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Advances in Glycoproteomics: Methods, Challenges, and Applications

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Abstract: ***Glycoproteomics is the large-scale study of glycosylated proteins, combining proteomics and glycobiology to characterize glycan modifications on proteins. It has emerged as a crucial field for understanding protein function and disease biomarkers, since protein glycosylation influences numerous biological processes. In this paper, we review the key methodologies enabling glycoproteomic analyses, including enrichment techniques and mass spectrometry strategies, and discuss the unique challenges posed by the complexity of glycans. We also highlight applications of glycoproteomics in biomedical research, such as the discovery of cancer biomarkers. The progress in analytical techniques and bioinformatics has greatly advanced glycoproteomics, paving the way for new insights into the role of protein glycosylation in health and disease.***

Keywords: Glycoproteomics, Protein Glycosylation, Mass Spectrometry, Enrichment Strategies, Biomarker Discovery, Post-translational Modifications, Proteomics

1. Introduction

Protein glycosylation involves the enzymatic attachment of carbohydrate chains (glycans) to proteins, and is one of the most prevalent post- translational modifications. Glycosylation is ubiquitous in cells and influences protein folding, stability, and cell signalling, playing critical roles in health and disease [1], [2]. The field of **glycoproteomics** has arisen to systematically analyse glycosylated proteins on a proteome-wide scale, with the goal of identifying which proteins are glycosylated, at which sites, and with what glycan structures [2], [3]. By combining techniques from proteomics and glycobiology, glycoproteomics provides insights into how glycosylation modulates biological processes and can reveal disease- associated glycan alterations. However, glycoproteomics has historically lagged behind standard proteomics due to the added complexity of glycans and analytical challenges [4], [5]. Glycan structures are highly diverse and heterogeneous; a single glycosylation site can carry many possible glycoforms, complicating analysis. In addition, glycans can be large and labile, making intact glycopeptides more difficult to detect and sequence by conventional mass spectrometry (MS) methods [5]. Despite these challenges, technological advances are closing the gap. Improved methods for glycopeptide enrichment and sensitive MS workflows have greatly enhanced the depth of glycoproteome coverage in recent years. Large scientific initiatives, such as the Human Proteome Project, have also underscored the importance of mapping protein glycosylation to fully understand the proteome [6]. As a result, glycoproteomics is rapidly expanding, enabling new biological discoveries and biomarker identification that were previously inaccessible. In the following sections, we discuss the methodologies that have fueled advances in glycoproteomics and examine key applications and future directions for this evolving field.

2. Methodologies

2.1 Sample Preparation and Enrichment

Comprehensive glycoproteomic analysis requires effective enrichment of glycosylated proteins or peptides from complex biological samples. Enrichment is necessary because glycopeptides are often low in abundance and can be suppressed by non-glycosylated peptides in mass spectrometric detection. A variety of strategies have been developed to selectively capture glycoproteins or glycopeptides prior to MS analysis.

2.2 Lectin affinity chromatography

Lectin affinity chromatography is one of the most widely used enrichment techniques. Lectins are proteins that bind specific glycan motifs. By passing a sample over an immobilized lectin column, glycoproteins containing the targeted glycan motifs can be captured. For example, concanavalin A (ConA) binds mannose-rich N-glycans and is commonly used to isolate N-glycoproteins [7]. Many lectins with different specificities (such as wheat germ agglutinin for sialylated or GlcNAc-terminated glycans, and others) have been applied in glycoproteomic workflows [7]. Because each lectin captures a subset of the glycoproteome, **multi-lectin** strategies have been introduced to improve coverage. Combining several lectins in parallel or sequentially can enrich a broader range of glycoproteins in one experiment [8]. High-performance multi-lectin affinity chromatography (HP-MLAC), which integrates multiple lectins in one column, has been shown to substantially increase the yield of glycoproteins from plasma and other samples. Multi- lectin approaches have enabled capture of a large portion of the plasma glycoproteome; for instance, using a set of lectins including ConA, Jacalin, and wheat germ agglutinin, over 50% of plasma glycoproteins could be retained in one study [8]. Nonetheless, lectin methods have limitations, as no single lectin or combination can bind all glycan structures present in a complex sample [7]. Often, highly specific lectins will miss glycoproteins that do not bear the recognized motif, and very broad- specificity lectins like ConA still cannot capture certain classes of glycans. Despite these caveats, lectin affinity remains a cornerstone of glycoproteomic sample preparation due to its simplicity and effectiveness for targeted subsets of glycoproteins.

In addition to lectin-based methods, several **chemical and chromatographic enrichment techniques** have been developed. Hydrophilic interaction chromatography

(**HILIC**) is a powerful approach that exploits the overall hydrophilicity of glycopeptides. Early work by Wada *et al.* demonstrated that glycopeptides could be isolated using HILIC and then characterized by multi-stage mass spectrometry. Subsequent improvements include using zwitterionic HILIC columns and adding ion-pairing agents to enhance retention of glycopeptides, which achieved more efficient enrichment and greater recovery [9], [10]. For example, Mysling *et al.* reported an ion-pairing HILIC solid-phase extraction method that provided highly effective glycopeptide enrichment from complex mixtures [10]. HILIC-based methods are now frequently employed either alone or in combination with lectin affinity to capture glycopeptides with diverse glycan moieties.

Another important strategy targets a specific glycan feature such as sialic acids. **Metal oxide affinity chromatography**, especially titanium dioxide (TiO_2), preferentially binds acidic moieties and has been adapted to enrich sialylated glycopeptides. Larsen *et al.* introduced a TiO_2-based method to selectively capture sialic acid–containing glycopeptides, enabling analysis of sialylated subsets of the glycoproteome [11]. Subsequent studies have compared and optimized methods for profiling sialylated glycopeptides, including variations of TiO_2 and other materials, to achieve more comprehensive coverage of sialylated species [32]. Such specialized enrichment is valuable because sialylated glycopeptides can be under-represented if not specifically targeted, due to their often higher polarity and labile nature.

Beyond affinity and chromatography, chemical capture methods have also contributed to glycopeptide enrichment. A notable example is the **hydrazide chemistry (glycotype) method**, sometimes called the "glyco-capture" approach. In this technique, glycoproteins are oxidized to convert cis-diol groups on glycans (typically on sialic acid or galactose residues) into aldehydes, which are then covalently coupled to hydrazide beads. After binding glycans to the solid support, the formerly glycosylated peptides are released (usually by proteolysis and PNGase F digestion, which cleaves N-glycans and in the process tags the N-glycosylation site Asn to Asp). This method effectively isolates peptides that were originally glycosylated, even though the glycans are removed in the process. Zhang *et al.* first described this strategy using hydrazide beads to capture N-linked glycopeptides, allowing identification of numerous glycosylation sites across the proteome [13]. The hydrazide-based enrichment has been widely applied and was instrumental in early large-scale N- glycosylation site mapping studies. Variations on this approach, including different chemistries to capture glycans or glycopeptides, continue to be an important part of the glycoproteomics toolkit.

It should be noted that each enrichment method has biases – no single technique captures all glycopeptides. Therefore, researchers often combine multiple methods to improve coverage of the glycoproteome. For example, a study might use lectin enrichment followed by HILIC to refine the glycopeptide fraction, or perform sequential captures targeting different glycan features. Additionally, careful sample preparation (e.g., depletion of high- abundance non-glycosylated proteins, optimization of protease digestion conditions) can significantly improve the detection of glycopeptides. Studies have been conducted to optimize such parameters; for instance, Berven *et al.* systematically evaluated conditions for glycopeptide capture from plasma to maximize yield and reproducibility [35]. Thanks to these advances in sample preparation, current glycoproteomics experiments can detect thousands of glycopeptides from complex samples where earlier analyses found only a few hundred. (Comprehensive reviews of enrichment strategies for glycoproteomics are available for further reading [35].)

2.3 Mass Spectrometry Analysis and Data Interpretation

Mass spectrometry is the central analytical technology for glycoproteomics, as it is for proteomics. However, the presence of glycans poses additional challenges for MS analysis. A glycopeptide consists of a peptide backbone with one or more glycan attachments, which affects its ionization, fragmentation, and detection. Specialized MS methodologies and instruments have therefore been developed to more effectively analyze glycopeptides.

One key consideration is the **fragmentation technique** used during tandem MS (MS/MS) sequencing of glycopeptides. In collision-based fragmentation methods such as collision-induced dissociation (CID) or higher-energy collisional dissociation (HCD), glycans tend to fragment preferentially before the peptide backbone. This produces abundant **oxonium ions** (low-mass ions derived from pieces of the glycan) and often results in the loss of the glycan as a neutral fragment, while yielding limited information about the peptide sequence or glycosylation site. As a consequence, early glycoproteomic studies using CID could detect the presence of glycopeptides (via diagnostic glycan fragment ions) but struggled to confidently identify which peptides carried the glycans or which glycosylation sites were occupied. Newer fragmentation methods address this issue. **Electron- transfer dissociation (ETD)** and related electron-based methods cleave peptide backbones preferentially, while leaving glycan modifications largely intact on the peptide fragment ions. ETD thereby preserves the site of glycosylation on fragments and provides sequence information necessary to identify the peptide and site [14], [15]. For instance, ETD was shown to be highly effective for mapping O-glycosylation sites, which are otherwise hard to determine if the glycan is lost during fragmentation [15]. In practice, a combination of fragmentation methods is often used: an MS/MS spectrum from HCD can confirm the presence and general composition of a glycan (through oxonium ions and glycan fragment patterns), whereas an ETD spectrum of the same precursor can pinpoint the modification site on the peptide backbone. Modern instruments allow **triggered** MS/MS workflows (e.g., triggering an ETD scan when glycan fragment ions are detected in an HCD scan), enabling automated acquisition of complementary spectra for each glycopeptide. Hybrid fragmentation techniques such as EThcD (a combination of ETD and mild collisional activation) have further improved glycopeptide analysis by concurrently providing glycan and peptide fragmentation information in a single spectrum. These advances in MS fragmentation have greatly increased the confidence and coverage of glycopeptide identification in glycoproteomics studies [14].

High-resolution and high-sensitivity mass spectrometers are particularly valuable for glycoproteomics. Glycopeptides often co-elute with many other peptides and exist in multiple charge states; resolving isotopic patterns and small mass differences (e.g., among glycan compositions) requires high mass accuracy. Instruments such as Orbitrap and time-of-flight mass analyzers have become standard, and the use of **tandem mass tags (TMT)** or other labeling techniques can facilitate multiplexed quantification of glycopeptides across samples, although quantifying glycopeptides remains challenging due to their lower ionization efficiency compared to naked peptides. Nonetheless, quantitative glycoproteomics is feasible – for example, by metabolic or enzymatic ^18O/^16O labeling of glycopeptides, relative quantification of glycoprotein levels has been demonstrated [23].

Equally important to the experimental techniques are the **bioinformatics tools** for interpreting glycoproteomics data. Identifying glycopeptides in MS data is significantly more complex than identifying unmodified peptides. The search space must consider various possible glycans at each potential glycosylation site, greatly increasing the number of candidate matches. Traditional database search engines were not designed for this, so specialized software has been developed. Tools like Byonic, pGlyco, MSFragger-Glyco and others use annotated glycan databases and clever search algorithms to match MS/MS spectra to glycopeptide candidates [16], [17]. Open database search strategies and hybrid approaches (combining glycan- neutral-loss analysis with peptide identification) have improved the sensitivity of glycopeptide identification [16]. In addition, controlling false discovery rates (FDR) in glycoproteomics requires tailored approaches, because the incorrect assignment of a glycan or site could still produce a seemingly plausible match. New scoring methods and target-decoy strategies specific to glycopeptides have been introduced to address this issue [33]. For instance, a multi-attribute scoring and FDR control method by Polasky *et al.* allowed more confident large-scale glycopeptide identifications by accounting for both peptide and glycan assignment accuracy [33]. Together with these software improvements, comprehensive glycan databases (listing biologically plausible glycans) and spectral libraries are increasingly used to aid identification. There has also been progress in automated **glycosylation site localization** algorithms, which assess the MS/MS evidence to pinpoint which amino acid (when multiple potential sites are present in a peptide) carries the glycan [24]. Such bioinformatics advances, along with higher-quality spectra from modern instruments, have dramatically expanded the glycoproteome coverage. Whereas a decade ago glycoproteomic studies might report identification of a few hundred glycopeptides, current state-of-the-art studies can identify tens of thousands of distinct glycopeptides in a single experiment. The continual development of computational tools is expected to further improve depth and reliability, making glycoproteomics data analysis more accessible to researchers.

3. Applications of Glycoproteomics

The ability to characterize glycosylation on a proteome-wide scale has opened new avenues in biology and medicine. One major application of glycoproteomics is in **biomarker discovery** for diseases. Changes in protein glycosylation are a hallmark of many diseases, especially cancer, inflammation, and genetic disorders of glycan metabolism. Glycoproteomic analyses of clinical samples can identify glycan alterations on proteins that may serve as biomarkers for diagnosis or targets for therapy. For example, researchers have applied glycoproteomic methods to serum or plasma from cancer patients to find glycosylation changes associated with tumors. Abbott *et al.* conducted a targeted glycoproteomic study of breast cancer and identified several glycoproteins with altered glycosylation as candidate biomarkers [20]. In another study, Ahn *et al.* used multi-lectin fractionation and high-resolution MS to detect low-abundance glycoproteins in serum, leading to the identification of tissue inhibitor of metalloproteinases 1 (TIMP1) as a potential cancer biomarker with elevated levels and distinct glycoforms in patients [21]. Altered fucosylation of serum glycoproteins has been linked to cancer as well; for instance, increased fucosylation on certain acute-phase proteins was observed in esophageal adenocarcinoma using a quantitative glycoproteomics approach [26]. Similarly, core fucosylation of E-cadherin was found to be higher in metastatic lung cancer, and a glycoproteomics- guided immunoassay was developed to measure this modified glycoprotein as a diagnostic marker [27]. These examples illustrate how glycoproteomics can reveal disease-associated glycan patterns that are invisible to routine proteomic or genomic analyses. Because many secreted or cell-surface proteins (which often end up as biomarkers in blood tests) are glycosylated, surveying the glycoproteome provides another layer of information to distinguish healthy and disease states. Indeed, glycoproteomics has been identified as a promising tool in the early detection of cancer and other diseases, complementing traditional proteomic biomarker discovery [19].

Glycoproteomics also has important applications in understanding **cellular biology and pathology** beyond biomarker discovery. In infectious disease research, glycoproteomic analysis of viral proteins (such as the spike glycoproteins of viruses like HIV or coronaviruses) helps elucidate how glycosylation shields the virus from the immune system or affects infectivity. In one recent example, top-down glycoproteomics was employed to compare the intact glycoforms of the SARS-CoV-2 spike protein variants, revealing how glycan profiles evolved in different viral strains. In microbiology, glycoproteomics has shed light on bacterial glycosylation systems, which can differ markedly from those in humans, thereby identifying potential vaccine targets or novel enzymatic pathways. In developmental biology, large-scale glycoproteomic studies have been used to track how the glycosylation of proteins changes during organismal development or cell differentiation, indicating glycosylation's role in these processes.

Another impactful area is **biopharmaceutical development**. Many therapeutics are glycoproteins (for example, monoclonal antibodies, erythropoietin, and other recombinant proteins). The efficacy and safety of these biotherapeutics can depend on their glycosylation patterns. Glycoproteomics provides analytical techniques to characterize the precise glycan structures on therapeutic

proteins and to ensure consistency in glycosylation across production batches. Advanced MS methods can profile site-specific glycosylation on a therapeutic antibody, for instance, to verify the presence of the correct N-glycan forms that confer desired effector functions. As an example, researchers have used glycoproteomic approaches to analyze the heterogeneity of glycoforms in biotherapeutic proteins and to monitor product quality during manufacturing. The combination of bottom-up and top-down glycoproteomic analyses can give a comprehensive view of a drug's glycosylation. Thus, glycoproteomics is now an integral part of biopharmaceutical quality control and development of "biosimilar" drugs, where matching the original product's glycosylation is critical.

Overall, applications of glycoproteomics are broadening as technology improves. From discovering disease biomarkers and therapeutic targets to investigating fundamental biological mechanisms, glycoproteomics adds a crucial dimension to our understanding of the proteome. Large-scale projects are increasingly incorporating glycoproteomic analyses; for example, some cancer genome atlas studies and proteome atlas efforts now include characterization of protein glycosylation to provide a more complete picture of molecular alterations in disease [18]. As data from such studies accumulate, there is also a push to integrate glycoproteomics data with other "omics" data (genomics, proteomics, glycomics) to build systems biology models that include glycosylation. The continued development of databases and tools for sharing glycoproteomics data will further facilitate its applications in diverse fields.

4. Conclusion

Glycoproteomics has matured rapidly into a key branch of proteomics, enabling the large-scale analysis of protein glycosylation. In this paper, we have reviewed the principal methodologies that make glycoproteomic investigations possible, including specialized enrichment techniques for glycopeptides and advanced mass spectrometry strategies for sequencing and identifying glycosylation sites. These technological advances, combined with powerful computational tools, have overcome many of the historical challenges in glycoproteomics. Researchers can now profile thousands of glycosylation sites and glycan structures in a given sample, uncovering patterns and changes that are critical for biological function.
Glycoproteomics studies have begun to yield important insights – for example, identifying glycosylation-based biomarkers of diseases and revealing how glycan modifications alter protein interactions and signaling.

Despite the progress, challenges remain. The extreme complexity of the glycoproteome means that current analyses still only scratch the surface of the possible glycan heterogeneity. O-glycoproteomics, in particular, continues to be a frontier due to the lack of a simple consensus sequence for O-glycosylation and the diversity of O-glycan structures [15]. Improvements in sample preparation (such as more efficient enrichment of O-glycopeptides), instrumentation (higher sensitivity and novel fragmentation methods), and data analysis (better algorithms for glycan identification and site localization) are actively being pursued to address these gaps. The field is also moving toward integrating **top-down** glycoproteomics (analysis of intact glycoproteins) with the more common bottom- up approach, which may provide a more holistic view of how combinations of glycosylation events on the same protein work together. Furthermore, cross-disciplinary efforts linking glycoproteomics with glycomics (free glycans analysis) and systems biology are expected to deepen our understanding of glycosylation in complex biological networks.

In conclusion, glycoproteomics is an essential and evolving discipline that extends the scope of proteomics into the realm of glycobiology. By characterizing the glycosylation of proteins on a system-wide scale, glycoproteomics offers unique insights into cellular physiology and disease pathology that cannot be obtained by examining proteins or glycans alone. Ongoing innovations in analytical methods and bioinformatics are continually improving the coverage and accuracy of glycoproteomic analyses. These advancements herald a future in which glycoproteomic profiling may become routine in both research and clinical diagnostics, ultimately contributing to more comprehensive biomarkers and a better understanding of the molecular underpinnings of health and disease.

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