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Review

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Mass Spectrometry Approaches to Glycomic and Glycoproteomic Analyses

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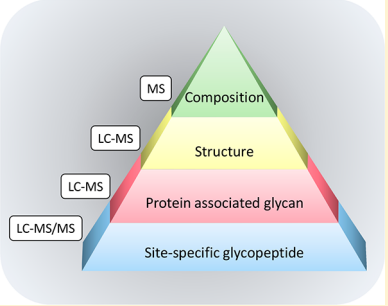
ABSTRACT: Glycomic and glycoproteomic analyses involve the characterization of oligosaccharides (glycans) conjugated to proteins. Glycans are produced through a complicated nontemplate driven process involving the competition of enzymes that extend the nascent chain. The large diversity of structures, the variations in polarity of the individual saccharide residues, and the poor ionization efficiencies of glycans all conspire to make the analysis arguably much more difficult than any other biopolymer. Furthermore, the large number of glycoforms associated with a specific protein site makes it more difficult to characterize than any post-translational modification. Nonetheless, there have been significant progress, and advanced separation and mass spectrometry methods have been at its center and the main reason for the progress. While glycomic and glycoproteomic analyses are still typically available only through highly specialized laboratories, new software and workflow is making it more accessible. This review focuses on the role of mass spectrometry and separation methods in advancing glycomic and glycoproteomic analyses. It describes the current state of the field and progress toward making it more available to the larger scientific community.



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1. INTRODUCTION

Glycosylation plays a key role in maintaining health and in diseases. While the importance of glycosylation has long been known, the ability to determine and quantitate structures have hindered the ability to obtain deeper and more refined details regarding the role of glycosylation. Indeed, the modification of proteins by glycosylation has been a long-standing problem hampered by the dearth of methods to characterize and quantitate glycans. Unlike the genome and the proteome, there is no template for the glycome. Glycans are produced by a set of competing enzymes with the addition of each monosaccharide dictated by the one before. Furthermore, there is no "completed" structure. The protein may exit anywhere along the glycosylation pathway producing a suite of structures that vary homogeneously by linkages, length, number of antennae, and composition. For these and other reasons, glycomic and glycoproteomic methods have not advanced as rapidly as genomic and proteomic methods. Nonetheless, there have been considerable recent improvements. Glycan profiling tools whether by mass to yield compositions or separation to yield structures measure the scope of the glycome, while proteomic and lipidomic methods are advancing to yield intact glycoconjugates. Mass spectrometry (MS) has been at the center of this effort. Mass spectrometry methods that yield accurate mass, structurally informative fragments, coupled to advanced separation methods including capillary electrophoresis (CE), high performance liquid chromatography (HPLC), and ultrahigh pressure liquid chromatography (UPLC) have contributed significantly to the effort.

Glycosylation is a post-translational modification of proteins, but glycans can also be found on lipids and as free compounds in, for example, human milk. Combined in various forms, it represents one of the most common types of modification of proteins and the one that is also most structurally complicated. On proteins, they add an additional level of information but play outsized roles on protein function.

Glycans are short carbohydrate chains consisting of a single monosaccharide to large polysaccharides consisting of thousands of saccharide units. The monosaccharide includes the most notably *O*-GlcNAc (*N*-acetylglucosamine),[1] however, other monosaccharides modifications have also been observed.[2] Large polysaccharides include polysialic acids and glycosaminoglycans (GAG).[3] This review will focus on those that are on serine or threonine, the *O*-glycans, and those on asparagine, the *N*-glycans. Those interested in other types of glycans such as *O*-GlcNAc and GAG are referred to other current reviews.[4,5]

Figure 1 illustrates the many levels of information associated with protein glycosylation. The glycans may be released and

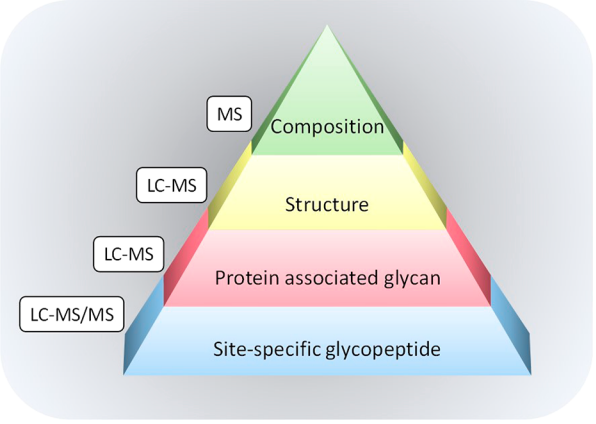

Figure 1. An illustration of the several levels of complexity in glycoprotein analysis.

measured by mass spectrometry to yield compositional information (the apex of the pyramid). The majority of early MS analyses of glycans have focused on composition because glycans can be released and profiled according to mass. The analysis yields the number of hexose (Glc, Gal, and Man), *N*-acetylhexosamine (GlcNAc and GalNAc), deoxyhexose (Fuc), and sialic acid (Sia or NeuAc and NeuGc). Although this information is limited, it is sufficient for observing, for example, the rise of fucose or sialic acid in *N*-glycans during biomarker discovery.

Glycans may be separated chromatographically and analyzed by mass spectrometry or spectrophotometric detection to yield the glycan profile, structurally separated and typically elucidated array of structures (Figure 1). Glycan profiles have increased our understanding of the large breadth in glycan structures from biological samples. A common early belief was that there are too many structures resulting in heterogeneity too great to perform comprehensive analysis. It was believed, for example, that the large number of possible structures were nearly infinite. Six oligosaccharides can indeed chemically produce 10^{12} possible combinations,[6] however, there is a limited number of glycosyltransferases, which severely limits the number of structures. The notion was perhaps first noted when HPLC using porous graphitized carbon stationary phase became available for oligosaccharides profiling. The analysis of free oligosaccharides in human milk (human milk oligosaccharides or HMOs) with LC-MS showed that rather than the potentially millions of structures thought to be present, it was observed that five different mothers produced less than 500 structures spanning

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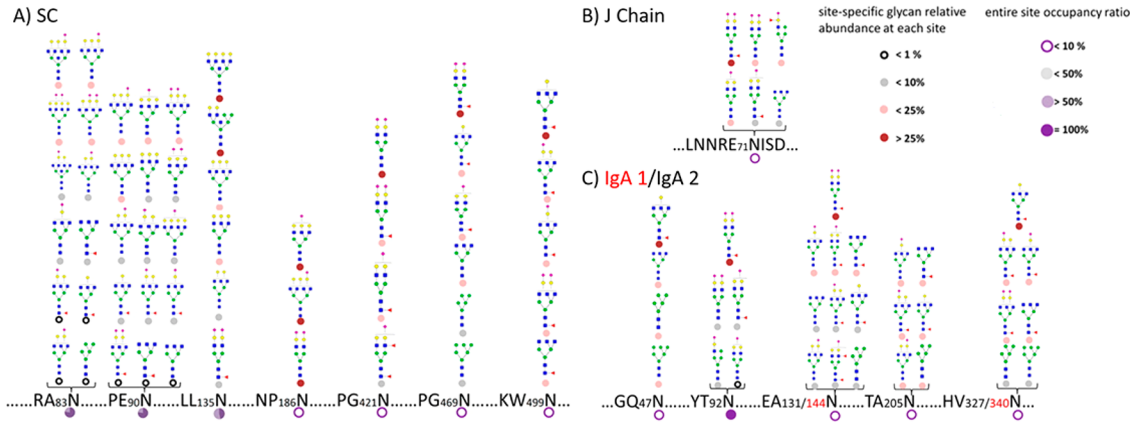

Figure 2. Site-specific glycosylation with occupancy information on secretory IgA from human milk. An example of the complexity of glycosylation even on a single protein. Reprinted with permission from ref 12. Copyright 2015 American Chemical Society.

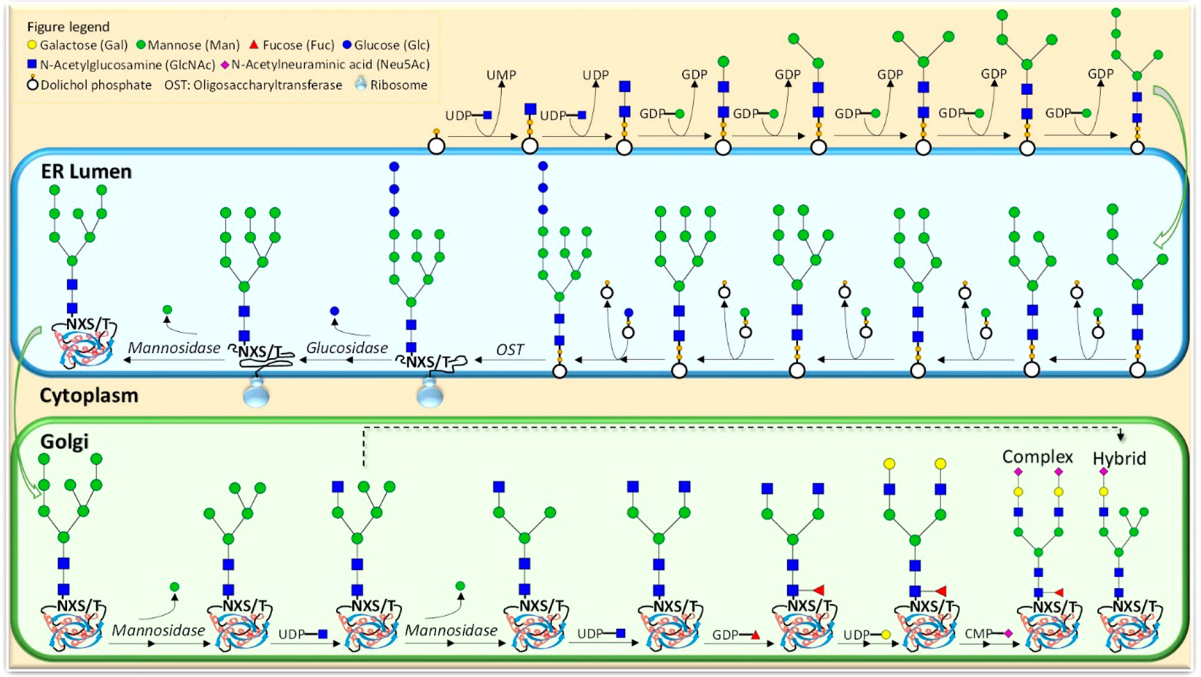

Figure 3. Mechanistic pathway for the formation of *N*-glycans.

over 5 orders of magnitude in abundances. A single mother produced approximately between 23 and 130 structures.[7]

Using a neural network of potential oligosaccharides, Kronewitter et al. deduced the different compositions that can be produced and hence the size of the serum *N*-glycome.[8] They estimated that there are less than 500 compositions employing standard monosaccharides found in humans. On the basis of this analysis, we assumed that the glycome is limited and can be characterized entirely using annotated glycan libraries. There have since been efforts to create comprehensive libraries of complete structures for serum *N*-glycans[9] and for human milk oligosaccharides.[10,11]

The glycans are associated with specific proteins providing large diversities among protein glycoforms (Figure 1). An example of the complexity of glycoproteins is illustrated with IgA, an abundant immune protein in blood and human milk (Figure 2).[12] The secretory IgA found in human milk is composed of the secretory component (SC), J chain, and IgA1 or IgA2, while IgA in blood is mainly monomeric. The permutations between the different glycoforms in the polypeptides units can be almost endless.

Protein enrichment followed by glycan release provide information regarding protein-specific glycosylation (lower tier of the pyramid in Figure 1). This method has been used extensively, for example, with immunoglobulin G (IgG), where the glycoprotein is enriched by protein G and the glycans are released from the enriched fraction.[13] This method has also been used to discover markers for a whole host of diseases including various cancers.[14]

Despite the difficulty in glycoproteomic analysis, the protein and the site-specific glycans can now be simultaneously characterized. An initial effort toward the extensive characterization of glycosylation on proteins using an automated software was first described by An et al. on a small set of proteins.[15] Recent glycoproteomic software are now commercially available that yield extensive characterization of intact glycopeptides.

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1.1. Understanding the Biological Pathways Is Necessary for Elucidating Structures

The key to developing methods for glycoproteomic analysis lies in understanding the glycosylation process and the products manufactured by cells. The structural properties of glycans are complicated and as such highly challenging from the perspective of structural elucidation. First, glycans may be subdivided based on the way they are attached to the peptide (*N*- or *O*-glycans as well as *O*-monosaccharide modifications). *N*-Glycans and *O*-glycans are produced in a series of competing enzymatic steps through nontemplate driven processes. The processes differ for the two glycan types. A recent review by Moremen et al. described well the diversity, structure, and function in vertebrate glycosylation.[16]

N-Glycans are produced at first in the endoplasmic reticulum on a lipid (dolichol) to yield ultimately a high mannose structure consisting of nine mannoses with a triglucose terminus (Figure 3). This structure is then transferred to a nascent polypeptide chain where it guides the protein folding process. When folding is complete, the glycan structure first losses the triglucose structure and a stepwise process disassembles the high mannose structure and rebuilds a variety of structures in the Golgi. Typically, *N*-glycans have a common core consisting of two GlcNAc residues attached to three mannoses. This core structure may be extended using multiple substitutions to form different branching patterns as well as a large number of linkages.[17] *N*-Glycans may be classified into three groups: the complex type *N*-glycans, the hybrid type *N*-glycans, and the high-mannose type *N*-glycans (Figure 4). On the basis of the biosynthesis of *N*-glycans, the

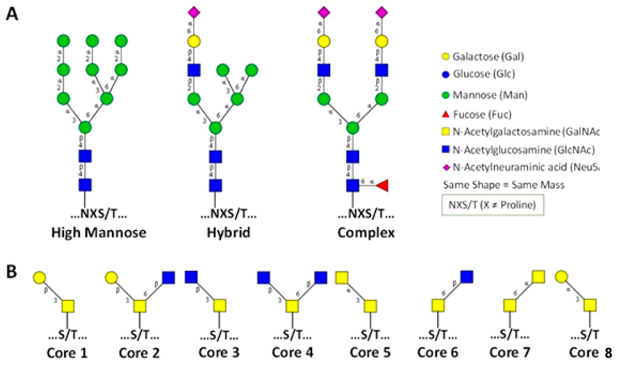

Figure 4. Variation in the *N*- and *O*-glycosylation in proteins. (A) Three types of *N*-glycans. (B) Eight core structures of *O*-glycans.

structures follow a strict regime involving a single core but differ in the composition and lengths of the antenna. Glycoproteomic analysis of *N*-glycans is further simplified by a consensus sequence for a glycosylation site of N-X-S/T, where N is asparagine, X is any amino acid but proline, and S/T is serine or threonine.

In contrast to *N*-glycans, *O*-glycans represent a greater challenge for structural analysis. There is no unique consensus sequence for *O*-glycosylation. There are several types of *O*-glycans including α-linked *O*-GalNAc, β-linked *O*-GlcNAc, α-linked *O*-fucose, α-linked *O*-mannose, β-linked *O*-xylose, α- or β-glucose glycans, and α- or β-linked *O*-galactose.[18] However, there have been many more studies performed on *O*-GalNAc and *O*-GlcNAc types of glycosylation due to their known biological functions. There are currently eight known core structures with the mucin-type *O*-glycans varying in length and in branching antennae (Figure 4).[19] The difficulty of *O*-glycan analysis also lies in the lack of universal enzymes to release *O*-glycans. There are enzymes that can release mono- and disaccharides from proteins but not larger more complicated structures. The current methods for release rely on chemical approaches that are discussed in the glycomics section below.

Despite the complexity of glycan synthesis, the resulting glycosylation appears to be biologically (phenotypically) conserved. The serum glycomic profile of an individual is highly reproducible in the structures and their abundances.[20] Furthermore, the glycoprotein receives considerable structural variability due to glycosylation. A protein such as IgG has been shown to have over 70 different glycoforms at one site.[21] A protein with a single polypeptide backbone and three glycosylation sites with 10 possible glycan structures at each site may have over a thousand unique glycoforms. It is arguably due to this complexity that glycans have been largely neglected in the large proteomic effort. Even at this point, 20 years after "proteomic" was coined,[22] glycoproteomic analysis remains far from routine compared to proteomics.

1.2. Previous Reviews on MS Methods for Glycomics and Glycoproteomics

While the majority of the analytical chemistry advancements over the last decades have focused on analyzing nucleic acids in the form of DNA and RNA and amino acids in the form of proteins and peptides, it has become evident that glycans are also important biopolymers and play important functions in areas as broad as embryonic development and evolution but also in essentially every area of cellular processing. As with all other "omics", it is clear that technological advances in analytics and informatics are major drivers for the in-depth analysis of glycans and glycoproteins. Mass spectrometry, in particular with its multitude ionization, fragmentation, and detection techniques, plays a central role in glycomic and glycoproteomic analyses.

There have been recent notable reviews in this area. A series of comprehensive reviews have been written by Harvey on glycans analyzed by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS).[23−27] Mulagapati et al. have described the analysis of *O*-glycosylation by current mass spectrometric methods.[28] Banazadeh et al. examined the recent advances in glycoprotein analysis by mass spectrometry with a focus on *N*-glycosylation.[29] Oligosaccharide analysis by MS was reviewed by Kailemia et al.[30] Although not in the scope of this review, but important to glycosylation nonetheless, Ma et al. reviewed the state of *O*-GlcNAc analysis in proteins and the proteome.[4] The concept of chemical glycoproteomics, which employs chemical approaches to glycoprotein analysis, was reviewed by Palaniappan et al.[31]

2. INSTRUMENTATION FOR GLYCOMIC AND GLYCOPROTEOMIC ANALYSES

2.1. Key Characteristics of MS Needs/Requirements

Mass spectrometry has several important features that make it ideal for glycomic and glycoproteomic analyses. It has the capability of providing structural information on small amounts of material. Limits of detection have reached femtomolar to attomolar levels for glycans, while the dynamic range is typically 4 or 5 orders of magnitude.[32,33] For an abundant signal at 100% relative intensity, signals with corresponding relative abundances of 0.01% are also observed. Structural analysis of glycans can be performed on abundances typically in the femtomolar range. There is no other analytical method that can provide such low limit of detection with the structural information. Further mass

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analyzers can be combined in tandem (e.g., QTOF and QIT-FTMS), and these hybrid mass analyzers can further improve the dynamic range.

The instrumental requirements for glycomics, glycoproteomics, and intact glycoprotein analysis are summarized in Figure 5.

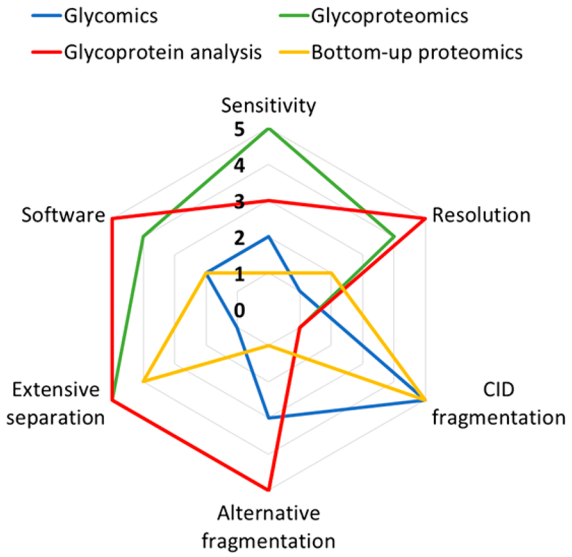

Figure 5. Spider graph showing the instrument requirements for glycomics, proteomics, glycoproteomics, and intact glycoprotein analysis.

For comparison, bottom-up proteomics is also included. Specifically for glycomics experiments, the sensitivity of the method should be slightly higher compared to bottom-up proteomics strategies due to the poorer ionization efficiency of glycans. The need for sensitivity is even higher for glycoproteomics experiments, where the glycan heterogeneity is also subdivided over the peptide backbones. In this regard, extensive separation is less of a requirement in glycomics experiments where glycan compositional information may be obtained by direct MALDI-TOF or MALDI-FTICR analysis of released glycans. However, it is needed for glycoproteomics and even more so for intact glycoprotein analysis, where isolation of a single glycoprotein is highly beneficial. Indeed, compositional glycomic analysis is typically performed using MALDI-MS, which can be configured for high-throughput applications. The requirement of LC or CE separation prior to MS analysis for glycopeptides and intact glycoproteins typically results in a loss of throughput. While CID fragmentation is traditionally the method of choice for bottom-up strategies, this fragmentation strategy may provide information in a glycomics experiment but is generally not sufficient in glycoproteomics and intact glycoprotein analysis. For better identification of intact glycopeptides and glycoproteins, alternative dissociation techniques such as ETD, EThcD, UVPD, or AI-ETD are needed to provide more comprehensive fragmentation of both the peptide backbone and the glycan. As a result, more extensive software tools are required in these experiments to assign the MS spectra for structural elucidation.

Mass analyzer performance also varies depending on the resolution, mass accuracy, and scan rate. Resolution is defined in mass spectrometry as $m/\Delta m$, where Δm is the full width at half-maximum. Because of the relatively large mass differences between monosaccharides, it was generally believed that high resolution was unnecessary for mass spectrometry. However, there is a need for accurate mass, particularly in global profiling of released glycans. High mass accuracy provides rapid differentiation of glycan peaks from nonglycan peaks. Peptides and even lipid contaminants can have masses that nominally corresponds to glycan compositions but are rapidly differentiated when the accurate masses are known. Fourier transform ion cyclotron resonance mass spectrometry (FTICR MS) was the earliest technique used for high resolution analysis of glycans.[34] *N*-Glycans and *O*-glycans released from serum proteins and analyzed by MALDI-FTICR illustrated the utility of global glycan profiling for disease and specifically cancer biomarker discovery.[35] Time-of-flight analyzers employed in QTOF and MALDI-TOF instruments have improved considerably in terms of resolution and are used for global glycan profiling with great success. More recently, orbitrap FT MS with high resolution and mass accuracy has also been used, however, more for glycoproteomic analysis.[36]

Glycoproteomic methods generally require high resolution to be most effective and are best performed with orbitrap MS. Both top-down and bottom-up methods require high resolution for more specific identification and elucidation of glycoforms. FTICR generally lacks the capability of fast LC-MS analysis and are not used for glycoproteomics, although there are numerous examples of glycoproteins analyzed by FTICR.[37] Quadrupole and TOF mass analyzers have high scan rates and are highly essential in high-throughput analyses to get reliable quantitative data. In glycoproteomics, these instruments are used to quantitate glycans and site-specific glycosylation.[38,39]

2.2. Ionization Considerations

Glycans contain labile residues such as fucose and sialic acid that might easily be fragmented either in-source or post-source during ionization. Soft ionization techniques that impart little excess energy and thus generate intact molecular ions are normally used for glycan and glycopeptide analyses. Milder source conditions are often needed for the analysis of glycans, especially native glycans, than those for peptides and small molecules.[40] Among the various ionization methods available, MALDI and ESI are the most widely used.

MALDI-MS has been extensively used for profiling of released glycans derived from biological mixtures. The energetic conditions of the process did not initially favor the formation of intact species. MALDI ionization produced in-source fragmentation that made it unsuitable for early high performance instruments such as FTICR MS.[41] Efforts were placed on finding matrices that yielded intact species. Permethylation was one of the methods used to produce intact species in both time-of-flight and FTICR MS. Partial derivatization including esterification of sialic acids achieved similar results in a more specific manner. For native compounds, post-collisional cooling was used in FTICR MS to remove the excess energy. Metastable dissociation of MALDI produced ions were studied by Ngoka et al. showed that ions generated by MALDI dissociated in the millisecond time scale.[42] Because oligosaccharides have weak gas-phase basicities, they were typically doped with alkali metals to yield quasimolecular ions. They further showed that alkali metals yielded variable rates of dissociation with the fastest corresponding to the smallest metal, thus lithium yielded the fastest rate. Similarly, Fenselau and co-workers showed that peptides produced by MALDI also decayed, with in-source collisional cooling found to minimize dissociation.[43] Later FTICR MS included collisional cooling to decrease fragmentation of

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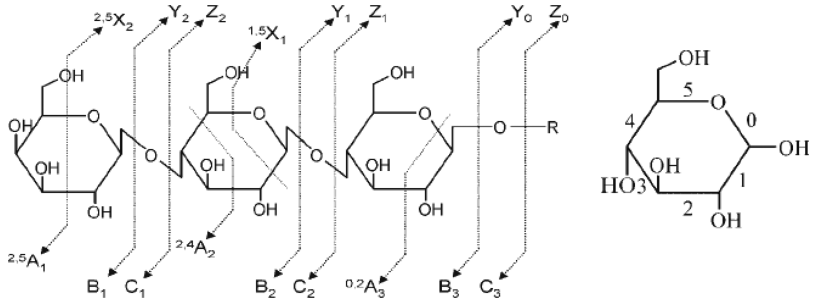

Figure 6. Fragmentation behavior of native oligosaccharides under collision-induced dissociation conditions. Reprinted with permission from ref 48. Copyright 2011 Wiley Online Library.

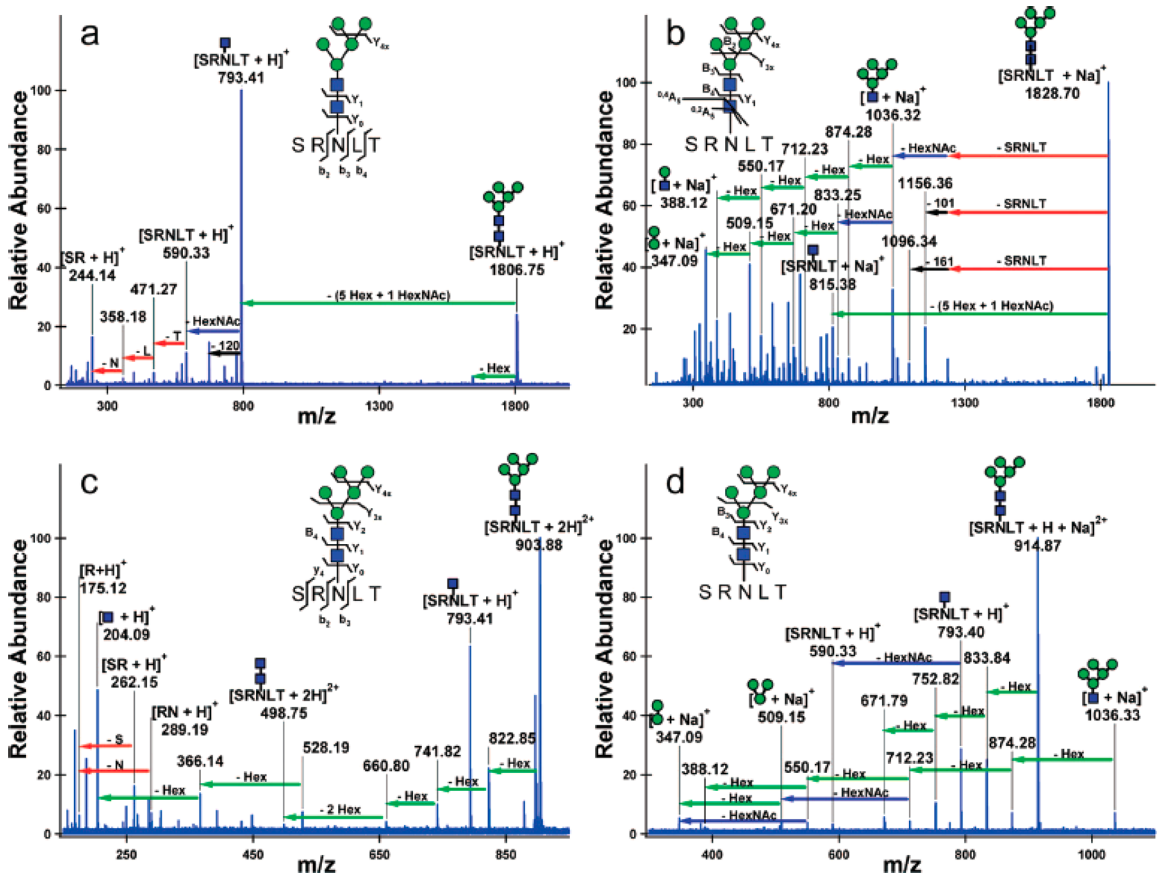

Figure 7. IRMPD of glycopeptides yields low energy fragmentation corresponding to glycan and peptide fragments. Protonated species yield information regarding the peptide, while sodiated species yield fragmentation regarding the glycan. Conditions can be varied to yield both glycan and peptide fragmentation for protonated species under CID and IRMPD. Reprinted with permission from ref 53. Copyright 2008 American Chemical Society.

oligosaccharide ions, thereby allowing native analysis of multiply sialylated and fucosylated species.[44]

MALDI ionization was used extensively for biomarker discovery. The first glycomic biomarker study of cancer was performed by MALDI.[45] MALDI-MS has the advantage of being more amenable to biological species containing salt residues. Indeed, sodium chloride was often used as dopant to yield sodium coordinated oligosaccharide ions.[41] MALDI-MS is used less for profiling because of the difficulty in mating with chromatography. The method is used more today for MS imaging of oligosaccharides as discussed further below.

Electrospray ionization (ESI) is more commonly used for glycan analysis due to its greater sensitivity and its ability to couple to liquid chromatography. ESI imparts less internal energy on the ion compared to MALDI. Unlike MALDI, where the neutral compounds are better ionized in the positive mode while sialylated compounds are better ionized in the negative mode, ESI can be used for both neutral and anionic native glycans in the positive mode. ESI is also most amenable for glycopeptides, however, glycosylation tends to diminish the glycopeptide signal compared to the peptide signal considerably.[46]

2.3. Fragmentation Methods

The ability to obtain fragments gives MS the structural elucidation capability with high sensitivity. Nearly every fragmentation method has been attempted with glycans and glycopeptides. They have been sufficiently characterized to determine the best methods for glycan and glycopeptide

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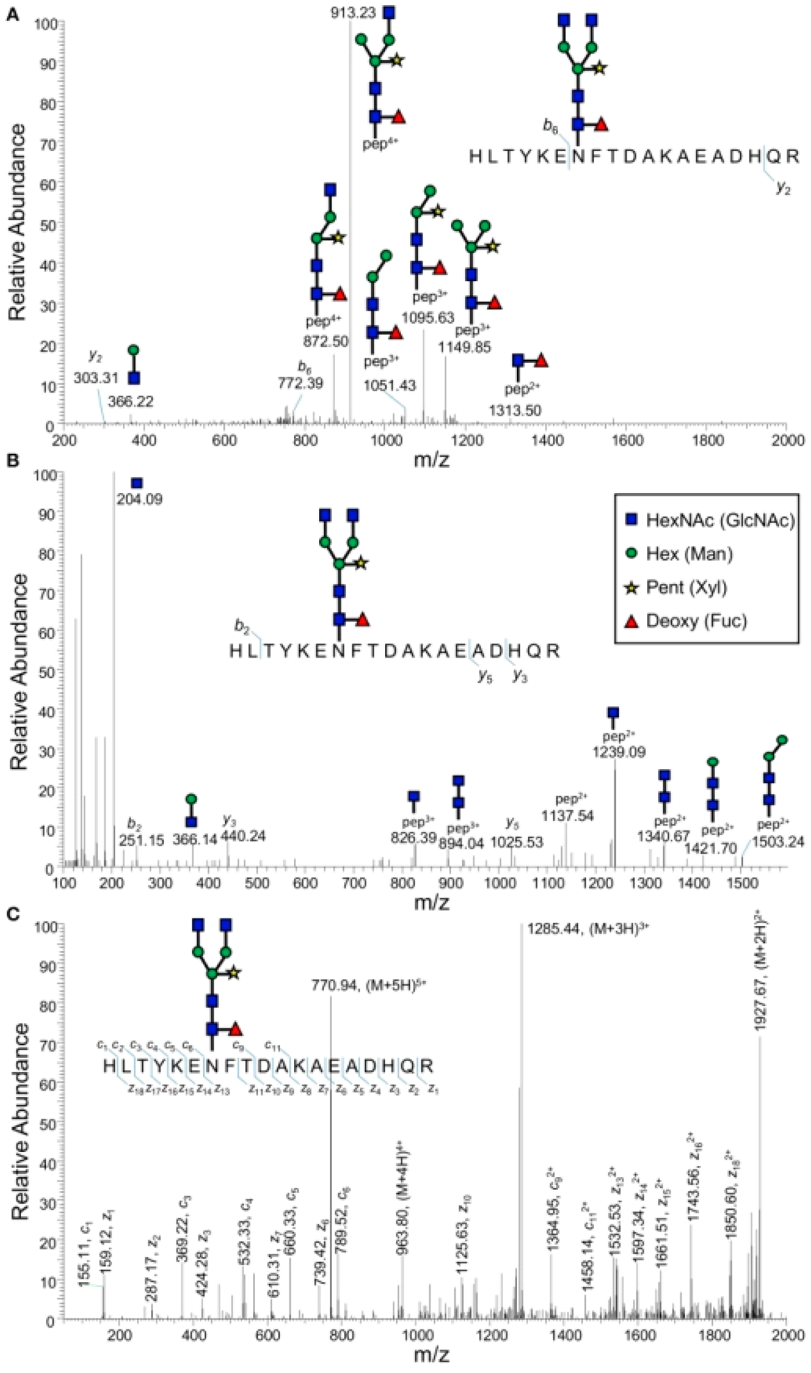

Figure 8. Comparison of (A) CID, (B) HCD, and (C) ETD fragmentation of an enriched plant glycopeptide. Reprinted with permission from ref 59. Under Creative Commons license (https://creativecommons.org/licenses/by/4.0/).

analyses. The methods for glycoproteins are discussed in greater detail below for top-down analysis.

2.3.1. Low Energy Dissociation Methods. Collision-induced dissociation (CID) involves the use of collisions with neutral gas molecules or surfaces to yield fragmentation of isolated ions. For glycans (and glycopeptides), collision induced dissociation with gases is more common. Surface induced dissociation is less common but yields the same results.[47] Infrared multiphoton dissociation (IRMPD) is a photon-based dissociation method with energetics similar to CID, yielding the same fragments and hence included in this group. The fragmentation of oligosaccharides under CID and IRMPD conditions have been reviewed extensively previously.[48]

Low energy tandem MS methods are typically employed in analyzers such as quadrupole and FTICR. The fragmentation behavior of *N*- and *O*-glycans follow regular rules of fragmentation (Figure 6). This includes primarily cleavage along glycosidic bonds for native glycans. Low energy dissociation methods follow the tendency order of the bond to break. Fucosylated glycans tend to lose fucose more readily, resulting in abundant fragment ions with fucose loss from the quasimolecular ion in immediate succession. Sialic acids are also readily lost when present and are even more labile than fucose. In the positive mode, the loss of sialic acids are the major fragment ions. In the negative mode, sialic acid is the charge containing

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unit. The most abundant fragment tends to be the lone negatively charged sialic acid.

An important issue with native glycans is the occurrence of migrating species during CID. Fucose tends to transfer with high probability from the termini to the reducing end in native oligosaccharides performed in the positive mode, resulting in the loss of internal residues. This is diminished significantly when the reducing end is reduced to the alditol or derivatized. The topic has been covered extensively in a previous review.[49]

Permethylated compounds yield fragments that diverge from native species. The permethylation reaction of oligosaccharides originates from the early work on carbohydrates, where permethylation of oligosaccharides allows thermally labile species to be more volatile.[50] For oligosaccharide analysis, proponents claim that it yields higher sensitivity. However, for native glycans, limits of detection as low as attomole and even zeptomole have been reported,[51] while no similar reports of permethylated species have been found. Fragments tend to concentrate along glycosidic bonds in permethylated species, however, cross-ring cleavages are also present in the tandem mass spectra. The use of MS^n where $n > 2$ has been employed as a method for obtaining linkage information.[52] Cross-ring cleavages generally identify the linkage position, however, they do not readily determine the stereochemistry nor the anomeric character of the linkage.

Fragmentation of glycopeptides is complicated by the presence of two chemically dissimilar groups. Peptides create their own difficulties under CID conditions. Glycan bonds are more labile than peptide bonds. Glycans also have significantly lower intrinsic basicity than peptides. For these reasons, both the ionization and the CID conditions of glycans differ significantly from that of glycopeptides. Early studies using both CID and IRMPD yielded predominantly glycan fragments but peptide fragments as well. Glycan fragmentations are low energy processes, and primarily B and Y ions produced by cleavages of glycosidic bonds are the most abundant fragments.[53] Charge states and coordinating cations varied the product ion in proportion depending on how the charge was retained. Sodiated species produced information primarily on the glycan sequence, while protonated species yielded both glycan and peptide information (Figure 7).[53] The same fragmentation patterns were reported for both *N*-glycopeptides and *O*-glycopeptides.[53,54]

2.3.2. High Energy Dissociation Methods. High energy dissociation methods include high energy CID, as well as electron activation methods and UV photodissociation. High energy CID, those involving keV energies, yield extensive dissociation and are available in two types of instrumentation, namely TOF/TOF and sector instruments. Sector instruments are no longer commonly used in bioanalysis and will not be discussed further. TOF/TOF can be used but are not directly coupled to chromatographic methods, which are necessary for separating glycan isomers. Furthermore, the precursor mass selecting capabilities of TOF/TOF instruments remain crude compared to the quadrupoles used in LC-MS methods.[55] These methods have not received wide utility in glycomic and glycoproteomic analysis.

Electron Aided Dissociation. Besides high energy CID, another widely used method for fragmentation of glycopeptides and glycans is electron aided dissociation, where fragments are due to interactions between electrons and multiply charged ions. The most common is electron transfer dissociation (ETD), where an electron in a molecular carrier is transferred to a precursor ion. For peptide cations, the fragment ions produced by ETD are mainly c and z ions along peptide backbone compared to the b and y ions produced by CID.[56] The method also yields fragmentation of the peptide backbone for glycopeptides, where CID of the same precursor ions yield more extensive glycan cleavages. ETD has the advantage of keeping the glycan moieties intact and retained on the amino acid while cleaving the peptide backbone(Figure 8).[57−59]

For both *N*- and *O*-glycopeptides, ETD has been shown to provide structurally characteristic fragment ions. Windwarder et al.[60] characterized the O-linked glycosylation of the recombinant membrane proximal (c/MAM) domain of neuropilin-1 using ETD MS/MS, revealing that the c/MAM domain is *O*-glycosylated and contains mainly disialylated core structure. *N*-Glycopeptides of recombinant polymeric human IgM protein were probed with ETD MS/MS, showing the heterogeneity of glycosylation on different glycosylation sites.[61]

ETD has also been combined with other dissociation methods to generate complementary information in different spectra of one precursor or more comprehensive fragmentation patterns in one spectrum. One commonly used hybrid dissociation method is the combination of electron-transfer and higher-energy collision dissociation (EThcD) whereby HCD fragmentation is further applied to all the ions generated from the ETD step.[62] It should be noted that HCD in the context of this review is only high energy CID and is used by the manufacturer to distinguish from a lower energy CID, which they term as CID. EThcD was first developed to obtain higher peptide sequence coverage, but was later applied to studying post-translational modifications (PTM) of proteins.

Often, the combination of several fragamentation methods provides better coverage than a single method. Parker et al. studied the glycosylation changes on adipocyte cell membrane under the state of insulin resistance using LC-MS/MS with triggered EThcD.[63] The MS/MS spectra of enriched *N*-glycopeptides were first collected with HCD. Then, the precursor ions with HexNAc fragments in the MS/MS spectra were reisolated and subjected to EThcD. To confirm the glycan structures of *N*-glycopeptides, CID was also conducted on the reisolated precursor ions. Due in part to the inefficiencies of triggering EThcD, the number of identified glycopeptides was lower than for HCD alone. The manual interpretation of EThcD spectra provided precise localization of glycosites. However, they identified more unique glycopeptides by combining HCD and EThcD data into one matching algorithm. By modifying the EThcD format to collect HCD and ETD fragments simultaneously, Yu et al. were able to perform the large-scale *N*-glycopeptide characterization of both human plasma and rat carotid artery samples.[64] With the improved fragmentation and shortened ETD reaction times, more *N*-glycopeptides were identified and mapped on their respective glycosylation sites.

Other electron aided dissociation methods that have been applied in LC-MS/MS for glycan or glycopeptide analysis include electron capture dissociation (ECD) and electron excitation dissociation (EED). ECD has been used together with IRMPD for characterizing tryptic *N*-glycopeptides in *Erythrina corallodendron* lectin, providing complementary structural information including peptide backbone fragmentation generated from ECD and monosaccharide composition generated from IRMPD.[65] Studies on improving the efficiency of ECD for glycopeptide dissociation have been performed by optimizing the electron energy ranging from 0.2 to 9 eV. Higher sequence coverages of complex *N*-glycans with sialylation were

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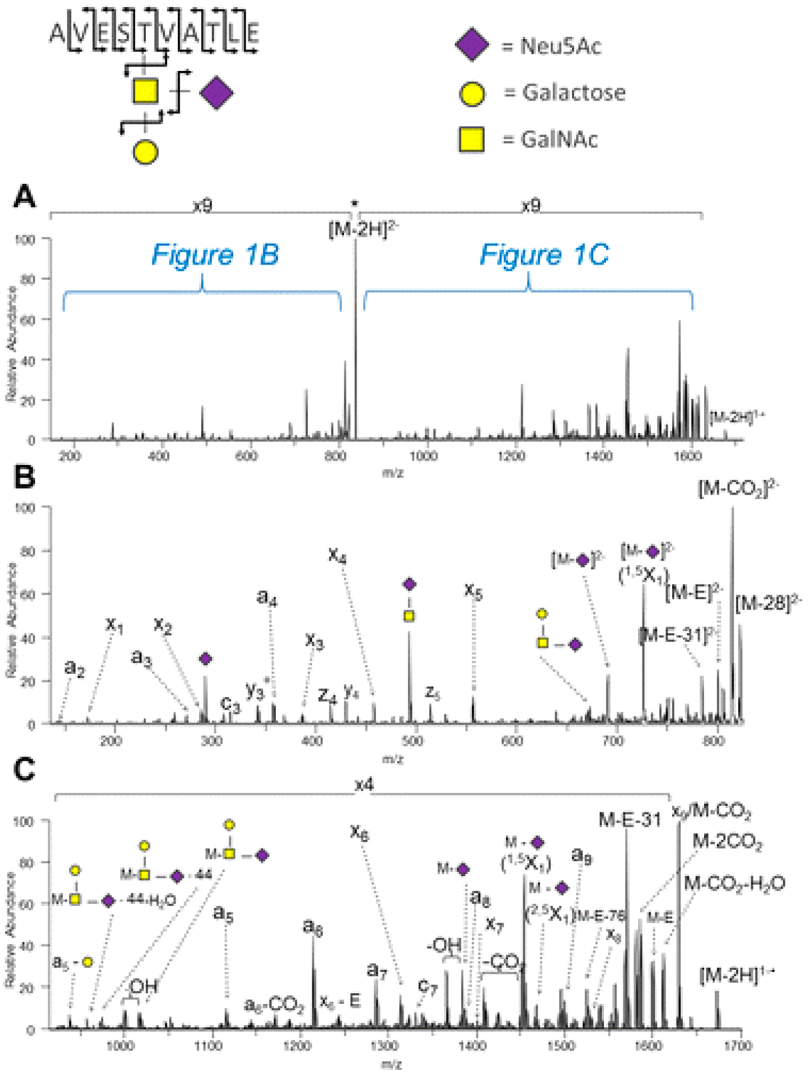

Figure 9. UVPD of a doubly deprotonated *O*-glycopeptide from κ casein yields fragmentation of both the glycan and peptide backbone. Reprinted with permission from ref 70. Copyright 2013 American Chemical Society.

obtained from "hot" ECD, which they defined as ECD at the higher energy range.[66]

Electron energy larger than 9 eV is considered as EED, which was found to generate even more fragments than hot ECD. To obtain efficient EED, decreasing the electron flux to minimize electron repulsion in the ion cyclotron resonance (ICR) cell was required to increase the electron interaction. This method was applied toward analyzing glycans with sialylation and fucosylation in tumor antigens.[67] An improvement of EED, compared to ECD or ETD, is that there is no charge reduction in EED so that glycans with lower charges can also be fragmented efficiently.[67]

Ultraviolet Photodissociation (UVPD). UVPD is an emerging new method that has great potential in both bottom-up and top-down glycoproteomics. Pulsed UV photons generated from lasers are used to excite ions to higher electronic states, raising the internal energy of the ion to the range of 3.5−7.9 eV.[68] This method differs from IRMPD, where the fragmentation mechanism follows lower energy dissociation pathways due to the small energy step size from lower photon energy. UVPD provides higher energy UV photons that excite ions to higher energy levels that generate new fragmentation pathways. Because the dissociation of ions takes place either right after the excitation or after energy redistribution, different dissociation pathways are accessed, resulting in more diverse fragmentation products. Two commonly used wavelengths in UVPD for analyzing biological macromolecules such as peptides, glycopeptides, and glycans are 157 and 193 nm.[68] For oligosaccharides, both glycosidic and cross-ring cleavages take place in UVPD, thereby generating more A and X-type ions, which is useful for determining the branching patterns of glycans.

Both N- and O-linked glycopeptides have been studied with UVPD mass spectrometry. Zhang and Reilly have employed 157 nm UVPD on N-linked glycopeptides, thereby generating more varied fragmentation of both peptide and *N*-glycan.[69] UVPD has also been employed on O-linked glycopeptides (Figure 9)[70] to yield a greater amount of fragmentation of both the peptide backbone and the glycan in contrast to CID.[71] Additionally, UVPD at 193 nm has been applied to oligosaccharides with labile groups such as sialic acids, thereby facilitating their locations in highly branched structures.[72] Middle-down UVPD at 193 nm was recently used as a promising strategy to characterize therapeutics by providing both the peptide sequence and the glycosylation site of immunoglobulin G.[73]

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3. CURRENT METHODS FOR COMPREHENSIVE GLYCOMICS

The rapid development of MS methods for glycomic analysis has made it possible to obtain comprehensive glycan profiles of biological samples. Glycomics is less complicated than glycoproteomics because the carrier proteins and glycosylation sites of the glycans are not considered. However, glycomics data alone can often identify aberrant glycosylation changes in diseases and facilitate the characterization of glycoproteins. This section will cover the essential techniques for glycomics experiments including strategies for sample preparation, glycan separation, and structural elucidation. Other emerging techniques such as ion mobility and imaging will also be discussed.

3.1. Strategies for Releasing Glycans

3.1.1. Enzymatic Release. A critical step in glycomic analysis is the efficient release of *N*- or *O*-glycans from glycoproteins or glycopeptides. Enzymatic digestion using endoglycosidase or glycoamidase is preferable because it can provide specific and complete sugar removal under mild conditions. The most common method for *N*-glycan release is enzymatic digestion using peptide-*N*-glycosidase F (PNGase F). Complete release of *N*-glycans from glycoproteins or glycopeptides in complex mixtures is normally achieved by overnight incubation with PNGase F at 37 °C. Alternatively, faster release can be accomplished within 10 min using microwave[74,75] or within 2 min using immobilized PNGase F under ultrasonication.[76] PNGase F works for all kinds of high-mannose, complex, and hybrid *N*-glycans except the ones with $\alpha(1,3)$-linked core fucose.[77] Another enzyme, PNGase A, can cleave *N*-glycans with or without $\alpha(1,3)$-linked core fucose residues. However, the efficiency of PNGase A in *N*-glycan release from glycoproteins is lower than that from glycopeptides. A novel bacterial enzyme, PNGase H^+, has recently been reported and proven to have combined advantages of PNGase F and A at an optimum pH of 2.6.[78] At such low pH, however, loss of sialic acid will occur. A variety of endoglycosidases that cleave between the two GlcNAc residues in the core region and leave one GlcNAc bound to the protein are also available. Unlike PNGase F and A, endoglycosidases have specificity for different types of glycans. For example, endoglycosidase H (Endo H) and Endo F1 cleave high-mannose and some hybrid-type oligosaccharides but not complex-type oligosaccharides. While Endo F2 cleaves high-mannose and biantennary complex-type oligosaccharides, and Endo F3 cleaves biantennary and triantennary complex-type oligosaccharides, especially the core-fucosylated structures.[79] Enzymatic release of *O*-glycans is more challenging due to both the diversity of core structures and the lack of enzymes with broad substrate specificity. Commercially available *O*-glycosidases are only able to catalyze the removal of core 1 or core 3 O-linked disaccharides from serine/threonine residues of glycoproteins.[80] *O*-glycans with extended chains need to be trimmed to core structures by exoglycosidases before releasing using *O*-glycosidases.

3.1.2. Chemical Release. For complete and unbiased release of glycans, especially *O*-glycans, numerous chemical release methods have been developed. Reductive β-elimination under alkaline conditions is the most commonly used liberation approach for the analysis of *O*-glycans. The released *O*-glycans in alditol forms can be characterized using MS-based methods. Other approaches such as hydrazinolysis and nonreductive β-elimination can be used to obtain *N*- and *O*-glycans in their nonreduced forms for subsequent or one-pot labeling of the reducing end using fluorescent or ultraviolet (UV) tags.[81,82] More recently, an oxidative strategy named oxidative release of natural glycans (ORNG) using NaClO from household bleach have been reported to liberate all types of glycans from glycoproteins and glycosphingolipids.[83] However, most of these chemical release methods are problematic because they can cause peeling of the released glycans, resulting in altered glycans compositions and poor reproducibility. Goso et al. recently evaluated the release of mucin-type *O*-glycans using hydrazine, ammonia, or sodium hydroxide treatment under nonreductive conditions.[84] Although peeling was minimized when the porcine gastric mucin or bovine fetuin samples were treated with hydrazine gas in the presence of malonic acid, "peeled" products were detected under all of the conditions. In addition, chemical release often introduces degradation of the polypeptide chain, making it impossible to analyze the deglycosylated proteins for glycoproteomic study.

3.2. Glycan Derivatization

Native glycans do not have a chromophore and cannot be directly monitored using fluorescence or UV detection. Label-free qualitative analysis can be achieved by obtaining the accurate masses of intact glycans using MS-based methods. As glycans are composed of monosaccharides with unique masses, the composition of a glycan represented by the numbers of monosaccharide units can be easily deduced. Quantitative analysis can be performed using the absolute ion counts of the compounds. However, due to the variation of glycan structures and existence of labile groups such as sialic acid, derivatization of glycans is often needed to improve the stability, ionization efficiency, and quantitation accuracy for glycomic analysis. A variety of glycan labeling strategies and their use in glycan separation and detection by mass spectrometry have been reviewed by Ruhaak et al.[85]

3.2.1. Derivatization of Reducing End. The most commonly used glycan labeling strategy involves derivatization of the reducing end through reductive amination. In this protocol, primary amine group from the label reacts with the aldehyde group of the glycan to form a secondary amine under reducing conditions. A variety of labels have been developed such as 2-aminobenzamide (2-AB), 2-aminobenzoic acid (2-AA), 2-aminopyridine (PA), 2-aminonaphthalene trisulfonic acid (ANTS), and 1-aminopyrene-3,6,8-trisulfonic acid (APTS).[86,87] These labels can add a chromophore or fluorophore to the glycan, making it possible to perform quantitation using UV or fluorescence detectors. When applied to MS, the introduced secondary amine group can greatly improve the ionization efficiency of glycans in positive ion mode. Besides reductive amination, labeling of the reducing terminus can also be conducted using 1-phenyl-3-methyl-5-pyrazolone (PMP) or its analogues through Michael addition reaction,[88,89] or alternatively using hydrazide labeling reagents such as (carboxymethyl)trimethylammonium chloride hydrazide (Girard's reagent T).[90,91]

Recent efforts in glycan reducing end labeling include the development of novel labels, improvement of sample preparation efficiency, and introduction of isotope tags for quantification. Hydrazinonicotinic acid (HYNIC) is a novel derivatization reagent developed by Jiao et al. for glycans analyzed by MALDI-MS.[92] Because HYNIC can also act as a matrix, removal of excess reagent and addition of matrix are not needed. Comparison of excess HYNIC with a traditional matrix, DHB, showed that peptide signal was greatly suppressed by HYNIC in this method,

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enabling direct analysis of the released glycans by MALDI. However, sample cleanup is still needed for LC-MS. Zhao et al. synthesized 10 hydrazino-*s*-triazine based labeling reagents and compared their efficiencies using maltoheptaose as model.[93] The most hydrophobic reagent, *n*-Pr_2N, was found to yield the most enhanced signal and applied to *N*-glycans released from human serum. Other new labels that can facilitate the glycan enrichment after derivatization were also investigated such as 4-aminophenylphosphate tag combined with Ti^{4+}-SPE[94] and heptadecafluoroundecylamine tag combined with fluorous SPE.[95] Jiang et al. developed a global solid-phase approach for the reductive amination of glycans by streamlined glycan extraction, derivatization, and purification on nonporous graphitized carbon sorbents.[96] They compared this method with traditional in-solution derivatization using multiple common labels including 2-AA, 2-AB, and 2-amino-*N*-(2-aminoethyl)-benzamide (AEAB) and found 20−30% increase in glycan discovery. Another strategy for simplifying sample preparation is the one-pot method for simultaneous release and labeling of glycans. This method is generally applied to *O*-glycans released by non-reductive *β*-elimination or hydrazinolysis that contain reducing terminus. Labels such as 2-AB and PMP either with or without deuterium label have been reported.[81,97,98] More recently, Yang et al. developed a glycoprotein immobilization for glycan extraction (GIG) method that combined a series of enzymatic and chemical reactions including the immobilization of glycoproteins by reductive amination, derivatization of sialic acids by carbodiimide coupling, release of *N*-glycans by PNGase F digestion, and release of *O*-glycans by *β*-elimination and one-pot PMP labeling (Figure 10).[99,100] The approach allowed for the simultaneous extraction and analysis of *N*- and *O*-glycans from biological samples using a solid support.

3.2.2. Perderivatization of Oligosaccharides. Permethylation is another glycan derivatization approach that can be used either alone or together with reducing end labeling approaches. In permethylation, hydrogens on the highly polar hydroxyl groups, amine groups, and carboxyl groups are converted to nonpolar methyl groups.[85] Compared to their native forms, permethylated glycans have improved stability and ionization efficiency and are more readily separated by reversed phase liquid chromatography.[29] The most widely used permethylation procedure was introduced by Ciucanu and Kerek using dimethyl sulfoxide and methyl iodide with solid hydroxide.[101] Modifications and improvements have been made to the protocol such as adding a trace of water to eliminate oxidative degradation[102] and performing online reaction using capillary reactors packed with powdered NaOH.[103] However, permethylation efficiency is affected by various factors such as differential reactivities of monosaccharides, oxidative degradation, and peeling of the glycans and repeatability of liquid−liquid extractions.[103]

Recent researches have been focusing on the automation of sample processing and improvement of efficiency. Shubhakar et al. developed an automated high-throughput protocol in 96-well microplate format using a liquid handling robot that can perform *N*-glycan release, enrichment, permethylation, and extraction.[104] Released IgG *N*-glycans analyzed by HILIC UHPLC after 2-AB labeling or by MALDI-TOF-MS after permethylation showed good correlation for abundant glycans, while slight variation was observed for sialylated glycans (Figure 11). This method was applied to derivatize and extract glycans from 96 biopharmaceutical samples in less than 5 h. Its applicability to complicated biological samples remains to be tested. Hu et al. discussed the low permethylation yield of HexNAc residues using traditional

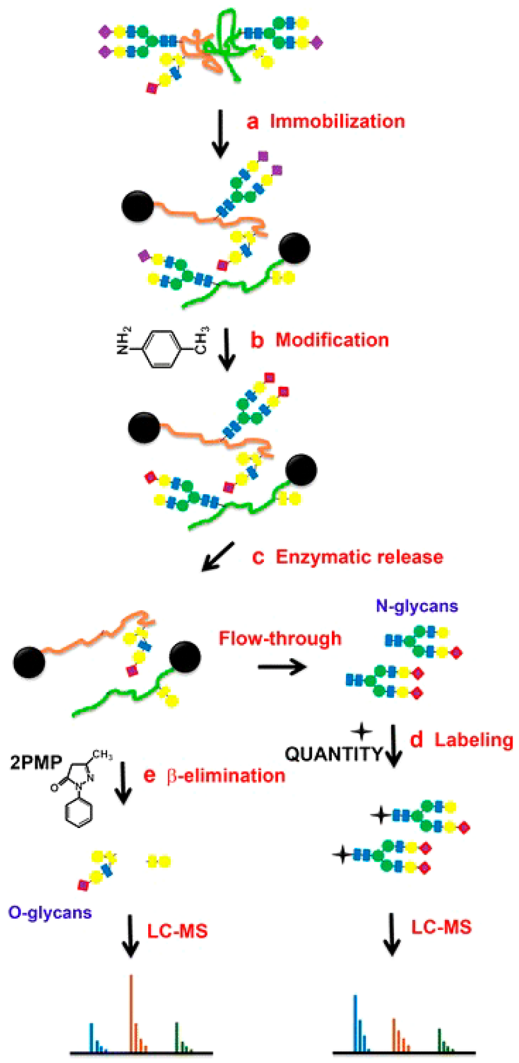

Figure 10. A chemoenzymatic method for sequential releases and analyses of N-linked and O-linked glycans. Reprinted with permission from ref 99. Under Creative Commons license (CC BY 4.0) (http://creativecommons.org/licenses/by/4.0/).

protocol and reported a spin column-free approach that provided comparable or better yields than some widely used spin column-based procedures.[105] Permethylation efficiencies of >98% for hexose and >99% for HexNAc residues were obtained for disaccharide standards before this method was applied to plasma samples collected from 20 breast cancer patients and age-matched controls. Nevertheless, the reaction efficiency for anionic residues such as sialic acid was not discussed in this research. The complicated matrix of biological samples, which may significantly affect the reaction efficiency, was also not considered. Incomplete conversion limits the dynamic range because the baseline is chemically noisy with partially methylated species. Furthermore, the issue of isomer separation of permethylated glycans has yet to be adequately addressed.

3.2.3. Linkage-Specific Derivatization of Sialic Acids. A recent advancement in glycan derivatization is the development of linkage-specific sialic acid derivatization strategies to stabilize the labile sialic acid residues and differentiate the $\alpha(2,3)$ and $\alpha(2,6)$ linkages directly by mass. The original approach was reported by Wheeler et al. in 2009, where the glycans were treated with methanol and 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-

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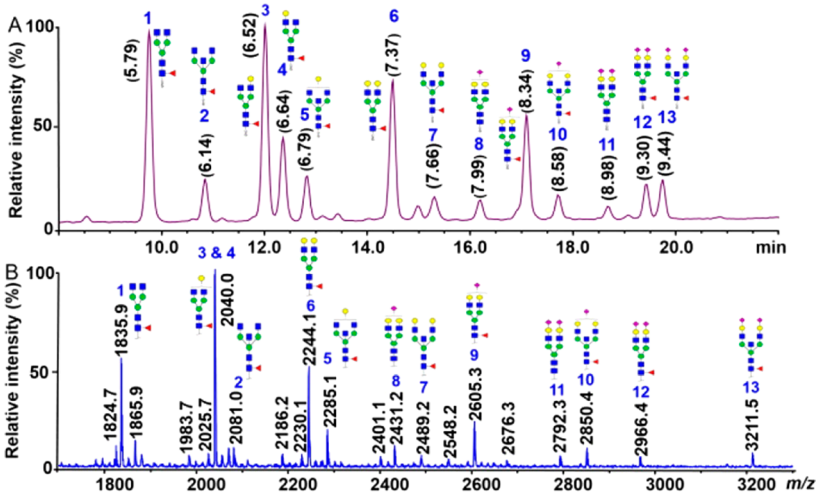

Figure 11. Comparison of IgG *N*-glycans prepared by an automated workflow using a liquid handling robot and analyzed by (A) HILIC UHPLC after 2-AB labeling and (B) MALDI-TOF-MS after permethylation. Reprinted with permission from ref 104. Copyright 2016 American Chemical Society.

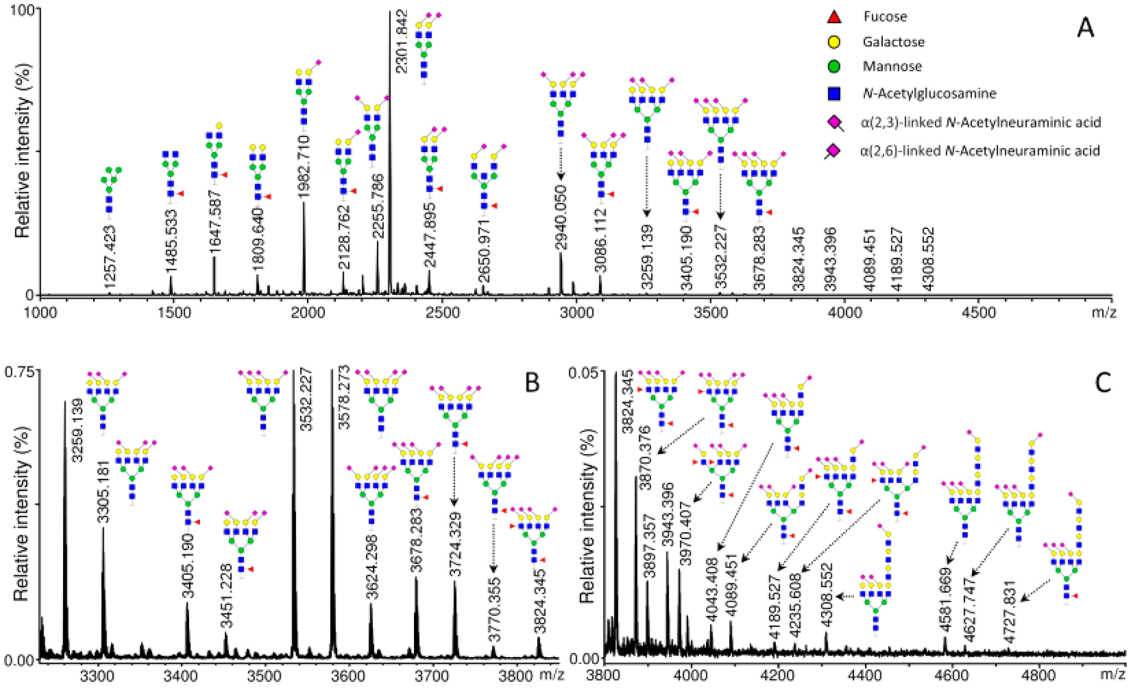

Figure 12. MALDI-TOF MS spectrum of human plasma *N*-glycans after linkage-specific sialic acid ethyl esterification. Reprinted with permission from ref 109. Copyright 2014 American Chemical Society.

4-methylmorpholinium chloride (DMT-MM) to produce methyl esters (+14 Da) for α(2,6)-linked sialic acids and lactones (−18 Da) for α(2,3)-linked sialic acids.[106] This method was applied to breast and other cancer serum samples using MALDI-TOF MS or HILIC-MS/MS analyses.[107,108] To reduce derivatization time and side reactions, Reiding et al. modified the method by using 1-ethyl-3-(3-(dimethylamino)propyl)-carbodiimide (EDC) and 1-hydroxybenzotriazole (HOBt) as activators to ethyl esterify α(2,6)-linked sialic acids in ethanolic solution under mild conditions.[109] Derivatized *N*-glycans from human plasma were analyzed by MALDI-TOF MS, and 77 glycan compositions corresponding to 108 glycan species with different sialic acid linkages were identified (Figure 12). It was also shown that the efficiency for lactonization of α(2,3)-linked sialic acids was higher in ethanol than in methanol. This method is more rapid and higher throughput compared to the original protocol, making it more readily used in large glycan studies.[110−112] However, because the lactones formed by intramolecular dehydration are not stable in aqueous solution, further derivatization such as permethylation and methylamidation is often performed.[113−115]

3.3. Separation Methods for Oligosaccharides

Glycans are composed of monosaccharide units that have unique masses. Monosaccharide composition of a glycan can be calculated using its accurate mass obtained by MS detection. However, structural isomers due to variations in the sequence and linkages between the monosaccharides cannot be fully differentiated by mass spectrometry. To characterize the structural heterogeneity of glycans and reduce the signal suppression from concurrent ions, an efficient separation technique is often needed for glycomic analysis, especially for

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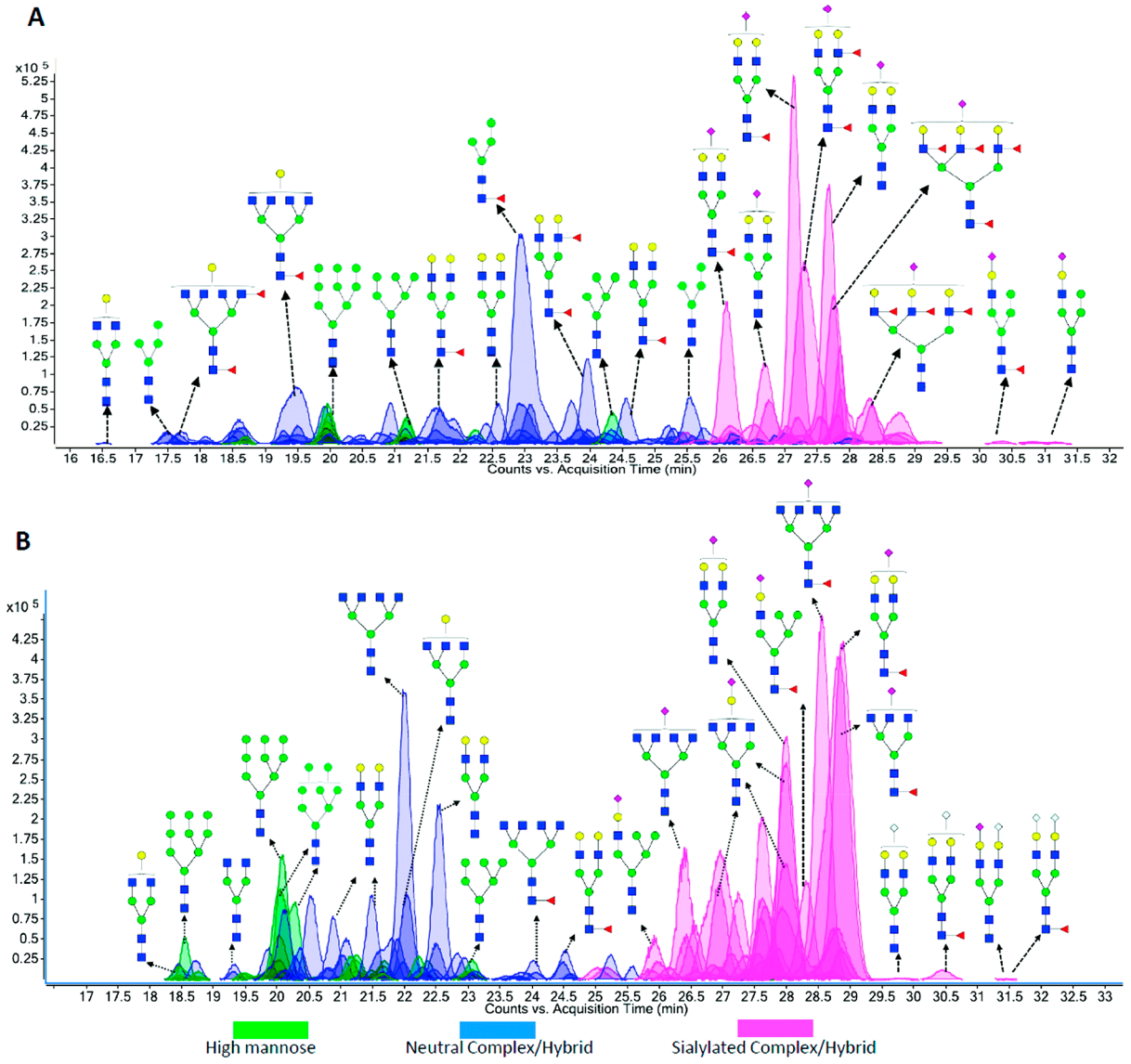

Figure 13. PGC separation of N-linked glycans released from (A) human milk and (B) bovine milk. Reprinted with permission from ref 135. Copyright 2012 American Chemical Society.

complicated biological samples that may contain hundreds if not thousands of glycan structures.

3.3.1. Liquid Chromatography of Oligosaccharides and Glycans. Liquid chromatography is currently the most prevalent glycan separation technique for several reasons: (1) Various methods are available for either native or derivatized glycans based on the interaction between glycans and stationary phases, such as RPLC, HILIC, and graphitized carbon LC. (2) Efficient isomeric separation can normally be achieved without the use of nonvolatile salts, making it compatible with MS detection. (3) When adapted to UHPLC and nanoLC systems, the separation efficiency and sensitivity can be further improved. Different chromatographic separation techniques have been applied to glycan analysis, including reversed-phase, normal-phase (hydrophilic interaction), and porous graphitized carbon (PGC) chromatography.

Reversed-Phase Liquid Chromatography. Reversed-phase liquid chromatography (RPLC) uses hydrophobic materials such as C18 or C8-bonded silica as stationary phase to retain nonpolar compounds. Native glycans are hydrophilic and not well retained in RPLC. Therefore, RPLC separation is only applied to glycans that are permethylated or derivatized with a hydrophobic tag on the reducing end (such as those described above). The strength of interaction between a glycan and the hydrophobic stationary phase is mainly determined by the tagging agent. However, monosaccharide composition and linkage also influence the retention, enabling separation of different glycan types and structural isomers. Pabst et al. compared the separation of IgG *N*-glycans derivatized by PA, 2-AB, and *p*-aminobenzoic acid ethyl ester (ABEE), respectively, using a C18 column.[86] PA and ABEE labeled glycans were better separated than 2-AB labeled ones, while the effect of galactosylation on elution order was opposite for PA and ABEE labeled glycans. Walker et al. derivatized plasma *N*-glycans with 4-phenethylbenzohydrazide (P2PGN) via hydrazone formation and compared the separation efficiencies of nanoflow RPLC and HILIC.[116] A total of 42 glycan compositions were detected by both methods, while 18 additional compositions were detected only by RPLC-MS with narrower peak widths. RPLC has also been applied to the separation of permethylated glycans.[117−119] Improved chroma-

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tographic resolution with increasing temperature[117] and linear correlation between the retention times and glucose units of *N*-glycans[118,119] were observed. A recent review by Vreeker and Wuhrer have discussed the application of reversed phase separation methods to released glycans.[120]

Hydrophilic Interaction Liquid Chromatography. Glycans contain multiple hydroxyl groups that can interact with HILIC stationary phases like amine, amide, or zwitterion-bonded silica through hydrogen bonding, dipole−dipole, or ion−dipole interaction. HILIC separation has been applied to the analysis of underivatized glycans derived from fetal bovine serum[106] and human plasma[121,122] in conjunction with MS analysis. Lam et al. built an online-coupled RP-HILIC system to separate peptides, glycopeptides, and glycans from a single injection.[123] Glycomics data obtained from the same sample could provide valuable complementary information for studying the glycoproteome. To improve the sensitivity and isomeric separation efficiency, however, glycans are often derivatized on the reducing end before HILIC analysis. With a UHPLC platform developed by Ahn et al. using a column packed with <2 μm amide sorbent, excellent separation on the basis of both sequence and linkage was achieved for 2-AB labeled glycans with fluorescence detection.[124] A total of 143 human serum *N*-glycans were separated and assigned with a 30 min HILIC gradient using fluorescence detection.[125] This platform has been automated, coupled to mass spectrometry, and widely applied to glycomic analysis of monoclonal antibodies.[126−128]

Porous Graphitized Carbon Chromatography. PGC is currently the most widely used stationary phase for the purification and separation of underivatized glycans. Comparison of PGC separation of reduced glycans and ZIC-HILIC separation of 2-AB derivatized glycans from monoclonal antibodies has demonstrated that PGC is a more efficient sorbent for the separation of isobaric glycans with various degrees of mannosylation and galactosylation.[129] PGC has been used extensively to separate sialylated *N*-glycans from bovine fetuin.[130] Up to six isomers were observed for a disialylated glycan composition. Isomers of sialylated *N*-glycans from serum[131,132] and cell lines[133,134] were also efficiently separated by PGC employing LC-MS platforms. The retention of a glycan on PGC is greatly dependent on its size, monosaccharide composition, and linkage. For example, sialylated and/or fucosylated complex type glycans generally elute later than high-mannose type glycans.[74,135] (Figure 13) Increased retention times are often associated with higher degrees of sialylation and fucosylation or lower degrees of mannosylation.[131] The α(2,3)-linked isomers of sialylated *N*-glycans were found to elute later compared with the α(2,6)-linked isomers.[133] Although peak broadening was occasionally observed on PGC for highly sialylated species,[121] Kronewitter et al. were able to characterize polysialylated glycans containing up to 18 sialic acid residues with a 60 cm graphite fused silica column.[136] Using improved sample preparation steps and optimized LC conditions, they identified 290 glycan compositions and observed 994 potential isomer peaks from human serum.

3.3.2. Capillary Electrophoresis of Oligosaccharides and Glycans. Capillary electrophoresis has long been recognized as a highly efficient separation technique that has been applied to various types of compounds. The analysis of native or labeled glycans has been accomplished by employing several electromigrative separation techniques, including capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE), micellar electrokinetic chromatography (MEKC), and capillary electrochromatography (CEC).[137] The migration rate of a glycan is determined by a number of factors including its own structure as well as the separation mechanism, strength of electric field, buffer pH, and column temperature. Guttman et al. studied the effect of column temperature on the structure specific separation of glycans using capillary gel electrophoresis[138] and greatly enhanced the separation of APTS labeled glycans of biotherapeutic interest using an optimized temperature gradient.[139] Feng et al. discovered the high orthogonalities between the CZE and MEKC separation of glycans and developed a multiplexing technique by combining these two mechanisms to achieve higher peak capacity for the analysis of complicated glycan mixtures.[140] The coupling of CE to MS, however, has been challenging due to the low flow rates employed in CE.[141] This issue has been partially addressed by the implement of sheath-flow[142,143] or sheathless-flow[144,145] interfaces with compromised sensitivity or reproducibility. Development of novel low-dilution sheath-flow or liquid junction interface designs that can improve sensitivity while creating stable spray is of great interest for CE-MS. Chen and co-workers reported a flow-through microvial interface[146] that was applied to underivatized *N*-glycans released from IgG and recombinant human erythropoietin (rHuEPO).[147] The presence of *O*-acetyl groups and polyLacNAc chains were observed for rHuEPO-derived glycans. Another type of interfaces are microfluidic devices that fully integrate CE separation system with a nanoESI source on a single chip.[148] These integrated devices do not require precise alignment by the user and are therefore more readily commercialized. Khatri et al. recently applied a commercial ZipChip CE-ESI device for the analysis of released glycans and glycopeptides from standard glycoproteins.[149] However, the application of microfluidic CE devices to glycans from complicated biological samples has not yet been widely pursued.

3.3.3. Ion Mobility of Oligosaccharides and Glycans. Ion mobility-mass spectrometry (IMS-MS) has been of great interest in the past decade for its potential applications in glycomic, proteomic, and glycoproteomic analyses. The ability of IMS to separate ions in the gas phase based on their differences in shape and charge makes it possible to resolve some structural isomers. A review by Gray et al. has discussed different types of ion mobility instruments and their applications in the analyses of free saccharides, released glycans, and glycoconjugates.[150] A more recent mini-review by Hofmann and Pagel explores the history and recent advancements of glycan analysis by IMS-MS.[151] Earlier efforts for the isomeric separation of carbohydrates were focused on mono-, di-, and trisaccharides from simple mixtures.[152−155] The differences of the compounds in shape can be represented by their collision cross sections (CCS). Fenn and McLean published a CCS database of 303 carbohydrates including three sets of isobaric isomers.[156] Certain structural patterns such as branching and 1−3 linkage versus 1−4 linkage could result in more compact structures and therefore shorter drift times.

Studying *N*-glycan isomers using IMS-MS is more challenging due to the high structural similarities accompanied by large variations in glycan structures. The existence of conformers, while fundamentally interesting, further interferes with structure assignment. The earliest studies on IMS-MS of *N*-glycans were published by Clemmer and co-workers in 2008.[157,158] Glycans from human serum were directly ionized by an ESI source and analyzed by IMS-MS with a 1 m long drift tube. Separated features were assigned based on their m/z and drift times and

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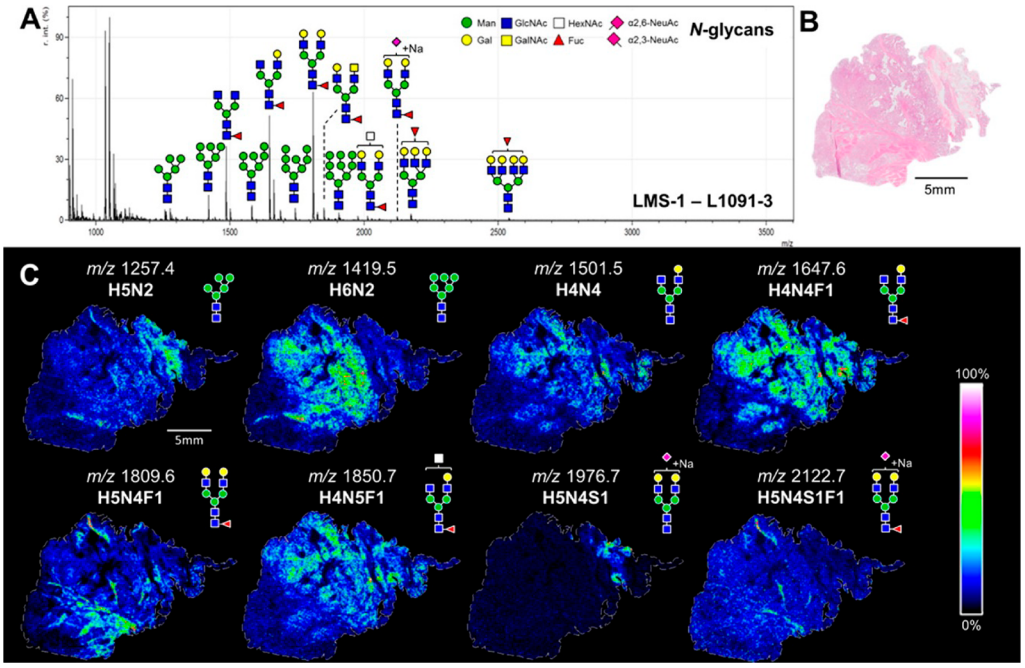

Figure 14. MALDI-IMS for visualizations of the distribution of various *N*-glycans throughout a leiomyosarcoma tissue. Reprinted with permission from ref 180. Copyright 2016 American Chemical Society.

compared between control and liver disease groups.[157] Another study by the same group focused on the separation of Man7 glycan isomers.[159] The broad drift time distribution of this composition was assigned to four potential isomers, which were distinguished by their fragment ion drift time distributions. To facilitate the identification and assignment of glycan structures and isomers, Pagel and Harvey reported a calibration protocol for calculating CCS values of sodiated *N*-glycans using traveling wave (TW) IMS instruments.[160] They published a CCS library for *N*-glycans released from standard glycoproteins. Certain structural isomers such as those for $Hex_4HexNAc_3$ and $Hex_5HexNAc_4$ could be distinguished based on their CCS values. The list was expanded to over 350 CCS values corresponding to 70 precursor glycan structures and added to a freely accessible database, GlycoMob.[161]

To increase the peak capacity and identify more structures, IMS-MS is coupled to LC as a complement to chromatographic separation.[162,163] However, IMS-MS currently has limitations as a separation technique for glycomic and glycoproteomic analyses. First, overlapping peak is often observed for isomers, especially for glycans, with only minor differences in the position or linkage of one monosaccharide residue.[164,165] Several strategies have been developed to increase the resolution of IMS separation. One effective method is to use metal cations as charge carriers because the formation of metal cation adducts with isomeric glycans can induce greater differences in the conformations and thereby improve separation by IMS.[166,167] Huang and Dodds systematically studied the ion-neutral collisional cross sections of carbohydrate isomers as group I[168] and group II[169] metal ion adducts. Different metal ion adducts were found to be useful in separating isomeric groups, while the most commonly used metal ion adduct, sodium, was not necessarily as effective for improving IMS resolution. Morrison et al. evaluated the effects of seven metal ion adducts on the separation of five isomeric tetrasaccharide-alditols.[170] Transition metal cations such as Co, Cu, and Mn notably improved the separation. Similar results were obtained by Zheng et al. for oligosaccharide standards and synthetic *O*-glycans.[171] Despite these improvements, baseline separation of glycan structural isomers by ion mobility remains difficult. Additionally, IMS-MS data is often further complicated by the existence of conformers that overlap with structural isomers.[172] Molecular modeling techniques, used to calculate theoretical cross sections, may provide insightful information regarding experimental collisional cross sections of the glycans.[158]

3.4. Imaging MS of Glycans

The glycan and glycoprotein distributions in living organisms are rarely homogeneous. The spatial distributions of glycans in tissues and in organisms can provide fundamental understanding of the glycobiology as well as locate specifically diseased areas. Imaging mass spectrometry (IMS) has emerged as a tool for this purpose owing to its high sensitivity and specificity for detecting glycan analytes.[173] Mass spectrometry techniques for imaging include secondary ion mass spectrometry (SIMS), MALDI-MS, desorption electrospray ionization mass spectrometry (DESI-MS), and direct analysis in real-time mass spectrometry (DART-MS). Among them, MALDI-MS is the most widely used method and has already been applied to a variety of biomolecules.[174] Imaging of glycans in tissues using MALDI-IMS is relatively new, but important progress has been made by Drake and co-workers in the past few years. The original workflow involved on-tissue release of *N*-glycans by spraying PNGase F onto prepared tissue slices, followed by matrix application and MALDI-IMS analysis.[175,176] Images of individual glycans could then be extracted to illustrate their distributions within the tissue (Figure 14). This method has been applied to tissues in different formats such as frozen tissues, formalin-fixed paraffin embedded (FFPE) tissue blocks, and tissue microarrays for biomarker analysis.[175,177,178] To stabilize labile sialic acid residues and characterize their linkages, in situ linkage specific derivatization was performed on FFPE tissues.[179] Additional multiply sialylated species with $m/z > 2500$ were detected after derivatization. A new application is a multimodal approach to sequentially analyze

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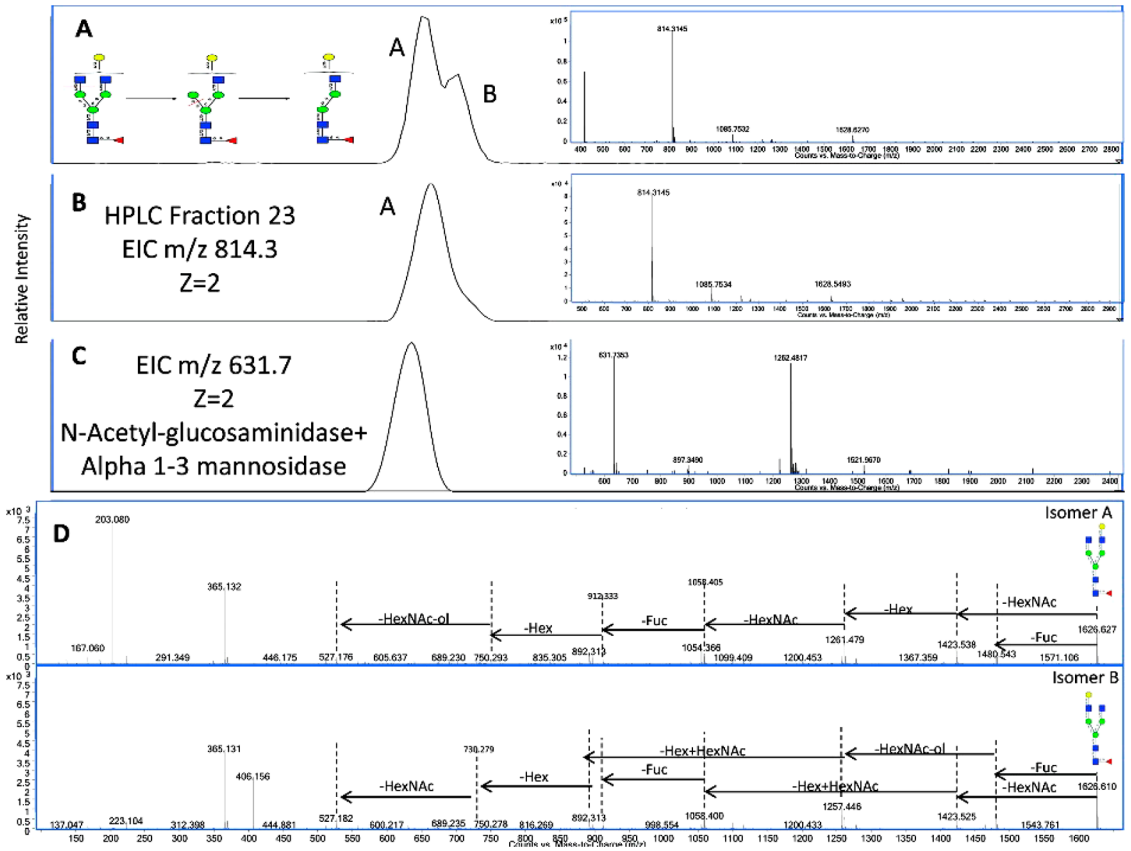

Figure 15. *N*-Glycan structural elucidation by exoglycosidase digestion and tandem MS. (A) EIC of m/z 814.29 shows two isomers for $Hex_4HexNAc_4Fuc_1$. (B) HPLC fractionation to isolate one isomer. (C) EIC of exoglycosidase digestion product with m/z 631.7. (D) Differential tandem MS spectra for the identified isomers. Reprinted with permission from ref 200. Copyright 2012 American Chemical Society.

N-glycans and proteins in the same tissue by digestions with both PNGase F and trypsin.[180] For more detailed structural identification, however, other techniques such as permethylation, exoglycosidase digestion, and LC-MS/MS are often necessary to provide complementary information for MALDI-IMS.[175,181]

3.5. Structural Elucidation of Glycans

Although separation and linkage-specific derivatization techniques can yield some structural information such as the linkage of sialic acid[109] and position of fucose,[182] the structural elucidation of glycans is still challenging due to the complexity and large variability in the structures. Complete structural elucidation requires extensive determinations of monosaccharide composition, sequence, branching, and the unique linkages of each monosaccharide residue. There are two commonly used approaches for structural analysis of *N*- and *O*-glycans. The first is based on tandem MS techniques including the low and high energy dissociation methods. The second one involves enzymatic digestion using exoglycosidases and detection by UV, fluorescence, or MS.

3.5.1. Structural Elucidation by Tandem MS. Several aspects of the data obtained from MS and tandem MS can be used to elucidate partial glycan structures. For example, monosaccharide composition of a glycan can be easily deduced from the accurate mass of the quasimolecular ion. The connectivities between monosaccharides can be determined by the sequential cleavage of glycosidic bonds under low-energy conditions such as CID and IRMPD,[183,184] and the glycosidic linkage positions can be obtained through cross-ring cleavages under high-energy fragmentation conditions such as electron dissociation methods (ExD) and UVPD.[72,185,186] Isomers having variable linkage of a single monosaccharide residue are identified by MS/MS based on the variations in distinct bond energies.[187−189] However, interpretation of glycan tandem MS spectra can be complicated by the rearrangement of monosaccharide residues, especially fucose, and the presence of spurious MS peaks.[190] One strategy to address this issue is to develop searchable MS^n spectra libraries by using specifically developed software tools. A universal software for annotating glycan structures based on tandem MS data is still lacking, perhaps due to the diversity of glycan structures and the large variations in fragmentation patterns under different conditions.

Tandem MS alone is not sufficient for complete structural elucidation of glycans because it cannot provide some essential information such as the anomeric nature of the glycosidic bonds and the stereochemistry of the monosaccharides. It can be used in combination with other techniques to enhance the efficiency and accuracy for glycan identification. Fellenberg et al. applied an integrated LC-MS/MS and 1D ^{1}H NMR workflow to the analysis of 10 desialylated underivatized glycans from bovine fibrinogen.[191] By comparing and combining data from both methods, the branching and linkage information partially assigned by MS/MS was further confirmed by an NMR database search. LC-MS profiles were additionally used to determine the sample purity for NMR interpretation. An improved method by the same group was able to extract the NMR spectra of *N*-glycans from heavily overlapping chromatographic separations by mathematically dissecting the NMR spectra obtained from chromatographic fractions.[192] Due to the limitations of NMR

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sensitivity, however, only abundant glycans could be unambiguously characterized.

Glycan microarray is another technique that can be combined with tandem MS for detailed structural analysis. An approach named metadata-assisted glycan sequencing (MAGS) was developed by Cummings and co-workers,[193] where undefined glycan arrays were interrogated by glycan-binding proteins to filter glycans with specific motifs or significant biological relevance. Additional structural information on specific glycans could be obtained further by tandem MS.

3.5.2. Structural Elucidation by Exoglycosidase Digestion. Exoglycosidases cleave glycosidic bonds of monosaccharide residues at the nonreducing end of oligosaccharides or polysaccharides. There is a variety of exoglycosidases; some have general reactivity, for example, they may cleave all terminal galactoses, while others are highly specific to one type of glycosidic linkage with defined anomeric character. Therefore, the linkage of a terminal monosaccharide residue in a glycan can be unambiguously determined by the detection of expected product after exoglycosidase digestion. Complete structure of a glycan can then be elucidated by sequential or enzyme matrix digestions using a series of exoglycosidases. The combination of exoglycosidase digestion and mass spectrometry was extensively used by Wu et al. to create a library of structures for human milk oligosaccharides.[10,11] This approach also has been widely used in conjunction with various separation and detection methods such as CE-LIF,[194,195] HPLC-LFD,[196] and LC-MS.[197] Exoglycosidase digestion has been combined in a rapid-throughput analytical HPLC platform called GlycoBase and autoGU by Rudd and co-workers.[198,199] GlycoBase is a database containing more than 350 2-AB-labeled N-linked glycan structures (117 from human serum) and their corresponding HILIC elution times expressed as glucose unit values. AutoGU automatically assigns glycan structures using combined data from a set of sequential exoglycosidase digestions. An experimental strategy combining these two tools helped less experienced glycobiologists to identify glycan structures from HPLC data without the need for tandem MS knowledge. The method has some drawbacks because limited isomer separation with HILIC columns can complicate the identification of coeluting isomeric glycans. Aberrant compounds from the matrices might also interfere with glycan analysis for complicated biological samples. Aldredge et al. addressed these issues by combining exoglycosidase digestion with nanoLC-MS/MS by employing PGC as stationary phase.[200] A *N*-glycan pool from human serum glycoprotein standards was fractionated by off-line HPLC and subjected to sequential exoglycosidase digestions. Compounds before and after digestion were analyzed by nanoLC-MS/MS to confirm the compositions by both accurate masses and tandem MS spectra (Figure 15). The effective separation with PGC and sensitive detection with nanoLC-MS/MS facilitated the construction of a serum *N*-glycome library that allowed rapid identification of exact glycan structures simply by matching their nanoLC retention times and accurate masses. The initial library contained over 300 entries with 50 completely elucidated structures derived from serum glycoprotein standards. A more refined library was later created with a larger number of elucidated structures using glycans released directly from human serum.[9] The relative abundances and variations in the *N*-glycans from several individuals were determined using this library. More recently, Abrahams et al. used a similar approach to assess the elution behavior of previously characterized *N*-glycans derived from standard glycoproteins on capillary PGC-LC-MS/MS.[201] The glycan retention times along with their associated MS/MS spectra were added to an elution centric database GlycoStore. Enzymatic digestion methods for glycan structural elucidation will continue to be developed and facilitate the extension of existing glycan libraries. These libraries will greatly improve the efficiency and accuracy of glycomics experiments in biomarker and biotherapeutic studies.

3.6. Minimum Information Required for a Glycomics Experiment (MIRAGE)

The rapid development of sample preparation methods, as well as separation, ionization, and detection techniques for glycomics studies has brought valuable tools for understanding the key roles of glycans in biological processes. However, the diversity of methods has also led to inconsistencies between laboratories and scientists in generating and reporting glycomics data. To establish standards for the glycobiology community, a group of scientists published the minimum information required for a glycomics experiment (MIRAGE) guidelines for reporting mass spectrometry-based glycoanalytic data.[202] The guidelines were developed according to the processes published in 2014.[203] Five major sections were described as essential information that authors should provide in their glycomics experiment reports; they include ion source conditions and software tools for data interpretation, etc. Recently, more MIRAGE guidelines for sample preparation[204] and for reporting glycan microarray-based data[204] were also added. Although these guidelines have not yet been widely applied to glycomics studies, they provide an important starting point and standardized criteria for authors and reviewers for handling glycoanalytic data.[205]

4. MS METHODS FOR GLYCOPROTEOMICS

The characterization of glycoproteins can provide both glycan and protein information, thereby linking glycomic and proteomic analyses. Glycoproteomics combines the tools of proteomic analysis with site-specific glycomic information. However, it also takes the difficulties in protein analysis and compounds them with the difficulties associated with glycan analysis.

The analyses of peptides and glycopeptides, as in bottom up, provide the simple route for the development of glycoproteomic platforms. Site-specific analysis of glycoproteins employing intact glycopeptides is challenging due to the presence of multiple glycosylation sites and the glycan heterogeneity associated with each site. Glycopeptide analysis by mass spectrometry is also complicated by several factors. Glycans tend to diminish severely the ionization efficiencies of the respective peptides. The many glycoforms distribute further the peptide signals over several species. Tandem MS methods were not sufficient to obtain both peptide and glycan information simultaneously.

Early attempts at glycoproteomics released glycans from the carrier proteins or peptides so that the glycans and deglycosylated peptides can be analyzed separately, or the glycans not at all.[206] This proteomic-centered approach simplified proteomics data but typically lost glycan information. However, with the emergence of newer fragmentation methods and bioinformatics tools focused on glycosylation, determining glycosite heterogeneity and occupancy has become more feasible. The improvements in enrichment and separation methods have also contributed greatly to the increased sensitivity and coverage.

4.1. Enrichment Methods for Sample Preparation

Enrichment methods have been used primarily to determine glycosylation in specific glycoproteins of interest. In this targeted

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approach, glycans and glycopeptides are released to obtain protein-specific glycosylation. In untargeted glycoproteomic approaches, glycopeptides are enriched to minimize competition for charge with the more abundant and more ionizable nonglycosylated peptides. Ion suppression from highly abundant peptides and glycan heterogeneity at the glycosylation site makes the detection and identification of glycoproteins challenging. To improve the sensitivity and coverage in glycoproteomic analysis, a diverse array of enrichment methods has been developed such as immunoaffinity and single-lectin affinity for targeted enrichment of one or a small group of glycoproteins, and multilectin affinity, HILIC, and covalent interaction-based methods for nonspecific enrichment. Several reviews have covered different aspects of these methods.[207−210]

4.1.1. Enrichment Based on Immunoaffinity. Antibodies or antibody-like agents, immobilized on beads or columns, are used to target glycoproteins through immunoprecipitation or immunoaffinity chromatography. The purified glycoproteins or glycopeptides are typically quantified by traditional immunoassays such as enzyme-linked immunosorbent assay (ELISA) but are increasingly probed by mass spectrometry techniques. The development and application of technologies combining immunoaffinity assays and mass spectrometry for quantitative proteomics in biological fluids have recently been reviewed by Borchers and co-workers.[211]

Immunoaffinity chromatography or immunoprecipitation as an enrichment step for subsequent MS analysis can greatly improve the sensitivity of MS detection for target glycoproteins. Immunoprecipitation using antibody-bound agarose beads has been used to obtain comprehensive glycan maps of selected glycoproteins. For example, Hong et al. used antihuman-IgA and antihuman-IgM bound to agarose to enrich IgA and IgM from human serum. Glycopeptide mapping was then performed on the enriched glycoproteins to identify glycopeptides that could be quantified by MRM.[20] A more common application of immunoaffinity enrichment is to purify specific glycoprotein of interest for biomarker discovery and validation. Recent examples include the characterization of clusterin in the plasma of clear cell renal cell carcinoma patients before and after curative nephrectomy,[212] glycoprofiling of intact transferrin for diagnosis and subtype identification of glycosylation in the congenital disorders, and[213] site-specific and linkage analyses of fucosylated *N*-glycans on haptoglobin in the sera of patients with various types of cancer.[214] In general, immunoaffinity enrichment can greatly improve the sensitivity for the detection of targeted protein or glycoprotein with acceptable reproducibility. Due to the high specificity of antibody capture and limited number of available antibodies, however, immuno-enrichment assays are not suited for untargeted glycoproteomics.

4.1.2. Enrichment Based on Lectin Affinity. Lectins are carbohydrate binding proteins that are widely used for selectively capturing and identifying glycoproteins. Unlike antibodies that may target individual proteins, each type of lectin can enrich a group of glycopeptides or glycoproteins with a unique glycan motif. Thus, lectin-based enrichment methods can provide a wider coverage of glycoproteome compared to immunoaffinity enrichment. Various lectin-based capturing techniques have been used in combination with mass spectrometry for glycoproteomic analysis, including lectin affinity chromatography, lectin microarrays, and lectin magnetic bead arrays.

Lectin affinity chromatography (LAC) can be performed using a single type of lectin to extract selectively one group of glycoforms or a combination of multiple types of lectins for the enrichment of a broader glycoproteome. Single-lectin affinity chromatography is generally applied to complicated biological samples such as serum, plasma, and tissue lysate to monitor specific glycosylation changes in deseases.[215,216] A variety of immobilized lectins with high specificities are commercially available for this purpose such as *Sambucus nigra* agglutinin (SNA) lectin for $\alpha(2,6)$ sialylated glycans and *Lens culinaris* agglutinin (LCA) lectin for core-fucosylated glycans.[216,217] To simplify data processing, the enriched glycoproteins were often deglycosylated before analysis, with the assumption that all of the identified proteins containing DXS/T concensus sequence were originally modified by the specific glycan epitope.[218] One problem with this approach is that the lectins may have broader specificity than expected, especially for complicated biological samples. The presence of naturally occurring aspartic acid and nonspecifically bound glycoproteins will often lead to misinterpretation of glycoproteomic data.

For more global analysis of glycoproteome, multilectin affinity chromatography (M-LAC) that can simultaneously capture glycoproteins with various glycan motifs was developed by mixing multiple lectins of interest. M-LAC can be combined with other depletion methods when applied to complicated biological samples such as human plasma.[219] For example, Gbormittah et al. incorporated 12 high-abundance proteins (12P) depletion with multilectin affinity enrichment by employing equal amounts of *Aleuria aurantia* lectin (AAL), SNA lectin, and *Phaseolus vulgaris* leucoagglutinin (PHA-L). Tryptic digests of M-LAC bound and unbound proteins were separately analyzed using nanoLC-MS/MS to compare the glycoproteome and proteome of clear cell renal cell carcinoma plasma before and after curative nephrectomy.[220] Alternatively, M-LAC bound glycoproteins could be eluted sequentially to fractionate glycoproteins specifically bound to each individual lectin. Totten et al. incorporated this approach with isotopic labeling during alkylation for relative quantitation of glycopeptides from two plasma samples.[221] The use of multiple lectins, however, still cannot fully resolve the glycopeptide loss caused by the biases of individual lectins during untargeted enrichment. Other complementary enrichment methods are often needed to completely cover different types of glycoforms.[222]

Lectin microarrays have proven to be a simple and rapid technique that has been applied to the high-throughput detection and differentiation of glycoforms for disease diagnostics.[223] When combined with in situ proteolysis and MALDI-TOF MS analysis, lectin array-based detection and identification of the specifically bound proteins can be achieved.[224−226] More recently, lectin magnetic bead array (LeMBA) platforms have been developed to facilitate the isolation of lectin-bound glycoproteins. Isolated glycoproteins can be readily eluted and digested before LC-MS/MS analysis.[227−229] Shah et al. reported a semiautomated LeMBA-based pipeline for serum glycoprotein biomarker discovery and candidate qualification.[230] Serum samples were incubated with magnetic beads coated with 20 different lectins to pull down glycoproteins, followed by on-bead trypsin digestion, proteomic analysis by nanoLC-MS/MS, and candidate glycoprotein quantitation by LC-MRM-MS. However, glycoproteome mapping and quantitation only performed at the protein level in this and other similar studies.[228]

4.1.3. Enrichment Based on HILIC. Glycopeptides contain hydrophilic glycan moieties and are thereby better retained on polar materials than nonglycosylated peptides. This feature allows glycopeptides to be isolated from the highly abundant and hydrophobic peptides using HILIC. Various HILIC stationary

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phases have been applied to glycopeptide enrichment such as silica-based functional groups including amine,[231] amide,[232] cyclodextrin,[233] maltose,[234,235] and zwitterion,[236−239] and polysaccharide-based materials including cellulose,[240] cotton wool,[241] and sepharose.[221,242,243] Compared to LAC, HILIC generally has broader glycan specificity that enables the enrichment of a wider range of glycopeptides. However, the strength of interaction between a glycopeptide and the HILIC material is largely dependent on the hydrophobicity of the underlying peptide backbone as well as the size and composition of the glycan moiety. Some *O*-glycopeptides and large tryptic glycopeptides are very weakly retained on HILIC and may be lost during enrichment.[244]

To increase the effectiveness of the enrichment and thereby increase the coverage of glycoproteome, efforts have been made to refine HILIC techniques by combining it with other enrichment methods. Electrostatic repulsion hydrophilic interaction chromatography (ERLIC), originally introduced by Alpert[245] for isolation of phosphopeptides, has become a popular technique for glycopeptide enrichment.[246−248] Its combined action of hydrophilic and electrostatic interactions allows for the simultaneous enrichment of neutral and sialylated glycopeptides.[127] Totten et al. recently compared the efficiencies of strong anion exchange ERLIC (SAX-ERLIC), M-LAC, and Sepharose-HILIC for the enrichment of *N*-glycopeptides from human plasma.[221] SAX-ERLIC was found to be the most robust method for glycopeptide enrichment, yielding over 800 unique glycopeptides from 95 plasma glycoproteins (Figure 16). This method could provide a potentially effective tool for system-wide site-specific mapping of the glycoproteome. Another refinement is the use of magnetic beads and nanoparticles functionalized with various hydrophilic ligands. This includes cross-linked cyclodextrin−metal−organic frameworks,[74] chitosan-coated magnetic colloidal nanocrystal clusters,[249] and graphene oxide−polyethylenimine-Au−L-Cys ZIC-HILIC nanocomposites.[250] The high biocompatibility and large surface area of these nanomaterials make them readily applicable to biological samples. However, no significant improvement in glycoproteome coverage has been reported for these materials compared to conventional HILIC approaches.

A more direct approach toward improving glycopeptide coverage is to use a combination of different enrichment regime. A study by Calvano et al. compared M-LAC and ZIC-HILIC enrichment protocols and discovered that different groups of glycopeptides were enriched when a single method was used.[238] A combined approach involving lectin enrichment of intact proteins followed by ZIC-HILIC enrichment of tryptic glycopeptides provided more comprehensive coverage of the serum glycoproteome. Other strategies such as TiO_2 chromatography and even chemical methods have also been used sequentially or in parallel with HILIC for glycopeptide enrichment.[251,252] The combination of different approaches can normally increase the number of captured glycopeptides dramatically. However, glycopeptide loss could also occur when enrichment methods with similar specificities are used sequentially such as the HILIC-ERLIC enrichment strategy tested by Zacharias et al.[127]

4.1.4. Enrichment Based on Covalent Interactions. Enrichment methods based on covalent interactions between glycopeptides and binding ligands are thought to be more universal and unbiased because chemical reactions are less affected by variations in glycan structures. The two most common types of glycopeptide enrichment involve boronic acid

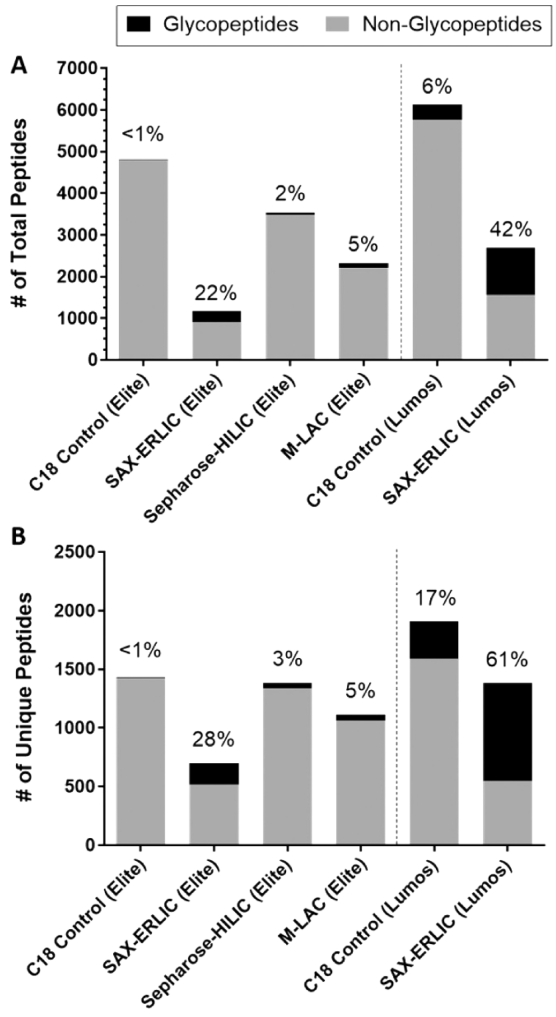

Figure 16. Comparison of different glycopeptide enrichment and detection methods showed ERLIC enrichment with HCD/ETD fragmention yielded the largest percentage and number of glycopeptides from palsma. Reprinted with permission from ref 36. Copyright 2017 American Chemical Society.

and hydrazide chemistry. Both methods produce covalent bonds between the *cis*-diol groups present in monosaccharides and the boronic acid or hydrazide groups, respectively. The major difference between these two methods lies in the reversibility of the reaction.[209] The five- or six-membered cyclic esters formed between boronic acids and glycopeptides can easily be dissociated under acidic conditions to release the captured glycopeptides intactly. The hydrazone formation between hydrazide groups and the aldehyde groups formed by oxidization of glycan *cis*-diols, however, is irreversible. Glycans must be cleaved by PNGase F or chemically to release the enriched peptides. In this method, only deglycosylated peptides are observed, while the glycan and perhaps even the site-specific information are lost. The same problems occur with other chemical enrichment methods such as reductive amination[253] and oxime click chemistry.[74] For this reason, only boronic acid chemistry is further discussed as a universal enrichment method for site-specific glyoproteomic analysis.

Various types of solid supports have been functionalized with boronic acids for the enrichment of glycoproteins or glycopeptides. Some functionalized materials including mesoporous silica,[254,255] magnetic nanoparticles,[147,256,257] and carbon

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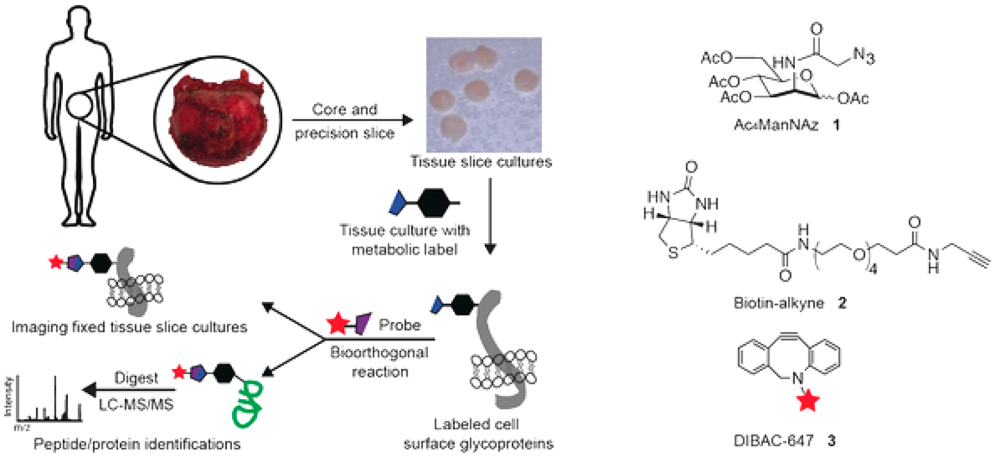

Figure 17. Bioorthogonal labeling of human prostate tissue slice cultures with azido sialic acids for imaging, enrichment, and LC-MS/MS analysis of sialoglycoproteins. Reprinted with permission from Spiciarich, ref 267. Copyright 2017 Wiley Online Library.

nanotubes[258] have been applied to the glyoproteomic analysis of complicated biological samples.[259,260] For example, magnetic beads conjugated with boronic acid were combined with stable isotope labeling of amino acids in cell culture (SILAC) to capture and quantitatively characterize glycoproteins in the secretome and whole cell lysates of yeast.[261,262] Although boronic acid chemistry is suitable for the enrichment of a wide range of glycoforms, its nonspecific binding of free saccharides and other compounds in the matrices can compete with glycopeptide capture and interfere with subsequent LC-MS/MS analysis. Novel materials such as dendrimeric boronic acid-functionalized magnetic nanoparticles may enhance the binding capacity and address this problem to some extent.[263] However, further improvement in specificity is still necessary.

Another emerging approach involves the use of bioorthogonal functional groups, such as azides, alkynes, or ketones for targeted labeling and enrichment of glycoproteins from cultured cells.[264−266] More recently, Spiciarich et al. applied this approach to human prostate cancer tissue.[267] Peracetylated *N*-azidoacetylmannosamine (Ac4ManNAz) was used in sliced normal and cancerous prostate tissue cultures to add the azide groups to the sialic acid residues on cell surface and secreted glycoproteins. The labeled sialoglycoproteins were then reacted with a biotin-alkyne probe, captured with avidin resin, digested with trypsin, and analyzed by LC-MS/MS. (Figure 17) These bioorthorgonal approaches have high specificity for enriching sialylated glycoproteins and other types of glycoproteins when proper metabolic labels are utilized. For global labeling of *N*-glycoproteome, for example, comparison of three sugar analogues, *N*-azidoacetylgalactosamine (GalNAz), *N*-azidoacetylglucosamine (GlcNAz), and *N*-azidoacetylmannosamine (ManNAz), revealed that GalNAz could yield the greatest number of glycoproteins and glycosylation sites for HepG2 liver cells.[268]

4.2. Separation Methods for Glycopeptides

4.2.1. Liquid Chromatography of Glycopeptides. Reversed-phase liquid chromatography (RPLC) employing a C18 column is the most widely used method for untargeted site-specific glycoproteome analysis. Separation of glycopeptides with RPLC is mainly based on the binding interaction with the peptide backbone and can be predicted based on the amino acid sequence.[269] The glycan has only minor influence in the interaction between glycopeptide and RPLC. The effect of glycan composition and structure on the retention behavior of glycopeptides was recently studied by Kozlik et al.[270] Reduction of the retention time was observed with increasing number of neutral monosaccharide units of glycans attached to the same peptide backbone (Figure 18). Relative retention times of different glycoforms could thereby be calculated and used as a qualitative parameter to reduce false positive rate of glycopeptide identification. However, care should be taken when using this prediction approach because the actual retention times of glycopeptides could be complicated by variations in chromatographic conditions and existence of sialic acids residues.[271]

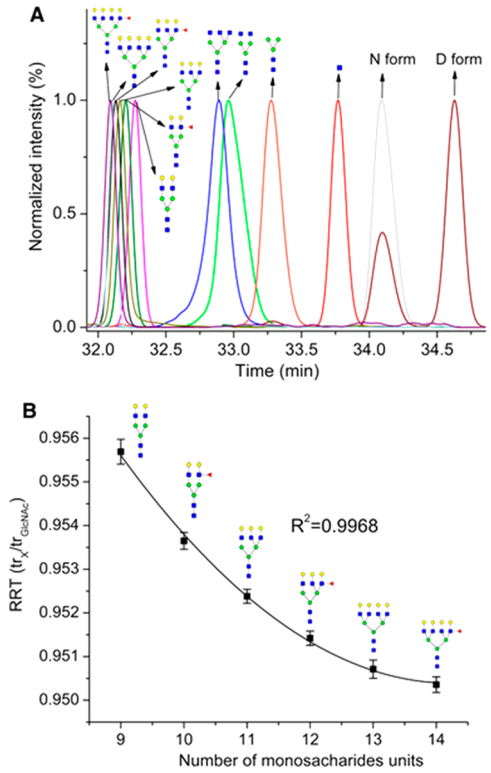

Figure 18. Analysis of the VVLHPNYSQVDIGLIK peptide of haptoglobin by nanoLC. Decreased retention of glycopeptides was observed with increasing number of neutral monosaccharide units. Reprinted with permission from ref 270. Copyright 2017 Wiley Online Library.

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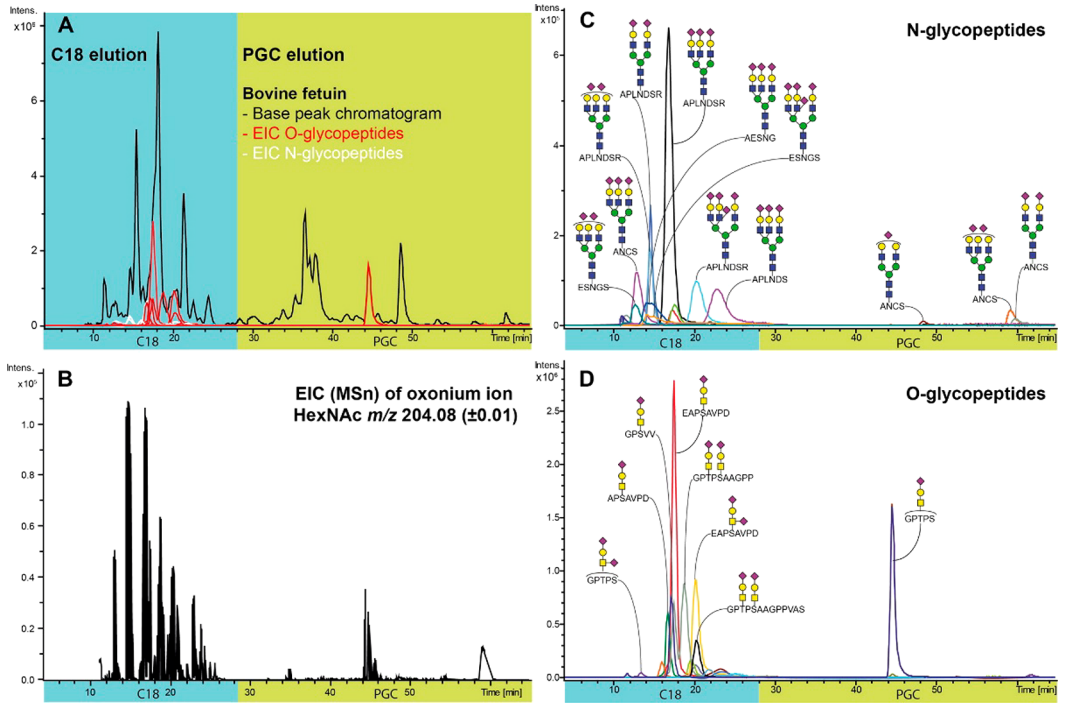

Figure 19. C18-PGC-LC-ESI-QTOF-MS/MS analysis of *N*- and *O*-glycopeptides from pronase digest of bovine fetuin. Reprinted with permission from ref 274. Copyright 2015 American Chemical Society.

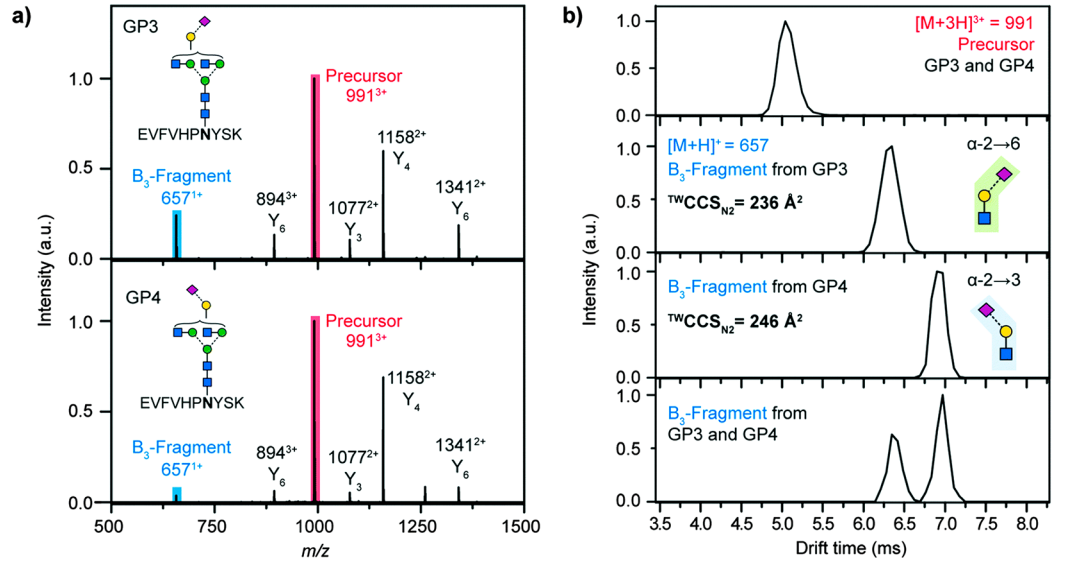

Figure 20. Distinguishing sialic acid linkage isomers on glycopeptides using CID fragmentation and subsequent IM-MS analysis. Reprinted with permission from ref 290. Under Creative Commons Attribution 3.0 license (https://creativecommons.org/licenses/by/3.0/).

The major drawbacks of RPLC for glycopeptide separation include the poor retention of highly hydrophilic glycopeptides and the limited separation of different glycoforms. To address these problems, orthogonal separation methods combining RPLC and PGC or HILIC have been developed and applied to site-specific analysis of *N*- and *O*-glycopeptides derived from trypsin[272,273] and pronase[274] digestion. Complementary separation by PGC or HILIC can efficiently recapture the hydrophilic glycopeptides that might be lost in a conventional RPLC-only approach (Figure 19). PGC or HILIC alone was also used to enrich and separate intact glycopeptides derived from specific and nonspecific protease digestion of glycoproteins.[275] Isomeric separation was achieved for both *N*-glycopeptides and *O*-glycopeptides, especially for those obtained from pronase digestion with short peptide backbones.[54,244,276−279] These methods are ideal for glycosylation site and structural heterogeneity mapping of purified glycoproteins. Although HILIC separation has also been used to separate IgG *N*-glycopeptides from human serum,[280] neither PGC nor HILIC alone is sufficient for comprehensive glycoproteome mapping because important information from the unretained hydrophobic peptides and glycopeptides are lost.

4.2.2. Capillary Electrophoresis of Glycopeptides. Early researches on capillary electrophoresis of glycopeptides have applied sheath-flow CE-MS to hydrophilic glycopeptides such as tryptic *N*-glycopeptides from ribonuclease B[281] and antithrom-

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bin[282] glycoprotein standards. Sanz-Nebot and co-workers have applied this approach to a series of studies on the characterization of *N*- and *O*-glycopeptides from recombinant human erythropoietin[283] and other glycoproteins.[284,285] They have also systematically studied the electrophoretic migration behavior of peptides and glycopeptides that can be used to simulate their migration and separation on CE.[286] However, the limitations of CE-MS discussed above for glycomics are further amplified for glycoproteomics because higher sensitivity and more robust separation are required to characterize the structural heterogeneity of glycopeptides. For example, comparison of CE-MS and μLC-MS showed that CE had poorer separation for glycopeptides when a surfactant was used to enhance enzymatic digestion.[284] To improve the sensitivity of CE-ESI-MS in glycopeptide analysis, Kammeijer et al. employed dopant enriched nitrogen gas with a coaxial gas flow to build a sheathless nanoESI interface.[287] The addition of dopant gas greatly facilitated the desolvation and ionization, thus achieving higher sensitivity for glycopeptide detection. More recently, Qu et al. reported a rapid CZE-ESI-MS method with an electrokinetically pumped sheath-flow nanospray interface for the analysis of glycopeptides from IgG and haptoglobin standards. The development of these novel interfaces has increased the applicability of CE to glycoproteomics as a complementary separation approach to RPLC.

4.2.3. Ion Mobility of Glycopeptides. Ion mobility separation of glycopeptide isomers is even more challenging than that of glycans due to the high structural similarities, numerous possibilities for isomerization, and lack of commercial standards. Isomeric separation of intact glycopeptides achieved so far include the separation of peptides modified with different monosaccharide residues (GlcNAc or GalNAc) at the same site,[288] GalNAc at different locations,[289] or a sialylated *N*-glycan at different sites,[290] and the separation of isomeric glycans attached to a single amino acid.[291] For *N*-glycopeptide isomers that differ only in the linkage of one monosaccharide residue, partial separation was achieved for their highly charged species.[292] Baseline separation is nearly impossible. A novel strategy to address this issue involves IM separation of the diagnostic isomeric product ions after fragmenting the glycopeptides using low-energy CID. This strategy has been used to distinguish sialic acid linkage isomers of synthesized *N*-glycopeptide standards (Figure 20)[290] and *N*- and *O*-glycopeptides derived from glycoprotein standards.[293] However, the application of this approach to complicated mixtures remains challenging because other coeluting sialylated glycopeptides will generate the same fragments.

4.3. Determination of Glycosylation Sites

The most common approach for identifying site-specific glycosylation involves a series of steps including enzymatic proteolysis of glycoproteins as well as chromatographic separation and mass spectrometry analysis of glycopeptides.[294] For enzymatic digestion, trypsin, which exclusively cleaves a protein at the C-terminus of arginine and lysine except when they are bound to a proline, is widely used. Because of its high specificity, trypsin digestion generates predictable peptide backbones. Furthermore, the ionization efficiency of tryptic digested glycopeptides are improved during MS analysis because a basic residue is contained in each peptide backbone. A huge drawback of trypsin digestion is the possibility of generating missed cleavages due to steric hindrance at the cleavage site near the glycosylation site.[295] The resulting glycopeptides can be too large for the current proteomic workflow. Additionally, some glycoproteins may not be compatible with trypsin digestion conditions.[296] These issues can make identification of glycosylation sites more challenging.

To minimize the limitations of trypsin digestion, nonspecific digestion using proteases like pronase and proteinase K can be applied.[20,297] These nonspecific proteases cleave glycoproteins into glycopeptides with shorter peptide backbones or non-glycosylated dipeptides and amino acids. The resulting glycopeptides can be further enriched by solid phase extraction using PGC prior to MS analysis. Compared to specific protease digestion using trypsin, nonspecific digestion could provide much better glycosite coverage but fewer glycoforms on some sites (Figure 21).[20] This method has been extensively used to

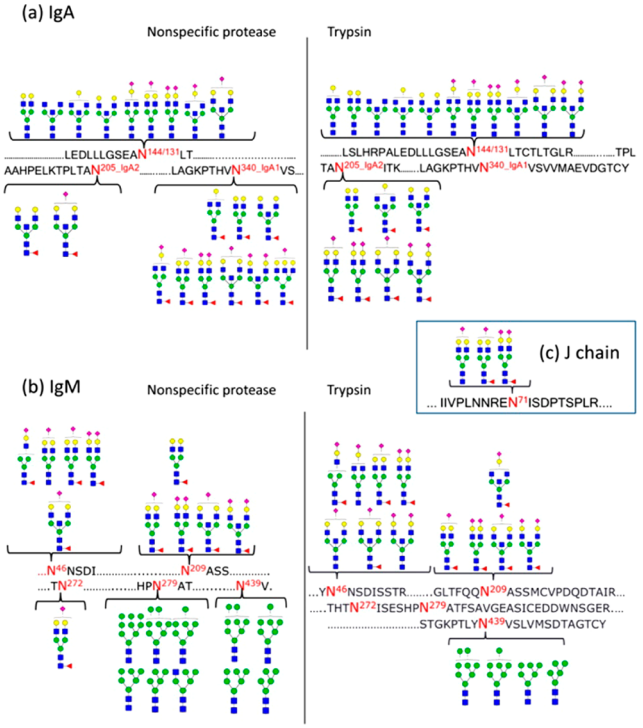

Figure 21. Comparison of nonspecific protease and trypsin for site-specific glycan mapping. Reprinted with permission from ref 20. Copyright 2015 American Chemical Society.

identify the site heterogeneity (*N*- and *O*-glycosylation site) of many glycoproteins.[298] However, it is limited mainly to the analysis of either one purified glycoprotein or small pools of glycoproteins because the resulting glycopeptides contain fewer amino acids. Multienzyme strategies employing combinations of specific proteases could also be used to improve glycosite identification. The combinations of trypsin and Glu-C as well as trypsin and chymotrypsin have been used to identify *O*-glycosylation sites in human serum proteins.[299] All of the methods mentioned above can be further improved by enriching glycoproteins prior to the digestion or by enriching glycopeptides after the digestion.

A simple approach for identifying glycosylation sites is to remove the glycans completely or partially using enzymatic means. The entire *N*-glycans can be cleaved off from the attached asparagine by peptide-*N*-glycosidase F (PNGase F), which converts asparagine into aspartic acid. Detection of aspartic acid would be considered as a direct indication of an occupied *N*-glycosylation site. However, deamidation of asparagine, a

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common post-translational modification, can result in the false positive identifications. This problem can be addressed by performing the deglycosylation in isotopically labeled water.[300] Another approach is to perform partial deglycosylation using endoglycosidases[301−303] or chemical methods,[304] thus leaving a single monosaccharide (GlcNAc) attached to the peptide while removing the rest of the glycan moiety. The resulting glycopeptide with a single GlcNAc is a valid indication of an occupied site.

4.4. Applications of Glycoproteomics

The development of LC-MS/MS techniques in glycoproteomics makes it more widely used for studying the pathogenesis of several different diseases including cancer[305−307] and neurological diseases.[308] In addition, glycoproteomics research can now be conducted on various types of biological samples such as blood,[309−311] urine,[312] cell lines,[313−315] and other tissues.[127,316] Many of these studies focus on the biomarker discovery for a specific disease by quantifying glycopeptides and determining glycosite occupancies.[317−319] Other studies expand the glycoproteome with regard to unique organisms.[320] While the application of glycoproteomics has expanded considerably, this section highlights more recent examples related to advances in dealing with various sample types with specific focus on diseases.

Goyallon et al. studied the site-specific glycosylation of proteins in cerebrospinal fluid using an approach that combines *N*-glycoproteomic and *N*-glycomic analyses.[321] The *N*-glycans from human serum and cerebrospinal fluid were released, permethylated, and analyzed with MALDI-TOF MS. Tryptic glycopeptides from the same samples were analyzed after enrichment as intact glycopeptides or deglycosylated peptides with a nanoLC-MS/MS system (Figure 22). In total, over 100

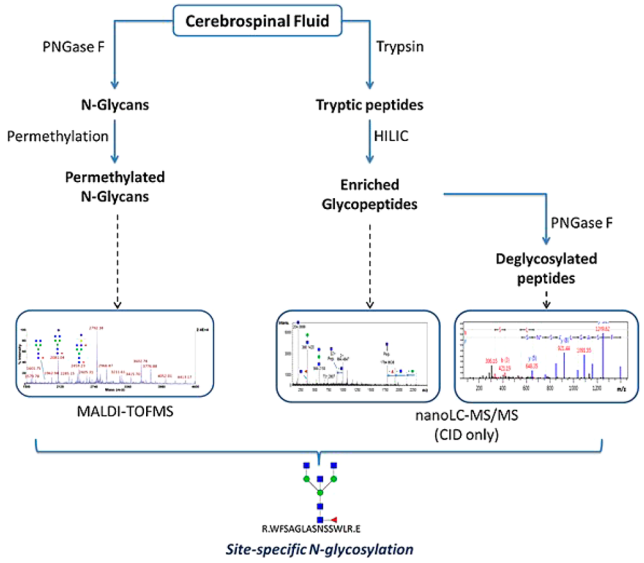

Figure 22. Overall strategy used for the site-specific glycopeptide analysis of cerebrospinal fluid samples. Reprinted with permission from ref 321. Copyright 2015 Wiley Online Library.

glycopeptides from 36 glycoproteins were identified in a pooled cerebrospinal fluid sample. Unique "brain-type" glycoforms were identified in this sample compared to those found in serum.

Glycoproteomic analysis of mouse and human embryonic stem cells (mESC and hESC) were performed by Stadlmann et al. to identify new proteins involved in the ricin toxicity.[322] Glycopeptides were enriched from digested cell lysis using HILIC cartridges and analyzed by nanoLC-MS/MS (Figure 23). By applying the method to mESC, the total number of

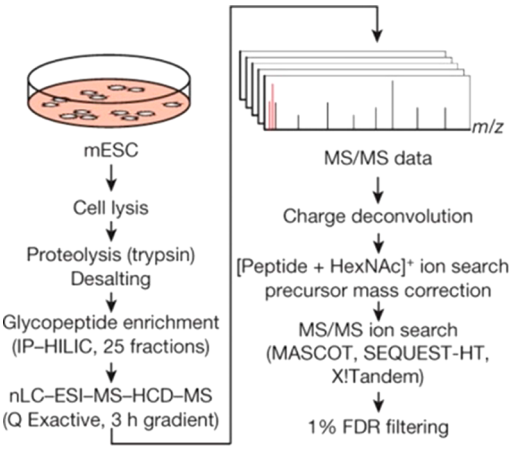

Figure 23. Workflow of glycoproteomics by combining proteomcis platform and novel algorism for intact glycopeptide identification. Reprinted with permission from ref 322. Copyright 2017 Springer Nature.

experimentally identified *N*-glycosylation sites was almost twice the number previously determined in the mouse glycoproteome. The method was also applied to hESC, and more novel glycoproteins were identified. The specificity of the method was validated by studying the glycoproteomes of Fut9 and Slc35c1 knockout cell lines, which are ricin-resistant. Several fucosylated glycoproteins that are essential in ricin toxicity resistance were revealed.

Mouse brain glycoproteome was also studied by Fang et al.[320] using multienzyme approaches by employing both the enzymes with high specificity such as trypsin and Glu-C and those with low specificity such as chymotrypsin and pepsin. Compared to the single-enzyme approach using only trypsin, the number of identified *N*-glycosylated sites was greatly improved with the multienzyme approach. The efficiencies of four glycopeptide enrichment methods, including hydrazide chemistry, TiO_2 chromatography, HILIC (Sepharose CL-4B), and ZIC-HILIC, were also evaluated in the study. While complementary *N*-glycosylation sites were obtained from different methods, HILIC and ZIC-HILIC yielded the largest numbers of identified glycopeptides. Furthermore, two different fractionation techniques, SCX prefractionation before ZIC-HILIC and bRP prefractionation after ZIC-HILIC, were also compared. Altogether, over 10000 *N*-glycopeptides from nearly 4000 proteins were identified in this study, revealing the large diversity of *N*-glycoproteome in mouse brain. Sample preparation methods optimized in these types of studies therein will be helpful for the further study of neurological diseases.

The *N*-glycoproteome of dried blood spot (DBS) samples has been studied by Choi et al. with a relatively simple sample preparation method.[323] The DBS samples were prepared and stored for different periods of time before analysis to evaluate the stability of *N*-glycoproteins. *N*-Glycopeptides were obtained after denaturation and tryptic digestion without depletion, desalting, or enrichment. With their optimal sample preparation method, more than 30 *N*-glycopeptides of 14 glycoproteins were quantified consistently over different storing periods. *N*-Glycan profiles of DBS samples have also been reported by Ruhaak et al.,

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showing the applicability of DBS for long-term and large cohort clinical studies.[324]

Glycoproteomic analyses have been performed extensively on cancer. One widely studied target is ovarian cancer, a disease that desperately needs good diagnostic markers. Ji et al. performed both proteomic and *N*-glycoproteomc analyses of ovarian cancer cell line OVCAR8 and its doxorubicin-resistant variant, NIC-ADR/RES, to unravel the underlying mechanism for multidrug resistance (MDR).[325] Quantitation of peptides and glycopeptides were conducted using stable isotope labeling of amino acids in cell culture (SILAC). After the enrichment of *N*-glycopeptides with HILIC, PNGase F was used to remove the *N*-glycans. The resulting deglycosylated peptides containing glycosites were analyzed with LC-MS/MS. Quantitative proteomic information was obtained simultaneously. Several peptides with *N*-glycosylation sites were found to be differentially expressed with and without changes in site occupancy, which may be relatible to MDR properties of ovarian cancer cells.

To understand the biology of epithelial ovarian carcinomas, Li et al. studied the overall variation of glycoproteome and site occupancy of glycoproteins in high-grade ovarian serous carcinoma (HGSC) and benign epithelial ovarian tumor.[326] Both tumor tissues and benign tissues were collected from female patients and homogenized for protein extraction. Glycopeptides were enriched using hydrazide chemistry and labeled with an isobaric tag for relative and absolute quantitation (iTRAQ) reagent. Samples were prefractionated on basic RPLC prior to analysis with LC-MS/MS to provide better coverage. Approximately 40 deglycosylated peptides showed significant changes between ovarian tumor and benign tumor, while the site occupancies of 10 glycopeptides varied, revealing that specific changes in glycosylation site occupancy may be potential biomarkers for ovarian cancer.

To obtain potential biomarkers of hepatocellular carcinoma (HCC), Liu et al. analyzed serum samples collected from patients with early HCC related to chronic hepatitis B virus (HBV) infection and controls.[327] Glycoproteins with $\alpha(2,3)$-linked sialic acid were enriched with *Maackia amurensis* lectin (MAL) and quantified using the iTRAQ labeling method on nanoLC-MS/MS. The quantitation results showed significant fold changes of eight proteins. The differential expression of these proteins were further validated by Western blotting, and galactin 3 binding protein (Gal-3BP) was selected for ELISA analysis as a candidate biomarker to identify HBV-related HCC.

5. SOFTWARE TOOLS FOR GLYCOMICS AND GLYCOPROTEOMICS

Mass spectrometry of glycans and glycoconjugates in biological samples generates large amounts of data. To fully interpret the data, bioinformatics tools are necessary. Unlike proteins and DNA, glycans and glycoproteins are highly heterogeneous due to their nontemplate biosynthesis. This makes it difficult to automatically annotate glycan and glycopeptide structures. The ability to assign peptide sequence, glycan composition, and glycosylation site simultaneously is challenging and considerably more difficult than elucidating point modifications on proteins such as phosphorylation. For the analyis, tandem MS data are usually deconvoluted and deisotoped. Effective searches need to be performed against both protein and glycan databases. The theoretical protein and glycan search space should also be defined properly to reduce false discovery rates (FDRs)[328] in the database searches. A recent review by Hu et al. has discussed the issues associated with interpretation of glycopeptide tandem MS data.[329]

Software for glycomic analysis should facilitate the annotation of structures of *N*-glycan, *O*-glycans, glycosaminoglycans, and other oligosaccharides from mass spectrometry data. Xu et al. recently developed GlycoMaid with a graphic user interface (GUI) for automatic interpretation of *N*-glycan data from MALDI-TOF MS based on the masses and the isotopic distribution of the signals.[83] The software was improved from a previously developed version using a matching glycan isotope abundance (mGIA) algorithm, enhancing the ability for deconvolution of overlapping isotopic distributions of glycans.

Other approaches produce glycan structures based on tandem mass spectrometry data. A machine learning tool developed by Kumozaki et al. conducts de novo sequencing of glycans based on fragmented glycan peaks in MS/MS spectra without database searches.[330] The fragmentation model of glycans is first established, and a solver using Lagrangian relaxation together with a dynamic programming technique was utilized. The algorithm scores were then optimized by making use of structured support vector machines (SVMs), which is a machine learning technique by studying the parameters for glycan structures using training data sets such as MS/MS spectra of known glycans. To annotate glycan structures more accurately, known glycan core structures including O-linked and N-linked glycans were more constrained. The algorithm enables the de novo sequencing of complicated glycan structures.

For glycoproteomic analysis, commercial and in-lab software have been developed. Byonic is arguably one of the most commonly used comercial software packages for interpreting the tandem MS data of peptides and glycopeptides.[331] With the target protein database and N-linked or O-linked glycan library, the software can perform automatic glycan search based on MS/MS spectra of glycopeptides without knowing glycosylation sites or glycan compositions. Besides glycosylation, several other modifications such as carbamidomethylation of cysteine (C [+57.021]) and oxidation of methionine (M [+15.995]) can be set as fixed or variable modifications on peptides. In addition, enzymes with variable specificities can be specified for in silico digestion when searching spectra. The results generated from Byonic include a summary of assigned peptides and a list of proteins. In the list of peptides, the possible variable and fixed modifications with their masses on each peptide and glycosylation modifications including glycan compositions and masses are illustrated. The charges of observed glycopeptide precursor ions are also listed. Several scores are used to evaluate the quality of the site-specific assignments of glycopeptides, including Delta Mod and Log Prob. The Delta Mod evaluates the quality of the assigned modification on the peptide and Log Prob shows the possibility that the assigned peptide sequence is random. Both of these two parameters are used in further data analysis to remove those poorly matched glycopeptides. The original spectra together with matched peptide fragments can be accessed from the software to check the coverage of protein sequence.

A software that was developed strictly for extensive site-mapping of glycans is GlycoPeptide Finder (GP Finder).[278] Instead of searching data based on specific or hemispecific enzymatic digestion, GP Finder can be used to annotate nonspecifically digested glycopeptides using enzymes such as pronase E by calculating all possibilities of peptide sequences after digestion. With nonspecific digestion, glycans connected to variable lengths of peptides are observed. Although the lengths of

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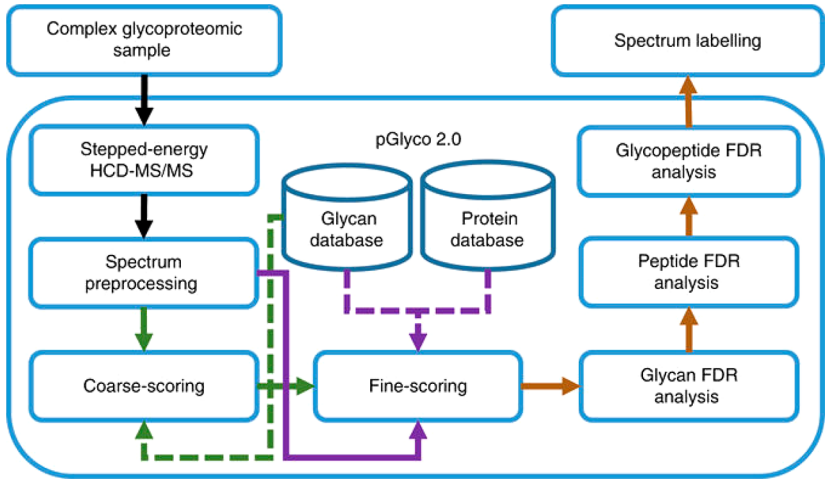

Figure 24. Overall flowchart of data collection for pGlyco 2.0 analysis. Reprinted with permission from ref 332. Under Creative Commons CC BY license (https://creativecommons.org/licenses/by/4.0/).

peptides generated in this way vary, digestion time can be regulated to control the variability of peptide lengths. In data analysis, glycopeptide candidates are filtered based on accurate mass-to-charge values, fragmentations, and diagnostic oxonium ions. A decoy data library generated by adding 11 Da residue to theoretical glycan compositions was used to evaluate the false discovery rate. This software has been applied to glycosylation mapping of bovine pancreatic ribonuclease (RNaseB) with a single glycosylation site, a mixture of three known glycoproteins with multiple sites, and several unknown glycoproteins in very-low-density lipoprotein (vLDL) nanoparticles.[278]

A more recently developed software for glycoproteomic analysis is pGlyco, which takes advantage of the features of orbitrap MS with a new algorithm to reduce the FDR of glycopeptide identification.[332,333] The software was applied to standard glycoprotein mixtures and mouse tissue samples using an Orbitrap Fusion instrument. In the analysis, HCD-MS/MS data were collected. Glycopeptide precursor ions were selected for further MS/MS or MS3 analysis when the diagnostic ion, 138.055, was detected in HCD tandem MS spectra (Figure 24). The CID tandem MS data contain mainly glycan fragments, and the structure of glycan was inferred using both HCD- and CID-MS/MS data. The MS3 spectrum of Y_1 ion, the peptide backbone with one HexNAc residue, contained peptide backbone fragments to yield the peptide sequence. The identified glycan structures were aligned with the peptide sequences based on retention times, generating a list of glycopeptides. The FDR was estimated by a novel spectrum-based decoy method where a decoy spectrum of the target glycopeptide was generated by modifying each peak with a random mass of 1−30 Da and matched to an experimental spectrum. With pGlyco, over 300 unique glycopeptides were identified in a mixture of six standard glycoproteins. Subsequently, approximately 10k nonredundant glycopeptides from 955 glycoproteins in mouse tissues were identified.

Earlier attempts for automated analysis include GlycoMaster DB,[334] SweetNET,[335] gFinder,[336] and Integrated GlycoProteomeAnalyzer[337] and the oldest GlycoX.[15] These software were developed for specific applications, while all lacked a method to properly determine the false positive rates.

In addition to these tools, databases of glycans and glycoproteins/glycopeptides have been developed as references for glycomic and glycoproteomic studies. Some provide tentative structure of glycans, such as GlyTouCan,[338] Carbohydrate Structure Database (CSDB),[339] and UniCorn.[340] GlyTouCan provides structures together with compositions and topologies of glycans, and accession numbers are assigned to glycans, making it more searching friendly. CSDB contains NMR spectra and other properties of both natural and derivatized glycans in plants; UniCorn provides theoretical structures of *N*-glycans that are derived by cleaving glycans with enzymes in different pathways of biosynthesis. There are also database related to specific research areas. For example, SugarBindDB is a database that covers the interactions between glycans and proteins such as human pathogens and adhesins.[341] For researchers learning about glycans using ion mobility mass spectrometry, GlycoMob would be useful in the way of providing CCS values, masses, and fragments of glycans.[161] Glycoprotein databases such as Unipep and dbOGAP include information on *N*-glycosylated proteins together with peptides and *O*-GlcNAcylated proteins, respectively.[342]

6. TOP-DOWN GLYCOPROTEOMICS

In comparison to the previously described strategies for the analysis of released glycans and glycopeptides using bottom-up proteomics approaches, top-down proteomics, or the analysis of intact proteins, has not yet found wide utility for glycoproteins. The reason is in part due to the relatively recent introduction of high resolution mass spectrometry (e.g., FTICR-MS, Orbitrap, and HR-TOF[343,344]), which is a requirement for top-down.[345] Advancements in top-down proteomics have recently been reviewed extensively.[346]

One of the most prominent advantages of top-down glycoproteomics is that the information is obtained on the whole protein so that full coverage of the protein can be achieved and almost all of the glycosylation sites can be identified. Meanwhile, these can hardly be achieved in bottom-up glycoproteomics due to difficulties with digestion, unfavorable conditions for HPLC separation, and MS ionization. The availability of the information on the whole protein has additional consequences in that specific protein variants or proteoforms,[347] which originate from the same gene but have undergone different processing and carry different PTMs (not only glycosylation but also phosphorylation, acetylation, and other PTMs), can be individually identified. With the recently increasing interest in personalized medicine,[348] which requires

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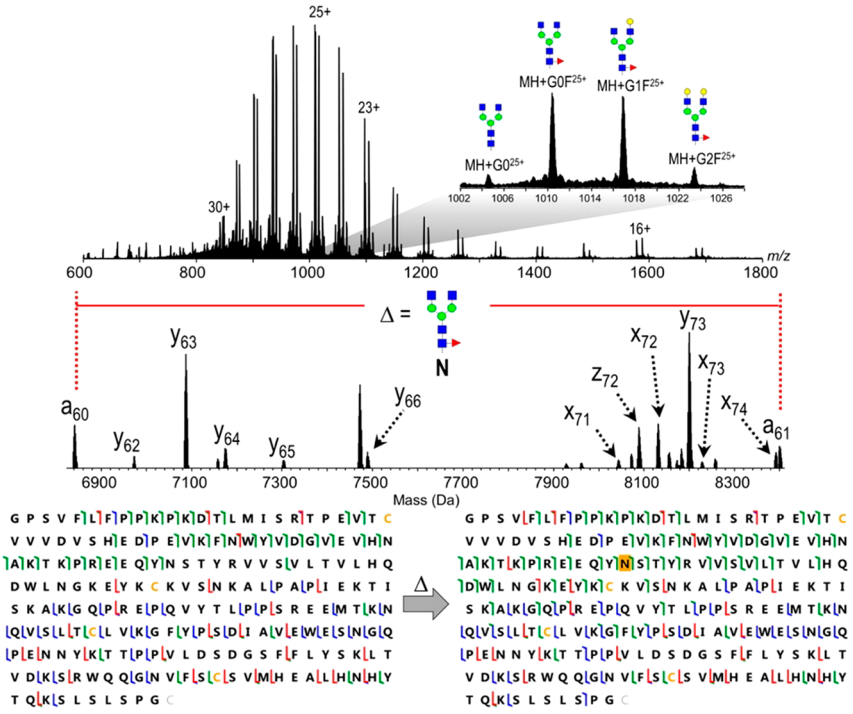

Figure 25. UVPD for middle-down analysis of a therapeutic monoclonal antibody. Reprinted with permission from ref 73. Copyright 2016 American Chemical Society.

knowledge of an individual's phenotype, the characterization of individual proteoforms could yield highly valuable information for diagnosis and treatment.

While this approach may be potentially powerful, the application of top-down proteomics for the characterization of protein glycosylation is limited because its use is constrained by several drawbacks. One of the most important limitation is the high complexity of the mass spectra obtained, which include not only the extensive charge envelope but also the complexity related with the glycan heterogeneity. Furthermore, the sensitivity in top-down proteomics is limited compared to bottom-up for several reasons. Peptides are small compared to intact proteins and are more effectively separated with C18 stationary phases. While separation and enrichment may be achieved for proteins using C4 or C8 material,[213,349] proteins are much less resolved, thus reducing the analytical sensitivity. Consequently, peptides from biological mixtures are often more easily analyzed, while the analysis of complex mixtures via top down is much more challenging. Thus, antibody-based enrichment of the target protein is typically required prior to top-down analysis.

Another limitation is the sheer size of the intact protein, which can affect tandem MS. The precursor signal can be divided over a vast number of fragments, resulting in the reduction of the sensitivity of the approach. Although this limitation can be ameliorated by increasing acquisition times, the typical requirement of more lengthy analysis time further limits multiplexing capabilities. The sensitivity can be further affected by the large number of vibrational modes. A large protein requiring significantly greater energy during tandem MS can accept more energy while simultaneously follows more numerous fragmentation pathways after activation. In contrast to peptides and glycopeptides which can often be sufficiently analyzed by CID, ETD or ECD are typically necessary for protein and glycoprotein fragmentation. The dissociation efficiencies of these techniques are generally much lower than CID due to the strong dependence on precursor charge density. The problem is compounded in glycoproteins, which are often in a low-charge state, resulting in lower fragment signal intensities and reduced analytical sensitivity.

Even with these major constraints, the method is finding early applications in the characterization of monoclonal antibodies (mABs).[350] mABs are often available in larger quantities and are extensively purified as part of the manufacturing process. Further thorough characterization is required prior to FDA approval. The most common representation carry a single site of glycosylation in the Fc part of the protein,[351] thus limiting the complexity due to glycan heterogeneity. A recent example is the study by Yang et al. with the analysis of three IgG4-Δhinge mutants at the intact protein and the released glycan levels.[352] In general, the predominant glycoforms detected by the top-down approach were in good agreement with the analysis of released glycans, although specific analysis of the released glycans yielded considerably more glycoforms.

CID and to a lesser extend ETD and ECD still yielded insufficient fragmentation information to completely characterize glycoproteins in top-down workflows. However, novel fragmentation strategies that are likely to be more beneficial for the analysis of protein glycosylation in a top-down manner have emerged. Ultraviolet photodissociation (UVPD) was introduced for the so-called middle-down analysis of a therapeutic monoclonal antibody (Figure 25).[73] In this analysis, restricted enzymatic digestion is employed to generate large peptides (~3−20 kDa) that are still amenable to high resolution LC-MS/MS analysis. It was shown that the rapid and high energy activation obtained through UVPD resulted in efficient cleavages of amino acid residues while maintaining the integrity of the more labile glycans, thereby allowing the confident localization of the glycan moieties. While this technique has been shown to provide very good protein coverage in top-down proteomics as

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well,[353] it has not yet been employed for analysis of large intact glycoproteins.

The ETD and ECD fragmentation techniques suffer from a phenomenon called nondissociative electron capture or transfer, particularly for larger molecules such as intact (glyco)-proteins.[354,355] Here, peptide or protein backbone cleavage occurs, but the product ions are held together by extensive intramolecular bonds, thus interfering with the generation of sequence information. Frese et al. introduced a combination of CID (or HCD) with ETD (EThcD), whereby a supplemental energy is applied to all ions formed by ETD to generate more informative spectra.[356] In a comparative study using top-down proteomics, it was shown that EThcD provided increased protein sequence coverage and facilitates greater phosphorylation site identification compared to ETD.[357] So far, EThcD has proven to be an attractive technology for glycopeptides identification,[358] but the fragmentation technology has still not yet successfully been applied to intact glycoprotein analysis.

The application of additional energetic activation in ECD, recently called "activated ion ECD" (AI-ECD) has been shown to increase the ECD product ion yield and has successfully been applied in top-down proteomics. The technique was shown to be beneficial for the analysis of IgA *O*-glycopeptides[359] and was likely to provide increased glycoprotein information compared to CID and ECD. Besides AI-ECD, there are attempts for the implementation of AI-ETD using infrared photoactivation in top-down proteomics.[360,361] Riley et al. showed that the implementation of this technique on a quadrupole-orbitrap-linear ion trap hybrid mass spectrometer (Orbitrap Fusion Lumos) resulted in nearly complete sequence coverages of intact proteins.[362,363] In the analysis of smaller proteins, AI-ETD was shown to outperform EThcD and provides at least similar protein coverage compared to UVPD. Because of the combination of the vibrational activation (through the IR laser) with the electron transfer driven activation, it is anticipated that AI-ETD will also provide more informative fragmentation in top-down glycoproteomics.

The previous examples were all performed using electrospray ionization. However, top-down glycoproteomics may also be employed using MALDI ionization. Nicolardi et al. conducted top-down glycoproteomics using MALDI-FTICR for the characterization of glycoforms of apolipoprotein C-III.[364] In this study, ultrahigh resolution MALDI-FTICR MS was employed to characterize the glycosylation profiles of apolipoprotein C-III from 96 serum samples. The differences between the samples were assessed using MALDI-FTICR MS, while the identification of the different proteoforms was performed with ESI-FTICR MS with CID fragmentation.

Although the above-described methods covered mainly the application of direct infusion or MALDI ionization to the analysis of intact glycoproteins, hyphenation of the mass spectrometry technique with liquid chromatography or capillary electrophoresis has also been applied. Specifically, Van Scherpenzeel et al. have reported a platform in which C8 reverse-phase HPLC was combined with HR-TOF-MS for the characterization of glycosylation profiles from intact transferrin.[213] The method was applied for the identification of patients with congenital glycosylation disorders based on the different top-down glycosylation profiles. Moreover, it provided potentially a sensitive technique for monitoring current and future therapies. A similar approach was applied for the in-depth characterization of two monoclonal antibodies.[349] Using this technology, mean CVs of 2.2% and 3.7% were obtained for intra- and interassay variability, respectively, showing that a top-down strategy can be adopted for quality control of the production process of mAb biopharmaceuticals.

Capillary electrophoresis using coated capillaries helps avoiding protein binding and may be useful for separating intact glycoproteins prior to MS analysis. One of the early examples includes CE-TOF-MS analysis of erythropoietin (EPO), which carries three *N*-glycosylation sites and one *O*-glycosylation site.[365] A similar method was used to assess the glycosylation of transferrin,[366] and more recently a 2D-CE method was employed for the detailed MS characterization of mAb charge variants.[367]

Prostate specific antigen (PSA) is currently applied as a marker for prostate cancer, however, suffering from limited specificity.[368] Recent reports indicate that PSA glycosylation may increase its specificity for prostate cancer,[369] thus sparking increased interest for the characterization of PSA glycosylation. The association of biomolecular research facilities (ABRF) recently conducted a comparative glycomics study using a PSA standard from semen,[370] and the top-down glycoproteomics was performed using HR-TOF-MS and orbitrap-MS, showing the potential application of the top-down glycoproteomics on the analysis of an individual glycoprotein with limited complexity. It was also concluded that the top-down glycoproteomics is promising for the analysis of smaller glycoproteins with a single glycosylation site. Although the detection of minor *N*-glycans yielded poor reproducibility, consistent identifications of all major and most intermediate abundance *N*-glycans were achieved. For more complex samples or larger glycoproteins, further evaluation on this technique is necessary.

7. QUANTITATION METHODS

The key to the eventual utility and understanding of glycosylation in biology and disease diagnosis lies in the ability to quantitate protein glycosylation. Quantification of protein glycosylation may be performed either at the relative or at the absolute level. Moreover, it may be determined at the glycan, the glycopeptide, or the glycoprotein levels, either in the forms of mixtures or a single glycoprotein. There has a been recent debate as to what strategy could provide the most useful results,[371−373] with arguments being made specifically for the relative quantification of protein glycosylation,[372] which for discovery purposes may be easier to develop. However, relative quantification does not allow for identification of changes in total glycosylation and is generally not accepted for translation to clinical diagnostics.[373] Similarly, as we previously indicated for identification, the quantification at the glycan level may be performed with more glycan structural identifications than the quantification at the glycopeptide level. However, when just glycans are quantified, information on the protein and site of attachment is lost, which is important to determine biological significance.[371] Therefore, the quantitative strategy (relative vs absolute quantitation and at which level) should be tailored toward the hypothesis in question and the expected results.

Similarly, protein and glycosylation quantitations may be performed at different levels of confidence depending on the required application. For example, if one wishes to assess whether a glycosylation pattern can differentiate healthy cells from cancer-derived cells in a discovery experiment, the precision requirements are different from a clinical test where the glycosylation on a specific glycopeptide can detect a certain disease. In relative quantitation, the ion abundances are directly compared between the two groups of samples, and only relative differences expressed in fold changes are reported. However, in

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absolute quantitation, absolute concentration is required and important, for example, for clinicians to make diagnosis. Typically, a targeted mass spectrometry approach is used in the latter, while nontargeted, full spectrum approaches are used in the first analysis. Clearly, there is a large gap in terms of performance between the relative quantification in the first method and absolute quantification in the clinical test. To bridge this gap, performance criteria have been developed for the translation of proteomics tests from discovery methods (tier 3) to clinical tests (tier 1).[374] Similar criteria are applicable for the development and subsequent translation of methods for glycosylation analysis.

7.1. Quantitation of Glycan Profiles

An individual's glycosylation profile is dependent on the actual glycan structures present on the proteins but also on the (relative) quantitation of the proteins carrying the glycosylation. Initially, the focus of quantitative glycosylation analysis has been on the analysis of the released glycans from individual glycoproteins,[375] a subproteome,[376] or the entire glycoproteome[377,378] of an organism. In these studies, *N*- and/or *O*-glycans are released using PNGase F or chemical means and assessed for their compositional, structural and quantitative changes. However, a major disadvantage is the lack of information regarding the sites of glycosylation, which hampers linking differential glycosylation results to biological function.

Quantitative glycomics may be performed without labeling or relatively to labeled glycans. Song et al. employed a label-free approach using nanoLC-Chip-Q-TOF-MS in combination with an in-house serum glycan library to simultaneously identify and quantify over 170 *N*-glycans from human serum.[9] The relative abundances of individual glycan structures were obtained (Figure 26). Similar label-free approaches have been successfully applied toward the identification of candidate markers for a large number of health and disease states.[332,377,379−383] However, the information on the glycans quantified using these approaches is only as good as its qualitative properties. Recent focus has especially been on improving the quantitation of sialic acid containing glycans and linkage specificity of sialic acids (α(2,3) vs α(2,6)-linked residues). Traditionally, permethylation has been used as a method to neutralize sialic acids and improve their stability in MS,[103] but variation in the number of methyl groups attached hampers this approach. More recent approaches enhance the analysis of sialic acid containing peptides by the selective modification of the sialic acid carboxyl group.[384] Using this same approach, linkage specific derivatization strategies were developed for sialic acids.[109] This derivatization approach might enable faster (relative) quantitative analysis of linkage specific differences, such as those identified on prostate specific antigen.[369]

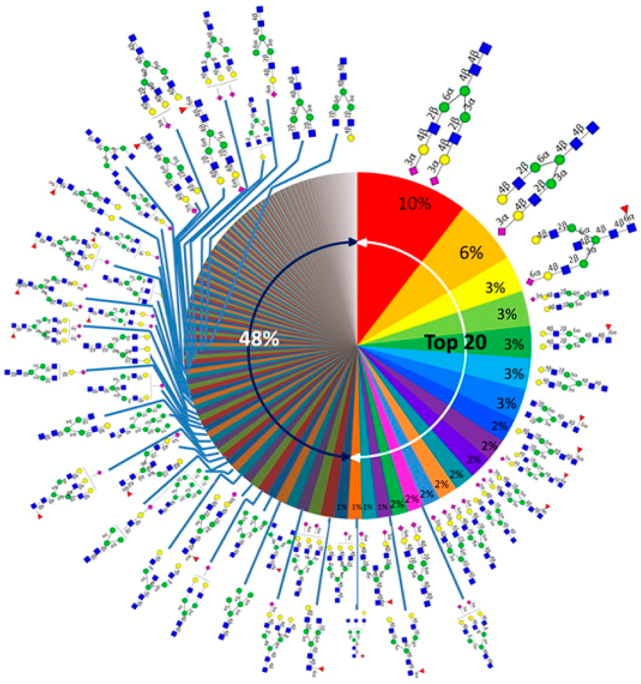

Figure 26. Relative abundances of human serum *N*-glycans averaged for nine individual sera. Reprinted with permission from ref 9. Copyright 2015 American Chemical Society.

To further enhance the relative quantitation of glycans, stable isotope enriched or more recently also isobaric labels have been introduced. These labels have recently been reviewed,[385] and the reader is referred there for a complete overview. Isotopic labeling strategies based on permethylation,[386,387] reductive amination using aminobenzamide[384,388,389] or alanine,[189,390−392] glycan reduction,[393] and ^{18}O incorporation by PNGaseF[393−395] have been reported for *N*-glycans. Similarly, a stable isotope labeled PMP labeling method was developed for *O*-glycans.[98,396] Reactions with tertiary amines,[397,398] $^{13}CH_3I/^{12}CH_2DI$ permethylation,[6] and most recently quaternary amines[399] were used to introduce isobaric tags. While these labeling strategies are valuable, they only allow for relative quantitation compared to other samples: ratios between the labels are being compared as representatives of the relative abundances of their reflecting glycans. As with all chemical modifications, the accuracy of the relative quantitation is highly dependent on the variability of the incorporation of the quantifying label.

An encouraging recent development is the synthesis of stable isotope labeled glycans (Figure 27).[400] By using the chemoenzymatic synthesis, a library of biantennary ^{13}C-labeled *N*-glycans with a mass increment of 8 Da was developed. The labeled glycans were quantified in absolute terms using NMR spectroscopy prior to their use as internal standards for the absolute quantitation of glycans derived from a monoclonal antibody by MALDI-TOF MS. The CVs obtained for the quantitation of eight glycans were below 14%. As pointed out by the authors, the complete liberation of *N*-glycans from the proteins by PNGaseF is a prerequisite for absolute quantitation using this method. It is anticipated that enlargement of the library will enable accurate quantitation of glycans in complex biological mixtures, which is required for the clinical application of glycans as biomarkers.

7.2. Quantitation of Glycosylation Site Occupancy

As stated previously, the decoration of proteins with *N*- or *O*-glycosylation is an enzymatic process that is dependent on the enzyme, glycosylation site, and substrate availability. Glycoprotein site occupancy may therefore influence its function. Quantification of glycosylation site occupancy may therefore reveal disease processes, and methods to do so were recently reviewed.[401]

The strategy that has categorically been used for the identification of differentially occupied glycoproteins is the analysis of deglycosylated peptides. In one approach, the glycans on the glycoproteins are irreversibly bound to beads, upon which the proteins are digested.[402] Nonglycosylated peptides are washed away, and the deglycosylated peptides are then released

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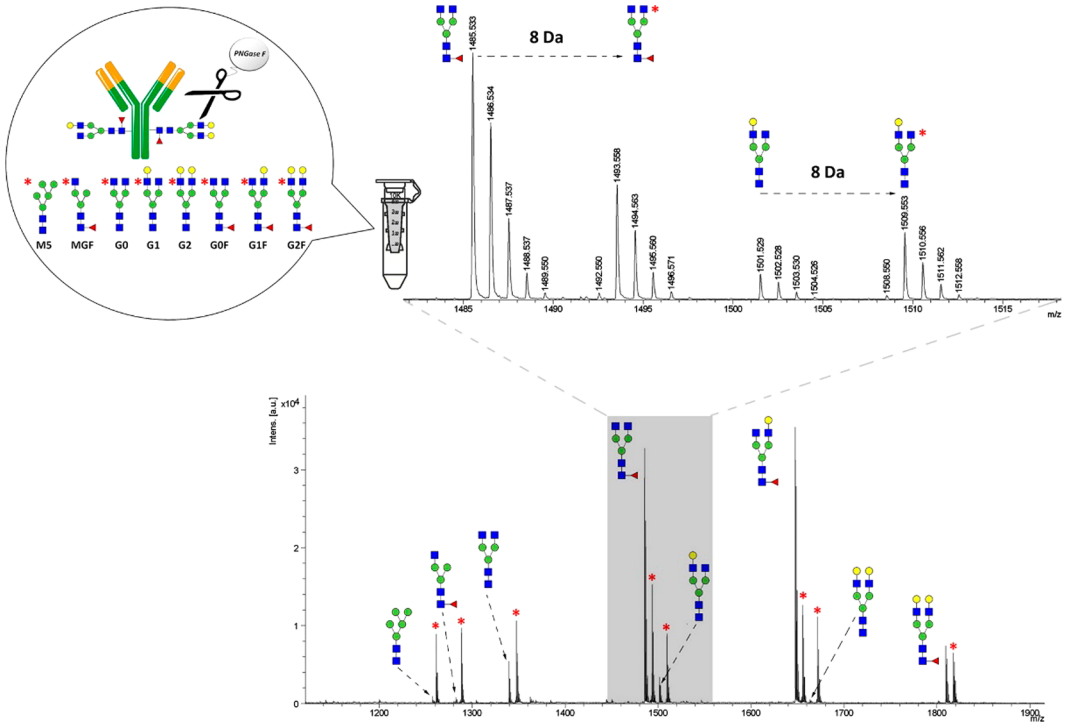

Figure 27. MALDI-TOF MS quantitation of IgG1 glycans using biantennary ^{13}C-labeled *N*-glycans as internal standards (marked with an asterisk). Reprinted with permission from ref 400. Copyright 2015 American Chemical Society.

from the beads using PNGaseF, thus removing the full glycan. Typically, this strategy has been employed in complicated samples such as tissue samples or other body fluids.[210,403] Recently, automated methods for the identification and label-free relative quantitation of site occupancy have been developed using data independent strategies such as SWATH[404] and SWAT, a targeted DIA approach.[405] In a comparative study, SWAT outperformed SWATH for the quantitation of peptides in linearity and sensitivity, and more deglycosylated peptide/peptide pairs could be quantified to assess the occupancy of the glycosylation site. The authors indicated that this was mainly due to increased specificity.[405]

The incorporation of an isotopic label could improve the quantitation of glycosylation site occupancy. A strategy using ^{18}O labeled Asp has been introduced for the potential diagnosis of CDG type-I patients[406] and was shown to differentiate patients with different levels of severity of type-I CDG. More recently, a targeted PRM based method for the quantitation of site occupancy of 43 sites of glycosylation was developed according to performance criteria established by the Clinical Proteomic Tumor Analysis Consortium (CPTAC).[210] Heavy stable isotope labeled peptides which incorporated ^{13}C and ^{15}N isotopes at the C-terminal K or R position of each tryptic peptide were used as internal standards, and site occupancy could be quantified with total assay CVs below 5%.

Importantly, the spontaneous deamidation of asparagine has been reported to occur during sample preparation.[407,408] Therefore, the strategies where PNGaseF is used for de-*N*-glycosylation may result in incorrect quantities due to spontaneous deamidation of asparagine residues, which may overestimate the glycosylation occupancy.[408] An alternative strategy has recently been reported where the unglycosylated portion of the glycoprotein is measured relative to the protein concentration.[409] By including SIL peptides for both the unglycosylated peptide as well as the peptide used for protein quantitation, the effects of deamidation are compensated, and by using this strategy, the site occupancy of fetuin was reported to be significantly lower than with a PNGaseF based deamidation strategy. A similar strategy was used to assess the site occupancy in a fusion protein.[410] Another strategy to perform glyan site occupancy is to use Endo H and/or Endo D exoglycosidases to deglycosyate glycoproteins. This approach performs partial deglycosylation while leaving single or small glycan moiety attached to the protein. Cao et al. used Endo H and PNGase F to quantify site occupancy in HIV-1 envelope glycoproteins.[411] With this strategy, different masses were obtained for glycopeptides with high mannose, complex, and no glycan.

Also, absolute quantitation of site occupancy is determined by using MRM. In summary, glycoprotein is proteolytically digested prior to deglycosylation with PNGase F. The signal intensity of the standard containing deglycosylated and nonglycosylated peptides were used to calculate the site occupancy. Yang et al. used this method to obtained site occupancy of recombinant monoclonal antibody drugs.[21]

7.3. Quantitation of Glycopeptides

The identification of protein- and site-specific changes in glycosylation provides a much more targeted approach compared to the analysis of all glycans from (a subset of) the glycoproteome.[371] The recent advances in the identification of glycopeptides have also opened up new opportunities for the site- and protein-specific quantitation. However, the quantitative analysis of protein- and site-specific glycosylation is only recently gaining attention. Strategies for glycopeptides quantitation can be label-free or relative to labeled standards and may be data-independent or targeted. The earlier reports of glycopeptide quantitation comprise label-free methods, where glycoproteins are isolated and glycopeptide extracted ion signal intensities are normalized either to one glycoform[412,413] or to the sum of all glycoforms.[414] While the strategy is most often applied to the

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analysis of *N*-glycopeptides, it has also been applied to *O*-glycopeptides.[413,415] In a recent interlaboratory study on the identification and quantitation of glycopeptides from α-1-acid glycoprotein, the interlaboratory variability of label-free relative quantitation based on total glycopeptide abundance for the 10 major glycopeptides was shown to be <25%.[416] Recent advances in the technology and software have significantly increased the number of glycopeptides (both *N*-glycopeptides[332] and *O*-glycopeptides[299]) identified in complicated mixtures such as tissue or biofluids. It is anticipated that such techniques will soon also allow for label-free quantitation based on extracted ion chromatograms.

In an alternative strategy, different lots of human chorionic gonadotropin (HCG) were compared for their glycosylation pattern by using tandem mass tag (TMT 10-plex) labeling,[417] which is traditionally used in comparative proteomics studies. By alternating HCD/ETD/CID fragmentation, 1000 *N*- and *O*-glycopeptides could be identified in at least one of the HCG samples. Of these, 167 glycopeptides were significantly differentially expressed based on reporter signal intensities of the TMT tags. While such an approach is only suitable due to the three-way fragmentation, the study shows that traditional proteomics TMT labeling is suitable for relative quantitation of glycopeptides in less complex mixtures.

7.3.1. Targeted Methods for Glycopeptide Quantitation. Targeted mass spectrometry techniques, specifically multiple reaction monitoring (MRM) on triple quadruple instruments[418] and parallel reaction monitoring (PRM) on orbitraps,[419] have been widely adopted in the proteomics community for the quantitative evaluation of proteins in clinical specimens. The application of targeted MS strategies in combination with stable isotope labeled (SIL) peptides[420] is now starting to be adopted for quantitation of proteins in clinical chemistry laboratories.[421,422] The application of targeted methods for (relative) glycopeptide quantitation is still in its infancy and was recently reviewed.[423,424] It could be concluded that MRM in combination with glycoprotein enrichment improves sensitivity and specificity of the quantitation of minor glycoforms, specifically after optimization of the instrument settings.

In targeted mass spectrometry approaches, the fragmentation behavior of the analyte is of great importance. Currently, the fragmentation of glycopeptides in targeted approaches is typically performed using CID. It is well established that the glycosidic bonds are more labile in CID than peptide bonds, resulting in fragmentation spectra dominated by B-type oxonium ions (glycan fragments) and potentially low amounts of fragments of the glycosylated peptide (y-type ions) (Figure 6).[38,425] This is different for oligomannose type glycopeptides, where CID fragmentation results in highly intense y-type ions, and B-ions are of much lower intensity.[20]

Therefore, initial studies toward the quantitation of glycopeptides employed the use of B-type oxonium ions (m/z 204, 274, 292, 366, 512, and/or 657). While these fragments are common to all glycopeptides, and thus do not provide high specificity, it was shown by Hong et al. that this strategy was suitable for the accurate quantitation of glycopeptides from IgG, IgA, and IgM directly from serum,[20,38] and later, Ruhaak et al. expanded the method with six other abundant glycoproteins (Figure 28).[426−429] A similar approach was used to quantify glycopeptides from human milk.[430] Similarly, B-type oxonium ions were applied for the quantitation of glycopeptides from haptoglobin isolated from serum.[431] In an alternative approach, more specific

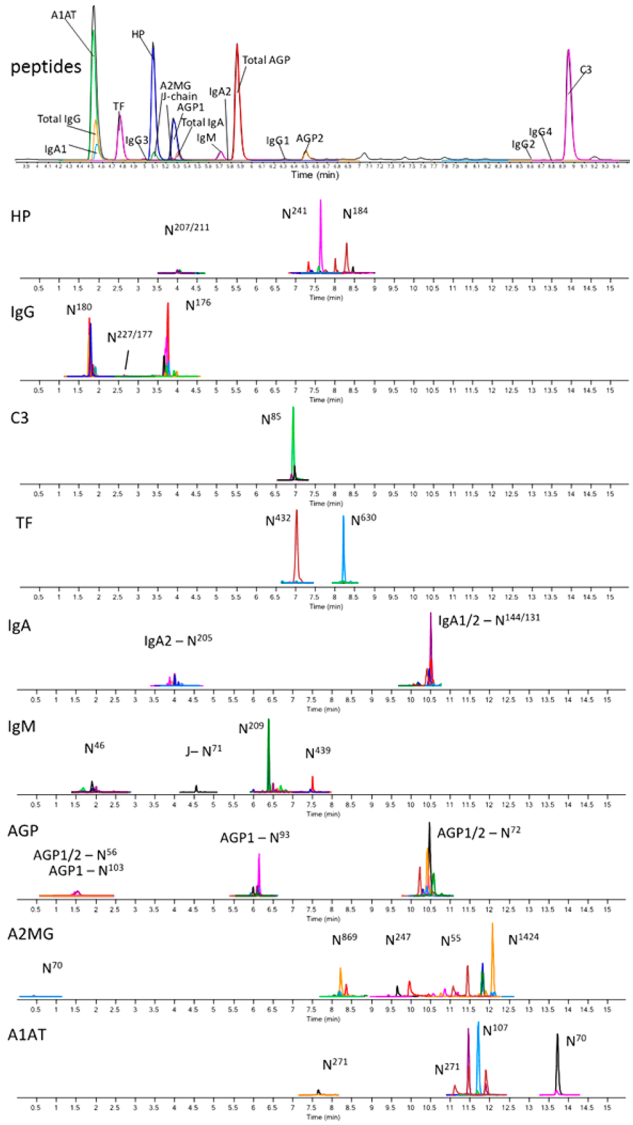

Figure 28. LC-MRM MS chromatograms for the relative quantitation of glycopeptides from abundant serum proteins. Both peptides (top) and glycopeptides can be monitored. Adapted from ref 427. Copyright 2018 American Chemical Society.

Y1 fragments were monitored to quantify glycopeptides from mouse serum.[432] More recently, a strategy in which Y0 fragments are monitored was proposed, because the Y1 fragments contain the innermost GlcNAc, which may be fucosylated, thus resulting in Y1+F fragments.[433] These fragment ions have also been used in DIA approaches for glycopeptides.[434,435]

However, similarly to quantitation of glycans, a reference is required to allow for the comparison of quantitative results between samples. Here, several approaches have been employed. One could argue to assess the glycosylation profile independent of the protein concentration; therefore, quantitation relative to the protein amount, as determined by the measurement of a nonglycosylated peptide, was employed. Using this strategy, differential glycosylation patterns could be identified in serum samples from ovarian cancer patients compared to healthy controls[426] as well as in samples from liver disease.[431,436] Alternatively, ratios between glycopeptides were used to identify altered expression of glycopeptides in patients with colorectal

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cancer and hepatocellular carcinoma,[437] and results were comparable to label-free MALDI-FTICR quantitation.

Recently, the use of isotopically labeled internal standards for the quantitation of glycopeptides from mABs was reported.[433] Tryptic digestion was performed in either $H_2{}^{16}O$ or $H_2{}^{18}O$ to induce a 2 Da mass difference, and glycopeptides were then analyzed using targeted mass spectrometry on a high-resolution instrument (triple TOF). Indeed, this is a promising strategy, but it has to be mentioned that a mass shift of 2 Da is limited and as such requires the use of high resolution instruments. The application of targeted methods for quantitation of glycopeptides is a promising strategy. However, for accurate absolute quantification and adoption in clinical chemistry tests, stable isotope labeled glycopeptides will be necessary.

7.3.2. Importance of Enzymatic Digestion. As with the quantitation of proteins,[374,438] the stable and complete enzymatic digestion of glycoproteins is of the highest importance for quantitation of protein glycosylation by means of its glycopeptides. While in protein quantitation a choice for an ideal peptide can typically be made, this is not the case for glycopeptides, where the specific peptide with the glycosylation site needs to be quantified. Specifically, the glycosylation may hamper or resist proteolytic digestion.[439] Recently, the preferential tryptic cleavage of high mannose, hybrid, $\alpha(2,3)$-sialylated, and bisected glycoforms on IgG was observed over the other most abundant neutral, fucosylated glycoforms.[440] This was most profoundly seen in native protein, but was not completely diminished by protein denaturation. Furthermore, mucins, which are highly *O*-glycosylated, have been reported to strongly resist proteolytic cleavage, which may even be part of their biological function.[441] Therefore, the efficiency of proteolytic cleavage, as well as glycopeptide degradation, needs to be taken into account when employing glycopeptides to quantify protein glycosylation.

Similarly, the variations in ionization efficiencies and fragmentation behavior between different glycopeptides are concerns for label-free relative quantitation. While the ionization efficiencies seem to be rather similar between neutral and sialylated glycopeptides in positive mode ESI-MS, MALDI based analysis may be somewhat more problematic as signals of sialylated glycans may be reduced due to ion suppression or in-source decay. Furthermore, when using MRM or PRM, fragments may form more or less easily, for example, fragment m/z 366 will form twice as much from a glycopeptide carrying a biantennary nonsialylated glycan than from a glycopeptide carrying a monoantennary glycan.[38] Therefore, care needs to be taken when representing relative abundances based on mass spectrometry results.

7.4. Quantitation of Glycoproteins

The quantitation of intact glycoproteins is still in its infancy, although it is anticipated that methods currently being developed for the quantitation of intact proteins will be equally applicable to intact glycoproteins. Because it is still not known what the specific effects of glycans on the ionization efficiency of glycoproteins are, the quantitation of the different glycoforms based solely on ion abundances may be limited. This approach cannot yet be applied to complicated mixtures, but approaches where glycoproteins are captured using antibodies, such as that currently applied to transferrin and apolipoprotein C-III[442,443] using mass spectrometry based immunoassays (MSIA),[444] have shown encouraging results. The technique is promising and may have more immediate applications with both individual glycoproteins and enriched fractions.

8. TOWARD RAPID-THROUGHPUT ANALYSIS

Although structural analyses of glycans and oligosaccharides remain challenging, there have been notable efforts in developing rapid-throughput methods. Rapid-throughput structural elucidation is likely not possible for oligosaccharides or any class of biomolecules, however, rapid identification can make rapid-throughput glycomics possible. This method has been used to study human milk oligosaccharides and childhood stunting. Totten et al. employed an HMO library created by Wu et al.[11,445] containing HPLC retention times and accurate masses to profile with quantiation HMOs from human milk samples.[446] They found that stunted children had mothers milk that were lower in fucosylated, sialylated, and total oligosaccharides compared to nonstunted subjects.[447] The analysis monitored over 300 structures in each run.

To observe cancer or disease specific changes in glycans and glycoproteins for discovery of biomarkers, large-sample-size studies are also needed, which further necessitates rapid-throughput quantitative methods for glycomics and glycoproteomics. Untargeted glycoproteomic mapping for large sample sets is still a challenge due to the lack of robust data processing software to identify and quantify glycopeptides concurrently in a biological sample. Although stable isotope labeling methods introduced above such as TMT 10-plex have been applied to the quantitation of glycopeptides, it could only be performed in 10-sample sets.[448] Rapid-throughput analysis can be performed with MRM in a targeted effort by quantitating glycosylation with site specificity in proteins. The speed and robustness of this methodology make it ideal for large sample sizes. It has been implemented to monitor site-specific glycosylation in serum proteins for ovarian cancer biomarker discovery in a study involving 168 subjects. A biomarker panel containing 11 glycoproteins was discovered and proved to increase the accuracy of ovarian cancer prediction when combined with CA-125, the most widely used biomarker for ovarian cancer.[426,427] This MRM method for quantitation of immunoglobulin glycopeptides also provides an ideal tool to study autoimmune-associated glycan alterations for autoimmune biomarker discovery.[449]

While methods for rapid and global glycoroteomic analysis need further improvements, rapid-throughput methods for released glycan analysis employing various platforms have been developed and employed to discover and validate disease biomarkers. MALDI-MS is one of the most widely used methods for rapid analysis and has recently been applied to the discovery of glycan biomarkers for various diseases such as gastric cancer[450] and hepatocellular carcinoma[451] with over 200 subjects. Furthermore, with emerging high-throughput permethylation and derivatization methods, the stability and ionization efficiency of sialylated glycans can be greatly improved.[109,452] UPLC with fluorescence detection is another technology for the analysis of the glycome in a rapid-throughput manner. Pucic et al. combined this method with a novel 96-well plate IgG isolation platform and applied it to characterize 2-AB-labeled *N*-glycans released from isolated IgG from plasma of 2298 individuals.[453] High variability in the 24 chromatographically separated glycans was observed between individuals. For simultaneous quantitation and in-depth structural characterization of glycans in complicated biological samples, however, high-throughput LC-MS methods are necessary. Ruhaak et al. demonstrated that chip-based nanoLC-TOF-MS is a highly stable and repeatable platform for

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large-scale serum *N*-glycan studies.[39] This platform was applied to an epithelial ovarian cancer cohort with approximately 300 samples[379] and a nonsmall cell lung cancer cohort with over 600 samples[454] for glycan biomarker discovery.

9. CONCLUSIONS AND FUTURE DIRECTIONS

New analytical methods and techniques are advancing glycomic and glycoproteomic analyses at a rapid rate. The next step for glycomics and glycoproteomics is to achieve rapid-throughput analysis with quantitation. Glycomic analysis has initially focused on obtaining monosaccharide composition. When used with the known biological synthesis, it could provide putative structures particularly for *N*-glycans, which is sufficient for determining whether a compound was fucosylated, sialylated, or mainly a high mannose type. However, it yielded little information regarding the sequences or the linkages of glycans. For complete structures, chromatographic separation is necessary to profile components of the glycome. However, de novo structural analysis of oligosaccharides is not feasible in a rapid-throughput manner. In this regard, comprehensive structural analysis may take the same path as proteomics and metabolomics, that is, creating a reference library of structures and identity elements for each entry. Toward this end, the creation of structural libraries with annotated structures and identifiers such as chromatographic retention times and tandem MS spectra would advance this process considerably.

Glycoproteomic analysis can now provide simultaneous identification of proteins and characterization of the associated glycoforms in an automated manner. As the bioinformatic software improves, characterization of site heterogeneity will improve, providing more sites and more glycoforms. The more challenging aspect will be the structural analysis of glycans associated with specific sites. This should be the target of future efforts.

The analytical methods are nonetheless sufficiently advanced to solve a host of biological problems in glycobiology. What is most welcomed is for those with little expertise in separation or mass spectrometry to take advantage of these tools and solve problems in diseases and fundamental biology. It is perhaps in this last challenge, where the greatest obstacles will be.

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Notes

The authors declare no competing financial interest.

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Gege Xu studied chemistry at Peking University in China, where she received her B.S. degree in 2013. She is currently completing her Ph.D. research at the University of California, Davis, under the supervision of Dr. Carlito B. Lebrilla. Her studies focus on developing qualitative and quantitative methods for the analysis of dietary saccharides and cell surface glycans and glycoproteins using LC-MS/MS.

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Elisha Goonatilleke received her B.S. in 2013 and continued with her Ph.D. at the University of California, Davis. Currently, she is a graduate student in the group of Dr. Carlito B. Lebrilla. Her field of study is in analytical chemistry with an emphasis on mass spectrometry. She focuses on the identification, characterization, and quantification of glycoproteins and oligosaccharides in human milk.

Carlito B. Lebrilla completed his B.S. from University of California, Irvine, and his Ph.D. from University of California, Berkeley. He was a NATO-NSF and an Alexander von Humboldt postdoctoral fellow with Prof. Helmut Schwarz at the Technical University in Berlin. He was also a UC President's postdoctoral fellow at the University of California, Irvine. He has been on the faculty at UC Davis since 1987 and is currently a Distinguished Professor in the Chemistry Department and in Biochemistry and Molecular Medicine. He is on the editorial board of several mass spectrometry journals. His research focus is on bioanalytical chemistry with an emphasis on oligosaccharides and glycoconjugates.

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ABBREVIATIONS

2-AA	2-aminobenzoic acid
2-AB	2-aminobenzamide
AI-ECD	activated ion ECD
CE	capillary electrophoresis
CID	collision induced dissociation
ECD	electron capture dissociation
EED	electron excitation dissociation
ETD	electron transfer dissociation
EThcD	electron-transfer/higher-energy collision dissociation
ERLIC	electrostatic repulsion hydrophilic interaction chromatography
ESI	electrospray ionization
FTICR	Fourier transform ion cyclotron resonance
HILIC	hydrophilic interaction liquid chromatography
HPLC	high performance liquid chromatography
HR	high resolution
IRMPD	infrared multiphoton dissociation
LAC	lectin affinity chromatography
mAB	monoclonal antibody
MALDI	matrix-assisted laser desorption/ionization
MRM	multiple reaction monitoring

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MS	mass spectrometry
PNGase F	peptide-*N*-glycosidase F
PGC	porous graphitized carbon
QQQ	triple quadrupole
RPLC	reversed-phase liquid chromatography
TOF	time-of-flight
UPLC	ultrahigh performance liquid chromatography
UVPD	ultraviolet photodissociation

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