGC-LC-MS/MS is capable of separating isomeric forms of N- and O-glycopeptides.[155] Additionally, the retention of isomeric forms, such as α2,3-sialoglycopeptides and α2,6-sialoglycopeptides can be controlled by adjusting the column temperature.[150] The optimal performance for resolving the glycopeptide isomers was at a column temperature of 75°C and a pH of 9.9. Stavenhagen et al. developed an integrated approach involving both a C18 column and an in-house made PGC column for the analysis of glycopeptide following pronase digestion.[149] This method enabled the retention of more hydrophobic glycopeptides on the C18 column, while the downstream PGC column facilitated the separation of more hydrophilic glycopeptides (referred to as C18-unbound glycopeptides), allowing glycopeptides of diverse peptide sequence lengths to be analyzed within a single analytical run. Yet, the broad application of PGC-LC glycoproteomics has been hindered by challenges associated with large glycopeptide elution, primarily due to the high retention of more hydrophobic species.[137] It should be emphasized that while the LC-based approaches mentioned above are conventionally conducted on conventional LC columns, they can also be effectively implemented using chip-based fluidic devices.[33,153,156]

Characterization of glycopeptides using CE-MS

Competitive alternative separation methods, apart from LC, are increasingly finding applications in the identification of glycans and glycopeptides. CE is an effective technique in glycoproteomics due to its notable features, including high sensitivity, superior peak capacity, rapidity with low sample consumption, and ability to resolve isomers.[42] Advances in technology have facilitated the integration of CE with MS, thereby allowing the combination of the high-resolution power of CE with the high sensitivity of MS analysis. Therefore, this technique can serve as a potent tool in glycoproteomics for separating glycopeptide isomers and potentially enhancing both reproducibility and sensitivity.[43,157,158] CE separates and characterizes the glycopeptides according to their charge and size. Consequently, the size and composition of glycans (and particularly the presence of sialic acid) influence the electrophoretic migration of glycopeptides.[42,159] This process enables the glycan-dependent separation of glycoforms sharing the same peptide backbone. In recent advancements, enhanced efficiency and detection capabilities in glycan separation were realized through the utilization of a double-layer polyvinyl alcohol-coated capillary column, where a more homogeneous, stable, and compact PVA film resulted in an improved separation process.[160]

Zaia group conducted comprehensive glycoproteomic analyses by utilizing a microfluidics-based CE-MS (ZipChip CE-ESI) approach that enabled the identification of glycopeptides, released glycans, and monosaccharides.[42] Tryptic-digested glycopeptides derived from human alpha-1-acid glycoprotein (AGP) were employed to assess variations in electrophoretic mobility between sialylated and non-sialylated species. According to the results, glycopeptides typically elute after 8 minutes, but non-glycosylated peptides elute within the first 10 minutes. Thanks to the robust interface between CE and MS, this study paved the way for exploring extended separation pathways with enhanced resolution capabilities. In another study, Kammeijer and co-workers established a CE-ESI-MS platform capable of distinguishing between α2,3- and α2,6-sialylated glycopeptides.[159] This differentiation of isomeric glycopeptides was achieved based on disparities in their electrophoretic mobilities, which are associated with variations in acidity levels. More recently, the Lageveen-Kammerijer group established a zero-flow method utilizing CE in combination with sialic acid derivatization and MS/MS, where the separation is carried out based on the electrophoretic

mobilities of the analytes by only the application of a separation voltage without the need for applied pressure.[44] This approach allowed structural characterization of isomeric N-glycans with high resolution and sensitivity, where 208 N-glycans were characterized in human plasma, with 57 compositions exhibiting multiple isomers.

Characterization of glycopeptides using IM-MS

IM-MS is a method that separates gaseous phase ionized molecules according to their mobility through a carrier gas, followed by analysis using a mass spectrometer.[161,162] In IM-MS, ions of varying collision cross-sections (CCS) move at various speeds inside a drift tube under the influence of an electric field, thereby causing smaller ions to travel faster than larger ones.[134,163] The assessment of CCS reveals the 3D configurations of analyte molecules in the gas phase, which are influenced by different factors such as ion size, charge, and shape, thus offering characteristic structural insights for each analyte molecule.[39,137,164] IM-MS is regarded as a complementary technique to chromatographic and electrophoretic methods in the separation of isomeric glycopeptide ions, since it has the potential to differentiate these ions by their charge, shape and distinct conformational size, resulting in different CCSs and drift times; two essential parameters for glycopeptide identification.[40,41,138,165] Gelb and co-workers recently explored the influence of glycan size, composition, and the charge states of glycopeptides on CCS.[162] Their findings highlighted the potential for isomeric separation via IM-MS due to the variations in three-dimensional shapes among different glycopeptide isomers. Pallister et al. recently utilized a high-throughput LC-FLR-IM-MS workflow to comprehensively differentiate glycan isomers, and also to separate several IgG positional glycopeptide isomers.[40] This was achieved through the utilization of both intact IM-MS and fragment-based IM-MS glycan sequencing, relying on the calculation of the CCS distribution (CCSD) for both intact glycopeptide isomers and their fragments. This innovative approach enabled the separation of two pairs of positional isomers, including 3-arm galactosylated and 6-arm galactosylated isomers, as well as tri-antennary F(6)A3 and bisecting F(6)A2B glycans. Additionally, direct IM-MS analysis has also demonstrated the capability to distinguish between isomeric O- and N-glycopeptides that differ solely in the position of the glycosylation site.[165,166] Additionally, it can distinguish between epimeric O-glycopeptides containing either Glc or Gal residues, or different Man or Glc residues on the same sites.[167]

In recent times, field asymmetric waveform ion mobility spectrometry (FAIMS), also known as differential mobility spectrometry (DMS), has been employed to improve peptide identification in LC-MS-based proteomic investigations. FAIMS operates by separating gas-phase ions transported by carrier gas through an asymmetric waveform electric field created between planar or curved electrodes. In a recent investigation, FAIMS demonstrated the capability to separate O-glycopeptide isomers obtained from mucins.[41] In the same report, the approach showed a good potential to separate a pair of O-linked glycan isomers, namely α- and β-GalNAc anomeric glycopeptides.

Isomeric separation of glycopeptides was performed by IM-MS. However, these investigations encountered limitations due to the microheterogeneity of the glycopeptide isomers and the moderate resolution of IM-MS. Furthermore, IM-MS glycoproteomics analyses mentioned above mostly concentrated on purified glycopeptides or model glycoproteins, while there is a lack of comprehensive investigation into the isomeric separation of glycopeptides from complex biological samples, highlighting the need for further exploration in future research endeavors. Despite this, IM-MS is still an attractive complementary approach to conventional LC-MS since it offers a secondary dimension of isomeric separation in structure-focused glycoproteomics.[138]

MALDI–MS analysis of glycopeptides

MALDI is a vaporization-ionization technique commonly employed for the analysis of complex biomolecules like peptides and protein mixtures.[4,35,168] In this method, the sample is combined with an appropriate organic matrix solution and then allowed to co-crystallize directly on specialized sample plates. MALDI has found extensive biological applications in glycomics and glycoproteomics, particularly in profiling released glycans derived from glycoproteins in biological mixtures.[169,170] This is attributed to its straightforward operation, and uncomplicated sample preparation protocols, enabling the screening of numerous samples efficiently. MALDI is frequently combined with time-of-flight (TOF) MS, which offers an extensive m/z range and rapid scan capabilities, rendering it a widely favored MS tool in clinical glycomic analysis.[21] Currently, the typical workflow for MALDI-MS analysis of glycoproteins follows a set of standardized steps, including protein denaturation, enzymatic digestion, release of N-linked glycans using PNGase F, permethylation with CH_3I, purification through diverse methods, mixing with a suitable organic matrix solution, and subsequent MALDI-MS analysis of the purified glycans.[171,172] These MALDI-based workflows have been effectively employed to generate glycan profiles indicative of disease onset or progression and for biomarker discovery.[173,174] For instance, they have been utilized in profiling high-mannose glycans during breast cancer progression,[13] and in investigating GALT (galactose-1-phosphate uridyltransferase) deficiency, which is associated with a transition from predominant high-mannose-type glycans in healthy individuals to complex type N-linked glycans in diseased individuals.[175] Moreover, the first glycomic biomarker study aimed at discovering potential biomarkers for ovarian cancer was conducted using MALDI-FTICR-MS, identifying 15 unique serum glycan markers present in all patients but absent in normal individ-

uals.[11] However, the low ionization efficiency due to the microheterogeneity of glycans and their hydrophilic nature, as well as the unfavorable energetic conditions inherent in the process hinder the formation of intact species. Therefore, research efforts have focused on identifying improved matrices capable of yielding intact species. Accordingly, various efficient matrices and co-matrices have been assessed, including 2,4,6-trihydroxyacetophenone (2,4,6-THAP),[176] α-cyano-4-hydroxycinnamic acid (CHCA),[177] 3-aminoquinoline/α-cyano-4-hydroxycinnamic acids (3-AQ/CHCA),[178] and 2,5-dihydroxybenzoic acid (2,5-DHB),[4,177,179,180] with the latter being the most commonly utilized matrix in glycomics and glycoproteomics.

Derivatization of glycan as well as labeling using diverse reagents to enhance detection, improvements in MALDI technology, and the establishment of high-throughput capacities have been important trends in glycan analysis by MALDI-MS.[173,181] Permethylation in MALDI-MS analysis, a valuable tool in glycan characterization, is a chemical modification technique commonly used in glycomics and glycoproteomics, which involves the methylation of all active hydroxyl groups (OH) on glycan structures typically using hydrophobic reagents like methyl iodide or methyl sulfate.[181-183] This modification increases the mass of the glycans and enhances their ionization efficiency in MS analysis. Moreover, permethylation helps to stabilize the glycans, improve their detection sensitivity, and facilitate structural characterization by MS techniques.[182-184] However, manual permethylation necessitates additional sample preparation steps, making it laborious and time-consuming, particularly when implemented to large sample numbers. Shubhakar et al. introduced a novel and high throughput workflow for N-glycan analysis by MALDI-TOF-MS based on an automated permethylation in a 96-well microplate configuration.[182] The automated and high-throughput permethylation facilitated the characterization and relative quantitation of glycans, glycoproteins, and biopharmaceutical samples such as IgG4 and rhEPO, with minimal side reactions. Moreover, glycan preparation and permethylation were completed within 5 hours, demonstrating the method's convenience, speed, and reliability. It was demonstrated that integrating the automated HT permethylation method with well-established data analysis procedures could enable comprehensive linkage and structural analysis of glycans, offering important applications in drug glycan profiling and clinical studies focused on glycan biomarkers.

The distribution of glycans and glycoproteins within living organisms is typically non-uniform. Studying glycan spatial distribution within biological matrices not only offers insights into fundamental aspects of glycobiology but also enables the identification of diseased regions with good precision.[37,185] MALDI imaging mass spectrometry (MALDI-IMS) is considered a valuable approach for this objective due to its great selectivity and sensitivity in glycomics.[36,37,81,186-190] The application of MALDI-IMS in imaging glycans in tissues is a relatively recent development, yet significant advancements have been achieved in recent years by Drake and coworkers who introduced a tissue-based glycan imaging approach enabling in situ N-glycan

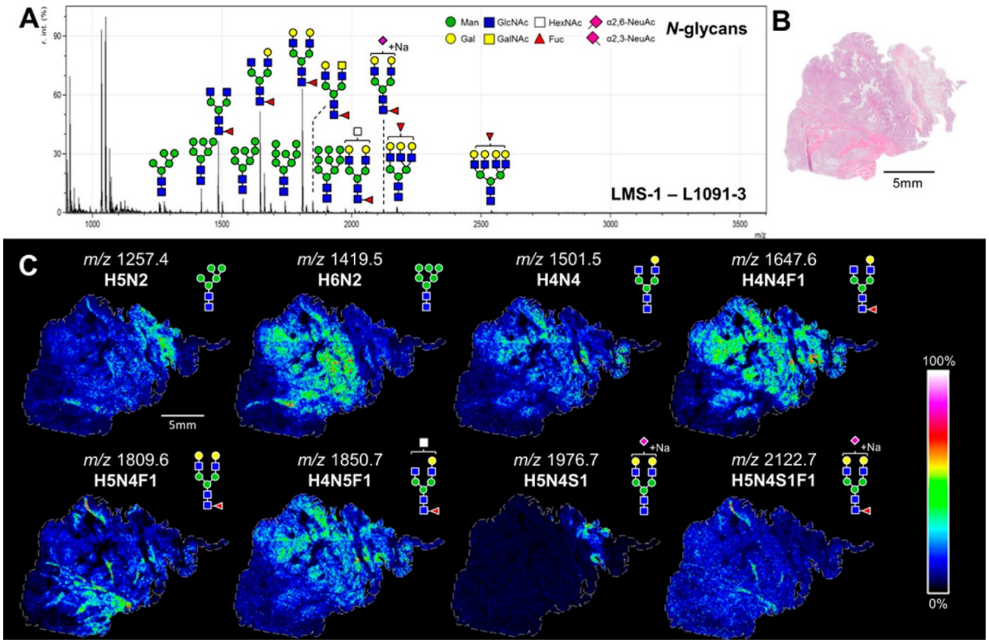

Figure 7. Overall average mass spectrum of the *N*-glycan from leiomyosarcoma tissue and visualizations of the distributed N-glycans on the tissue by MALDI-IMS. Reproduced with permission from Heijs et al.[186] (2016).

analysis on tissues, both qualitatively and quantitatively.[36,187,189] This process entailed N-glycan release by applying PNGase F onto the pretreated tissue specimen, and implementation of organic matrix solution, followed by MALDI-IMS analysis. Afterward, images depicting the spatial distributions of individual glycans within the tissue could be extracted for visualization (see Figure 7).[186] By combining specific enzymatic digestions with MALD-IMS, West et al. successfully visualized core- and branch-fucosylated glycan isomers on cancer tissues.[37] This approach has been extended to various tissue formats including frozen tissues, tissue microarrays for biomarker analysis, and formalin-fixed paraffin-embedded (FFPE) tissue blocks.[187-189]

While MALDI-MS or MALDI-IMS can differentiate between glycan isomers through enzymatic digestions or specific derivatizations, their capability is limited to resolving certain glycan isomers like sialic acid linkage isomers or fucosylation isomers. To achieve a more comprehensive characterization of glycan and glycopeptide isomers, further methodologies such as LC-MS/MS analysis, exoglycosidase digestion, and permethylation are frequently required which can offer complementary data for MALDI-IMS analysis.

Strategies for releasing glycans

A crucial stage in glycoproteomics involves the effective release of N-glycans or O-glycans from glycoproteins or glycopeptides. Typically, the current glycomics workflow includes protein denaturation, enzymatic digestion, and glycopeptide enrichment followed by specific and regulated release of N- or O-glycans. However, the process of glycan release from purified glycoproteins or those within complex biological samples has long been known to be time-intensive. For instance, the enzymatic release of N-glycans usually requires overnight incubation at 37°C, whereas more intricate and labor-intensive protocols are utilized for the effective release of O-glycans. However, recent advancements have introduced several methods enabling the efficient release of both N- and O-glycans within significantly shorter timeframes, while employing less laborious procedures.

Enzymatic release

For glycan release by enzymatic digestion, the use of endoglycosidase or glycoamidase is favored due to its ability to achieve precise and thorough sugar elimination in gentle environments, thereby preserving the structural integrity of glycans, peptides, and their substitutions.[6,191] The most commonly used approach for releasing N-glycans from mammalian glycoproteins has relied on the use of peptide-N-glycosidase F (PNGase F), which cleaves the bond between the first GlcNAc residue of the glycan and the glycosylated asparagine and now available from many commercial suppliers. Conventional enzymatic digestion using PNGase F typically involves an overnight incubation at 37°C to fully release N-glycans from glycopeptides in biological samples, leading to a significant deceleration in the sample preparation process. Alternative approaches were introduced for a more rapid release of N-glycans within 10 min by utilizing microwave-assisted methods or within 2 minutes[192,193] by employing immobilized PNGase F coupled with ultrasonication.[194] Moreover, Waters and Prozyme have launched two kits employing a high concentration of enzyme-compatible surfactant along with a higher incubation temperature, facilitating fast and efficient enzymatic release of N-glycans.[27,31] Complete release of N-glycans can be achieved within minutes using either approach. PNGase F is effective in cleaving high-mannose, complex, and hybrid-type N-glycans; however, it exhibits limited efficiency in releasing N-glycans with a core α-1-3-fucose and is not suitable for numerous microbial N-glycans.[195] On the other hand, PNGase A is another enzyme that is derived from almonds, and shows good efficiency in releasing core α 1-3-fucosylated N-glycans, thereby making it useful for certain glycomic analyses, especially those involving plant or insect-derived proteins.[196,197] However, its cleavage efficiency may not be as high as that of PNGase F, particularly for releasing N-glycans from mammalian glycoproteins. Therefore, PNGase F is typically preferred when working with glycoproteins sourced from mammals, whereas PNGase A is recommended for proteins derived from plants or insects.[170,198] A novel super acidic bacterial enzyme (PNGase H^+), originating from the soil bacterium *R. cellulosilytica* has been recently documented and demonstrated unique properties resembling both PNGase F and A when operated at an optimal pH of 2.6.[199] However, it is important to note that the use of this enzyme at such a low pH can result in the loss of sialic acid.[199] Other endoglycosidases are now also available, which specifically cleave the glycosidic bond between two N-acetylglucosamine (GlcNAc) residues within the core region of glycans, resulting in the retention of one GlcNAc residue covalently attached to the protein. Unlike PNGase F and A, endoglycosidase H (Endo H) specifically targets high-mannose and some hybrid glycans but is not effective against complex N-linked glycans.[200,201] Endo F2 selectively targets high mannose and biantennary complex-type glycan for cleavage, whereas Endo F3 specifically exhibits specificity towards biantennary and triantennary complex-type glycan, with a particular affinity for core-fucosylated structures.[202,203]

The enzymatic release of O-glycans presents unique challenges compared to N-glycans and requires careful consideration of the glycan structure as well as the availability of appropriate enzymes to ensure efficient and selective glycan cleavage.[6,185] Commercially available O-glycosidases, such as endo-α-N-acetylgalactosaminidase primarily target core 1 or core 3 O-linked disaccharides from serine/threonine residues of glycoproteins.[204] This specificity limits

their utility for the removal of other types of O-glycans with extended chains or different core structures. To address this limitation, O-glycans with extended chains may need to be trimmed to core structures using exoglycosidases, such as β-galactosidase or β-N-acetylglucosaminidase before being released by O-glycosidases.[205,206] This process is essential to remove sugar residues from glycans, making the glycans accessible to O-glycosidases for release and enabling the determination of glycan structures and composition.

Chemical release

Various chemical methods have been developed to achieve comprehensive and unbiased release of glycans, particularly O-glycans, from glycoproteins using chemical reagents. One commonly used method for the analysis of O-glycans involves reductive β-elimination where the glycoprotein is subjected to alkaline conditions, leading to the elimination of the glycan moiety as an unsaturated sugar residue so that it can be characterized using MS-based methods.[207-209] Another approach is based on hydrazinolysis, where glycoproteins are treated with hydrazine under mild conditions to selectively cleave the glycan chains at the glycosidic bonds, releasing them from the protein backbone.[210] Hydrazinolysis has been demonstrated as effective and unbiased, making it convenient for the quantitative and nonselective release of glycans.[210-212] However, its application necessitates specialized chemical handling protocols, potentially limiting its broader utility.[6] This approach, as well as ammonia-based nonreductive β-elimination, is employed to release nonreduced forms of N- and O-glycans for subsequent or one-pot labeling of the reducing end with ultraviolet (UV) or fluorescent tags.[210,211]

A novel oxidative approach termed the oxidative release of natural glycans "ORNG", has emerged as an effective method for liberating diverse glycan structures from glycoproteins and glycosphingolipids.[213] This method utilizes sodium hypochlorite (NaClO), a compound commonly found in household bleach, to achieve comprehensive and unbiased glycan release. Unlike traditional enzymatic or chemical methods, ORNG has shown promise in efficiently cleaving various types of glycans, including O-glycans, N-glycans, and glycosphingolipids, from glycoconjugates.[213]

Although chemical release methods have proven to be valuable in analyzing glycan structures, particularly those that cannot be easily cleaved enzymatically, many of these methods are problematic. One significant concern is the potential for peeling of the released glycans during the release process, leading to alterations in glycan compositions and reduced reproducibility of results.[185,214] Additionally, chemical release procedures frequently result in degradation of the polypeptide chain, rendering it unsuitable for subsequent analysis in glycoproteomic studies.[6,170] These limitations highlight the challenges associated with chemical release methods and underscore the need for further refinement by careful optimization of reaction conditions to ensure accurate and reliable glycan analysis while preserving the integrity of the glycoprotein samples.

Database search for glycomics and glycoproteomics

Analyzing glycans and glycopeptides in biological samples using MS generally produces vast quantities of data. One of the most challenging aspects of characterizing glycans and intact glycopeptides is the accurate interpretation of the generated spectra. In comparison to the well-established field of proteomics where the utilization of software tools for data analysis has become a common practice, bioinformatic tools for glycomics and glycoproteomics are relatively underdeveloped.[4,185] Manual identification of glycans and glycopeptides is highly accurate, yet it is a labor-intensive process primarily because of the diverse glycan structures and the significant differences in fragmentation patterns under varying conditions.[215,216] It becomes even increasingly challenging and less feasible when applied to the identification of glycans and glycopeptides in complex biological matrices. Furthermore, a key difficulty in glycoproteomic analysis lies in precisely identifying both the peptide and glycan constituents. If these glycopeptides possess similar glycans, they are likely to coelute together, presenting additional challenges to the identification process.[4,27]

High throughput and reliable bioinformatic tools offer a solution to simplify the above-mentioned challenges, as these tools can automatically and accurately identify glycans and glycopeptides. By utilizing these advanced tools, the process becomes more efficient and accurate, enabling researchers to overcome the complexities associated with identifying and quantifying these compounds.[217-219] To date, various bioinformatic tools, encompassing both commercially available and open-source programs, have been developed to identify glycans and intact glycopeptides. Zhou group has recently introduced GlycoMaid software, featuring a user-friendly graphic interface, designed for the automated interpretation of N-glycan data obtained from the analysis of Chinese Hamster Ovary (CHO) samples by MALDI-TOF MS.[220] This software utilizes masses and isotopic distribution of signals for analysis, thereby improving the capacity for deconvolution of overlapping isotopic distributions of glycans. Overall, this tool demonstrated substantial enhancements in sensitivity, accuracy, and user-friendliness, facilitating the automatic identification and annotation of glycan profiles. Byonic is probably the most widely utilized commercial software package in the field of glycoproteomics research for the interpretation of the MS data of peptides and glycopeptides.[221] Byonic employs sophisticated algorithms to perform database searches and identify glycopeptides without prior knowledge of glycan masses or glycosylation sites by considering the target protein database and N-linked or O-linked glycan library. This approach enhances the accuracy of glycopeptide identifica-

tion by accounting for the complexity and heterogeneity of glycosylation patterns. Byonic also offers various features such as customizable search parameters, visualization tools, and statistical analysis options, making it a versatile and valuable tool for researchers studying protein glycosylation.

GlycoWorkBench is one of the most user-friendly and appreciated tools in the structural analysis of glycans, particularly through the interpretation of MS data.[217] The software assesses a collection of structures suggested by the user by comparing the theoretical list of fragment masses associated with these structures to the list of peaks obtained from the spectrum. It offers tools and features to annotate glycan spectra, analyze fragmentation patterns, and compare experimental data with existing glycan structure databases. Through these functionalities, GlycoWorkBench assists in identifying and characterizing complex glycan structures present in biological samples.[218]

MSFragger-Glyco was recently introduced by the Nesvizhskii group as a glycoproteomics mode of the MSFragger search engine for rapid and sensitive identification of N- and O-linked glycopeptides, as well as facilitating open glycan searches in glycoproteomics analyses. open glycan searches.[222] With significantly improved annotation of glycopeptide spectra, coupled with ultrafast index-based scoring, this open-source software enables extensive interrogation of highly complex and challenging glycoproteomics data for hundreds of glycan compositions and several variable modifications and also understanding the important biological role of glycosylation in health and disease.[222]

The accuracy of intact glycopeptide identification is further enhanced by pGlyco 2.0, a software that contributed greatly to glycoproteomic analysis.[29,88,223] pGlyco 2.0 was designed for the identification of intact glycopeptides from tandem MS data and enabled the estimation of the false discovery rate (FDR) of glycans, peptides, and glycopeptides simultaneously. This comprehensive approach offers functionalities for glycan composition analysis, glycopeptide identification, and quantification of glycosylation patterns, ensuring a higher level of accuracy in identifying intact glycopeptides. Quantitative analysis was possible with $^{15}N/^{13}C$ metabolically labeled glycoproteome samples to validate the identification of glycopeptides.[29] By utilizing the selective ZIC-HILIC enrichment approach, optimized step-HCD collision, and advanced algorithm of pGlyco 2.0, the authors generated a comprehensive glycoproteomic dataset, which included 10,009 unique site-specific N-glycans identified on 1,988 glycosylation sites from 955 glycoproteins across five mouse tissues.[29] More recently, pGlyco 2.0 was also applied to the identification of intact N-glycopeptides in mouse brain tissue to understand glycosylation in Alzheimer's disease (AD), where a total of 3524 intact N-glycopeptides corresponding to 722 glycoproteins, and 1493 N-glycosites were successfully characterized.[15] The latest version "pGlyco 3.0" has demonstrated outstanding performance in terms of search speed, and has shown high levels of accuracy and adaptability for glycopeptide search involving monosaccharide modifications.[224] Based on an optimized ZIC-HILIC-HCD-Orbitrap method, this software tool successfully identified 609 glycopeptides and 235 glycoproteins, outperforming MSFragger-Glyco which only identified 336 glycopeptides and 137 glycoproteins.[84]

GlycoPeptide Finder (GP Finder) is a software tool designed specifically for comprehensive site mapping of glycans.[225] Unlike methods reliant on specific or hemi specific enzymatic digestion, GP Finder enables the annotation of nonspecifically digested glycopeptides using enzymes like pronase E, which generates smaller glycopeptides. This approach involves calculating all possible peptide sequences following digestion, allowing for the observation of glycans connected to peptides of varying lengths.

Glyco-Decipher is another recently developed software tool for the analysis and interpretation of glycoproteomics data of N-linked glycopeptides.[226] The software performs peptide matching without relying on a glycan database and utilizes the fragmentation pattern of shared peptide backbones in glycopeptides to enhance spectrum interpretation. This approach allows for unbiassed glycan profiling and facilitates the identification of unforeseen glycans associated with modifications. By allowing comprehensive characterization of site-specific protein glycosylation, Glyco-Decipher demonstrates significant potential for enhancing glycoproteomics analysis in biological research.

In a study published in 2022, Mao et al. developed a powerful proteomic tool called MS-Decipher for the analysis and interpretation of glycoproteomics data of O-linked glycopeptides.[227] MS-Decipher demonstrated the same sensitivity and confidence in peptide identification compared to other traditional database searching software, and even better performance for obtaining more O-glycopeptide spectrum matches.[228] MS-Decipher has built-in visualization tools "user-friendly graphic interface" for the visualization of O-glycopeptide-spectra matches and provides good support for result visualization, thus making it easier to operate.[227,228]

In addition to the abovementioned bioinformatic tools, many other earlier attempts have been carried out to establish reliable software that can automatically identify glycans and glycopeptides, such as Glyco Master DB,[229] SweetNET,[230] Sweet-Heart,[231] GPQuest,[232] GlycoPAT,[233] Glyco-DIA,[234] GlycoMine,[235] GlypNiroO,[236] glyXtool^{MS},[219] GlycoBinder,[237] GlyFragWork,[238] etc. Nevertheless, the majority of these software programs were designed with a narrow focus on particular applications, while lacking an effective approach to accurately ascertain the rates of false positives. Most academic software tools are typically open-source and tailored to specific requirements, but may not receive frequent updates. Furthermore, they often lack intuitive graphic design, which can hinder user-friendliness. On the other hand, commercial bioinformatic tools are

designed to be more user-friendly, undergo continuous development, and be regularly updated to meet evolving research requirements.

Conclusion and future outlook

In conclusion, this review has explored the recent research and accomplishments in the analysis of protein glycosylation within complex biological matrices, by emphasizing the latest developments in both the enrichment procedures and mass spectrometric analysis of glycoproteins and glycopeptides. By investigating various enrichment strategies coupled with cutting-edge mass spectrometry techniques, researchers have achieved remarkable progress in glycomic and glycoproteomic analyses. Emerging trends indicate a shift towards the utilization of improved and more selective efficient enrichment methods based on novel materials like MOFs, smart polymeric-based devices, and other functional composite nanomaterials due to their superior physicochemical characteristics. The accuracy, sensitivity, and speed of protein glycosylation profiling have been further improved through the use of advanced bioinformatic tools in conjunction with powerful analytical techniques for the separation of glycan and glycopeptide isomers. While significant progress has been witnessed in the field, challenges still exist in the current methodologies. The efficiency of O-glycopeptide enrichment remains not quite optimal, often causing a loss of intact O-glycan structure details in many existing techniques. Enhanced O-glycopeptide enrichment methods capable of preserving the structures are highly required to advance O-glycoproteomics forward. Moreover, integrated workflows facilitating simultaneous analysis of N- and O-glycosylation are increasingly in demand. With advancements in bioinformatic software, the identification of site heterogeneity is expected to enhance, resulting in an increased number of sites and glycoforms. The more laborious aspect is the structural study of diverse glycan compositions linked to a specific glycosylation site, which should be the objective of future endeavors. Additionally, future directions in profiling glycans and glycoproteins should include more reliable isomer differentiation tools with high-resolution power, including ion mobility, capillary electrophoresis, and infrared spectroscopy.

Conflict of Interest

The authors declare no conflict of interest.

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