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High-sensitivity Analytical Approaches for the Structural Characterization of Glycoproteins

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1. INTRODUCTION

1.1. General Considerations

Structural intricacies of carbohydrate molecules and their propensity to form varied linkages, substitutions, and branching patterns have fascinated many generations of chemists, as have the three-dimensional aspects of carbohydrate interactions with other biomolecules. The steadily increasing biochemical knowledge in this area has further added to the increasing importance of the field now referred to as “glycobiology” or, more generally, “glycoscience”. Yet, most of the emphasis over the last 50 years or so has been on two other classes of important biopolymers, namely nucleic acids and proteins. However, in the “post-genomic era”, complex carbohydrates can no longer be neglected, as it is becoming clear to many scientists that most mammalian proteins are glycosylated, and microbial systems and plants can have their own unique monosaccharide building blocks and special ways they can be interconnected and branched into unusual structures. Throughout evolution and the development of living organisms, glycoconjugates must have played major roles, no doubt due to their unusual biological selectivities, which, in turn, could well be due to the enormous information capacity of the “sugar code”.[1,2]

Throughout the 1980s, the multilateral importance of glycoconjugates in biology and medicine was recognized,[3-6] albeit with an understanding that only new methodological approaches and systematic investigations would further define new vistas and provide intimate knowledge of how complex carbohydrates participate in all life processes. Today’s glycoscience is a multidisciplinary undertaking in which chemistry is expected to have an important role to describe the most complex structural aspects of sugars and their conjugates with other biological molecules. While the biological and biomedical relevance of studying glycosylation and sugar–protein and sugar–sugar interactions will undoubtedly be guided by

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advances in other respective fields (immunology, cancer research, parasitology, cell biology, and developmental biology, among others), the chemical disciplines' two major tasks are to (a) isolate and structurally characterize biologically important glycoconjugates and (b) synthesize carbohydrate structures for biochemical investigations, enabling technologies and medical applications and providing new therapeutics. While the goals and directions of carbohydrate synthesis have been summarized elsewhere,[7-11] the focus of our review has been on *glycoanalytical chemistry*. The synthetic and bioanalytical directions are not mutually exclusive, as new structural findings will undoubtedly provide further rationale for synthetic efforts and these, in turn, the availability of standards for structural verification.

Since publication of the review on structural investigations of glycoconjugates at high sensitivity[12] in these pages a decade ago, the field of analytical glycobiology has seen dramatic changes in its scope and depth. It is widely appreciated within the glycoscience community and increasingly by others that both new techniques and instrumentation and the established (albeit optimized) analytical methodologies have played very important roles in advancing the science of glycoconjugates to its current stage. Due to their different physical and chemical characteristics, the main classes of glycoconjugates, i.e. glycoproteins, glycolipids, polysaccharides, and proteoglycans with their highly charged constituents, glycosaminoglycans, demand somewhat specialized analytical and structural elucidation approaches. Our review will largely be focused on *glycoproteins* and their associated *glycans*, hoping that other scientists will describe the analytical aspects of the remaining glycoconjugate biomolecules elsewhere.

The early advances in proteomics, the scientific area mostly preoccupied with identification and structural characterization of proteins, have led to diverse activities in protein post-transitional modifications (PTMs), which are often associated with important biological activities. Glycosylation of proteins is arguably the most widely spread and functionally most intriguing PTM in nature. It is already known that certain glycosylation patterns in proteins give rise to functional variance, with far-reaching consequences for health-disease issues, immunological disorders, toxicity effects, microbial invasion processes, etc. To investigate any of these highly important processes in sufficient molecular detail, analytical techniques capable of a high degree of structural elucidation and measurement sensitivity are currently needed.

Within the plethora of new "-omics fields" (genomics, transcriptomics, lipidomics, metabolomics, etc.), the fields of *glycoproteomics* and *glycomics* have started to assume their respectable roles. Analytical glycobiology, representing both glycomics and glycoproteomics, now shares access to new measurement technologies that enable characterization and quantification of molecular processes in living organisms. Extensive glycomic and glycoproteomic data that can nowadays be generated with modern techniques and instrumentation are likely to enrich the "systems biology" approach.[13-17] Both fields have started to contribute substantially to a better understanding of multicellular interactions in eukaryotic systems and important issues pertaining to human health and disease.[18-23] Additionally, the long-held view that glycosylation is unimportant in prokaryotic systems is no longer defensible.[24,25]

Since our previous review[12] in this journal, much progress has been achieved in terms of methodological developments toward better, more informative, and more sensitive measurements of glycoproteins and their glycan components. In addition, many conceptually important applications of new tools already point to the future needs for dealing with the enormous complexity of glycopeptides and oligosaccharide mixtures extracted from biological tissues and physiological fluids. The relatively recent interest of the pharmaceutical and biotech industries in recombinant glycoproteins, such as monoclonal antibodies, for treatment of cancer and other diseases,[26-30] demands the use and further development of glycomic and glycoproteomic analytical procedures as well. Similarly to our previous report,[12] the current review has been organized to discuss separately recent advances in glycoproteomics and glycomics, dealing first with the isolation and direct analysis of glycoproteins, followed by the description of advances in glycopeptide analysis and determination of the sites of glycosylation, and moving toward the analysis of complex glycan mixtures. Even more today than 10 years ago, mass spectrometry (MS) is the most prominent methodology in the arsenal of glycoprotein analysis tools. A number of new MS techniques, previously unexplored or insufficiently developed, are now at the center of attention of glycobiologists. At the sensitivity levels required by contemporary glycobiology, MS and tandem MS (MS^n) techniques are currently the only means to provide reliable structural information. Carbohydrate derivatization (chemical modification of carbohydrates at microscale) uniquely enables certain MS measurements in terms of enhanced sensitivity and structural information.

Due to the enormous “chemical space” for carbohydrate structural complexity,[1,2] MS alone, no matter how sophisticated, is unlikely to provide all needed answers. However, in combinations with modern separation methodologies (different forms of chromatography and electrophoresis) that provide unique component resolution in time and space, MS detection and identification capabilities become enormously enriched. The past decade has seen substantial improvements in the chromatographic analysis of complex carbohydrates: (1) transition from the conventional-scale columns to capillary column dimensions, or even microchips, with the resulting gains in mass sensitivity of measurements; and (2) rapidly increasing use of stable and reliable hydrophilic column materials and graphitized carbon adsorbents. Further advances in capillary chromatographic separations pertain to effective resolution of very complex mixtures as well as the frequently needed separation of different isomers. Chromatographic advances of the recent years also relate to simple purifications of samples (analysis steps now often referred to as solid-phase extraction, or SPE) or the more sophisticated microcolumn lectin or affinity materials needed in group separations and preconcentration of certain glycoproteins for analysis. The past decade has also witnessed a rapid development of glycan array technologies, in which the surface-bound glycan structures (either synthesized or isolated from natural mixtures) are presented to glycan-binding proteins in biological samples.[31-33] While these enabling technologies are novel and exciting, they will not be covered in this review, which primarily emphasizes techniques leading to structural elucidation of glycoproteins. Likewise, immunologically based measurements will not be discussed.

1.2. Biological and Medical Rationales for Investigating Glycosylated Structures

Glycosylation provides additional structural diversity to the already specialized protein molecules. Besides the relatively simple roles of glycans in protein folding, the displays of glycans on protein surfaces and different glycosylation sites all result in very sophisticated structures needed for multilateral functions that glycosylated proteins assume in biological cells. Through the additions of different monosaccharide units during the enzyme-catalyzed biosynthetic steps, very precise structural entities are formed and further processed inside a living cell through a fine-tuned action of various specific glycosidases and glycosyltransferases.[34,35] In a eukaryotic cell, glycosylated proteins are found in virtually all cellular compartments, serving different biomolecular functions. Many glycosidases and glycosyltransferases responsible for the assembly and modification of glycans in the endoplasmic reticulum and the Golgi apparatus have been genomically identified. According to the recent estimates,[36] approximately 2% of the human genome encodes proteins dedicated to biosynthesis and degradation of glycans. A fairly detailed account on glycosyltransferases and their genetic basis has been provided.[37] However, while transcriptomic profiling[38] provides a useful tool to glycobiologists, painstaking progress toward understanding the different aspects of cellular glycosylation has largely been achieved due to a number of analytical tools for glycan structural characterization. As glycan biosynthesis is not directly subjected to a template-driven process, there are interpretation difficulties for transcriptomics, giving further credence to the value of direct bioanalytical measurements which can ascertain a structural type and its precise quantification.

In a brief, eloquent review, Hart and Copeland[39] capture the current understanding of the importance of glycans in mammalian cellular biology: inside a cell, glycans regulate quality control, turnover, and protein trafficking among organelles, and additionally, through their dynamic, reversible nature, O-linked *N*-acetylglucosamine (*O*-GlcNAc) entities participate in signaling, gene expression, and response to stress.[40,41] Considerably more complex glycan structures are incorporated on the cellular surface in different receptor functions, controlling cell growth, cytokinesis, and cell differentiation, but also cell-cell recognition, cellular adhesion, and metastasis. It is now well established that certain glycans on cellular surfaces are recognized by bacteria and viruses, including the extensively studied HIV infection-related phenomenon.[42-44] The question arises as to how many specialized glycan structures, their combinations, and structural arrangements are needed to fulfill the myriad of tasks of a specialized mammalian cell. From the extremely high number of glycan structures that the cellular machinery might hypothetically produce, the functional arguments[45] seem to restrict the numbers to less than 10,000 structures, which is still a considerable task for analytical profiling and measurements. Knowing the distinctly selective carbohydrate structures will, in turn, facilitate detection and further characterization of carbohydrate-binding proteins which recognize oligosaccharides (soluble lectins, antibodies, enzymes, cell surface proteins, etc.), as has already happened during the past decade with the discoveries in the area of galectins, selectins, and siglecs. To find new types of glycan ligands through structural and bioanalytical investigations is both exciting and necessary for the future of the field.

Unusual types of glycosylation have been associated with human diseases, particularly cancer, for several decades. Numerous investigations in recent times link aberrantly glycosylated structures to many known diseases and metabolic disorders, ranging from congenital disorders of glycosylation[46] to cardiovascular disorders, diabetes, and cancer.[47] Cancer cells are known to evade normal growth by changing their surface glycan structures and thereby also avoid detection by the immune system.[48] Capturing glycoprotein disease biomarkers through different research strategies and determining the difference between glycosylation in physiological and disease states has now involved a great number of laboratories. While distinguishing other inflammatory diseases from cancer is currently problematic, the recent applications of glycomic profiling to different types of cancers appear encouraging for the future of diagnostic and prognostic measurements.[49,50] In these investigations, common physiological fluids (blood serum, plasma, cyst fluids, and urine) have nominally been used for highly sensitive analyses, but other biological materials, such as cell lines or tumor biopsies, may also be applicable.

During the past decade, substantial strides have been made toward a more complete understanding of the roles of glycosylation in the immune system. The diverse set of cells mediating both the innate and adaptive immunity engage glycoproteins with both *N*- and *O*-linked glycans.[51] It is now becoming evident that the immune cell differentiation, activation, and death are associated with substantial changes in glycosylation. The glycan modifications appear tightly controlled: even a slight structural modification in IgG, such as an addition of a sialyl residue, can convert this key molecule from a pro-inflammatory to an anti-inflammatory agent.[52] In particular, sialylation and fucosylation of key structures in different immunologically active cells[51] can involve different glycan isomerism, driving cell-to-cell recognition and binding of the key lectins (siglecs, C-type lectins, and galectins).

The recent reviews enthusiastically endorse the use of modern glycomic and glycoproteomic techniques to study the intricacies of the immune system.[53,54] Applications of highly sensitive MS have already been demonstrated in the structural characterization of neutrophils[55] and the comparative analyses of human eosinophils, basophils, and mast cells.[56] Not surprisingly, the most commonly studied molecule is currently IgG, with its different chains,[57,58] but highly sensitive MS-based approaches will undoubtedly be applied to other immunoglobulins in the near future. The characterization and in-depth comparative studies of the less abundant Ig isotypes can benefit from preconcentration through microaffinity systems.[59] Glycobiology of the immune system is clearly a frontier scientific area necessitating application of the best -omics technologies for both the benefits of a better understanding of important healthrelated issues and developing targeted therapeutics.

Whereas glycomics and glycoproteomics have been most actively pursued methodologies in the studies of mammalian (multicellular) systems, the new investigations on glycosylation in prokaryotic systems are advancing rapidly. Apparently, the glycoproteins of bacteria can feature both O-linked and N-linked glycosylation and usual monosaccharides in their glycan structures.[24,25,60-64] A further increase of interest in the area of microbial and parasitic glycobiology is expected due to the importance of host-parasite interactions based on sugar recognition.

As mentioned previously, the biotechnology industry has presented a need for robust analytical methods that are both sensitive and highly reproducible to measure the glycosylation profiles of purified proteins, particularly for monoclonal antibodies. Antibodies have emerged as a promising class of therapeutics in oncology, chronic inflammation, cardiovascular, disease, and infectious diseases. The attached glycans play important biological and physicochemical roles such as resistance against proteases, elongation of the circulatory halflife *in vivo*, and potential antibody-dependent cytotoxicity.[65-69] Furthermore, antigenic epitopes potentially can be introduced during the cell line-based manufacturing process, which involves nonhuman animal materials.[70] Currently, a large number of publications can be found in the literature describing various methods for the analysis of recombinant antibody glycans. Most of these have been developed with the highperformance liquid chromatography (HPLC),[71,72] capillary electrophoresis with laser-induced fluorescence (CE-LIF),[70,73-76] and liquid chromatography–mass spectrometry (LC-MS)[72,77-80] analytical platforms, though some have described matrix-assisted laser-desorption/ionization mass spectrometry (MALDI-MS)[79,81] or capillary electrophoresis-mass spectrometry (CE-MS)[82-84] approaches. While HPLC and CE-LIF-based techniques can provide excellent quantitative reproducibility, as well as resolution and sensitivity, a further need for direct structural identification leads to the pursuit of the tandem techniques that incorporate mass-spectrometric detection schemes.[85-87] According to current guidelines, antibody pharmaceuticals must be demonstrated to meet applicable quality requirements to ensure safety, purity, and potency, including an examination of glycan distribution and the potential impacts of glycoform on function,[88] but there are no specific regulations for the glycan content of these important biologics. It is possible that, as the research community gains a deeper understanding of the physiological roles of specific glycans, the standards for regulation of antibody pharmaceuticals could become more stringent and, thereby, further drive the development of robust and optimized approaches to thoroughly characterize glycosylation of the purified proteins.

Since specific glycan expression is a characteristic feature of all developing biological tissues, it is not surprising that mass spectrometry is finding its applications across different fields of developmental biology, analyzing the samples from different model organisms (bacteria, yeast, drosophila, plants, fish, etc.). Together with the increasing capabilities to clone and knock out glycosylation-related genes, MS and the use of isotopic labeling are rapidly advancing the knowledge of these fields. This situation has been acknowledged in a review article by Wilson et al., found in the book edited by Gabius,[2] and particularly featured in a study of the *N*-glycosylation developmental aspects of the *Drosophila melanogaster* embryo.[89]

2. CARBOHYDRATE STRUCTURES AND NOMENCLATURE

Carbohydrates are conjugated to their respective protein backbones through different amino acid side chains; the literature accounts for at least nine different residues[90] that are capable of accepting a monoor oligosaccharides. By far, the most commonly glycosylated residues are asparagine, which may have an oligosaccharide associated with its side chain amide nitrogen, resulting in the N-linked class of glycans, and serine and threonine residues, in

which the oxygen of their hydroxyl groups can have an attached carbohydrate. These are known as the O-linked class of glycans. Less commonly, oligosaccharides may be linked to several other amino acids. Among these infrequently glycosylated amino acids, N-linked glucose monosaccharides may be attached through a β-linkage to arginine residues of certain proteins expressed by sweet corn,[91] and some evidence exists that N-linked glycans may be attached to glutamine residues in recombinant immunoglobulins expressed in Chinese hamster ovary (CHO) cells.[92] Unusual amino acids associated with O-linked glycans may be found in collagens, where galactose monosaccharides are attached through a β-type linkage to hydroxylysine residues[4] and glycosylated hydroxyproline, modified by arabinose, is found in plant cell walls.[93] This amino acid has also been found to be galactosylated in wheat endosperm.[94] Another less commonly encountered type of glycosylation is C-mannosylation, in which a mannose monosaccharide is attached at the C-2 position of a tryptophan residue. This modification has been reported for several proteins, including ribonuclease A[95] and thrombospondin.[96] One of the most recently discovered amino acids to be amenable to glycosylation is cysteine.[97] In the glycopeptide sublancin, a glucose unit attached to its thiol side chain was shown to be critical for its antimicrobial properties.

Beyond simply being attached to different amino acids, N- and O-linked glycans have other notable differences. N-linked structures are most commonly found within the amino acid consensus sequence of NXS/T, where X is any amino acid except proline; while bacteria frequently have an extended sequon of D/EXNXS/T.[98] However, in rare cases, N-linked glycans have been observed outside of the consensus sequence, as in the case of recombinant bovine trypsin expressed in maize[99] and recombinant immunoglobulins expressed in CHO cells.[92] Conversely, O-linked glycans are not associated with a particular amino acid sequence. Therefore, any serine or threonine residue can be thought of as a potential site of O-glycosylation. However, in many mucin-like proteins, certain regions in their amino acid sequences tend to be enriched with serine and threonine residues and are heavily O-glycosylated.

Structurally, *N*- and *O*-glycans are also quite different. The N-linked glycans have a common chitobiose core composed of $(GlcNAc)_2Man_3$ (GlcNAc: *N*-acetylglucosamine; Man: mannose), and other monosaccharides extend from the α1-3- and α1-6-branched core mannose units, including Man, galactose (Gal), GlcNAc, fucose (Fuc), and sialic acids, usually *N*-acetylneuraminic acid (NANA) or *N*-glycolylneuraminic acid (NGNA), to complete the structure. In plants, xylose (Xyl) monomers are often present. On the basis of their specific structural features, N-linked glycans are subcategorized into three main classes. The high-mannose class of glycans is that in which the core mannose monosaccharides are branched and extended with only other mannose units, as shown in Figure 1. The so-called “complex” glycans are those that have GlcNAc monosaccharides attached to the core mannose units, creating branches or antennae, and they may be extended with galactose units and lactosamine structures (GlcNAc-Gal disaccharides) and capped with sialic acids. Fucose monosaccharides may be incorporated as substituents on either a branch or the core of these types of structures. Further structural diversity of this glycan class is introduced by attaching a β1-4-linked GlcNAc unit to the β1-4-linked mannose of

the core (Figure 1). A combination of the complex and high-mannose classes is known as the hybrid glycans (Figure 1). In contrast to the N-linked glycans, O-attached carbohydrates do not have a common core structure. Rather, there are eight routinely encountered cores that are represented in Figure 2. Similarly to the N-linked class, *O*-glycans may be branched and extended.

While most frequently an *N*-acetylgalactosamine (GalNAc) residue is used to anchor an O-linked glycan to the protein, other monosaccharides have also been shown to function in this role. In many cytosolic and nuclear proteins, a single GlcNAc moiety is directly attached to the side chain of a serine or threonine residue,[100] and this modification has been implicated as playing a critical role in cancer cell growth and proliferation.[101] O-linked fucose has been found in epidermal growth factor-like repeats and has been identified on thrombospondin,[96] while O-linked mannose units have been found in the human and rabbit forms of the protein α-dystroglycan.[102,103] Clearly, while the majority of both N- and O-linked glycans seemingly follow a set of biological “rules”, numerous exceptions do occur in nature, and while rare, the importance of their physiological roles cannot be denied.

Adding to the overall complexity of a given glycoprotein is the “microheterogeneity” for sites-of-glycosylation. For a population of given glycoproteins, rarely is a single site occupied only by a single carbohydrate structure; rather, multiple glycans are frequently associated with glycosylation sites. A classic example of a microheterogeneity is bovine ribonuclease B, which has a single site of glycosylation, yet it is modified by high-mannose glycans possessing five to nine of these monosaccharides. Further complexity is added, since some potential sites of glycosylation remain vacant, while others are only partially occupied. Still others are reversibly modified; that is to say, they are glycosylated during part of the protein’s life cycle and vacant at other times. Thus, a complete characterization of a glycoprotein is a truly daunting task when a thorough structural elucidation of the carbohydrates is required, along with locating the sites-of-glycosylation, their levels of occupancy, and their microheterogeneities.

Given the overall complexity associated with carbohydrates, it is important for researchers in the field to communicate accurately by displaying simple representations or “cartoons” of glycan structures. Two main notation schemes have been proposed:[104] the Consortium for Functional Glycomics[105] (CFG) nomenclature scheme and the Oxford–Dublin system.[106] Both notations use different geometric symbols to represent different monosaccharides, and both are found in the current literature. One of the attractive features of the CFG system is the recommendation to use the same shape for different monosaccharides that have the same mass. For example, hexoses are represented by circles and the different isomeric hexoses are indicated by different colors, such as green for mannose and yellow for galactose. Similarly, derivatives of a given monosaccharide are of the same color; for example, glucose and GlcNAc are both blue. Alternatively, since some confusion may arise in publications using black- and-white images, the Oxford–Dublin system recommends using different shapes for each monosaccharide. Additionally, linkage information is conveyed in this scheme by positioning extending residues at different locations on the carbohydrate (see Figure 1) to which they are attached, and the anomeric configuration, either α or β, is indicated by solid or dashed lines, respectively. Representations of oligosaccharides using either nomenclature

system may be quickly drawn using Glycoworkbench,[107] a tool developed by the EUROCarbDB initiative. This software tool, available at http://download.glycoworkbench.org/, has a number of other very useful functions.

3. GLYCOPROTEOMICS

Perhaps the greatest analytical challenge for glycoproteomic (and proteomic) investigations of biological mixtures remains the inherent complexity of the samples and the associated difficulties with detection, quantitative measurement, and structural characterization of low-abundant glycoproteins. For profiling hundreds to thousands of analytes, the use of at least two-dimensional separation technologies appears mandatory. In the case of glycoproteomics, methodologies that selectively isolate glycosylated species in these biological samples are vital, since they are otherwise frequently masked by nonglycosylated molecules during analysis. It is evident from the recent publications, methods and protocols, and reviews, that various combinations of LC, CE, and MS techniques are applicable in the search for the best protein and peptide mapping strategies.[108-110] Additionally, traditional 2-D gel electrophoresis and other modified gel-based methods continue to be utilized and further developed.[111-114] While these approaches are generally applicable to the field of proteomics (and glycoproteomics), they will not be covered in detail in this review, which will focus, rather, on techniques that have been developed specifically for the fractionation, enrichment, and preconcentration of glycoproteins and glycopeptides.

In both proteomics and glycoproteomics, the now routine immunodepletion strategies are employed for removal of the most abundant proteins. Unfortunately, due to the vast stratification of protein concentrations in materials such as blood serum (10+ orders of magnitude), this approach frequently reveals the next “layer” of proteins, while not greatly improving the detectability of the majority of underlying species.[115] However, immunodepletion can be nonetheless beneficial as a preliminary step in the excavation of minor sample components, especially when it is followed by enrichment strategies that target specific groups of (or even individual) glycoproteins. Affinity chromatography, employed for both enrichment and depletion, is now a vital component of most glycoproteomic analytical platforms.

A global glycoproteome-enrichment approach may be suitable for the initial profiling of a biological sample, while a semitargeted strategy is beneficial for examination of an already identified sub-glycoproteome of interest. General enrichment strategies exploit lectins or chemical groups that have an affinity for common glycan epitopes, such as constituent residues of the chitobiose core in N-linked glycans, for example. Conversely, rarer glycan moieties, such as tri- and tetra-antennary structures can be targeted with specific lectins,[116,117] thereby enriching a narrower spectrum of the glycoproteome. Through combinations of affinity chromatography and LC-MS/MS, a discovery of patterns or individual markers that discriminate biologically relevant sample populations, such as disease states, developmental stages, or genetically derived phenotypes, may be possible. Eventually, following the identification of specific glycoproteins that are of interest for a particular study, immunoaffinity chromatography can facilitate the isolation and characterization of individual glycoproteins. Readers interested in developing their own

immunoaffinity techniques are directed to the established body of literature, including two particularly informative and instructional texts.[118,119]

3.1. Lectin Affinity Chromatography

For several decades, lectins have been used to recognize carbohydrate moieties and employ these interactions in isolating glycoconjugates.[120] In 1970, Aspberg et al.[121] and Lloyd[122] first developed lectin affinity enrichment of glycoproteins. Its potential in a chromatographic format was demonstrated by Cuatrecasas and Tell,[123] who prepared lectin–sepharose media according to a previously described protocol from Cuatrecasas[124] and packed them in glass columns (i.e., repurposed Pasteur pipets). In this seminal investigation,[123] it was demonstrated that two lectins, wheat germ agglutinin (WGA) and concanavalin A (Con A), could immobilize insulin receptors of liver cell membranes, while washing them with large amounts of buffer, and then force an elution by the application of buffers containing competitive monosaccharides specific to each lectin, an approach that is still successfully practiced in today's laboratories, albeit at much smaller dimensions in many cases. One of the primary advantages of lectins for glycoanalysis is the functional diversity of this class of glycan-binding molecules for a variety of different carbohydrate motifs.[125-127] In a recent review, Fanayan et al. estimated that there are 160 easily obtainable lectins, with more than 60 being commercially available.[128] In principle, it should be possible to select lectins for any particular application, based on their unique binding properties, which can be advantageous for an enrichment of a subset of the glycomolecules in a richly and diversely glycosylated biological sample. Additionally, multiple lectins may be used either in a serial enrichment[129,130] or simultaneously, as in a multilectin enrichment format[131] to provide further control of the contents of the enriched sample pool(s). While characterizations have been performed to determine the specificities of the most commonly used lectins, such as Con A,[132] the binding specificity of many is not firmly established because of the difficulty associated with generating a comprehensive set of carbohydrate probes. Moreover, the majority of studies thus far were conducted in the 1970s and 1980s, while more recently it has become possible to evaluate specificities with wellcharacterized, complex glycans that closely resemble the target structures in human-derived samples.[127] Nonetheless, the research community has done much to describe the specificities of those lectins that are commonly useful (Table 1). Frequently, comparative analyses are performed to demonstrate the preference for one structure over another, which can give an indication of the expected performance of a lectin for a particular enrichment application.[133,134] A common misconception and potential pitfall for experimentalists, however, is to assume that a lectin with a described glycan specificity will effectively enrich any glycoproteins known to display the target glycan, with no additional considerations for the protein–protein interaction.[135] It is important to account for the orientation of a glycan in relation to the tertiary and quaternary structure of the protein on which it resides. As a prominent example, human IgG, which displays a number of complex biantennary glycans that are readily captured by Con A in their unattached state, is not efficiently enriched by the lectin,[136-139] as the glycans are primarily displayed in the Fc domain of the antibody, where they are intertwined with the polypeptide backbone and only minimally solvent-accessible. Therefore, when possible, a lectin's ability to enrich a target glycoprotein from a complex sample should be tested before proceeding with enrichment of precious samples.

While lectins have been used at length for glycan-oriented enrichment procedures in the past, their potential as highperformance, quantitative bioanalytical research tools has been developed only during the last several years. The implementation of lectin stationary phases immobilized on rigid support materials, such as silica particles[140,141] and monolithic polymers,[142] has provided suitable affinity columns for on-line multidimensional LC glycoproteomic platforms that operate at high pressures.[143] Furthermore, the scale of lectin enrichment experiments has been reduced through the application of microbore columns,[141] ≤1 mm i.d., which lowers sample consumption while improving recovery. In this manner, in biomedical glycoproteomic investigations that screen microliter volumes of blood sera, it has become feasible to observe and quantitatively compare[144,145] several hundred constituents in lectin-enriched fractions.[141,143,146-149] Following the development of microscale lectin affinity techniques for the enrichment of glycoproteins in biological materials, this approach has been the basis for a multitude of glycoproteomic investigations that aim to characterize the subglycoproteomes of a variety of biological materials derived from humans, including urine,[150,151] saliva,[152] organ tissues,[153] and, most frequently, blood serum.[143,153-158]

In a study of serum minor glycoproteins,[143] Madera et al. published a multidimensional LC platform for on-line lectin enrichment followed by reversed-phase (RP) LC fractionation of enriched glycoproteins in blood serum, for which the valve configuration can be seen in Figure 3. The serum samples had been immunodepleted of six highly abundant proteins prior to lectin enrichment. High-performance affinity chromatography (HPAC) with lectins was performed using microcolumns that contained one each of four common lectins: Con A, L-*Phaseolus vulgaris* (L-PHA), *Sambucus nigra* agglutinin (SNA), or *Ulex europaeus* agglutinin (UEA-I). The lectins were chosen based on their complementary specificities (see Table 1), with the intent of enriching a diverse spectrum of the serum glycoproteome. The lectin microcolumns were packed with macroporous lectin-functionalized spherical silica particles (10 μm particle diameter, 1000 Å pore diameter) according to a previously described procedure.[141] The enriched glycoproteins were on-line desalted and then subjected to RPLC with a C_8 stationary phase. Reversed-phase elution fractions were collected in a 96-well plate, where they were trypsinized and subjected to LC-MS/MS shotgun proteomic analysis. From a 16-μg amount of immunodepleted serum proteins, 271 glycoproteins were identified. A comparison of proteins identified from enrichment with the different lectins revealed that 98, 104, 89, and 102 total proteins and 47, 48, 48, and 48 unique proteins were identified from enrichment with Con A, SNA, UEA-I, and L-PHA, respectively. The results indicated that such lectins have both overlapping and selective properties. A qualitative comparison of the glycoproteomic profiles from the enrichment with each of the lectins indicated that, while their molecular weights ranged from <10 to >800 kDa, the majority were less than 200 kDa for each lectin, and they had p*I* values predominantly in the range 5–7. In their concluding remarks, the authors noted the value of a multimethodological separation platform—exemplified by their combination of immunodepletion, lectin enrichment, and RPLC fractionation of glycoproteins, and finally RPLC-MS/MS of trypsinized peptides—for the arduous task of discovering glycoprotein biomarkers in complex materials. They also observed that, in addition to a separative platform such as this, it would be necessary to implement a means of quantitatively

analyzing the LC-MS data for identified glycoproteins, which was addressed in a following publication.[159] The multimethodological quantitative approach was then applied to a study of pooled serum samples from esophageal cancer patients.[154]

In a 2006 study by Zhao et al., lectin enrichment was incorporated in another extensive multimethodological approach to serum glycoproteomics.[155] The lectins wheat germ agglutinin (WGA), SNA, and *Maackia amurensis* lectin (MAL), bound to agarose media, were used to enrich sialylated glycoproteins from the serum of pancreatic cancer patients that had been immunodepleted of 12 major proteins. Aliquots (50 μL) of immunodepleted serum were lectin-enriched in spin columns and eluted with appropriate mono-/disaccharides, 0.5 M *N*-acetylglucosamine in the case of WGA and 0.3 M lactose for SNA and MAL. The enriched glycoprotein pools were fractionated using a nonporous silica reversed-phase (NPS-RP) C_{18} column, followed by SDS–polyacrylamide gel electrophoresis (PAGE) of individual fractions. A peak was observed in the NPS-RPLC that was apparently different in healthy and pancreatic cancer patients for each of the three lectin-enriched samples. SDS-PAGE identified two bands at 60 kDa and 85 kDa, and an in-gel trypsin digestion was performed for MS analysis, which identified the protein as plasma protease C1 inhibitor (C1INH). In a subsequent publication,[160] the authors further investigated the sera of pancreatic cancer patients for differences in glycosylation, utilizing a “double lectin” enrichment approach. Serum protein samples (25 mg) were subjected to Con A-agarose enrichment in a 5-mL packed column. Next, the Con A-bound fractions were trypsin-digested, followed by a second Con A enrichment to preconcentrate glycopeptides. Glycopeptides were deglycosylated with PNGase F and glycan profiles measured by MALDI-MS, while the sites of glycosylation were predicted by identifying sites where aspartic acid had replaced asparagine (i.e., became deamidated) as a consequence of PNGase F digestion. These results indicated an increased frequency of highly branched glycans and fucose residues in the pancreatic cancer samples.

An example of targeted glycoproteomic analysis through lectin enrichment of a less common biological material can be found in a recent investigation of pancreatic cyst fluids.[161] The samples, which were collected by a fine needle aspiration of the cystic lesions, intraoperatively, to avoid peripheral contamination, were highly variable, with inconsistent coloring and viscosity, in addition to variable protein compositions and total content. After a combination of filtration and buffer-exchanging steps were applied, relatively clear fluids were obtained for glycomic and glycoproteomic profiling. MS-based glycomic analysis of these samples showed them to have many of the same glycans that are routinely observed in serum profiles; however, in a few of the fluids that were associated with a higher risk of malignant transformation, a number of hyperfucosylated glycans (unusual structures) possessing two to six fucose residues on a single structure were identified (Figure 4). Following an untargeted proteomic analysis to provide baseline information, a glycoproteomic profiling workflow was modified to include *Aleuria aurantia* lectin (AAL) for the identification of the glycoproteins that were hyperfucosylated. A label-free quantitative comparison of the nonenriched and AAL-enriched proteomic profiles identified several glycoproteins that were overexpressed. These included pancreatic α-amylase, triacylglycerol lipase, and elastase-3A, which were 22.4-, 20.2-, and 11.2-fold

overabundant in the hyperfucosylated samples, following AAL enrichment (refer to Table 2). This study illustrates the advantages of performing glycomic and glycoproteomic investigations in the same laboratory. It represents a less usual approach, i.e. glycomic profiling first, as a guide for subsequent glycoproteomic studies, whereas most investigators conduct proteomic studies first, targeting glycosylation later, which is more tedious.

With a better understanding of how the lectin preconcentrators work as critical components of the overall analytical schemes, further advances in glycoproteomic profiling can hopefully be realized. For comparative studies, as needed in virtually all topical applications of medical glycobiology, it is essential to secure adequate quantitative reliability in every step of a glycoproteomic workflow. It is thus desirable to utilize small-scale formats for the lectin enrichment step to ensure a quantitative recovery of the enriched sample components. Due to the relatively weak interactions between most lectins and their target carbohydrate moieties (approximate K_d range: 10^{-4} to 10^{-7} M), the best enrichment support materials provide a very high *accessible* surface area, while also exhibiting a fast rate of mass transfer. Furthermore, coupling schemes that yield high lectin densities provide superior binding capacities and can greatly improve the avidity of the stationary phase with target glycoproteins through simultaneous interactions with multiple sites of glycosylation (multivalency).[162] In this regard, monolithic columns are eminently suitable for this type of work, but the current rapid development of various new materials may also lead to the discovery of supports that offer their own unique advantages. As an example of these efforts, a novel particulate silica material (1.6 μm diameter) containing an extensive, sponge-like network of macropores has been utilized to reproducibly enrich important glycoproteins from a single microliter of whole blood serum or an equivalent amount of albumin- and IgG-depleted serum using Con A and *Aleuria aurantia* lectin (AAL).[163]

It is critical to ensure that the lectin preconcentration step does not become a bottleneck in a quantitative glycoproteomic procedure. While recent data[145] with Con A indicates that adequate analytical reproducibility can be achieved in label-free quantitative proteomics, rigorous standardization steps must be followed for all lectin-based procedures.

3.2. Lectin Arrays

Lectin-based visualization of glycosylation patterns in an array format has emerged as a promising, complementary approach to direct measurement of glycoconjugates by mass spectrometry. In general, microarrays offer rapid analysis of a high number of samples, becoming thus suitable for clinical studies, in which general glycosylation patterns may be able to discriminate between sample groups. Although some lectins exhibit preferential affinity for rare oligosaccharide compositions and even specific glycosidic linkages, “lectin-only” arrays are inherently limited, similarly to other affinity staining approaches, such as Western blotting, where an immunological stain may identify a protein but not the nature of its glycosylation. A lectin stain/array may identify interesting patterns of protein glycosylation but not facilitate the identification of the specific proteins so modified. However, through a combination of lectins (or glycan-specific antibodies) and protein antibodies, it is possible to probe the specific glycosylation of individual glycoproteins. Furthermore, side-by-side comparisons of “sandwich” enzyme-linked immunosorbent

assays (ELISA) and “sandwich” antibody–antigen–lectin assays allow researchers to identify when glycoproteins are aberrantly expressed or when their glycosylation itself is substantially altered.

A variety of analytical approaches have been described for creating lectin microarrays, though it is likely that a few of these will be most widely adopted, in particular those for which the necessary fabrication technologies are commercially available, such as the inkjet printer-based arrays that may be printed on nitrocellulose microscope slides.[164] Regardless, the diverse formats that have been reported offer unique advantages that may be beneficial for specialized applications. Here follows an account of several unique lectin microarray strategies published in recent years.

Zheng et al. described a method for fabrication of a lectin array on a thin gold film. An *N*-hydroxysuccinimidyl (NHS) ester alkyl disulfide was used to form a self-assembled monolayer with an amine-reactive surface.[165] In a follow-up publication, the method was exploited for the characterization of cell surface carbohydrates through phase contrast microscopic observation of cell binding to the lectins Con A, L-PHA, *Helix pomatia* agglutinin (HPA), MAA (a mixture of MAL and *Maackia amurensis* lectin II (MAH)), soy bean agglutinin (SBA), SNA, and WGA.[166] Additionally, the density of cells bound to each lectin was measured using the publicly available NIH ImageJ processing software (http://rsb.info.nih.gov/ij/). A comparison of five tumorigenic breast cancer cell lines (four of which exhibited high metastatic potential) and a line of healthy epithelial cells was performed. All cell lines bound in high density to Con A, which was expected, but they also all bound to L-PHA, with the highest density binding observed by the healthy epithelial cells. This was unexpected, as L-PHA exhibits specificity for the β1-6 branch of tri- and tetraantennary complex *N*-glycans, which have been observed in higher abundance on tumor cells.[167,168] The HPA lectin, on the other hand, was observed to bind to four of five cancer lines, but it did not bind to the nontumorigenic cells. HPA is said to preferentially bind α-linked GalNAc, so this observation was interpreted as an indication that there is a higher prevalence of these glycans on the tumorigenic cells. HPA binding had also been previously associated with metastasis.[169]

An alternative approach to lectin array design was described by Koshi et al., who utilized fluorescently-labeled lectins immobilized in a hydrogel.[170] The hydrogel was prepared according to a previously described procedure,[171] with slight modifications. One-microliter aliquots of the fluorescently-labeled lectins were incubated with the hydrogel array spots under “semiwet” conditions that allowed the lectins to noncovalently become embedded in the gel. The immobilized lectins were AAL, Con A, *Griffonia simplicifolia* lectin II (GSL-II), UEA-I, and WGA. Next a microliter of a fluorescence quencher conjugated to a carbohydrate for which each lectin has a known affinity, e.g., a Man-2-appended dabsyl compound in the case of Con A, was incubated with the lectin-hydrogel spots. Through a method termed bimolecular fluorescence quenching/recovery (BFQR), the fluorescence signal for the immobilized lectins could then be recovered by the application of a sample mixture, which contained glycoconjugates that displaced the carbohydrate quenchers. Through this approach, it is theoretically possible to apply any glycosylated sample material with no requisite preparation or chemical labeling step. To demonstrate this flexibility, the

hydrogel arrays were used for the detection of monosaccharides and oligosaccharides, standard glycoproteins, and carbohydrates derived from the cell lysates of six mammalian and two bacterial cell lines. Following the lectin imaging of the cell lysates, a quantitative evaluation of the recovered fluorescence intensity for each lectin was measured. A multivariate statistical comparison of similarity between the different cell lines (using the signals from all six lectins to compare the eight cell lysates) was visualized as a Euclidian distance matrix and a dendrogram (Figure 5). The dendrogram, in particular, highlights that this six-lectin array was able to differentiate between the two bacterial cell lines, NM522 and JM109, and the six lines of mammalian origin, thus demonstrating the potential of this lectin array for pattern recognition of glycosylation. It is also a sophisticated methodology, in which the hydrogel spots and carbohydrate quenchers, of which there were five, needed to be synthesized in-house. This limits the likelihood of widespread adoption of a lectin BFQR strategy by the medical research community, though it remains an attractive option when possible.

For visualizing glycosylation on specific proteins in complex mixtures, Chen et al. reported an approach analogous to the socalled “sandwich” ELISA, replacing antibody-based detection with various biotinylated lectin probes.[172] The microarrays were printed using a piezoelectric noncontact printer to spot 350 pL droplets of an antibody solution against the desired glycoprotein on a nitrocellulose-coated microscope slide. In a crucial next step, the glycans on the printed antibodies were derivatized with a cysteine–glycine (Cys-Gly) dipeptide to block the potential interaction with the lectins, which, following considerable optimization, was demonstrated to greatly diminish the prevalence of nonspecific signaling. On each slide, 48 arrays could be printed, with each array consisting of 36–48 antibodies (and control proteins) spotted in triplicate. The prepared microarrays were then incubated for 1 h at room temperature with either purified glycoproteins or 10-folddiluted serum samples, a 7-μL volume in either case. Next, a 7-μL volume of a biotinylated lectin/glycan antibody was added and incubated for an additional hour, followed by washing and drying. A 7-μL aliquot of a streptavidin–phycoerythrin reporter was applied to each microarray, and after a final wash, the fluorescence emission at 570 nm was detected using a microarray scanner. As a proof-of-principle, the method was applied to a small-scale study of notable proteins in serum samples from pancreatic cancer patients (N = 23) and healthy subjects (N = 23). The two proteins studied were carcinoembryonic antigen cell-adhesion molecule (CEACAM) and mucin-1 (MUC1), which have both been previously linked to pancreatic cancer, including possible differences with MUC1 glycosylation.[173,174] By capturing these proteins with immunopurification and then probing their glycosylation, it was observed that the two lectins AAL and WGA and the carbohydrate antibody for the sialylated Lewis a (SLe^a) antigen (also called carbohydrate antigen 19-9, cancer antigen 19-9, or CA19-9) were all bound in higher density to the cancer sera. However, after correcting for the concentration of the two glycoproteins, the CA19-9 antibody reported the only statistically significant change in glycosylation. Although a relatively low number of samples was analyzed in this work, it demonstrates that the microarray format is readily scalable for larger, clinical investigations. As with routine ELISA protocols, antibody-based lectin microarrays are excellent tools for targeted glycan characterization, when one has *a priori* knowledge of the interesting glycoproteins.

An analytically elegant approach to lectin microarrays was reported by Kuno et al.[175] based on an evanescent-field fluorescence-detection scheme.[176,177] Lectins were immobilized on an epoxy-coated glass slide, followed by a 1% bovine serum albumin (BSA) blocking solution that was added to prevent nonspecific binding of samples. A sample containing Cy3-labeled (fluorescently-labeled) glycoproteins was then applied to each spot on the array. An evanescent field, which is only propagated a very short distance from the sensor surface (100–200 nm) was applied to measure the fluorescence intensity of the glycoproteins from the sample that were bound to the lectin-coated surface. Unbound fluorescently-tagged molecules were, presumably, not detected because they were not in close enough proximity to the surface. Because a sample can be measured *in situ*, it is not necessary to wash away unbound fluorescent compounds in the sample prior to measurement. In the case of lectin–carbohydrate interactions, this feature is particularly beneficial, as it facilitates the use of many lectins with relatively weak binding affinities ($K_d > 10^{-6}$ M) that may be less suitable for affinity chromatography or similar techniques. Additionally, it is demonstrated that this *in situ* approach is appropriate for glycopeptide binding, despite the lower avidities of glycopeptides compared to glycoproteins. In a demonstration of the versatility of the strategy, the glycosylation of the four standard glycoproteins, mouse laminin (mLam), bovine transferrin (bTf), asialofetuin (ASF), and horseradish peroxidase (HRP), was surveyed with 39 different lectins. As with any array approach, the discriminating power of a lectin array is enhanced by increasing the number of lectins with unique specificities that are surveyed. Following the initial publication, where standard glycoproteins were used to validate the method, a study was performed using various CHO and murine cell lines.[178] In this work, 43 lectins were used to probe cell surface glycosylation of the cultured cells from CHO and several related glycosylation-defective mutants, as well as the primary splenocytes from wild-type (WT) and β1-3-*N*-acetylglucosaminyltransferase II knockout (β3GnT2KO) mice. The live cells were labeled with 10 μM cell-tracker orange CMRA reagents, which were metabolically converted to fluorescent derivatives inside the cells. The fluorescent signal plateaued after 30 min of incubation at 37 °C. The results were discussed in the context of the previously described glycosylation profiles of the different cell lines, and they were generally found to agree. As an example, CHO cells are known to display a high density of Siaα2-3-linked but not Siaα2-6-linked species,[179,180] and the fluorescent signal on MAL was strong, while it was very low on SNA. The murine splenocytes from WT and β3GnTKO knockout mice were also readily differentiated. The tomato lectin, *Lycopersicon esculentum* lectin (LEL), which binds to *N*-acetylglucosamine and β-lactosamine extensions, bound a high density of WT cells compared to β3GnT2KO variants (the latter of which lack polylactosamine extensions). Because the evanescent-field fluorescence measurements are made in a wet environment, as opposed to the dry formats of many other arrays, this design is one of the few lectin arrays that can be used to measure glycosylation in living cells.

4. GENERAL GLYCOPROTEIN/GLYCOPEPTIDE FRACTIONATION STRATEGIES

It is sometimes preferable to perform an indiscriminate preconcentration of all glycoconjugates in a mixture. For an initial glycoproteomic survey of an uncommon biological material, it can be valuable to measure the profile of the whole glycoproteome in

order to guide subsequent investigations of interesting subglycoproteomes. Alternatively, the glycopeptides from a prefractionated/purified glycoprotein proteolytic digest may be captured (and thus isolated from nonglycopeptides) to greatly enhance their ionization in MS. Several strategies have been developed for general enrichment of glycoconjugates.

4.1. Boronate-based Materials

Boronic acid-functionalized materials have been investigated as an option for glycocapture as a result of their unique ability to form reversible, covalent bonds with monosaccharides that feature vicinal diols.[181-183] Microscale variations of this approach have been demonstrated for the enrichment of glycopeptides from standard glycoproteins,[184-186] though they have only rarely been applied to glycoproteomic studies of biologically interesting samples.[187] Because of their unique, universal “lectin-like” properties, boronic acids (sometimes referred to as boronolectins) have also demonstrated potential for the enrichment of nonenzymatically glycated proteins[188] and peptides.[189] In general, though, boronic acids have not been widely exploited for preclinical affinity chromatography work because of their weak binding constants ($K_a \sim 10^3$ M).[190]

4.2. Hydrazide Capture

A popular approach for the isolation of glycocopeptides is to use hydrazide-coated beads, as described by Aebersold and coworkers.[191] Vicinal diols in the *cis* configuration on monosaccharide residues are oxidized to aldehydes in the presence of 15 mM $NaIO_4$ for 1 h at room temperature. The glycospecific capture results from a covalent hydrazone formation between hydrazide groups on the surface of a support medium and the aldehyde-modified carbohydrates. Although the periodate oxidation is performed under relatively mild conditions, it is possible that a polypeptide containing a primary amine and a vicinal hydroxyl, e.g. an N-terminal serine, will also be oxidized and thereby coenriched.[192] However, the likelihood of this is low if the glycoproteins are not digested prior to oxidation, but it becomes more prevalent if oxidation is performed on a proteolytic digest. When intact glycoproteins are oxidized and applied to the hydrazide media, it is possible to enzymatically remove nonglycosylated peptides by addition of a protease that cleaves them from the covalently bound glycopeptides. For elution of bound N-glycosylated species, PNGase F is added to cleave the (previously) glycosylated sample components only, while oxidation side products remain on the medium. The approach has been applied to glycoproteomic analysis of many complex materials, including saliva,[193] plasma,[194,195] blood platelets,[196] liver tissue,[197] and T and B cells.[198] While this covalent capture strategy represents a very effective approach for select applications, it is unsuitable for direct measurement of glycan moieties, which cannot be quantitatively recovered from the support material. Furthermore, it necessitates the enzymatic cleavage of *N*-glycans from their attachment sites, so it is not possible to analyze intact glycoconjugates, e.g. glycoproteins or glycopeptides, thereafter.

Following the initial publication of a hydrazide enrichment method,[191] a modified protocol was reported in 2007 by a different laboratory,[199] while Aebersold and co-workers reported an SPE-based protocol following their original publication.[200] To begin with, samples were digested with trypsin prior to periodate oxidation, unlike in the original method, where

whole glycoproteins were oxidized. The other major difference was that, following oxidation, excess sodium periodate was quenched by the addition of 20 mM sodium sulfite, where a SPE step had been used to remove excess periodate in the original paper. The modified approach was tested on standard glycoproteins and an ovarian cancer cell line. The specificity of the approach was evaluated on the basis of the number of glycopeptides identified by bottom-up proteomic LC-MS/MS analyses of the captured and uncaptured sample fractions. The standard glycoprotein mixture, comprised of invertase (yeast), α-1-antitrypsin (human), conalbumin (chicken), ribonuclease B (bovine), and ovalbumin (chicken), contained a total of 20 N-glycosylation sites. Of these, 15 were identified in the hydrazide-enriched fraction, while only one *N*-glycopeptide, originating from ovalbumin, was identified in the unbound fraction. Furthermore, it was previously documented that the potential N-linked site on the peptide found in the unbound fraction is not always occupied.[201] A similar evaluation was performed with ovarian cancer cell lysates, where two samples, each of 500 and 800 μg of protein digest, were enriched. In a single enriched fraction, a total of 311 unique peptides were identified, mapping to 156 different proteins. Among identified peptides, 286 (92%) contained the N-X-T/S consensus sequon. A total of 302 proteins were identified in the enriched fractions from the four experiments, and the glycopeptide specificity was 91.0 ± 1.6% for all experiments. The excellent specificity and good sensitivity of the glycoproteomic approach were in part attributed to the modifications made to the originally published hydrazide enrichment protocol. First, by denaturing and tryptically digesting glycoproteins prior to oxidation, internally oriented glycans were solvent-exposed and, thus, more likely to be oxidized and, thus, enriched. Second, quenching excess periodate with sodium sulfite, as opposed to SPE, facilitated a one-pot enrichment, whereby the hydrazide medium was added directly to the oxidized glycopeptides.

A 2009 study by Blake et al. described the application of hydrazide enrichment of glycopeptides[191,200] to confirm the glycosylation profile of hemagglutinin from three selected strains of the H5N1 influenza virus (i.e., “bird flu”).[202] Hemagglutinin, a membrane-bound glycoprotein on the virus surface that is involved in the initial binding to host cell receptors, is the primary antigen in commercial vaccines against seasonal influenza. A 50-μL aliquot of hydrazide–agarose gel slurry in water (50:50, v/v) was used to enrich glycopeptides, where carbohydrate diols had been oxidized to aldehydes with 12 mM $NaIO_4$ for 1 h in the dark at 4 °C. After removal of unbound sample components, the hydrazide-linked glycopeptides were released by addition of 1500 U of PNGase F with end-over-end rotation at 37 °C for 4 h. Collected glycopeptides were measured by a typical RPLC-MS/MS bottom-up proteomic approach. In a parallel set of experiments, another aliquot of the virus-derived peptides and glycopeptides was fractionated by hydrophilic interaction chromatography (HILIC) to enrich glycopeptides prior to bottom-up proteomics; for these samples, no hydrazide enrichment was performed. As expected, the HILIC enrichment followed by MS/MS of *intact* glycopeptides yielded precursor masses for the intact molecules, but the fragmentation spectra were dominated by spectral peaks derived from glycan fragmentation. (See section 5 for an explanation of MS-based glycopeptide fragmentation experiments.) Conversely, in the experiments where glycopeptides were isolated by hydrazide capture and subsequently deglycosylated, they could be sequenced *de*

novo, on the basis of the clear amino acid fragmentation patterns. Furthermore, sites-of-glycosylation were identified on the basis of the Asn → Asp modification introduced during PNGase F digestion. It was also noted that, even though the influenza RNA codes for only 11 proteins, a tryptic peptide mixture of this sample generated a fairly complex RPLC-MS chromatogram. In contrast, the selectivity of the hydrazide capture was demonstrated by a representative base-peak chromatogram for the hydrazide-enriched sample in which all of the major peaks observed were derived from glycopeptides (Figure 6). With the combination of the intact and deglycosylated MS data, it was possible to deduce the masses of the glycans on the intact structures. Through manual inspection of the glycan fragmentation patterns and mass matching to a table of theoretical glycan masses, the glycan profiles at each of the six *N*-glycosites were characterized. The authors identified sites of particular interest, namely site 4 of the Vietnam and Indonesia strains and site 3 of the bar-headed goose strains, which would be worthy targets for further investigation, as they exhibited high degrees of microheterogeneity.

As part of a quantitative N-linked glycoproteomic study of myocardial ischemia in rat hearts, three methods were used for glycopeptide enrichment from heart tissue, including hydrazide, HILIC, and titanium dioxide.[203] Following enrichment, glycopeptides were quantified by isotopic labeling and detection by LC-MS/MS. While the HILIC enrichment resulted in the highest number of identified glycopeptides, each of the three enrichment strategies were said to contribute a substantial number of unique identifications. In total, 1556 nonredundant N-glycosylation sites on 972 proteins were identified and quantified. Accompanied by a detailed discussion of the biological implications of their findings, which is beyond the scope of this review, the authors demonstrated that targeting glycoproteins is a valuable approach to study disease-induced tissue remodeling. Methodologically, they analyzed the mode of data acquisition and interpretation and made several comments widely applicable to other studies that have utilized similar techniques to study glycosylation. Identification was based on the observation of deamidated asparagines (Δ + 0.986 Da) in the consensus sequon for N-glycosylation while database-searching MS/MS fragmentation spectra. Although the Asn → Asp modification is a product of PNGase F digestion (and thus a marker for N-glycosylation), it is also a documented *in vivo* modification of nonglycosylated Asn.[204] The endogenous modification is most prevalent when Asn is followed by Gly or Ser. As such, a control was implemented to estimate false-discovery of glycopeptides. Glycopeptides enriched by TiO_2 and zwitterionic-HILIC (ZIC-HILIC) were subjected to LC-MS/MS without first being deglycosylated by PNGase F. From these experiments, 44 peptides were identified with the consensus sequon, leading to a false discovery estimation of 2.8%. Notably, 25 (56.8%) of the 44 contained N-G or N-S as the first two residues in the N-linked glycosylation motif. Additionally, more than 100 peptides were identified containing deamidated asparagine in a position other than the consensus sequon. Incidentally, the frequency of false discovery of glycopeptides by deamidation could be lower following hydrazide enrichment, where the specificity of capture is very high, and harsh washing conditions may be used to remove nonglycosylated species, owing to the stability of the covalent linkage between carbohydrates and the hydrazide resin. Nonetheless, in exploratory investigations, the practice of identifying glycopeptides following enzymatic deglycosylation should be accompanied by additional confirmatory

experiments, such as the various MS techniques described in section 5 for a site-of-glycosylation analysis. Many other studies that utilized the same strategy for glycopeptide identification have not included any control experiments akin to those implemented in this example, and it is likely that the number of false-positive identifications have been underestimated as a result.

Aiming to analyze a more analytically challenging class of glycans, a 2010 paper by Klement et al. described an approach for the enrichment of *O*-GlcNAc by hydrazide affinity chromatography.[205] The major difference in *O*-GlcNAc enrichment and *N*-glycan enrichment is the reduced reactivity of the vicinal hydroxyls located at C3 and C4 on GlcNAc (compared to galactose), which are in the *trans* configuration. However, by elevating the temperature to 37 °C, it was possible to oxidize these hydroxyls to aldehydes with 20 mM $NaIO_4$. The reaction was allowed to proceed for 6 h in the dark. Two hydrazide-functionalized resins, agarose and silica, were tested, and no recognizable difference in their performance was reported. Following optimization of the procedure with a standard glycopeptide, it was applied to the oxidation of the *O*-GlcNAc-modified protein, α-crystallin. The glycoprotein oxidation was performed in the presence of SDS and guanidine hydrochloride, and better results were achieved with SDS. Additional oxidative damage to the protein structure from the prolonged oxidation procedure was not investigated, although a partial oxidation of cysteine and methionine residues was observed during MS experiments. The hydrazide-linked α-crystallin was trypsin-digested, and unattached peptides were washed away. For the release of the bound *O*-GlcNAc-modified peptides, a standard β-elimination procedure for cleavage between the monosaccharide and Ser/Thr side chain was evaluated along with three methods for hydrazone bond cleavage to release the oxime derivatives of captured glycopeptides. The β-elimination was moderately successful and was used in combination with a Michael addition in 50 mM cysteamine hydrochloride for a site-of-glycosylation analysis as described previously by Wells et al.[206] (see section 5 for a brief description). For hydrazone cleavage, a periodate release that had been successful in solution proved inefficient for solid-phase removal. An attempted acidic cleavage resulted in a partial loss of the sugar moiety and a decrease in sensitivity. A third method that called for an overnight incubation with 200 mM hydroxylamine hydrochloride in mildly acidic conditions, 50 mM sodium acetate (pH 5), demonstrated a considerably better performance for the release of the intact oximes. The enrichment protocol, in combination with both the β-elimination and hydroxylamine release methods, were applied to an investigation of the O-GlcNAc modifications of the proteasome purified from *Drosophila melanogaster*. The enriched *O*-glycopeptides were analyzed by MALDI/LC-ESI TOF-MS techniques. Six GlcNAc-modified glycopeptides, including one that was asparagine-linked, were identified from five different proteins. The site of glycosylation was unambiguously assigned by collision-induced dissociation (CID), electron-transfer dissociation (ETD) or β-elimination/Michael addition (BEMAD) for five of the six glycopeptides (see section 5 for a description of these techniques). Interestingly, no *O*-GlcNAc modifications were identified on proteasomal subunits, but rather on interacting partners of the proteasome. The number of MS/MS spectra that were assigned to O-GlcNAc glycopeptides accounted for only 4% of the total MS/MS spectra collected. It was suggested

that a large contribution of interfering analytes can be attributed to N-terminal oxidation of serine/threonine residues during the prolonged periodate oxidation procedure.

Finally, it is important to note that the primary shortcoming of the hydrazide enrichment technique, namely that glycans themselves cannot be recovered from the hydrazide-functionalized resin, was partially circumvented in a publication from Nilsson et al. in 2009.[207] The paper described the utility of a mild periodate oxidation step, in which the glycerols of sialic acids were selectively oxidized by incubation with 2 mM periodic acid for 10 min at 0 °C. Oxidation was then quenched by the addition of excess glycerol. Following hydrazide enrichment and proteolytic digestion, with appropriate washing steps as needed, the captured glycopeptides were released by acid hydrolysis in 0.1 M formic acid at 80 °C for 1 h. This release cleaved the glycosidic bonds linking sialic acid residues to the glycopeptides, so sialic acids themselves were not recovered. This approach offers a means to enrich all sialylated glycoproteins, including those with both N- and O-linked sialylated glycans. As such, it is one of the few approaches available for analysis of O-linked glycoproteins and glycopeptides. In the report from Nilsson et al., the procedure was applied to the analysis of glycoproteins in cerebrospinal fluid, which resulted in the identification of 36 N-linked and 44 O-linked glycosylation sites.

4.3. Sialic Acid Enrichment by TiO_2

A dearth of chemically-based methods for targeting mammalian carbohydrates is available, primarily because few functional groups are unique to glycans, while the biological specificity of antibodies and lectins are vehicles for selective enrichment. Nonetheless, researchers have continued to pursue “non-biological” methods for their simplicity and, presumably, their cost effectiveness. Aside from hydrazine chemistry, a recent and notable contribution to this line of techniques was reported by Larsen et al. for the titanium dioxide-based enrichment of sialylated analytes.[208] Titanium dioxide is regularly used for enrichment of phosphopeptides,[209,210] but if these are dephosphorylated by enzymatic treatment with alkaline phosphatase, the binding interaction between sialic acid, either free or present at the nonreducing end of a glycan, with titanium dioxide is highly efficient. The negatively-charged sialic acid contains both carboxylic acids and hydroxyl groups, and it likely binds to the titanium dioxide via a multipoint interaction.[208] Acidic amino acids and neutral glycopeptides would likely also exhibit some affinity for the titanium dioxide, but an additive in the binding buffer can competitively inhibit these weaker interactions, while not disrupting the sialic acid binding. Through an optimization study, Larsen et al. determined that, following dephosphorylation, 5% trifluoroacetic acid (TFA) and 1 M glycolic acid limited nonspecific binding of amino acids and facilitated a highly specific enrichment of sialylated glycostructures. Following their capture, sialylated species were eluted in aqueous ammonia, pH = 11. The efficacy of the protocol for profiling the “sialiome” was demonstrated, in principle, by the enrichment of sialylated glycopeptides from tryptic digests of standard glycoproteins, immunodepleted human plasma, and saliva.[208,211] While the acidic buffer additives successfully inhibited the binding of acidic amino acids, it is important to consider that sulfated glycostructures, if present, would likely also be enriched by this approach, which could be advantageous or not, depending on the contents of the sample and aims of the glycosylation study. Although the method has not been widely

adopted as yet, a further evaluation by Wohlgemuth et al., who used a standard mixture containing the heavily sialylated glycoproteins fetuin and α-1-acid glycoprotein (AGP) for testing, determined that the method was highly specific on the basis of parallel experiments with and without neuraminidase digestion prior to the enrichment step.[212] All glycopeptides were identified in the aliquot that was not treated with neuraminidase, while none were in the desialylated sample.

4.4. Metabolic Labeling of Glycans

A major limitation for chemical affinity enrichment is that carbohydrates contain few unique functional groups that are not observed in other classes of biomolecules, such as proteins or nucleic acids. Thus, chemical enrichment strategies for glycans either often suffer from a limited specificity or, as is the case with the hydrazide capture, may prove too harsh for the necessary glycan characterization in some applications. However, an interesting alternative has emerged, by which it is possible to add unique functional groups specifically to glycoconjugates and, thus, provide additional possibilities for enrichment strategies and/or glycan imaging. Utilizing the specific biosynthetic pathway of an organism, two different research groups have demonstrated that it is feasible to incorporate azide-modified monosaccharides into glycoconjugates *in vivo* or *ex vivo* via a Staudinger ligation[213] and both copper-catalyzed[214] and copper-free click chemistries.[215] Sawa et al. described that, by means of the fucose salvage pathway, an azide-or alkyne-modified GDP-fucose analog may be substituted for the natural fucose through the action of fucosyl transferases.[214] Once incorporated, the fucose analogs were “clicked” to different naphthalamide probes for fluorescence imaging. Importantly, the labeling reaction itself was fluorogenic, so labeled residues fluoresced intensely, while unreacted reagents did not. The specificity of the fluorescent staining was demonstrated with AGP, which was incubated with either an azido- or alkyne-modified fucose analog or natural fucose (control) in the presence of α-1,3-fucosyltransferases II–VII. After an incubation period, the fluorogenic labeling reaction was conducted on each sample. To mimic a likely application of the technique, samples were then subjected to SDS-PAGE. UV-illumination of the glycoprotein bands revealed intense fluorescence signals for the samples incubated with fucose analogs, while the AGP incubated with natural fucose did not illuminate. *In vitro* fluorescent “staining” of Jurkat cells further highlighted the specificity of the bioorthogonal “light-switch” fluorescence reaction, as well as the potential for differential imaging of fucosylation events on cell surfaces. This technology could be of considerable value for disease research, particularly cancer studies where aberrant fucosylation has been widely implicated.[216-220]

Alternatively, a copper-free click strategy was reported by Baskin et al., in which metabolically incorporated azide-modified sugars (e.g., azido-sialic acid, SiaNAz) were labeled with a fluorescently-tagged cyclooctyne.[215] The reaction proceeded with comparable kinetics to the copper-catalyzed version, and it was successfully applied to a rapid labeling of azido glycans on the cellular membrane of live Jurkat cells within a few minutes. Significantly, no apparent toxicity was observed following this labeling procedure, indicating that it could be suitable for *in vivo* time-resolved imaging of glycosylation patterns in living organisms. In two following publications from the Bertozzi laboratory, *in vivo* imaging was demonstrated with *C. elegans* (nematode)[221] and zebrafish in early stage

development.[222] In addition to labeling with fluorescent probes *in vivo*, a sample of cell lysates from azidosugar-labeled *C. elegans* was reacted with a phosphine-FLAG peptide, called a “FLAG-tag”, that enabled affinity purification and Western-blot imaging of individual azidosugar-modified glycoconjugates with an anti-FLAG antibody.

In the case of the zebrafish, time-resolved glycosylation events were measured at several time points over the course of the first 120 h, postfertilization, by utilizing different difluorinated cyclooctyne reagents with different wavelengths of maximum fluorescence emission. This elegant bioorthogonal strategy to glycan imaging appears very promising and can have a large impact on glycobiological research in the future. However, in its current developmental stages, the considerable synthetic effort that is devoted to generating the cyclooctyne reagent may limit its immediate widespread adoption.

Metabolic labeling is an exciting new direction for glycocentric biological studies, with a promising potential as an alternative approach for affinity purification and also as a tool for imaging live organisms, particularly when the organismal tissues are relatively transparent. In the reported literature, the imaged cells and tissues have typically been incubated with the modified sugars between one and three days prior to fluorescent labeling; however, the time required for incorporation of the SiaNAz residue into CHO cells in the report from Baskin et al. was estimated to be between 15 and 30 min, on the basis of the observed saturation of the fluorescent signal at 30 min.[222] While the rate of incorporation does not yet facilitate real-time imaging, it appears suitable for comparisons of molecular dynamics in biological systems over the course of several hours or more.

The bioorthogonality of the azide and alkyne functional groups offers a unique chemical specificity that may be applied to great benefit. Considering the often promiscuous, yet occasionally highly specific nature of many glycan-binding molecules, it remains unclear how modifications to the carbohydrates themselves may impact various modes of glycobiological research. Considering the rules that govern specificities of glycan-binding molecules remain obscure, it is unclear how modifications to the carbohydrates themselves may impact various modes of glycobiological research.

4.5. Hydrophilic Interaction Chromatography

Some of the earliest applications of hydrophilic interaction chromatography (HILIC), reported in the 1970s, were for the separation of carbohydrates;[223,224] however, the technique was not called HILIC until Alpert coined the term in 1990.[225] HILIC exploits polar interactions, often in the form of hydrogen bonding, between analytes and a polar stationary phase, while also demonstrating selectivity based on ion-dipole and purely ionic interactions in cases where the stationary phase is charged.[226] Moreover, it has been shown that there is a static layer of water adjacent to the stationary phase surface, leading many researchers to assert a liquid/liquid partition chromatography mechanism for analyte retention.[225,227,228] A more recent in-depth review by Hemström and Irgum suggested that HILIC employs a “multimodal” retention mechanism, with liquid–liquid partitioning and Coulombic and hydrogen bonding adsorption events all contributing to the separation.[229]

In this mode of chromatography, samples are loaded in a relatively nonpolar mobile phase, e.g. 80/20 acetonitrile/water, and then eluted by increasing the percentage of water. Elution of the retained analytes with water is a defining characteristic of HILIC, distinguishing it from normal-phase liquid chromatography (NPLC), which utilizes more nonpolar solvents. Numerous stationary phases have been employed for HILIC, including silanols, diols, amines, amides, and various cationic, anionic, and zwitterionic functional groups. Amine-functionalized media have been used extensively in the past two decades for carbohydrate enrichment because of their strongly adsorptive retention mechanism,[230-236] but some column materials suffer from a high reactivity with reducing carbohydrates,[237] resulting in a poor sample recovery, irreproducible retention times,[238] and a loss of binding capacity over time.[239] While they are still employed occasionally for sample preparation in a batch mode,[240] amino stationary phases have largely been replaced in HPLC columns by more robust materials. The commercialized zwitterionic and amide phases have been used extensively in the recent literature for glycoconjugate analyses.[211,238,241-248] In some cases, high-resolution HILIC can be achieved,[226,243,248,249] providing a more tunable option for separation of glycoconjugates based on their glycan moieties than the binary capture strategies (e.g., boronic acid or lectin affinity), though implementing HILIC for solid-phase extraction (SPE) also remains a popular preanalytical step in multistage sample preparations. The substantial role of HILIC in glycan separations and glycomic applications will be further explained in sections 4.5 and 8.3 of this review. The remainder of this section is dedicated to applications of HILIC for glycoconjugate investigations, particularly glycoproteins and glycopeptides.

HILIC SPE for glycoconjugate enrichment prior to MS detection has been widely adopted by the research community over the past decade, particularly for the analysis of glycopeptides and glycoproteins. An early example of this approach was published by Wada et al., who reported the utility of microcrystalline cellulose and Sepharose CL-4B media for SPE of glycopeptides from standard glycoprotein samples and also from total blood serum glycoproteins.[250] The loading solvent was 1-butanol/ethanol/water (4/1/1, v/v/v), and the eluting solvent was ethanol/water (1/1, v/v) for all enrichment experiments. The cellulose enrichment method was a batch-mode variation of a column-based protocol from Shimizu et al. for a preparation of hydrazinolyzed *N*-glycans.[251] The cellulose enrichment was tested with a 1-mg bed of cellulose and 100 μg of tryptic digest of human transferrin, and while it was reported that tryptic glycopeptides were detected in the elution fraction, there remained significant contamination from nonglycosylated peptides. In contrast, when 200 μg of transferrin were incubated with 30 μL of Sepharose CL-4B, multiple glycoforms of the two known *N*-glycopeptides were isolated from the mixture, with only a single contaminating, nonglycosylated peptide. The recovery of transferrin glycopeptides was estimated to be between 30–50%. Because elution was performed in a salt-free solution (50% ethanol), it was a simple task to dry the sample and confirm the identities of the glycosylated peptides by MALDI-MS. Sepharose enrichment followed by MALDI-MS was reported for transferrin and two additional standard proteins, human IgG and β-2 glycoprotein 1. A sample of human blood serum tryptic digest was subjected to glycopeptide enrichment with Sepharose CL-4B, followed by LC fractionation. MS analysis of one of the LC fractions,

including CID fragmentation, was used to identify a glycopeptide from the β chain of haptoglobin.

A study published a year later applied the Sepharose enrichment technique to the analysis of additional standard glycoproteins, including plasma and cellular fibronectins.[252] The SPE solvents were modified from the previous work to improve the enrichment specificity; the loading solvent was a more organic mixture of 1-butanol/ethanol/water at a volume ratio of 5/1/1 and was further modified by 1 mM of either $MnCl_2$, $CaCl_2$, $CoCl_2$, $NiSO_4$, $CuCl_2$, or $ZnCl_2$, while the elution was still performed with ethanol/water (1/1, v/v). Divalent metal ions were added because it was hypothesized that they could enhance glycopeptide binding. The adjusted solvent conditions allowed improved recovery, ranging from 50 to 70%. Among the interesting results from this work were the identification of a new site of O-glycosylation on plasma fibronectin at Thr279 and a new site of N-glycosylation on apolipoprotein B at Asn2560 (Asn2533 of the mature protein).

Wohlgemuth et al. evaluated the performance of various HILIC stationary phases, including amino, underivatized silica, microcrystalline cellulose, sulfobetaine, and amide media, as well as hydrazine chemistry and TiO_2 for enrichment of N-linked glycopeptides.[212] An equimolar mixture of trypsin-digested bovine fetuin, ribonuclease B, bovine α-1-acid glycoprotein, bovine serum albumin, histone, and human IgG was subjected to each enrichment stationary phase, and the enriched sample components were digested with PNGase F and measured by reversed phase LC-MS/MS. On the basis of the number of identified *N*-glycopeptides, their signal-to-noise ratios, and the number of nonspecifically bound peptides, a qualitative evaluation of each medium was described. The authors concluded that, among the HILIC phases, the commercially available ZIC-HILIC (sulfobetaine) and the TSKgel Amide-80 (amide) materials enabled a comprehensive, controllable, and enhanced analysis of protein glycosylation. Furthermore, the specificities of these two media were excellent for an unbiased enrichment of glycopeptides, while the TiO_2 was efficient for capturing sialylated species. In contrast, hydrazine enrichment resulted in a lower peptide recovery, while necessitating a more complex enrichment scheme. Certainly, both the zwitterionic and amide stationary phases have been exploited extensively in the recently reported literature. A review by Wuhrer and co-workers that describes advances in HILIC for structural glycomics emphasizes this point.[253] A table in the 2011 review lists recent applications of HILIC for oligosaccharide and glycopeptide analysis. The table includes 42 citations, 35 (83%) of which utilized one of these two stationary phases.

A recent report by Gilar et al.[248] demonstrates the current state-of-the-art capability of HILIC for glycopeptide characterization, performed in a UPLC format with both UV- and MS-based detection simultaneously. This work utilized a 150 mm × 2.1 mm column with an amide stationary phase, BEH glycan from Waters Corp., which is a 1.7-μm bridged-ethyl hybrid (BEH) silica-based particle. The mobile phase solvents were 10 mM ammonium formate in water, pH 4.5 (solvent A), and 100 mM ammonium formate/acetonitrile mixed 1/9 (solvent B), except for separation of the bovine fetuin digest, where 0.5% formic acid (FA) in water (solvent A) and 0.5% FA in acetonitrile (solvent B) were used. For one of the glycoproteins, a humanized monoclonal antibody (Trastuzumab), several glycoforms of the

EEQYNSTYR peptide were baseline-resolved from one another over only a 5-min window of the gradient program (Figure 7). More strikingly, two peaks each for the positional (structural) isomers of two of the glycoforms, a complex biantennary monogalactosylated glycan (G1a and G1b) and a complex biantennary monogalactosylated fucosylated glycan (G1Fa and G1Fb), were nearly baseline-resolved as well. The efficient separation of trypsin-digested peptides from bovine fetuin, which contains a complex mixture of both N- and O-linked glycopeptides, was also demonstrated (Figure 8). A pair of experiments, in which the tryptic digest was not treated or treated with PNGase F prior to ultraperformance liquid chromatography–mass spectrometry (UPLC-MS), illustrated that the N-linked glycopeptides eluted from the column last. The extracted-ion chromatogram (XIC) for the common sialylated oxonium ion at an *m*/*z* value of 657 was used to further confirm the elution times for the glycopeptides, identifying both sialylated N- and O-linked glycoforms. In conjunction with the total-ion chromatogram from the PNGase F-digested sample, it was possible to infer the elution window for the O-linked glycopeptides. A theoretical mass list was used to identify individual glycopeptides by accurate mass assignment, which resulted in the characterization of 25 N-linked glycoforms across 4 sites of glycosylation and 9 O-linked glycoforms on a single site.

4.6. ZIC-HILIC

Zwitterionic chromatography–hydrophilic interaction chromatography (ZIC-HILIC) provides a positively and negatively doubly-charged stationary phase (at neutral pH), which can engage in charge–charge and charge–dipole interactions with mobile-phase electrolytes as well as analyte surface groups. A conceptualization of a sulfobetaine-functionalized silica particle demonstrates how a zwitterionic surface may generate a charged liquid layer near the stationary phase surface (Figure 9), creating a zone of high ionic strength for polar and charged interactions with a glycan, specifically for a sialic acid moiety in the illustration. Both attractive and repulsive electrostatic interactions contribute to the separative displacement of the analytes. Furthermore, the electrolyte concentration in the mobile phase can be adjusted to enhance resolution in some cases, as demonstrated by Takegawa et al.[226] In addition to HPLC formats, ZIC-HILIC has also been exploited for fractionation approaches. It is desirable to enrich glycoconjugates from a complex mixture that contains many additional nonglycosylated components, which frequently interfere with or entirely prevent the measurement of glycosylated species with MS. While it is advisable to first evaluate an enrichment protocol with a standard glycoprotein or mixture of glycoproteins, the benefits for the analysis of real biological materials are demonstrated by an investigation of a suitably complex sample such as blood plasma/serum.[242,254] Hägglund et al. devised a method by which a ZIC-HILIC medium was packed into GELoader tips,[242] analogous to the previously described methods for creating reversed-phase[255] and graphite powder microcolumns.[256] Samples were loaded onto the columns and washed in acetonitrile/water/formic acid (80/19.5/0.5, v/v/v), and then the captured species were eluted in water/formic acid (99.5/0.5, v/v). The glycopeptides were digested with a mixture of exoglycosidases, followed by Endo D/H,[257] which systematically trimmed the *N*-glycans, leaving only the asparagine-linked *N*-acetylglucosamine (and an α-linked fucose if one was present), which was then analyzed by LC-MS/MS using *N*-acetylglucosamine (203.08 Da) and fucosylated *N*-acetylglucosamine (349.14 Da) as variable modifications to identify the glycopeptides

through database searching. The methodology facilitates the discovery of *N*-glycopeptides, including a site-of-glycosylation analysis, for glycoproteins that have had their amino acid sequences mapped previously. This premise was first tested first with a simple mixture of trypsin-digested peptides/glycopeptides from the standard glycoproteins bovine ribonuclease B, bovine fetuin, human α-1-acid glycoproteins 1 and 2, chicken ovalbumin, and chicken ovomucoid was subjected to the HILIC-Endo D/H characterization protocol and compared to a sample subjected only to Endo D/H. Following the Endo D/H strategy, 32 nonglycosylated peptides and 7 glycopeptides were identified, while the HILIC-Endo D/H method identified only 2 nonglycosylated peptides and 8 glycopeptides. The HILIC columns were able to substantially enrich the glycopeptide fraction of the sample, but unfortunately the enrichment did not lead to the identification of significantly more glycopeptides by LC-MS/MS, leading the authors to describe the method as “partially successful”. Next, the approach was applied to a sample of human plasma that had been previously enriched in glycoproteins by lectin-affinity chromatography with Con A. The approach successfully identified 62 tryptic glycopeptides and 12 semitryptic peptides from 37 different glycoproteins in the HILIC-enriched fraction. Strikingly, only one glycopeptide (from the Ig α-1 chain C region) was identified in the flow-through fraction, and it was also present in the enriched fraction. In addition, one glycopeptide in the Ig γ-2 chain C region and three glycopeptides in the MAC-2 binding protein precursor were identified that had not been previously annotated as sites of glycosylation (though they contained the consensus N-X-T/S amino acid sequon, where X is not proline) in the SWISS-PROT database.

While polar HILIC strategies can discriminate glycopeptides from nonglycosylated peptides based on the hydrophilicity of the glycan moieties, ZIC-HILIC suffers from a strong interaction between the charged functional groups on the polypeptide backbone as well as the side chains, diminishing the relative impact of the glycan hydrophilicity and, thus, the specificity of the enrichment for glycoconjugates. As such, ZIC-HILIC can struggle to discriminate nonglycosylated peptides that are highly charged/hydrophilic from glycopeptides. The background signal from nonglycosylated peptides in a serum sample can then suppress ionization and potentially mask MS signals of coenriched glycopeptides. However, the use of ion-pairing reagents reportedly affects the proportional hydrophilicity of nonglycosylated peptides to a greater extent than that of glycopeptides (the hydroxyl groups are unaffected),[258] facilitating a higher purity enrichment of glycopeptides. Notably, more hydrophobic ion-pairing reagents, such as trifluoroacetic acid (TFA), are preferred to the similar but smaller molecules, such as formic acid (FA), as they appear to more substantially diminish the overlap of nonglycosylated peptides in the glycopeptide fraction.[259,260] This can be of paramount significance for mass-spectrometric detection of glycopeptides, which may be otherwise masked by non-glycosylated species during MS ionization events. Mysling et al. reported that, for the quantitative comparison of 600 plasma glycopeptides measured by RPLC-ESI-MS, the ion current of the glycopeptides increased 3.7-fold when 1% TFA was used rather than 2% FA during ZIC-HILIC fractionation.[254]

A paper from Picariello et al. described the enrichment of glycoproteins from human milk[261] using GELoader pipet tips that were packed with the ZIC-HILIC, 200 Å, 10-μm particles from SeQuant AB according to the method from Hägglund et al.[242] The milk proteins,

which had been precipitated from the whole sample and dried, were digested with trypsin and applied to the in-house constructed ZIC-HILIC extractors in acetonitrile/water/formic acid (80/19/1, v/v/v), washed twice with the same buffer solution, and eluted in two steps with 0.5% formic acid followed by pure water. The *N*-glycopeptides were deglycosylated and identified using a combination of MALDI-TOF- and LC-ESI-MS/MS shotgun proteomic approaches. Because glycosylated asparagines are converted to aspartic acids following PNGase F digestion, it was possible to identify sites of N-glycosylation through MASCOT database searching with aspartic acid selected as a variable modification. This enrichment and measurement approach resulted in the identification of 32 glycoproteins and 63 sites of N-glycosylation on them.

5. MASS-SPECTRAL CHARACTERIZATION OF GLYCOPROTEINS AND GLYCOPEPTIDES

Many approaches (lectin staining, carbohydrate-specific staining, etc.) have been used to probe glycoproteins in an attempt to acquire information pertaining to the overall structural nature of the molecules, including possible glycan types and glycosylation levels/alterations. However, by far, the most powerful analytical technique remains mass spectrometry (MS). While a structural characterization of the micro- and macro-heterogeneity of glycoforms on glycoproteins may be achieved for purified glycoproteins with low to moderate levels of glycosylation,[246,262,263] and optimized protocols have recently been reported for these analytical procedures,[264] the task of characterizing glycosylation in complex mixtures of glycoproteins provides several unique challenges regarding their separation and subsequent measurement with MS. Even though the overall goal is to most often characterize, either in a qualitative or quantitative sense, glycoproteins of interest, most researchers using MS approaches prefer to analyze peptides. While a structural characterization at the protein level may be possible for certain small glycoproteins with low levels of glycosylation,[262] several unique challenges encountered during a separation of intact proteins and their subsequent MS interrogation may complicate the overall analysis. Since the technologies for these methods have been deemed by many researches as more well-developed and robust for peptides, these are generally the preferred analytes.

One well-established technique in peptide characterization is tandem MS, often referred to as “MS/MS” or “MS^n”. Through the fragmentation of a glycopeptide via an appropriate fragmentation method, several key pieces of data relating to the overall nature of a glycopeptide may be obtained. Since both “components” (the peptide backbone and its associated carbohydrate) of a glycopeptide are equally important, a thorough elucidation of its entire structure should include not only a determination of the peptide’s amino acid sequence, but also an exhaustive characterization of its carbohydrate(s). Ideally, a further identification of the site of glycosylation is also desirable, and its occupancy level should be indicated, since not all sites may be occupied, or their levels may fluctuate in response to various stimuli, for example, a disease condition. Additionally, multiple carbohydrates may be associated with a single glycosylation site, leading to a microheterogeneity, and it is not a requirement for all structures to be represented at a given moment during a protein’s lifetime. As discussed in this section, several tandem MS methods have been developed and

applied to glycopeptide characterization studies. Frequently, a single tandem MS approach provides only a few key pieces of evidence about the overall structure of a glycopeptide, so a combination of multiple techniques is typically necessary for a comprehensive analysis.

5.1. Collision-induced Dissociation

One of the earliest tandem MS methods to be developed was collision-induced dissociation (CID), and it is still arguably the most widely applied approach to generate diagnostic fragment ions, though other tandem MS approaches that have been introduced more recently hold great potential for aiding in glycopeptide characterization. During a CID fragmentation experiment, the internal energy of the analytes is increased and they experience numerous collisions with an inert buffer gas. Once enough energy has been deposited to the molecule, bond scission occurs. Because the energy barrier of dissociation for the glycopeptide's carbohydrates glycosidic linkages of is typically lower than that for the amide bonds connecting the amino acids of the peptide backbone, the resulting spectrum is dominated by the so-called carbohydrate B- and Y-type cleavages,[265-268] according to the now widely accepted nomenclature first proposed by Domon and Costello.[269] This nomenclature system is schematically represented as Figure 10. Following the apparent sequential neutral loss of carbohydrate components, the resulting series of ions, generally observed with reductions in their overall charge state from the precursor ion, may be used to "reconstruct" the original carbohydrate,[265-267,270,271] as is demonstrated with the CID spectrum of a haptoglobin tryptic peptide shown in Figure 11a. Interestingly, some information pertaining to the isomeric possibilities of a glycan may also be acquired through this type of analysis.[267] This was demonstrated with haptoglobin tryptic peptides, where core-fucosylated structures seemed to generate Y-type ions and outer-arm fucosylation tended to favor B-type fragments. Since diagnostic peptide fragments are seldom observed when multiply-charged glycopeptide ions are subjected to a fragmentation method,[266] CID's main value may be its ability to assist in a carbohydrate's structural characterization while it remains attached to the peptide backbone. When CID experiments do not yield extensive peptide backbone fragmentation, complementary fragmentation strategies, for example electron capture-dissociation (ECD) or electron-transfer dissociation (ETD), are needed to induce adequate decomposition. Alternatively, sub-parts-per-million (ppm) mass accuracy of the precursor *m/z* may also be used in certain cases to determine the amino acid sequence.

In addition to the carbohydrate B- and Y-type ions, several hexose-*N*-acetylhexosamine (abbreviated as Hex and HexNAc, respectively) and Hex oxonium-type fragment ions are reliably generated and are usually observed at *m*/*z* values of 366 and 204, respectively, as described by Carr and co-workers in one of the first studies of glycopeptides to use ESI interfaced to a triple-quadrupole instrument.[265] These ions were then used as "signatures" to easily identify tryptic glycopeptides in LC-MS data for the analysis of soluble complement receptor type I, a 240 kDa protein with 25 sites of potential N-linked glycosylation. The technique of monitoring these and other oxonium ions (observed at *m*/*z* values of 292 and 657 for sialic acid and hexNAc-hex-sialic acid fragments, respectively) has proven to be quite sensitive, as it may indicate the presence of low-abundance glycopeptides; sub-picomole levels of tryptic glycopeptides of the monoclonal antibody drug trastuzumab were detected in a complex mixture when nanoflow LC was coupled to an ESI-based ion-trap

instrument.[272] Even more ultrasensitive measurements, those in the low femtomole range and below, have been possible for haptoglobin tryptic glycopeptides when searching LC-MS data for these types of ions.[267] Particularly for unknown samples, using diagnostic oxonium ions to locate glycopeptides has proven to be very effective, and this approach is still routinely used to indicate the presence of glycopeptides fragmented by CID, infrared multiphoton dissociation (IRMPD), source-induced dissociations (SID),[273] and the higher-energy collisional dissociation (HCD).

While most frequently performed in a mass spectrometer's positive-ion mode of operation, tandem MS analyses conducted in the negative-ion mode can provide complementary information,[274,275] which may prove to be useful in the total characterization of glycopeptides. The benefits of performing fragmentations in both ion modes were demonstrated for neutral and sialylated egg yolk glycopeptides electrosprayed into a linear ion trap/TOF instrument.[274] As reported previously by several investigators, the dominant fragmentation pathway resulted in the disassembly of the glycosidic bonds in a positive-mode CID. However, when the same peptides were fragmented under negative-ion mode CID conditions, several ions attributed to the fragmentation of the peptide backbone were recorded, in addition to two cross-ring fragments[274] that resulted from the scission of two bonds across a single monosaccharide unit. Likewise, negative-mode CID of the N-linked glycopeptides derived from bovine lactoferrin and the *O*-glycopeptides originating from bovine κ casein, generated by a digestion using immobilized Pronase, also exhibited adequate fragmentation of the peptide backbone when analyzed by a MALDI FT-ICR instrument,[275] with the CID analysis in this study performed in the ICR cell. Cross-ring fragmentation of the innermost GlcNAc residue was also observed for both of these proteins, with minimal dissociation occurring throughout the remaining carbohydrate. While most of the glycans associated with lactoferrin were high-mannose type and were readily detected in the positive mode, the *O*-glycans attached to κ casein were often decorated with multiple sialic acid residues, and the sensitivity was significantly enhanced in the negative mode.

In the previous examples, MS/tandem MS experiments were performed on instruments utilizing ESI coupled to ion-trap or quadrupole instruments, at least due in part to the ease with which these mass spectrometers can be coupled to liquid-based separations for the analyses of complex mixtures. However, the developments of MALDI-based tandem TOF and QTOF instruments in the late 1990s and early 2000s allowed fragmentation experiments to be conducted routinely on analytes ionized by this method. In one of the earliest studies to fragment MALDI-generated glycopeptides, Wuhrer et al. demonstrated that several singly-charged glycopeptides derived from horseradish peroxidase exhibited an extensive fragmentation of the peptide backbone.[276] In many cases, sufficient dissociation occurred to accurately determine the amino acid sequence, and in some instances, the carbohydrate remained attached to the peptide backbone, allowing the site of glycosylation to be determined. In addition, a few ionic products that could be associated with glycosidic bond breakages, with one of the most commonly observed cleavages being a $^{0,2}X_1$ ion, were recorded. The resulting piece of the glycan appeared to remain associated with the peptide backbone and could be beneficial in marking the site of glycosylation. Similar results were

obtained using a QTOF instrument for glycopeptides associated with β_2-glycoprotein I and those acquired from blood serum glycoproteins.[250] As with the previous study,[276] the Y_1-type ion (peptide + GlcNAc) seemed to be a quite common product. Additionally, other peptide fragments were observed, though not as many as observed by Wuhrer and co-workers,[276] and more extensive dissociation of the carbohydrate appeared to occur.[250] Interestingly, MALDI tandem TOF examinations of the much larger glycopeptides (those with masses greater than ~5000 Da) associated with ovalbumin and asialofetuin demonstrated primarily glycosidic bond cleavages,[277] though the lower *m*/*z* values were not reported and still allowed for the possibility that some fragmentation of the peptide backbone occurred. Once again, the strong signals for the Y_1 ionic fragment were recorded.[277] Similar results were reported for the fragmentation of glycopeptides associated with tissue inhibitor of metalloproteinases-1 (TIMP-1), purified by ZIC-HILIC and analyzed by a MALDI QTOF instrument.[258] While glycosidic bond cleavages were the favored pathways, several ions attributed to the peptide backbone were observed, along with cross-ring fragmentations across the internal GlcNAc units, another apparently preferred fragmentation pathway. Given the significant variation of chemistries possible due to the multitude of permutations in various combinations of amino acid sequences, the remaining fragmentations may be hard to predict, ranging from a nearly complete dissociation of the peptide backbone to the observation of only a few diagnostic fragments. These combined results seemingly agree with a report by Lebrilla and co-workers discussing several factors that influence the fragmentation of glycopeptides using IRMPD.[278]

Several studies have investigated a number of parameters that may alter the dissociation pathways of glycopeptides that researchers may consider useful to obtain the desired information. Some of these are more easily implemented, such as, for example, the choice of the MALDI matrix, while others, such as the charge state of an ion, are more difficult to influence. Different voltages determining the energy of fragmentation have also been used to alter the fragmentation of glycopeptide ions. Thus, a number of factors need to be considered and potentially optimized to obtain the desired information from a CID experiment.

One of the most straightforward approaches to alter fragmentation patterns is through the use of different MALDI matrices,[279] as was demonstrated for a standard glycopeptide ionized using the “cool” 2,5-dihydroxybenzoic acid (2,5-DHB) matrix and the “hot” α-cyano-4-hydroxycinnamic acid (CHCA) matrix. When 2,5-DHB-ionized analyte was subjected to CID, a preferred site of fragmentation was reported to be between the two GlcNAc units of the chitobiose core, producing the Y_1 ion. Extensive fragmentation of the peptide backbone also occurred, as indicated by the presence of a series of *y*- and *b**-type ions, which facilitated the identification of the site of glycosylation. A complementary fragmentation pattern was recorded using CHCA as the matrix. In this situation, only limited fragmentation throughout the peptide backbone occurred, while the carbohydrate underwent a thorough decomposition, allowing the structure of the glycan to be determined.

To further influence the fragmentation behavior of glycopeptides, various instrumental parameters may be adjusted, as was demonstrated by Roepstorff and co-workers using an ESI-based QTOF instrument.[280] Through a careful optimization of the dissociation

parameters, the diagnostic oxonium ions could be preferentially generated, which could then be used to search for glycopeptides in a complex mixture. Taking advantage of the higher resolution, and as a direct consequence, mass accuracy, offered by the TOF mass analyzer, a narrower “mass window” could be used when attempting to locate the oxonium ions, so that the nonspecific signals that may have previously indicated the presence of a glycopeptide could be minimized. Further, by increasing the collisional energy by ~20% for a MALDI-based QTOF instrument, the peptide backbone could be fragmented for the glycopeptides derived from tomato proteins.[281] In this study, the authors reported that first a sequential decomposition of the glycan occurred, followed by fragmentation of the peptide backbone. Evidence for this mechanism was the lack of carbohydrate fragments that remained attached to the peptide backbone.

A number of other factors have been shown to influence the fragmentation patterns for IRMPD-based experiments. Perhaps not totally surprisingly, multiply-protonated glycopeptides derived from ribonuclease B resulted in more extensive fragmentation of both the peptide backbone and the carbohydrate,[278] most likely due to a second mobile proton that is free to traverse the peptide backbone and induce dissociation, while the first ionizing proton remains sequestered at the peptide’s most basic site.[282] Regardless of charge, however, one of the most intense fragments was the Y_1 ion. Interestingly, the charge carrier seemingly greatly influenced the fragmentation patterns. While the singly-protonated glycopeptide produced a spectrum that contained several *b*-type ions resulting from the fragmentation of the peptide backbone, the singly-sodiated spectrum was dominated by products resulting from the cleavages of various glycosidic bonds, including the $^{0,4}A_5$ and $^{0,2}A_5$ cleavages across the innermost GlcNAc unit. The doubly-charged analogue with 1 sodium and 1 proton produced “simpler” spectra but still contained sufficient information to elucidate the glycan structure. In this scenario, no peptide backbone ions or the cross-ring fragments were noted. Additionally, the peptide’s amino acid sequence was also found to have an effect on the fragmentation patterns. When basic amino acids were present, as in the case of the products of a digestion with trypsin, fragmentation of the GlcNAc–GlcNAc bond, resulting in the Y_1 ion, was one of the major products in both singly and doubly protonated analytes. Interestingly, the sodiated spectra for glycopeptides with and without basic amino acids seemed to be quite comparable, with extensive fragmentation of the carbohydrate occurring, including crossring fragmentation in both cases, and with no fragments associated with the peptide being observed. The authors attributed this to the apparent difficulties of these types of amino acids to effectively sequester sodium ions.

5.2. CID Used in Conjunction with Accurate Mass Measurments

CID in combination with high-resolution MS data has also been proven to be an effective approach to facilitate glycopeptide discovery in complex mixtures. In a recent study of the membrane-bound glycoproteins from the select infectious agent, *Francisella tularensis* subsp. *holarctica*, Balonová et al.[111] characterized two glycopeptides from a novel virulence factor and achieved identification through high-resolution LC-FT-ICR MS source-induced dissociation (SID).[273] Following the enzymatic digestion, bottom-up proteomics was performed on the glycoprotein, FTH_0069, that had been isolated by 2-D gel electrophoresis. SID was used to monitor characteristic glycan oxonium ions, and, with *a*

priori knowledge of the amino acid sequence and glycan structure, a list of theoretical glycopeptide accurate masses was compared to the observed glycopeptides, leading to their identities, achieved exclusively through sub-ppm mass accuracy.

It is clear that high-resolution MS data are vital to this type of analysis, but the considerable preparative expertise that was necessary to isolate the target glycoproteins from bacterial cell lysates through density-based fractionation, liquid extraction of membrane-bound glycoproteins, and 2D-gel electrophoresis prior to the use of bottom-up proteomics was a prerequisite to the achieved ionization of the glycopeptides.[111] This work exemplifies the potential of multidimensional sample fractionation and separation techniques in combination with MS detection as a means to achieve a clearer understanding of individual, biologically interesting glycoproteins, emphasizing that the current analytical glycobiology often remains a multimethodological task, as was the case a decade ago.[12]

5.3. Electron-based Dissociations of Glycopeptides

Alternative approaches to molecular fragmentation are the electron-based methods, including electron-capture dissociation (ECD)[283,284] and electron-transfer dissociation (ETD).[285,286] ECD involves the capture of a thermalized electron and is performed in an ICR cell,[287] while the analogous version of ETD is performed in an ion-trap instrument.[285,288] To induce fragmentation in ETD, an electron-transfer vehicle, gas-phase fluoranthene, which is most commonly used in commercially available instruments, accepts an electron thermalized by a methane buffer gas. A peptide then abstracts the electron, resulting in bond cleavage. While the exact mechanism(s) of bond cleavage are still a topic of debate,[287,289] these methods are effective in breaking the N–C_α bond, generating a series of *c*’ and $z^\bullet$ ions. Since this approach to fragmentation is a “chemical reaction”, a peptide’s vibrational energy is not increased. Thus, labile post-translational modifications, including phosphorylation and glycosylation, generally remain attached to the peptide and are largely unaffected by the fragmentation process, though in some cases, with both approaches, radical-initiated processes may lead to the cleavage of carbohydrate bonds, resulting in a loss of the glycan.[270,290]

Both of these approaches have found several applications in the analyses of N - and O - linked glycosylation.[241,267,270,271,291-299] When used in conjunction with a “heating” tandem MS technique,[270,271,291,295] mainly CID or IRMPD, the carbohydrate typically undergoes extensive fragmentation, though it seems that high-mannose structures may not be as effectively dissociated by IRMPD[295] as by CID.[271] The electron-based fragmentation method may then be applied to fragment the peptide backbone to determine the amino acid sequence.[270,271,291,295] Figure 11b presents an ETD spectrum of an N-linked tryptic glycopeptide derived from haptoglobin. Importantly, in this example, the glycan remains attached to the peptide backbone and the site of glycosylation can be determined by the mass difference between c_5 and c_6 ions. A combination of the two complementary approaches may be used to more fully characterize a glycopeptide[270,271,291,295] (compare parts a and b of Figure 11).

One drawback to the electron-based fragmentation methods is an apparent *m*/*z* limitation. While multiply-charged glycopeptide ions with *m*/z values of up to about 1,000 could be

successfully fragmented,[271,299] the main reaction above this approximate value was just a nondissociative electron transfer in ETD; this phenomenon was also observed by other groups.[300,301] Since many glycopeptides result in multiplycharged ions with *m*/*z* values above this threshold, the basic approach to this fragmentation method needs to be modified. Since it appears that noncovalent interactions hinder the detection of the generated fragments,[300] the ETD parameters have been adapted to include a gentle CID-type of activation,[299,301] increasing the number of diagnostic fragment ions for such peptides. In one example from the Karger laboratory,[299] a glycopeptide generated by the Lys-C digestion of epidermal growth factor receptor (EGFR) was detected as a +5 ion with an *m*/*z* value of 1142.73. The ETD-only fragmentation of this large glycopeptide allowed only 9 of the 36 amino acids to be determined. However, upon activation of a charge-reduced species generated during the ETD process, 20 amino acids were determined, and the correct peptide sequence was determined through database searching.

Without modifications to the current instrumentation, these methods may be most effective for glycopeptides featuring smaller carbohydrates, such as truncated N-linked structures or those N-linked glycans attached to bacterial glycopeptides,[241] or O-linked glycopeptides with smaller oligosaccharide chains. Indeed, several recent examples have shown the utility of this method for the characterization of this important, and analytically challenging, class of glycopeptides. Unlike N-linked glycopeptides, the O-linked structures do not readily yield a consensus sequence to indicate the site of modification. Further compounding the analytical difficulties, O-glycosylation is frequently found in areas rich in serine and threonine that have a high degree of site occupancy. For these determinations, ECD and ETD have proven to be valuable tools, as a recent publication demonstrates for several large, multiply glycosylated, highly charged mucin-originated *O*-glycopeptides,[302] where ETD fragmentation assisted in determining the amino acid sequence and the site of glycosylation. Interestingly, this work showed a high degree of peptide fragmentation for the glycopeptides modified with neutral glycans, while those with sialylated structures tended to produce fewer fragments.[302] Similarly, activated-ion electron-based approaches have been applied to the hinge-region *O*-glycopeptides of a galactose-deficient IgA1 myeloma protein, a mimic of IgA1 found in patients diagnosed with IgA nephropathy.[303] This study conclusively mapped the sites-of-glycosylation and the carbohydrates occupying each spot. On the basis of these results, the preferential sites for galactose deficiencies could be determined.[303] Another investigation utilizing ETD revealed that a significant decrease in the levels of GalNAc attached to IgA1 *O*-glycopeptides was observed in patients diagnosed with rheumatoid arthritis,[304] a change most commonly associated with IgG. Additionally, a combination of CID and ETD has been used to determine the glycans attached to three sites of O-glycosylation of β-amyloid precursor protein secreted by CHO cells.[297] This protein, typically found in the amyloid plaques associated with Alzheimer's disease, possesses 27 serine and 39 threonine residues in its secreted form. Using ETD, the authors were able to conclusively identify threonine residues 291 and 292 in the peptide spanning amino acids 289–302 (sequence: VPTTAASTPDAVDK) as those being glycosylated. Additionally, several Core 1-type structures were identified, ranging from a single HexNAc unit to a HexNAc-Hex-sialylated structure. Interestingly, while both sites could be modified, and in several cases both were modified simultaneously, only threonine-292 was found to be

occupied in all of the glycopeptides identified. Similarly, the peptide spanning residues 574–587 (sequence: GLTTRPGSGLTNIK), with four possible sites of O-glycosylation, was found to be modified at threonine-276 with two different structures.

The modification of the hydroxyl side chains of serine and threonine residues by GlcNAc residues is also an important PTM to monitor, as it possibly modulates a number of physiological processes, including gene-silencing and nutrient and stress-sensing, and it may be involved in Alzheimer's disease and diabetes. Identifying sites of *O*-GlcNAcylation is key to understanding the biological interactions of this common monosaccharide addition. Using ETD, a total of 58 sites of *O*-GlcNAcylation were found in an analysis of a murine postsynaptic density (PSD) pseudoorganelle,[305] a significant increase over the total number identified by ECD combined with a β-elimination/Michael addition procedure.[306] A total of 28 of these sites were located on the protein Bassoon, and three of these sites were previously believed to be phosphorylated. This seemingly points to an interplay between these two modifications, and EC/TD may be important in monitoring this biological process. Interestingly, eight sites of N-linked GlcNAc units were reported.

Additional biological applications of ETD involved the determination of the sites of reversible O-glycosylation in cortical neuron proteins from embryonic rats.[307] In this study, an engineered β-1,4-galactosyltransferase was utilized to selectively label the C4 hydroxyl of a GlcNAc unit with a ketone-containing galactose derivative, which was then further reacted with an aminooxy-biotin analogue. Through the use of biotin–avidin chromatography, peptides containing an O-linked GlcNAc could be purified. Prior to disruption, the cells were treated with PUGNAc (*O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-*N*-phenylcarbamate), a molecule that inhibits the activity level of β-*N*-acetylglucosaminidase. Quantitation was achieved through the dimethyl labeling[308] of the N-termini and the ε-amino group of lysine residues. Using this combined approach, a total of seven peptides were determined to undergo reversible O-GlcNAcylation, including four proteins that had not been known previously to be capable of this reaction. The amino acid sequences of these peptides were determined through ETD.

ETD has also been able to locate and confirm unexpected sites of glycosylation. While possessing the same activity as bovine pancreatic trypsin, which is not decorated with carbohydrates, this same proteolytic enzyme recombinantly expressed in maize (given the trade name "TrypZean") has been implicated as being glycosylated.[309] However, the site of modification could not be determined. CID analysis indicated that the tryptic peptide, with an amino acid sequence of SIVHPSYNSNTLNNDIMLIK covering amino acid residues 70–89, could be modified.[99] Since this peptide lacks the consensus sequon for N-linked glycosylation, O-associated carbohydrates were suspected, as four possible sites are present in this peptide. Surprisingly, ETD revealed that the glycan ($HexNAc_2(Fuc)Man_3Xyl$) was attached to ASN-79. Further ETD analyses of peptides generated by pepsin confirmed these results.

In the past several years, noninvasive approaches for biomarker discovery have been explored, including the analyses of urine, which may be an important physiological fluid for glycobiologists to study. In one study, nearly 500 proteins were found to be common in

samples provided by several individuals deemed as healthy.[310] Of these, 20 of the most abundant (excluding serum albumin) are thought to be glycosylated, comprising nearly two-thirds of this proteome by mass.[310] In a more focused study of the glycoproteins present in this fluid, sialylated glycoproteins were selectively enriched through a coupling of the glycoproteins to hydrazide beads,[292] followed by tryptic digestion, and a subsequent mild acid hydrolysis to release the glycopeptides. Through a combination of CID-MS^3 and ECD fragmentation experiments, 58 N-linked and 63 O-linked glycopeptides corresponding to 53 urinary glycoproteins were characterized. This data allowed 40 of the O-linked sites to be unambiguously located. The information gained from the CID-MS^2 experiments revealed that the main N-linked glycan structure could be tentatively assigned as biantennary, while the main O-linked structure was a desialylated Hex-HexNAc carbohydrate.

5.4. Higher-energy Collisional Dissociation

The development of an orbital-trapping mass analyzer[311] and its later commercialization[312] as the LTQ Orbitrap by Thermo Scientific has significantly benefited researchers in the life science areas. In this mass analyzer, ions are trapped in an electrostatic field and oscillate around a spindle at their harmonic frequency, analogous to FT-ICR instruments. While the resolution offered by this instrument may not quite match that of an FT-ICR, the Orbitrap allows ions to be detected at significantly higher resolution than those obtained with traditional ion-trap instruments and with much improved mass accuracies (2 to 5 parts-per-million being commonly reported)[312] without the need for superconducting magnets. An additional feature unique to the Orbitrap is the so-called “C-trap”,[313] a device used to store ions following their ejection from the instrument’s linear ion trap and inject them as small discrete pulses into the orbital trap mass analyzer. While it seems that the original intention of this trap was to act as an assistant to improve the overall performance of the orbital trap,[313] Olsen et al. quickly realized that fragmentation could be performed in this region of the mass spectrometer and at higher energies than in the linear ion trap.[314] Later, an octopole was installed to improve the trapping efficiency for ions with low *m*/*z* values.[314] Termed higher-energy collisional dissociation (HCD), dissociations initiated in this region of the instrument are not subjected to the 1/3 *m*/*z* cutoff limit that plagues traditional ion traps. Thus, smaller ionic fragments are detected.[314]

HCD fragmentation of glycopeptides has been reported and seems to be quite beneficial in glycopeptide characterization. As first investigated by Segu and Mechref,[315] this approach can be used to assist in the determination of glycopeptide amino acid sequences. Using tryptically-digested glycoprotein standards, the resulting peptide mixtures were first separated by reversed-phase nanoflow LC and subjected to a traditional CID analysis that resulted in an extensive fragmentation of the associated carbohydrate, enabling a structural characterization of the carbohydrate moiety. The complementary HCD method also caused extensive fragmentation of the carbohydrate, though a different pattern was observed (i.e., smaller fragments corresponding to monosaccharide or disaccharide fragments appeared as the more intense features). In addition to several smaller oxonium fragments being present, the Y_1 ion (peptide + a single GlcNAc unit) was commonly one of the most abundant ions, as can be seen in Figure 12. To reveal its amino acid sequence, this ion was isolated and subjected to a second HCD fragmentation, resulting in extensive fragmentation and

ultimately allowing its amino acid sequence to be determined. While this work focused on glycoprotein standards, database searching of the MS^3 data could be used to determine the identities of unknown glycopeptides.

A similar approach was used to identify the sites of N-linked glycosylation of the glycopeptides derived from the bacteria *Campylobacter jujuni*.[241] As in the previously discussed studies, a CID analysis proved very useful in determining the constituent monosaccharides of the glycan: five *N*-acetylhexosamine units, a hexose monosaccharide, and a bacillosamine residue directly connected to the peptide backbone. However, the CID process did not generate any ions diagnostic of the amino acid sequence, so that an alternative fragmentation approach was needed. Upon HCD fragmentation, a slightly different dissociation pattern was observed than that reported by Segu and Mechref. In these spectra, the most intense ion generally appeared to be due to the peptide backbone deglycosylated by the HCD process, though the Y_1 ion (peptide + bacillosamine) was also easily observed. Interestingly, the HCD spectra of bacterial glycopeptides also exhibited extensive fragmentation of the peptide backbone and were sufficient to determine the sequence of amino acids. On the basis of these data, it seemed possible to tentatively assign the site of glycosylation based on the extended glycosylation motif of (D/E)XNX(S/T), where X may be any amino acid except proline. CID/ETD was also utilized in this study, and in total, 75 sites-of-glycosylation were determined, with 49 unique to the CID/HCD method and 26 being located through the CID/ETD method.

HCD has further been applied to detailed structural investigations of trace-level (sub-fmol) glycopeptides.[316] To achieve this, a 3-step data analysis procedure based on high mass accuracy (sub-2.5 ppm) was developed. This procedure first required the identification of potential glycopeptide ions and was achieved by scanning the HCD spectra for diagnostic oxonium ions with sub-ppm mass accuracies, followed by a characterization of the peptide backbone that was facilitated by the Y_1 ion (which was generally one of the most intense glycopeptide ions above an *m*/*z* value of about 500). A characterization of the associated glycan was achieved by subtracting the mass of the peptide from the corresponding glycopeptide. Using this approach, the authors of this study confidently detected 88 previously uncharacterized glycopeptides derived from a mixture of hen egg proteins and two unreported sites-of-glycosylation were identified for ovoglycoprotein, which was present at low femtomole abundances. Substantial microheterogenities were observed at the two previously unknown sites-of-glycosylation for ovomucoid. While this approach seems to be a “step in the right direction” for a rapid characterization of a large glycopeptide pool, the authors noted several potential weaknesses with this method that need to be resolved. Among them was the lack of diagnostic carbohydrate fragments observed in the HCD spectra, an issue that could possibly be rectified by performing a CID analysis.[241,315] A further limitation may be a definitive determination of the site of attachment of the glycan, particularly from glycopeptides possessing more than one site of modification. This problem could be solved by performing an ETD fragmentation, as suggested by the authors. For largescale studies, further problems may arise for identifications based solely on accurate mass.[317]

Peptides modified by O-linked GlcNAc have also been sequenced using a combination of HCD and ETD.[318] In this study, model peptides containing O-linked GlcNAc units, along with proteins derived from the cell line HEK293T enriched by monoclonal antibodies against *O*-GlcNAc, were investigated. Peptides possessing an *O*-GlcNAc were subjected to an HCD fragmentation, seemingly to confirm its presence by the generation of the oxonium ion present at an *m*/*z* value of 204 and its fragment ions. In this investigation, the HCD process produced only a limited number of cleavages associated with the peptide backbone, limiting the number of successful identifications by database searching. Therefore, to more effectively fragment the peptide backbone, ETD was employed. Using the combination of these two techniques, the authors identified 83 sites modified by O-linked GlcNAc units on 172 glycopeptides associated with 13 proteins. Only 13 of the sites had been previously assigned.

A further refinement of the sequencing method for O-linked glycopeptides involves the use of diagnostic oxonium ions to act as “triggers” for ETD experiments.[319] In this investigation, three “SimpleCell” lines were engineered to express truncated O-linked glycans, either the Tn moiety or the extended sialyl Tn analogue. Following a lectin weak-affinity chromatographic enrichment of the Tn-modified tryptic glycopeptides and a subsequent nanoflow LC separation, the glycopeptides were subjected to an HCD procedure. If the diagnostic ion at an *m*/*z* value of 204.09 was detected, the ETD process was initiated for that glycopeptide. Using this procedure, a total of 275 unique glycopeptides from 148 proteins were identified, and over 400 sites of glycosylation were located.

In a similar way, oxonium ions produced during an HCD fragmentation were used to begin[320] a supplemental activation ETD procedure[299,301] for the analysis of N-linked glycosylation of model glycoproteins. In this experimental design, unenriched proteolytic digests of bovine ribonuclease B (digested with Endoproteinase C) and human IgG (digested using trypsin) were first subjected to a ZIC-HILIC nanoscale LC separation. For the analysis of ribonuclease B, an ETD fragmentation was initiated only if ions were observed at *m*/*z* values of 204.09 and/or 366.14. Using these parameters, a total of 139 ETD events were triggered, with 33 of the resulting spectra corresponding to known glycopeptides, as based on a manual interpretation of the resulting spectra. Database searching was also performed by three different databases using the Man_{5-9} glycans as variable modifications; however, only a relatively small subset of the spectra were correctly identified. The authors attributed this finding to issues associated with the algorithms used to search ETD data.[321] The HCD fragmentation of IgG peptides initiated 273 ETD events. Of these, 27 corresponded to known glycopeptides. An additional 78 spectra were triggered, although the fragmentation data could not conclusively identify these analyte ions as being glycopeptides. In total, 69 spectra could be linked to known glycoforms of IgG glycopeptides, and 126 recordings were classified as being glycopeptides, though their amino acid sequences could not be determined.

5.5. UV Photodissociation

The use of 157 nm UV light has also been shown to be able to fragment both the peptide backbone and the associated carbohydrate.[322] This MALDI tandem TOF study

demonstrated extensive peptide backbone fragmentation with many x-, y-, v-, and w-type ions being recorded. Due to the presence of the v- and w-ions, isomeric amino acids (i.e., leucine and isoleucine) could be identified confidently. In addition, several cross-ring fragments associated with the carbohydrate were observed, which could allow for linkage-specific information to be obtained. Though as the authors conceded, due to the extensive fragmentation patterns reported, interpretation of the spectra can be challenging and may prove to be quite difficult for an unknown glycopeptide.

5.6. Bioinformatics for Tandem MS Data of Glycopeptides

Having successfully determined the presence of a glycopeptide, the next major task is the interpretation of the tandem MS data. For proteomic data sets, this is generally a straightforward process through database searching using one of, or in some cases, a combination of, several search engines. These routinely used algorithms allow the user to include a number of “simple” post-translational modifications into the search criteria. Unfortunately, due to a number of reasons, including the vast array of possible glycan structures, recorded fragmentation patterns, and the expected exorbitantly long searching times, in practice, this has not yet proven to be a feasible approach for glycopeptides. At the present time, the challenging undertaking of spectral interpretation for these types of analytes is most frequently performed manually and is frequently very time-consuming and requires advanced levels of skill for an accurate interpretation. Fortunately, in recent years, a number of algorithms developed specifically for glycopeptide interpretation have been designed to assist in this task. Several of them are publicly available. The first of these tools, GlycoMod,[323] can predict possible carbohydrate structures for a known glycopeptide or glycoprotein sequence and may be accessed through the Expasy.org Web site. A further advancement in this area was the Glycominer software.[324] This algorithm attempts to determine both the peptide sequence and the structure of the carbohydrate, with the initial study showing very promising results and few false-positives. Currently, it appears that this utility is compatible with the Waters, Thermo, and Kratos instruments. The GlycoX algorithm[325] developed in the Lebrilla laboratory uses high mass accuracy data. For example, using data acquired from an FT-type instrument, this program can predict both the site of modification and the carbohydrate structure. Accurate results were obtained for standard glycoproteins as well as those with unknown identities. This software was listed as available upon request. Glycospectrascan uses MS data rather than tandem MS information to identify glycopeptides and allows for multiply-charged ions to be entered, but it requires a knowledge of the potential N- and O-linked glycan structures, as well as the glycopeptide masses in a given sample. However, very good results were returned for the analysis of human secretory IgA. Glycopep grader,[326] seemingly an “upgrade” of Glyco DB,[327] offers a scoring function and relies heavily on the identification of the Y_1 ion (peptide + GlcNAc). This program was successfully used to characterize, in terms of both peptide and the attached carbohydrate, several glycoprotein standards, including ribonuclease B and asialofetuin. However, an *a priori* knowledge of the possible glycopeptide’s amino acid sequence is still a requirement. In addition to these, several other algorithms have been reported but are not yet publicly available.[328-330]

5.7. Multiple Reaction Monitoring

Tandem MS methods are generally applied for structural characterization/verification purposes, but they may also be used to precisely quantitate different analytes, including glycopeptides at high sensitivity, using a triple quadrupole mass spectrometer employing multiple (or single) reaction monitoring (MRM or SRM) techniques. In an MRM experiment, the first quadrupole is scanned for the selected ion(s) of interest over selected time frames during an LC analysis. These specific ions are transmitted to the second quadrupole, where they are subjected to a CID fragmentation. Selected fragments that are very specific to the precursor of interest, commonly referred to as transitions, are scanned using the third quadrupole. Thus, these methods are very specific to the analyte(s) of interest. One of the earliest applications of this method was to accurately quantitate the levels of vancomycin, a glycopeptide antiobiotic used to treat Gram-positive bacterial infections, in rat blood serum samples.[331] Later, this method was used to quantitate the level of the protein tissue inhibitor of metalloproteinase-1 (TIMP-1) in a patient diagnosed with colorectal cancer.[332] In this study, serum proteins with β1-6-linked GlcNAc units on the α1-6-linked mannose, commonly observed in many cancers, were enriched using the lectin phytohemagglutinin-L_4 and subsequently digested with trypsin. Using the stable isotope standards and capture by antipeptide antibodies (SISCAPA) method coupled with MRM techniques, low attomole amounts of TIMP-1 were reported and the aberrantly glycosylated protein was estimated to have a concentration of 0.8 ng/mL. An aliquot of only 1.7 μL of serum was used in this experiment. While nonglycosylated peptides were targeted, this is a very impressive study of a potentially clinically important glycoprotein.

MRM methods have further been applied to sialylated glycopeptides to identify potential indicators of diabetes in mouse serum.[333] In this investigation, serum samples were first digested with trypsin, and the “glycerol tail” of the sialic acid residues was selectively oxidized[334] and conjugated to hydrazide-activated supports. After removing unbound peptides, the immobilized analytes were released using ice-cold 1 M HCl and the resulting aldehyde group was reductively amidated with 2-aminopyridine. Using this approach and monitoring for the Y_1 ion as a key transition, sialylated glycopeptides originating from immunoglobulin gamma-2B, serotransferrin, murinoglobulin, α-2-macroglobulin, and serine protease inhibitor were found to be increased significantly in their expression levels in the diabetic mice.[333]

A similar enrichment approach/MRM analysis of sialylated glycopeptides derived from prostate-specific antigen (PSA) was employed in an attempt to improve the overall predictive ability of this protein for prostate cancer.[335] By studying PSA originating from both tumor and healthy tissues, the limited ability of this glycoprotein alone to act as a reliable indicator of prostate cancer was demonstrated. Interestingly, an examination of the overall glycosylation levels also produced results with limited diagnostic ability. However, an analysis of enriched (formerly) sialylated glycopeptides demonstrated that these glycopeptides were elevated in their abundance levels in the tumor tissues. Unfortunately, due to the desialylation, any information pertaining to the overall degree of sialylation was lost. Therefore, there appears to be a need to perform these types of studies on the

glycopeptides (with the glycan still attached) to obtain the maximum amount of information related to a peptide of interest.

In an attempt to keep part of the glycan attached to the peptide backbone and to monitor core fucosylation, serum glycoprotein glycopeptides enriched using LCH (also referred to as LCA) sepharose 4B were subjected to a digestion using Endo F3,[336] an enzyme which cleaves the β1-4 linkage connecting the two GlcNAc units of the core, and MRM was used to monitor the levels of core fucosylation. When this approach was applied to control serum samples and patients diagnosed with hepatocellular carcinoma (HCC), several peptides associated with Ig α-2 chain C, hemopexin precursor, and ceruloplasmin precursor were recorded with increased abundances. While only a handful of peptides were reportedly diagnostic of the disease, the small sample used in this study set may have "skewed" the statistical analysis; therefore, a larger sample set needs to be analyzed to confirm the potential of this method. Regardless, this approach appears to be very promising for a higher-throughput monitoring of core-fucosylation for HCC.

Most recently, the Mechref laboratory has taken on the study of MRM measurements of fully glycosylated glycopeptides using a label-free quantitation approach,[337] in contrast to the other studies that generally used isotopically labeled analogues of the target analyte for quantitation. Oxonium ions were used as the transitions, which were better produced at a collision energy of 40%. Several fully glycosylated glycopeptides were successfully quantitated from depleted blood serum with very good standard deviation values for three analyses.

5.8. Analysis of Deglycosylated Peptides

Perhaps because of some of the analytical challenges associated with the analysis of glycopeptides with their attached carbohydrate(s), many researchers prefer to conduct experiments on deglycosylated analytes or partially deglycosylated peptides. Some of these techniques involve the enzymatic removal of N-linked glycans in ^{18}O-labeled water[338] or a partial enzymatic degradation of the N-linked structures facilitated by β-*N*-acetylglucosaminidases (in particular, Endo-M,[339] a member of a series of endoglycosidases which cleaves the β1-4 glycosidic bond connecting the GlcNAc units of the chitobiose core with varying specificities toward glycan structures; for example, Endo F_2 and Endo M are reactive toward highmannose and biantennary structures, while Endo H is reactive toward high mannose and hybrid glycans). The removal of an O-linked carbohydrate via a β-elimination mechanism followed by a Michael addition has also been utilized to mark the site of glycosylation. Each of these experiments may provide unique information, facilitating quantitation or indicating the site of glycosylation. Additionally, the removal of the carbohydrate has been deemed by many investigators to help improve the overall mass-spectral performance through an improved ionization yield/detection of glycopeptides, which generally do not ionize with the same efficiencies as their nonglycosylated counterparts. The removal of the oligosaccharide may also allow for database searching of tandem MS data for peptide identification. Unfortunately, the price for these benefits is the loss of information relating the structures of the attached glycans.

One method to generate deglycosylated N-linked peptides for mass-spectral interrogation is through an enzymatic removal of the oligosaccharide chain by PNGase F, as will be discussed in sections 6.1–6.1.1 of this review. This digestion procedure converts asparagine residues to aspartic acids via a deamidation mechanism by the addition of an oxygen atom from the surrounding water. This modification increases the mass of the peptide by 0.9840 Da over its predicted mass. While this may be useful to a first approximation to identify sites-of-glycosylation, added confidence may be gained by performing this reaction in "heavy" water,[338,340] causing a mass increase of 2.9882 Da. Even fairly recently, with the improved technology associated with the latest mass spectrometers and methodological developments, enzymatic releases of N-linked carbohydrates catalyzed by PNGase F for site of glycosylation determinations are still performed in large-scale studies.[341] However, it appears that extreme care must be exercised during the sample handling and processing, and the results of these types of experiments need to be interpreted with caution. If subjected to a tryptic digestion, the trypsin must be completely deactivated to prevent a partial incorporation of ^{18}O at the C-terminus, which could cause ambiguous results,[342] unless this conversion is desired and allowed to proceed to completion.[340] When trypsin is allowed to quantitatively label C-termini with ^{18}O, glycopeptides deamidated by PNGase F reflect a high degree of accuracy with the expected ratios when compared to those digested in $H_2\ ^{16}O$.[340] An additional concern is the possibility of chemical deamidation that may be caused by various sample handling/preparation steps, further complicating an interpretation of the data.[343] This mechanism was highlighted by a study of membrane-bound proteins derived from *Escherichia coli*, a bacterium lacking N-glycosylation machinery. Employing widely used methods, 391 deamidated peptides were detected following a treatment with PNGase F and 584 were observed when the sample was treated with PNGase A. Since these enzymes have different optimal pH values (PNGase F's is slightly basic, while that for PNGase A is slightly acidic), there seems to be a pH dependence on the deamidation mechanism. Interestingly, several of these deamidation sites, indicated by the incorporation of ^{18}O, were located in the N-glycosylation motif,[343] which could easily be erroneously assigned as N-glycosylation sites. The deamidation mechanism seems to be especially prevalent if an asparagine residue is followed by a glycine.[343]

A partial deglycosylation procedure, leaving a single GlcNAc unit attached to the peptide, may be beneficial to indicate the site of glycosylation, which may be accomplished through a treatment with the exoglycosidase Endo-M.[339] Following the digestion, the overall mass of the peptide is increased by 203.08 Da, and the GlcNAc moiety should more definitively indicate the site of glycosylation. Additionally, tandem MS data for peptides subjected to this digestion can be searched against a database and should reduce the number of false positive deamidation hits. However, a certain level of care must also be taken with this procedure, since complicated glycan structures may inhibit the activity of Endo-M.[344] Core fucosylation also seems to render this enzyme as inactive. To circumvent this potential problem, glycopeptide samples may be simultaneously treated with a cocktail of exoglycosidases, and it was reported that the resulting spectra appeared to be an order of magnitude more intense than those acquired for samples digested with Endo-M alone. If combined with HCD to confirm the presence of core GlcNAc through the detection of the

oxonium ion present at an *m*/*z* value of 204, this procedure would be quite promising to determine glycopeptide sequences and sites-of-glycosylation.

An effective sequencing of O-linked glycopeptides and a confident determination of the site of attachment by mass spectrometry have proven to be a difficult task, in large part due to the lack of a universal enzyme that removes these structures from the peptide backbone. Further compounding the challenges associated with *O*-glycopeptide analysis is that these types of glycans are commonly located in regions of proteins with high densities of serine and threonine residues and may thus complicate a definitive determination of the site of glycosylation. To address some of these obstacles, many researchers have turned to the β-elimination/Michael addition (BEMAD) approach. The initial approaches utilized sodium hydroxide as the agent to induce β-elimination, which proved to be effective at removing the glycan while converting serine and threonine residues to dehydroalanine and dehydrobutyric acid, respectively, as indicated by the shift in mass by 1 Da.[345-347] While there appeared to be no degradation of the peptide backbone, in particular if a reducing agent was not included in the reaction mixture,[348] the high concentrations of salts used in this procedure required extensive purification,[345,347] which increased sample handling and consequently sample losses. Thus, the overall sensitivity of the method was somewhat limited. Further evolution of the method substituted ammonium hydroxide for sodium hydroxide,[346] an alteration which substantially improved the sensitivity of the method by eliminating the need for extensive sample purification. Drawing upon the evidence that an NH_2 group can added to the dehydrated peptide, de-O-glycosylations were attempted in methylamine and ethylamine in an attempt to further accentuate the mass difference at the site of O-glycosylation.[349] While both of these methods seemed to induce O-deglycosylation, neither was capable of a quantitative release, and methylamine appeared to catalyze increased nonspecific peptide backbone scissions, especially at longer reaction times.[349] In a further adaptation of this protocol, the dimethylamine-mediated release of O-linked glycans has been performed under microwave radiation.[350] While the reaction could be accomplished in about 30 min, only about 75% of the resulting peptides became modified by the dimethylamido group, with the other approximately 25% remaining in their dehydrated state. Interestingly, a quantitative release of *O*-glycans was reported in 2 h using gaseous methylamine, and this reaction proceeded at a much higher rate than releases conducted using ammonia vapor.[351]

As a result of the addition of methylamine to the serine or threonine side chains, tandem MS experiments could easily identify the site of glycosylation.[351] Using the dimethylamine analogue in the solution, it appeared that quantitative release of *O*-glycans was possible and the dehydrated serine and threonine residues were subjected to a condensation reaction with ethanethiol.[352] Because the peptides in this study were further reacted with a succidimidyl ester of tris(2,4,6-trimethoxyphenyl)phosphine (TMPP) to create a permanent positive charge at their N-termini, the resulting MS^2 recordings displayed a series of *a*-type fragment ions upon CID, and the ethanethiol tag appeared to be stable and was deemed as an appropriate marker of the site of glycosylation.[352] In a similar study, β-mercaptoethanol was used as the nucleophile when methylamine was used to induce β-elimination.[353] Similarly to the study using peptides modified with ethanethiol,[352] the β-mercaptoethanol tag appeared to be stable under MS^2 conditions. In an interesting modification, dithiothreitol (DTT) was

used as a nucleophile to indicate the site of O-glycosylation. Due to its bifunctional characteristics, formerly O-glycosylated peptides could be enriched using thiol-activated Sepharose gel.[354,355] Further, through the use of deuterated DTT, multiple samples could be monitored for their differential expressions in a single MS analysis.[355]

A general problem that has been observed is the resistance of particular glycopeptides to β-elimination.[352,356] It appears that modified threonine residues on the N-terminal side of a proline are particularly resistant and slightly harsher conditions may be required for a successful β-elimination.[352]

6. GLYCOMICS

One of the latest members in the "-omics" family of the life sciences is glycomics, an area that has recently received significant attention. Its recent considerable recognition as a key member of this broad collection of varied scientific disciplines is mainly due to the realization of numerous studies that a protein's glycan components are often the crucial functional determinants of biological events. With further methodological and technological developments and improvements in instrumentation, glycomics is rapidly positioning itself to become one of the important fields in addressing some key biological and medical questions.

While direct measurements of only a collection of carbohydrates may seem at a first glance to be somewhat limited in that the information relating to the overall integrated function and structure of a given glycoprotein is lost due to the requisite deglycosylation step, such measurements have a certain practical appeal as regards the following: (a) oligosaccharides are often *the* crucial functional elements in cellular and biomolecular interactions; (b) glycomic profiling techniques are inherently faster and methodologically easier to multiplex than the currently available proteomic approaches; and (c) the dynamic concentration ranges for glycans appear to be not nearly as broad as those typically observed for proteins in biological samples. However, we do not yet know what are the exact limits for glycans' meaningful physiological concentrations, and the measurement and reliable quantitation of glycans at very trace levels still remains a difficult task. Yet there is increasing evidence that these low-abundance structures are among the most important for the biomedical community to study in detail.

6.1. Glycan Release Procedures

At the heart of any glycomics experiment is the dissociation of the carbohydrates from their protein(s), whether it be a purified monoclonal antibody to be used as a therapeutic agent or a complex mixture of (glyco)proteins extracted from a biological source. The representative array of oligosaccharides (glycans) is subsequently displayed as a "glycomic profile" or "glycomic map" through a suitable bioanalytical technique. A quantitative and reproducible release of oligosaccharides from glycoproteins has always been a significant and difficult issue in glycobiology. It has gained an even greater importance in the high-sensitivity requirements of today's glycomic profiling, particularly for biomedical applications. The chemical release procedures used earlier, such as hydrazinolysis or the classical β-elimination in an alkaline medium, have now mostly been replaced by the more gentle

enzymatic deglycosylation (use of *N*-glycanases) for asparagine-linked glycans[357,358] or microscale chemical release procedures[359-361] for threonine/serine-linked oligosaccharides. It is now generally agreed that *N*-glycans are "easier" to cleave from proteins than *O*-glycans, largely due to the availability of peptide-*N*-glycosidases F and A (PNGase F and PNGase A) and other glycanases and exoglycosidases, which reliably cleave a broad range of substrates, regardless of their glycan substitution, with only a few exceptions noted, for example, N-linked glycans derived from bacterial proteins.[63]

6.1.1. Enzymatic Release of N-Linked Structures—The most straightforward, methodologically simplest, and most reproducible approach to free carbohydrates from their proteins is through an enzymatic treatment. Though a number of enzymes possessing endoglycosidase activity may be used, PNGase F[362] and PNGase A are most commonly used for this purpose. PNGase F is generally effective at releasing N-linked structures in mammalian systems, while glycomic studies of plants, insects, and other forms of life that often have core fucose monosaccharides attached as α1-3 are best treated with PNGase A, since PNGase F is insensitive toward this linkage.[363] These enzymes catalyze the cleavage of the amide bond of the side chain of the asparagine residue and substitute an oxygen molecule for the nitrogen, resulting in the deamidation of the asparagine residue. Due to its conversion to an aspartic acid, the mass of the deamidated peptide is approximately 0.98 Da greater than the expected mass for the amino acid sequence of the glycopeptide and in some cases may be used to indicate the location of the glycan. The carbohydrate is released as a glycosylamine, with the amino group at the N-terminus being quickly converted to a hydroxyl moiety, although, through carefully controlled reaction buffer conditions, the amino group may be preserved, providing a convenient site for modification.[364,365]

For high-sensitivity measurements, enzymatic release procedures are often performed for extended periods of time. Oftentimes, up to 24 h or longer are required to achieve the highest possible digestion efficiency. However, for large-scale studies, such as those in clinical or industrial settings, where hundreds, if not thousands, of samples may need to be analyzed in a single study, the throughput of the release procedure needs to be improved. One interesting approach to reduce the digestion time involves the use of ultra-high-pressure cycling, which subjects proteins to pressures of up to 30 kpsi.[366] Even at these conditions, PNGase F appears to be unaffected, while many glycoproteins are sufficiently denatured to be deglycosylated in as little as 20 min.[366] Similarly to proteolytic digestions in proteomics, the enzymatic cleave of oligosaccharides may be assisted using microwave radiation; a complete removal of the glycans from monoclonal antibody drugs has been achieved in as little as 10 min, while up to 1 h was required for other glycoprotein standards.[367,368]

Since each of the routinely used glycanases has a certain level of specificity, an enzymatic approach that could be applied to nearly any situation is highly desirable. To realize this idea, several research groups have explored the unique properties of the cocktail of proteolytic enzymes derived from *Streptomyces griseus*, known commercially as "Pronase", to isolate N-linked glycans from their respective proteins. This mixture of enzymes ultimately digests proteins to amino acids, and a complete digestion will result in single asparagine residues linked to their carbohydrate. Because of this property, Pronase may be applied in situations where no other suitable enzyme is available for deglycosylation, as

demonstrated in an interesting example where it was used to recover bacterial N-linked glycans.[63] Since glycans in this domain of life may be attached through a bacillosamine monosaccharide,[63] these types of N-linked glycans are resistant to PNGase F/A treatment. Additionally, Pronase has also been recommended as an alternative to PNGase F[369] for the analysis of other N-linked glycans. However, when Pronase is applied to the glycoconjugates, a complete digestion of the proteins to single amino acids is often required, and digestion times of up to 48 h may be needed. Fortunately, the incubation times can be significantly reduced using Pronase immobilized on solid supports,[370,371] resulting in efficient digestions in only a few minutes, a time scale that is compatible with on-line digestions for direct LC-MS analyses.[370]

6.1.2. *O*-Glycan Release Procedures—As opposed to the enzymes that cleave a wide variety of N-linked glycans, a single enzyme with a broad specificity toward O-linked structures is not readily available for this purpose, ostensibly due to the diversity of O-linked core structures, as indicated in Figure 2. However, unsubstituted core 1 structures may be removed using endo-α-*N*-acetylgalactosaminidase,[372] more commonly known as *O*-glycanase. Consequently, chemical release approaches are most often used for a general removal of *O*-glycans, with a β-elimination performed in an alkaline medium being among the most popular.[373-375] Even though this method was originally developed to release O-linked glycans, in certain situations, N-linked glycans may also be cleaved.[376] In the classical approach,[373] glycans are treated with a mild solution of sodium hydroxide (0.05–0.1 M), and high concentrations of sodium borohydride are needed to convert the released glycans to their alditol forms in order to prevent the peeling reactions that cause the sequential loss of monosaccharides from the reducing end.[373] Therefore, oligosaccharides released using this procedure require extensive purification, and consequently, this method has only been moderately successful for trace-level analyses. Since sodium hydroxide may cause damage to the peptide/protein backbone, milder reaction conditions using ammonia have been explored to preserve the integrity of the protein while still enabling an efficient β-elimination.[345,346,348] Further fine-tuning of the ammonia-based method substituted ammonia-borane complex as the reducing agent,[359,360] which can be removed without using a solid-phase extraction medium. Rather, excess ammonia-borane complex is first reacted with an appropriate acid and the resulting borane salts are converted to their volatile methyl esters with methanol and then removed under vacuum.[360] Consequently, enhancements in the method's sensitivity over the conventional methods were achieved. As with other β-elimination release protocols, the ammonia/ammonia-borane complex procedure generates glycans in their alditol (i.e., reduced) form. While this is not necessarily a problem for MS-based analyses, per se, this becomes an issue for other analytical methods used to display glycomic traces that require chromophores or fluorophores. To meet the needs of these techniques, including UV- and laser-induced fluorescence (LIF)-based detection schemes used in LC and CE or CEC, a nonreductive β-elimination protocol[359] was developed that regenerates the reducing end, thus enabling the attachment of various amino-based chromophores or fluorophores. While a certain amount of sensitivity was sacrificed with this method, its compatibility with other detection schemes is clearly valuable.

Other more gentle release conditions using mild alkylamines to induce β-elimination have been explored, including ethylamine, which resulted in glycans with a free reducing end.[377] Unfortunately, several degradation products were observed that were attributed to peeling reactions, most likely due to the lack of the inclusion of a reducing agent. However, this approach led to further developments for alkylamine-based protocols, including a procedure using dimethylamine[350] coupled with microwave radiation. Using this method, a release efficiency of over 95% was achieved in just over 1 h at 70 °C and was shown to be more efficient when compared to the classical procedure using NaOH. While the initial development of the method utilized peptides with attached *O*-GalNac, later experiments demonstrated this method was also as effective as the traditional method of release for the O-linked glycans associated with bovine fetuin, indicating this technique could have more universal applications.

In a shift away from the chemical methods, Pronase has been explored as a way to recover *O*-glycans for analysis. In this procedure, *O*-glycans linked to their serine or threonine residues were permethylated and released via a β-elimination mechanism,[378] while N-linked structures remained attached to their amino acid. Interestingly, the free amino group of the N-termini of asparagine residues underwent a β-elimination procedure of their own.[63] Thus, this procedure allows O-linked glycans to be easily differentiated from N-attached structures when both are present in the same spectra. When compared directly to the samples prepared using other *O*-glycan release techniques, the MALDI-TOF MS signals associated with the Pronase-digested samples were typically 10–20 times more intense, as demonstrated in Figure 13, which compares the Pronased-based method directly to the “classical” approach employing sodium hydroxide and sodium borohydride, the ammonia/ammonia borane complex method conducted on glycoproteins, and the ammonia/ammonia borane complex method performed on glycoprotein tryptic digests. Each sample was then permethylated using methyl iodide with different levels of deuterium substitutions, as discussed in section 7.1 of this review, to allow a direct comparison of the methods. When the enzymatic/chemical approach was applied to a 1-μg aliquot of bile-salt-stimulated lipase (BSSL), a large, heavily O-glycosylated protein isolated from human breast milk, 40 glycan structures were uniquely detected due to this method in comparison to other approaches.[379-381] In total, 75 O-linked oligosaccharides were identified from this glycoprotein.

6.2. Mass Spectrometry

Similar to other -omics fields, mass spectrometry is one of the key developments that has accelerated the field of glycomics. While other analytical techniques, for example, capillary electrophoresis (CE) and capillary electrochromatography (CEC), have provided significant contributions to the field in their own right, and are often used in conjunction with mass spectrometry,[382-384] the relatively high throughput and adequate sensitivity, coupled with the important mass information generated by MS-based experiments allows for more precise characterizations. However, in certain cases ambiguities may arise, which may be at least partly resolved by performing a tandem MS fragmentation experiment to verify a possible structure.

While a number of “modern” ionization methods have been developed to introduce biomolecules as intact analytes into the gas phase, the two that are used almost exclusively for the analysis of carbohydrates are MALDI and ESI, with MALDI arguably being the more popular. Generally considered to be the more sensitive approach, particularly for underivatized glycans, the key to a successful MALDI analysis is the selection of an appropriate matrix. While 2,5-dihydroxybenzoic acid has been widely used for a number of years,[385] several new matrices with very attractive properties, in terms of a general improvement of the ionic signal strength recorded and in some cases enhanced tandem MS fragmentation patterns, have been developed in recent years.

Given the importance of a number of trace-level glycans, the ability to reliably detect and quantitate low-abundance analytes is of keen interest to the biomedical community. Thus, a driving force behind the development of new matrices, and their desirable additives, is an enhancement of the overall ionic signal. In an interesting series of investigations, Perreault and co-workers studied the effects of adding aniline and its derivatives to 2,5-DHB.[386-388] By including these agents, an on-plate derivatization of the reducing end was accomplished under nonreductive amination conditions (i.e., the product remained as a Schiff base). While the initial attempts resulted only in about 50% derivatization after 10 min,[386] more intense MALDI signals were observed, which could be attributed to a possible comatrix effect or, as the authors noted, a more uniform distribution of matrix crystals following the inclusion of the additive. An additional benefit of this matrix combination was an enhancement of two tandem MS cleavages across the innermost GlcNAc monosaccharide, i.e. the so-called “crossring” fragments. Because an incomplete derivatization would act to compromise the overall sensitivity of the measurement, dimethylaniline[387] was later used as a matrix dopant. Because of the structural feature of this amine, its reactions with the reducing end of a carbohydrate are minimized, thus effectively circumventing the issue of multiple peaks being detected for a single analyte due to a partial derivatization. As with aniline, improvements in the uniformity of the matrix crystals were observed, resulting in measurements in the femtomole range.

Alternatively, aniline has been used to synthesize a solid ionic matrix from α-cyano-4-hydroxycinnamic acid (CHCA).[389] When compared to CHCA only, improved MALDI signals were recorded for a number of classes of biological compounds, including synthetic polymers, amino acids, peptides, proteins, and carbohydrates. When the oligosaccharide standard raffinose was subjected to the aniline–CHCA salt, the intact sugar was detected as a sodium adduct with minimal decomposition reactions, such as a loss of water, occurring. Such mechanisms were much more prevalent when CHCA alone was used. In general, similar levels of sensitivity for carbohydrates with this matrix and 2,5-DHB were observed. Moreover, lower laser powers were acceptable using the aniline–CHCA matrix, which led to improved spectral resolution.

Since the aniline-based investigation indicated that an on plate derivatization may be beneficial, other studies have focused on achieving a more complete derivatization using different classes of molecules. This effect was observed with procaine and procainamide used as matrix additives for 2,5-DHB.[390] With an extended spot drying time, resulting in a longer reaction period, the carbohydrates were modified at their reducing ends, with the

acidified procaine as the cause of a nearly complete derivatization yield. Similarly to aniline, the inclusion of these additives also led to a more uniform distribution of matrix crystals. While the overall ionic signal strength was comparable to using just 2,5-DHB, two additional important benefits were noted: the inclusion of an additive seemed to suppress noise levels, while the derivatized oligosaccharides were detected as being mainly protonated; and tandem MS experiments produced unique fragmentation patterns.

Perhaps the most attractive of the (co)matrices that modifies the carbohydrate is 3-aminoquinoline.[391] Following a careful optimization of a number of different parameters, including organic solvent, pH and the inorganic acid used, concentration of reagent, and the reaction time, a quantitative on-plate conversion was achieved in approximately 1 h. This matrix was suitable for both positive- and negative-mode detection, while as little as 1 fmol of analyte was detected as a nitrate adduct in the negative-ion mode. When compared to another matrix, harmine, negative mode fragmentations resulted in a more intense cross-ring fragmentation.

Additional groups have focused on evaluating other small molecules for their abilities to function as MALDI matrices. The flavonoid isoliquiritigenin (4,2′,4′-trihydroxychalcone) was shown to be an effective matrix that displayed several advantages when compared to the traditionally used 2,5-DHB.[392] In addition to improved signal-to-noise ratios at relatively high amounts of analyte (in the pmol range), adequate spectra were also acquired at high-salt situations, including in the presence of 8 M urea and 5 M NaCl. Under these conditions, no signal was detected when 2,5-DHB was used as the matrix. This new matrix may allow investigators to streamline sample preparation protocols by reducing the number of sample purification steps that are commonly required when other matrices are used. Interestingly, isoliquiritigenin may act as a slightly “hotter” matrix than 2,5-DHB, as based on the increased levels of cross-ring fragmentation observed in tandem MS spectra.

Ionic liquids are an interesting class of compounds that have found several applications across many different analytical areas, now including their use as MALDI matrices.[393] A number of potential advantages have been proposed for this class of compounds. One main benefit could be the more universal distribution of the sample throughout the spot, which would minimize the number of “hot” and “cold” spots commonly found in matrices in their crystalline form[393] and better shot-to-shot and spot-to-spot reproducibilities.[394] One of the first studies to develop an ionic liquid for carbohydrate analysis was conducted by Laremore, Zhang, and Linhardt, who developed a 1,1,3,3-tetramethylguanidimium salt of CHCA, termed G_2CHCA, to analyze dermatan sulfate and chondroitin sulfate.[395] These types of molecules tend to be difficult to analyze by MALDI due to the loss of their associated sulfate group(s). However, such mechanisms were significantly suppressed when the ionic liquid was used as the matrix. Additionally, more intense signals were recorded with this matrix.

In a similar way, *p*-coumaric acid has been reacted with 1,1,3,3-tetramethylguanidine to give the ionic liquid called G_3CA.[396] As with the G_2CHCA matrix, G_3CA was also effective at preventing the loss of sulfate groups attached to various sulfated oligosaccharides. Additionally, a nearly complete series of tandem MS fragment ions was reported in the

negative-mode tandem MS experiment for only 1 fmol of these types of analytes. This matrix also appeared to be an improvement upon 2,5-DHB for sialylated structures. Using a biantennary, disialylated structure, only a completely desialylated ion was detected using 2,5-DHB; that is, the intact molecule was not observed. Though some desialylation still occurred using G_3CA, the loss of sialic acids was markedly reduced and allowed the intact ion to be recorded. Additionally, encouraging reports for the analysis of heparan and heparin sulfate have been described using an ionic liquid synthesized from 2-(4-hydroxyphenylazo)-benzoic acid (HABA) and 1,1,3,3-tetramethylguanidine.[397]

Most recently, negative-mode applications of the G_3CA matrix have been explored by anion doping of the matrix.[398] A number of different anions were monitored for both their MS and tandem MS performances. For sensitive MS measurements, the BF_4^- ion was determined to be the best option, with femtomole limits being reported. However, for an efficient tandem MS fragmentation, the anion must abstract a proton and the nitrate anion was reported to induce a more complete decomposition of the carbohydrate structure.

Further improvements in the sensitivity of MALDI measurements have been reported for an ionic liquid synthesized by reacting 3-aminoquinoline with CHCA.[399] In this study, the authors claimed 10 amol of a monosialylated glycan could be detected in the negative-ion mode and fragmented by tandem MS methods, though much more convincing results were shown for 500 amol amounts.

6.3. Tandem MS Methods

While mass-spectral profiles obtained at high sensitivity provide significant clues about the overall identity of a possible glycan, many of the observed signals at particular *m*/*z* values arise from a combination of several isomeric possibilities. This structural diversity may be associated with different positional isomers, for example α1-3-vs α1-6-linked fucose units and sialic acids being located on different branches in structures that are less-than fully sialylated, or more subtle changes, such as different sialic acid linkages (α2-3 vs α2-6). Since MS analyses can be thought of as being “insensitive” toward these types of structural differences, tandem MS experiments are oftentimes required to ascertain the fine details required for a more complete characterization of a given oligosaccharide.

Fragmentation can be induced by several different approaches that can be roughly grouped into the “heating” techniques, which increase the bond vibrational energy and include low-energy CID and infrared multiphoton dissociation (IRMPD), and those that result in excited electronic states, such as high-energy CID, UV-based photofragmentation, and electron-capture/transfer and electron-displacement dissociations. Regardless of the fragmentation, the nomenclature first proposed by Domon and Costello[269] in 1988 is used widely to describe the types of detected fragments (refer to Figure 10).

CID experiments have been used extensively for glycan characterization and may be in the low-energy (a few volts to a few hundred volts) or high-energy (in the kilovolt range) regime. Low-energy CID is performed in ion-trap instruments and, similarly to MALDI post-source decay (PSD) analyses where certain analytes may undergo a spontaneous decomposition, it generates glycosidic bond cleavages between adjacent monosaccharide

units. The products of these reactions result in the formation of B and C ions if the detected fragment contains the reducing end, or Y and Z ions if the ionizing charge is retained at the nonreducing end. Bond cleavages of this type are thought to proceed through charge-directed mechanisms.[400,401] Conversely, CID performed on modern MALDI tandem TOF instruments can produce spectra containing the so-called cross-ring fragments of permethylated[363,364] and native glycans.[402] These fragments require the scission of two bonds across a single monosaccharide unit, and the resulting fragments are referred to as A or X ions. Ions of this type are useful in determining linkage and branching patterns.[403]

With the suspected importance of glycan isomers in different states-of-health, it is not surprising that other tandem MS methods have been developed for their structural analyses. One of these approaches is the sequential MS method that employs multiple stages of tandem MS (MS^n). Following a CID experiment, a resulting ambiguous fragment ion of interest is isolated and subjected to a subsequent round of fragmentation. This process is repeated as many times as deemed necessary. Eventually, fragments that are specific to a particular isomer are detected. In one of the first demonstrations of the ability of this technique, a triantennary-tetrasialylated glycan from bovine fetuin was analyzed and a sequential MS was able to pinpoint the location of a fourth sialic acid as being located on the antenna of the α1-6 arm.[404] Later, this approach was used to distinguish different glycan isomers associated with ovalbumin and to differentiate different isomeric structures of commercially available and humanized IgGs.[405] It was further used to differentiate glycan isomers in metastatic and nonmetastatic brain cancer cells lines.[406] While this procedure may be useful in the discovery of new isomers of well-studied glycans, as in the high-mannose glycans derived from bovine ribonuclease B,[407] such suspected new isomers should be verified through other analytical approaches.[408] This approach may be helpful to definitively deduce glycan structures, but the time-scales required may not be compatible with chromatographic separations and may be confined to direct-infusion experiments, which consume significantly more sample.

While many tandem MS experiments have been conducted in the positive-ion mode, fragmentations may also be performed in the negative-ion mode. They have been shown to provide spectra rich with structural details that can differentiate glycan isomers, for example, bisecting structures from the more highly branched complex carbohydrates.[409] In the first reports using ESI, negative ions of neutral (i.e., nonsialylated) glycans were generated which were then stabilized by an appropriate anion.[410-412] Nitrate ions have been found to give the most intense signals, while minimizing in-source decay processes.[410] The resulting tandem MS spectra of the negatively-charged ions have been found to contain numerous A-type cross-ring fragments, that were needed to identify different isomers, and C-type ions,[410-412] as opposed to the B and Y ions generated by the CID in the positive-ion mode. This type of fragmentation is shown in Figure 14 for bi-, tri-, and tetra-antennary glycans. While the fragmentation for neutral glycans seems to be quite efficient (due to the deprotonation of a hydroxyl group), sialylated structures, where the charge is associated with the carboxylate group, require significantly more energy to be fragmented to the same extent,[413] as demonstrated with a series of milk oligosaccharides. Due to the fragile nature of the bond connecting sialic acid units to the remainder of the oligosaccharide, negative-

mode spectra of sialylated glycans tended to show an intense B_1 ion. Additionally, cross-ring fragments across the GlcNac units of the core were observed along with a few glycosidic cleavages. Interestingly, upon esterification of the sialic acids, a more complete dissociation occurred, allowing the structure of the glycan to be determined more definitively. Further information present in these types of spectra provided some clues about the linkages of sialylated structures.[414] Sialic acids linked in an α2-6-fashion frequently produced the $^{0,2}A_7$ fragment, while those associated as α2-3 did not have such a diagnostic ion.

The concept of fragmentation in the negative-ion mode has been further extended to MALDI-based experiments, a method where it is much more difficult to generate negatively-charged ions for neutral structures. However, nitrate ions were again demonstrated to be effective for this purpose, with the resulting tandem MS spectra containing numerous cross-ring fragments. Additionally, C-type ions were observed, similarly to the negative-mode ESI tandem MS spectra.[415] Alternatively, glycans tagged with 2-aminobenzamide may be detected in their deprotonated form. The tandem MS spectra of these types of glycans resulted in extensive $^{1,3}A$ cross-ring fragments, likely due to the tag inclusion.[416] Additionally, a stabilization of the highly labile fucose units was noted.

Stabilization of fucose units in the negative-ion mode can be advantageous, as it is shown that caution must be used when assigning a definitive structure to glycans featuring fucosylation, when based on positive-ion fragmentation. This particular carbohydrate is “infamous” for its ability to migrate to different locations, or “scramble,” throughout the glycan structure in both MALDI-based and ESI-based experiments, even though the time frames for fragmentation are significantly different.[417] This reaction is seemingly more prominent for protonated analytes than sodiated structures, while the transfer seems to occur mainly between different arms. A migration from the core to an outer arm was not readily observable. The mechanism for this transfer requires a free hydroxyl group,[418] so that a modification that “blocks” hydroxyl groups, such as the permethylation reaction, discussed below, should prevent this type of rearrangement.

Alternatively, the use of electromagnetic radiation to induce the fragmentation of glycans has been explored in both IR and UV frequencies. While CID and infrared multiphoton dissociation (IRMPD) both result in increased vibrational energy, differences between the two techniques were observed for high-mannose-type glycans when both fragmentations were performed in an ICR cell.[419] It was reported that the CID spectra were generally of lower quality, displaying only a few fragments that could be attributed to glycosidic bond cleavages. It seems at least plausible that the pressures used for CID in an ICR could have been too low to induce a more complete fragmentation, as these types of glycans have been shown to dissociate with a high efficiency in ion-trap instruments.[271] At best, a definitive structural assignment would have been difficult using the CID data obtained in the ICR cell. However, sufficient fragmentation occurred during the IRMPD to allow the structure to be determined. Extensive fragmentation by IRMPD was also observed for several other complex-type *N*-glycans as well as O-linked glycans.

Higher-energy UV photofragmentation techniques using wavelengths of 157, 193, and 355 nm have shown promise for a thorough characterization of carbohydrates. Using an ion-trap MS, permethylated glycans were subjected to 157 nm radiation, and the high-energy nature of this technique resulted in an abundance of many cross-ring fragments.[420,421] Since this fragmentation was performed in an ion trap, MS^n experiments could be performed and could be used to further confirm the structures of isomeric glycan structures.[421] Using a slightly longer wavelength for photofragmentation, experiments utilizing 193 nm light also produced an extensive array of cross-ring fragments of deprotonated sialylated glycans.[422] When compared to a CID spectrum, the photofragmentation recording showed several unique ions, including the loss of the triol moiety present with sialic acids.

Interestingly, it seems that different reducing end tags alter the observed UV-photofragmentation patterns.[423] While both hydrazide and reductive amination tags both resulted in crossring fragmentation, UV-induced photofragmentation of the hydrazide-conjugated oligosaccharides resulted in more ^{2,4}A-type cross-ring cleavage ions, while reductively aminated oligosaccharides produced mainly ^{0,1}A-type ions. Several other ions, mainly A/C- and B/Y-type fragments, were observed, albeit at much lower intensities. On the other hand, CID of the same structures showed only a few low-intensity fragments.

Another wavelength reported for UV-photofragmentation of glycans tagged with different fluorophores is 355 nm.[424] While CID generally produced Y-type fragments, photofragmentation resulted in a series of A- and C-type ions (i.e., nonreducing end fragments). Three different tags, 6-aminoquinoline (6-AQ), 2-amino-9(10H)-acridone (AMAC), and 7-aminomethylcoumarin (AMC), resulted in very similar dissociation profiles and, in general, produced a more efficient photon absorption and subsequent dissociation than 2-aminobenzamide (2-AB).

Just as the electron-based methods have provided alternative fragmentation pathways allowing detailed structural characterizations to be performed in proteomic studies, similar methods are also finding applications in glycomics. Perhaps not surprisingly, these methods generally produce complementary fragmentation patterns when compared to CID or IRMPD, and a combination of the data obtained from these methods may assist in a more complete characterization. In one of the first of these studies, electron-capture dissociation was used to fragment carbohydrates adducted with different metal ions.[425] For certain carbohydrates, such as, for example, maltoheptaose, cross-ring cleavages were the dominant fragmentation pathway. However, N-linked structures with their higher degrees of branching did not initially show the same fragmentation efficiency, which was attributed to the increased intermolecular interactions that kept the produced fragments bound together as a complex. However, upon irradiation with IRMPD, the generated ions acquired enough energy to overcome the forces keeping them associated, and many cross-ring fragments were detected. The overall efficiency of the fragmentation process was demonstrated to be at least partly influenced by the cation, with the important parameters being the coordination number and the second ionization potential. It seemed that Mg^{2+} and Co^{2+} coordinated species produced more informative spectra than Ca^{2+} and Zn^{2+} coordinated species.

Additionally, ETD has been performed on a series of milk oligosaccharides,[426] with similar results being acquired. Many of the oligosaccharides coordinated to metal ions in this study showed extensive fragmentation, with many cross-ring X-type fragments being recorded. As with the ECD experiments, the fragmentation efficiency was also dependent on the metal ion used with this method, with the most complete fragmentations being generally observed for magnesium.

While it seems that the branched structures may result in a lower ion yield for ECD experiments, carbohydrates, both neutral and sialylated, which fragmented through electrondetachment dissociation, did not seem to suffer the same effect.[427,428] As with ECD, extensive cross-ring fragmentation was the preferred pathway for the deprotonated species used in this study.[427] In an attempt to further influence the fragmentation patterns, different reducing end-tags were investigated.[428] While 2-anthranilic acid (2-AA)-labeled structures resulted in intense ionic signals in the negative-ion mode, their cross-ring fragmentations were suppressed when compared to the native structures. Similar results were noted for carbohydrates labeled with 2-AB, though more cross-ring fragments were observed in comparison to 2-AA-labeled oligosaccharides.

6.4. Ion-mobility Spectrometry

In the past several years, carbohydrate studies conducted with ion mobility spectrometers (IMS) coupled to MS have shown encouraging results. The initial studies of oligosaccharides using this analytical approach were performed on in-house designed and constructed instruments using a drift tube filled with a neutral buffer gas. In this instrumental configuration, ionized gas-phase analytes are subjected to a series of collisions with the buffer gas under low electric field conditions, and ions with the same *m*/*z* values are separated on the basis of their unique collisional cross sections, a value which is related to the overall shape of the molecule. If their cross sections are sufficiently different, this technique may resolve isomeric structures, an area of interest in analytical glycobiology, as the abundances of different isomeric glycans may be associated with the different states-of-health in disease studies. While this type of instrumental configuration is still employed in many research laboratories, the introduction of a commercial instrument[429] using a “traveling wave”[430] to induce separation allows nearly any laboratory to capitalize on the advantages of this approach. This direction is finding increasing applications in glycomic analyses. Regardless of the method used for analyte mobility-based separations, the data acquired seem to be quite comparable.[431] The use of high-field asymmetric waveform ion mobility spectrometry (FAIMS) has been examined[432,433] for carbohydrate analysis, though this approach has not achieved the same level of popularity as the drift tube and traveling wave methods.

One of the earliest studies of carbohydrate isomers by IMS was reported in 1997 by Liu and Clemmer.[434] Using a direct infusion approach, a solution containing the trisaccharides melezitose, a structure resembling a “branched” oligosaccharide, and raffinose, a more linear carbohydrate, was introduced via electrospray ionization into a drift tube containing nitrogen at a pressure of ~3 Torr at 300 K by electrospray ionization. While some separation of these isomeric structures (drift times of 2.135 and 2.162 ms were reported for melezitose

and raffinose, respectively) occurred, it seemed that further optimization of the experimental and/or instrumentational conditions was required to achieve a more efficient separation. Nonetheless, this communication introduced IMS to the field of carbohydrate analysis and indicated that isomeric resolution, in a short amount of time, may be possible.

The first report of a liquid-phase separation of carbohydrates prior to an IMS analysis was published in the following year.[435] In this study, microbore high-performance liquid chromatography (HPLC) was employed using C_{18} as the stationary phase for the separation of various permutations of a series of 21 carbohydrates consisting of simple carbohydrates, sugar alcohols, and amino sugars. The focus of this work centered around the coupling of HPLC to IMS, and subsequent massspectral analyses for further characterization were not performed. On the basis of sufficiently different reduced mobilities (K_o values greater than 0.02), the authors of this communication concluded that several isomeric analytes could be resolved from one another, including D-glucose (K_o = 1.25), fructose (K_o = 1.31), and D-(+)-galactose (K_o = 1.28). Similar conclusions were also reported for the disaccharide isomeric pairs of β-D-maltose (K_o = 0.70) and lactose (K_o = 1.37) and for sucrose (K_o = 1.00) and D-(+)-cellobiose (K_o = 0.68). Interestingly, the amino sugars D-mannosamine (K_o = 1.29) and D-galactosamine (K_o = 1.27) could not be resolved by IMS. However, these structures were well separated from D-(+)-glucosamine (K_o = 1.37). These researchers also determined that a few hundred femtomoles to several picomoles of starting material were required to be recorded with a S/N ratio of 3. Interestingly, these values seem to be comparable to the requirements of modern commercial instruments.[431]

Operating the drift tube at elevated pressures has been recently shown to increase the resolving power of a stand-alone IMS instrument,[436] and this approach may be a useful way to improve the resolution of isomeric carbohydrates.[437] To investigate this possibility, a series of isomeric disaccharides, including several *O*-glycans derived from mucins, and structurally related trisaccharides were analyzed using a drift tube operated at an atmospheric pressure of buffer gas.[437] While not completely resolved, the IMS analysis of solution containing equimolar amounts of α-D-GalNAc-(1–6)-D-Gal-NAc-ol and α-D-GalNAc-(1-3)-D-GalNAc-ol resulted in a resolution of 1.12 and was sufficient to clearly identify the presence of the two isobaric analytes. An even higher resolution, 2.11, was produced by the baseline separation of β-D-GlcNAc-(1–6)-D-GalNAc-ol and β-D-GlcNAc-(1–3)-D-GalNAc-ol using this approach. Slightly larger oligosaccharides also seemed to benefit by using elevated gas pressures in the drift tube, which was noted by the analysis of melezitose, raffinose, and isomaltotriose. Each of these structures was baseline-resolved from one another using the atmospheric approach.

Further improvements in the resolution of glycoconjugates was the topic of an interesting study which examined the effects of the chemical nature of the ionizing agent and the influence of different buffer gases used in the drift tube on the IMS instrument.[438] This work demonstrated that the separation may be improved, or, conversely, compromised, by the choice of different cationizing agent. Using methyl-α- and methyl-β-D-galactopyranosides, the separation factor (defined as the ratio of the faster drift time to the slower drift time of two isomers) was determined for a series of metal ions and metal ion complexes. Lead(II) complexed with one acetate ion produced the highest separation factor (1.07), while the

frequently used sodium adducts resulted in an intermediate value (1.04), and it appeared that both of these ionizing agents could result in baseline separations. Calcium and Hg^{2+}, both complexed with one acetate group, resulted in the lowest values (1.01 for both). While some overlap of the analytes seemingly occurred when these metal complexes were used, sufficient separation occurred to allow for the visualization of both components of the mixture. The need to select an appropriate ion or ion complex for a high-resolution separation was further demonstrated using methyl-α- and methyl-β-galactopyranosides and methyl-α- and methyl-β-glucoopyranosides. When sodium ions were responsible for the ionization, the galactopyranosides were baseline-resolved, but an overlap of the analytes was observed for cobalt adducts. However, the opposite was observed for the analysis of methyl-α- and methyl-β-glucopyranosides. Similarly, the buffer gas used also influences the separation performance. As a general trend for sodium-adducted methyl glycosides, using helium as the buffer gas resulted in the highest separation factors, those for nitrogen and carbon dioxide were intermediate, and argon most often produced the lowest values. Taken collectively, these results demonstrate the challenges associated with the separation of isomeric structures by IMS and the need to carefully optimize the experimental conditions to achieve the desired results.

While time-of-flight (TOF) mass analyzers are frequently used in the coupling of IMS and MS, and fragmentation experiments may be performed on these types of instruments,[439,440] quadrupole mass analyzers have also been explored,[441] with one of the main advantages being their ability to perform MS^n experiments for enhanced confidence in the structural elucidation of isomeric carbohydrates. The need for performing higher-order MS fragmentation experiments was shown with the analysis of GlcNAc-(β1–6)-Gal-ol and GlcNAc-(β1–3)-Gal-ol. IMS was able to nearly baseline-resolve these structures, and in this analysis, the resulting MS/MS spectra were sufficiently different to positively identify the constituent of each peak. However, some isomeric structures may produce very similar tandem MS fragmentation patterns and performing higher orders of fragmentation may be required to definitively discern the identity of an analyte. This situation was exemplified with raffinose and melezitose. While these isomeric structures were well-resolved from one another, their subsequent MS/MS spectra were very similar, making an unequivocal determination of the specific structure difficult. However, by isolating a particular fragment common to both carbohydrates and performing an MS^3 experiment, the correct identity of each peak's analyte could be determined.

While much of the work focused on the successful analysis of isomeric structures has centered around "smaller" carbohydrates, N-linked isomeric structures derived from model glycoproteins have also been analyzed by IMS.[442] In a study of permethylated glycans derived from ovalbumin, three distinct features were repeatedly and reproducibly observed for an IMS feature, and it was determined that the composition of this glycan was H_5N_4, where H is a general hexose and N is a general *N*-acetylhexosamine. The authors proposed that these features correspond to isomeric structures for this sequence. Their conclusions were further supported by molecular modeling studies and lowest-energy theoretical cross sections that matched the experimental values within 1.5%.

Extending the concept of using IMS to analyze N-linked oligosaccharides to serum-derived glycans, this method has been used to differentiate different states-of-health.[443,444] In the first report for this approach, a monosialylated, biantennary structure was suggested as a possible marker of liver disease, based on a supervised principal component analysis (PCA).[443] This publication also indicated one of the main challenges of this approach: whether the features observed in the spectra are isomers or conformers. A follow-up study using 10 glycans in a supervised PCA (with *a priori* knowledge) demonstrated an improved discrimination of the various sample groups. While the data in this study could be collected in 2 min per sample, only 17 structures were observed, highlighting the trade-off between throughput and sensitivity. Further detailed studies using this approach, coupled with rigorous statistical evaluations, should be performed to truly demonstrate the exciting potential of this approach for a rapid analysis of different isomeric possibilities, including the changes in the location of fucose residues in different states-of-health.

Another research group has also applied IMS using a MALDI source to introduce carbohydrates into the gas phase for the analysis of positional and structural isomers and was able to propose certain drift time characteristics based on the glycosidic linkages and branching patterns present in various isobaric structures.[445] The observed drift time for a trisaccharide composed of 1-3 linkages (isomer 1) was shorter than that of a trisaccharide with its monosaccharides attached in a 1-4 manner (isomer 2), indicating that isomer 1 had a more compact gas-phase configuration. Further, a branched pentasaccharide, lacto-*N*-fucopentaose 2 (LNFP2), resulted in a shorter drift time, indicating a more compact gas-phase structure, than its corresponding linear analogue, lacto-*N*-fucopentaose 1 (LNFP1). This group reported that isomers with more significant structural differences are more readily resolved. This was demonstrated by a pair of trisaccharides composed of only 1-3 or 1-4 linkages and a pair of tetrasaccharides with one sugar consisting of only 1-3 linkages, while the second carbohydrate was composed of two monosaccharides associated in a 1-3 manner and one unit attached as a 1-4 linkage. It is worth noting that these experiments were performed at low drift tube pressures (~3–5 Torr of helium), and resolution is expected to increase at higher pressures.[436]

In an interesting application of IMS-MS, a high throughput simultaneous glycoproteomic/glycomic method been proposed.[446] In this method, the glycoprotein standard ribonuclease B was digested first with trypsin, followed by PNGase F to release the glycans, and subsequently analyzed by both MALDI- and ESI-based IMS methods. Using this direct analysis, the MALDI-based approach resulted in a sequence coverage of 43.5%, and all five high mannose glycans associated with ribonuclease B were detected. When this mixture was introduced into the drift tube by ESI, the amino acid sequence coverage was increased to 71.8%. However, the associated glycans underwent significant fragmentation, and only products of these reactions were observed.

A similar approach has also been employed to study the glycosylation of the N-linked glycans associated with the human immunodeficiency virus protein gp120 that was expressed in different cell lines subjected to inhibition of remodeling.[447] In this study, glycan profiles with high signal-to-noise (S/N) ratios in an IMS instrument could be acquired from the analysis of the crude digestion mixture; no sample cleanup was needed.

This could be due to the different regions of the drift space where the peptides and glycans are located, as shown in Figure 15a. By minimizing, or eliminating, the oftentimes required sample purification procedures for other analytical methods, the possibility of reliably analyzing lower-abundance carbohydrates exists. The advantages of the direct-analysis by the IMS approach were highlighted by a comparison of an ESI spectrum that indicated several carbohydrates were present, albeit with very low S/N ratios (see Figure 15b). Following an IMS separation, the S/N ratios were much improved, and the spectra were very similar to those acquired by a MALDI instrument (compare Figure 15c and d). This technique also demonstrated very good sensitivity, allowing high-quality profiles to be collected at sub-microgram levels of starting material.

In an attempt to shift carbohydrates into a region not populated by analytes, perhaps to move them away from peptides during a simultaneous peptide/glycan analysis, the oligosaccharides may be derivatized by a number of different reagents, such as boronic acids.[448] In this particular study, ferrocene boronic acid (FBA) and 4-[(2′,6′-diisopropylphenoxy)methyl]phenylboronic acid (PBA) were used to modify a series of oligosaccharides. Due to its compact nature, PBA was selected to shift carbohydrates to a higher mobility region, and such an effect occurred. Conversely, FBA was selected to retain derivatized sugars in the drift tube, but this was not experimentally observed.

Additionally, the mobilities of carbohydrates may be altered through noncovalent interactions with different tripeptides.[449] Given the flexible nature of peptides, these types of molecules could bind to carbohydrate isomers with different conformations and thus alter their collisional cross sections to different degrees. The potential value of this method was demonstrated for a series of disaccharides. While leucrose and trehalose were sufficiently resolved from one another without the presence of the peptide reagent, palatinose and melibiose exhibited nearly identical drift times. However, when trihistidine was introduced to the carbohydrate solution and electrosprayed into the drift tube, the resulting IMS profile for these particular disaccharides showed them to be baseline-resolved.

7. DERIVATIZATIONS TO IMPROVE MASS-SPECTRAL PERFORMANCE

In many ways, carbohydrates may be thought of as particularly difficult analytes to effectively measure by different analytical techniques. Given the lack of an appropriate chromophore, highly sensitive measurements following a liquid-based separation are difficult. Thus, carbohydrates are often modified to enhance their detection. Likewise, carbohydrates may not be ideal analytes for MS-based measurements. Their inherent hydrophilic nature may result in inefficient desolvation mechanisms during ESI, leading to lower-than-desired signal strengths. Compounding the sensitivity issues are the in-source and post-source decay reactions that are commonly encountered, particularly for sialylated and fucosylated structures. Such processes complicate data interpretation and may lead to ambiguous results. Fortunately, a number of modifications, many of which were summarized in a recent extensive review,[450] may be made to the glycan structure to aid in their overall analysis. Serendipitously, carbohydrates have several potential sites for derivatization, including their hydroxyl groups, reducing ends, and even their associated

sialic acids. A variety of derivatizations may be made to the glycan depending on the nature of the experiment.

7.1. Carbohydrate Permethylation

Permethylation has become one of the most popular derivatizations of carbohydrates. This modification transforms hydroxyl groups present on a glycan to methoxide moieties, esterifies the carboxylate of sialic acid residues, and adds a methyl group to the nitrogen of the *N*-acetyl groups of GlcNAc, GalNAc, and sialic acid monosaccharides. Permethylation offers several advantages, including (i) an improved sensitivity of 10 to 20 times over the native glycans; (ii) converting acidic structures to neutral analytes that permits the complete glycomic profile to be recorded in the positive-ion mode; (iii) enhanced cross-ring fragmentation during tandem MS procedures, enabling a more definitive structural characterization; (iv) preservation of monosaccharide linkages/locations (e.g., the migration of fucose units is blocked); and (v) making the resulting glycans sufficiently hydrophobic to permit their separation by reversed-phase LC, if needed.

The “modern day” approach to permethylation employing dimethylsulfoxide (DMSO) and methyl iodide can be traced back to 1964 with the developments pioneered by Hakomori,[451] while updated versions performed in a slurry of NaOH were introduced later.[452,453] Unfortunately, during the recovery of the permethylated analytes with this approach, peeling reactions may occur due to very basic pH conditions, thus limiting the overall sensitivity of the method. Consequently, this approach to permethylation may not meet the demands of modern-day biomedical research. To circumvent these degradation reactions, and to attain a higher sensitivity, a “solid-phase” approach to permethylation using “reactors” has been introduced.[454,455] In this approach, excess sodium hydroxide, present as discrete beads, is easily removed from the carbohydrate-containing solution. Thus, the pH experienced by the analytes is more controlled. First performed in capillaries, significant improvements in the sensitivity were observed.[454] Later, through a reoptimization of the reaction conditions, a spin-column approach was developed with comparable sensitivities that allowed for a much higher sample throughput.[455] Further improvements of the ionic signals recorded were achieved through an on-line recovery followed by an LC-ESI analysis.[456] By employing this setup, a total of 73 glycans were detected, compared to 63 using the liquid–liquid procedure.

Regrettably, permethylation in DMSO still presents some analytical challenges. For chromatographic purposes, it is generally deemed desirable to deal with glycans in their alditol (i.e., “reduced”) states to prevent the resolution of the α and β anomeric configurations present at their reducing ends. Through an interaction with DMSO, the closed-ring structure may be regenerated,[453] thus leading to complicated spectra with compromised detection limits due to multiple ions being detected for a single analyte. Moreover, a series of +30-Da artifacts are frequently observed due to the formation of iodomethyl methyl ether.[457] This compound, generated by reaction between methyl iodide and DMSO, is reactive toward hydroxyl groups and, once again, complicates the overall spectral interpretation (see Figure 16a) However, both of these reactions are minimized by performing the permethylation reaction in an alternative solvent, for example *N*,*N*-dimethylacetamide[453] or *N*,*N*-dimethylformamide,[458] as seen in Figure 16b.

The analysis of permethylated oligosaccharides has also been successfully applied to those structures modified with phosphate or sulfate groups. Both of these moieties seem to be stable throughout the permethylation procedure, with the phosphate group becoming singly or doubly esterified.[459,460] Most probably, extended reaction times would ensure a complete esterification of the phosphate group. Conversely, sulfate groups attached to a carbohydrate are unaffected by the permethylation procedure and retain their negative charge.[460,461] To detect sulfated glycans in a mass spectrometer's positive-ion mode, a "double-permethylation" procedure has been developed.[461] In this procedure, the sulfated glycans were permethylated and the sulfate group was then chemically removed via a treatment with acidified methanol. The samples were permethylated a second time using deuterated methyl iodide to label the site of sulfation.[461] Additionally, following the first permethylation step, sulfated glycans may be fractionated on the basis of their degree of sulfation only, since sialic acids are rendered as neutral, by strong-anion exchange chromatography.[462] Following a desalting procedure, the sulfate group was chemically removed and the site of sulfation was indicated using deuterated methyl iodide. An important class of sulfated glycoconjugates are the sulfated heparins, and advances have been made in the methods used for their permethylation.[463] Given that some types of analytes tend to be highly sulfated, they oftentimes exhibit only a limited solubility in DSMO. However, when converted to triethylammonium salts, heparin sulfates are much more amenable to entering the solution phase, thus improving their permethylation yields.

While this reaction is practiced in many laboratories worldwide, there are certain cases where permethylation-based schemes may not be the best approach. This is exemplified by the analysis of O-acetylated sialic acids, where the acetyl group may be located in the 4, 7, 8, or 9 positions.[464] These modifications are quite labile; therefore, acidic or basic conditions throughout the sample preparation/analysis procedures should be avoided to preserve the native structure of the sialic acid. Recently, glycans derived from erythropoietin demonstrated bearing this type of modification were successfully analyzed using LC-MS employing graphitized carbon as the stationary phase using 10 mM concentrations of ammonium bicarbonate in the mobile phases.[465]

7.2. Modifications of Sialic Acids

Sialic-acid moieties have been shown to be very labile[466] under MALDI conditions, and a number of different approaches have been developed to stabilize this unit. One of the earliest analytical methods to stabilize sialic acid was through esterification,[467] the conversion of the carboxylate group to a methyl ester that eliminated the acidic proton responsible for its decomposition. After esterification using methyl iodide in dimethyl sulfoxide, sialylated structures were detected as intact species. The modification of a sialic acid to its methyl ester also offered several secondary advantages. Both acidic (negatively charged) and neutral structures could be analyzed simultaneously in the positive-ion mode after esterification. In addition, esterified analytes were not present as a series of multiple metal ion adducts that are often associated with just a single acidic analyte. Later, the esterification reaction was applied to the products of Pronase digestions of N-linked glycoproteins[468] that resulted in glycans linked to only a single asparagine unit. The subsequent esterification through treatment with methyl iodide also generated a quaternary ammonium moiety, a

permanent positive charge, at the N-terminus of the amino acid. Studies with model proteins indicated that the sensitivity of these modified structures was 10 times that of their native counterparts.

By using an activating agent, other modifications may be introduced to the carboxylate by making this group more susceptible to a modification through a nucleophilic attack. One such reagent is 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride (DMT-MM),[469] which may be used in an esterification reaction when methanol is used as the nucleophile.[470] In this method, sialic acids linked as α2-6 became esterified while those that were α2-3 linked resulted in the spontaneous formation of lactones, preventing their modification. This differential labeling resulted in a 32-Da mass difference between the two structures and allowed a discrimination between α2-3- and α2-6-linked sialic acids. Thus, it seems possible to quantitate differences in sialic acid linkages using this procedure. In a different report, sialic acids activated by DMT-MM were subsequently treated with ammonium chloride, resulting in an amidation of the carboxylate.[469] These species resulted in more stable analytes under MALDI conditions when compared to their native counterparts. Tandem MS analyses of the amidated structures resulted in a more complete set of fragment ions, and the resulting spectra were less complicated than those of native glycans, a consequence of the elimination of salt adducts. The amidation reaction has been further modified to allow the distinction between α2-3- and α2-6-linked sialic acids (the previous study focused only on those sialic acids linked as α2-6), followed by a subsequent permethylation.[471] For permethylation-based platforms, amidation was deemed necessary, since permethylation esterifies carboxylate groups and lactones are unstable at the basic pH conditions used for this derivatization. An example of this overall analytical process is demonstrated in Figure 17, which presents the glycomic profiles for an amidated glycan from a control patient and a woman diagnosed with late-stage breast cancer. This figure further demonstrates the ability of this method to show differences in sialic acid linkages in different states-of-health.

In certain situations, a nonspecific amidation procedure is desirable and may be achieved using acetohydrazide derivatives.[472] Following this reaction, the sensitivity of the modified structures was increased 6-fold when compared to native glycans, with a limit-of-detection of 1 fmol being reported. However, tandem MS procedures resulted in an intense peak corresponding to the loss of a sialic acid. The authors suggested that the proton of the amide is slightly acidic and may be responsible for this phenomenon under tandem MS conditions. Similarly, a nonspecific methylamidation[473] of both linkage types of sialic acids may be accomplished by first activating the carboxylate group with the reagent (7-azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyAOP). This activator was chosen due to its ability to overcome the steric hindrance effects that often result in incomplete conversions of α2-3-associated sialic acids. Using positive-mode MALDI conditions, the sialic acid groups were stable; no decomposition was detected.

7.3. Modifications at the Reducing End To Improve MS Sensitivity

The reducing end of a glycan is in a dynamic equilibrium between the closed-ring structure and an aldehyde and provides a convenient location for derivatization. Many different

derivatization reactions based on reductive amination[474] have been designed to introduce different groups at this location. In these reactions, the aldehyde form of the carbohydrate, whose level is enhanced by performing the reaction in acidic conditions, is conjugated with an amine, resulting in a Schiff base, which is subsequently reduced with sodium cyanoborohydride, though 2-picoline-borane[475] and sodium triacetoxyborohydride[476] have been proposed as less toxic alternatives for such purposes. Among the benefits of these reactions are an improved MS sensitivity and, in some cases, enhanced tandem MS fragmentation performance.

Several groups have investigated the use of UV chromophores that were originally utilized in liquid-chromatographic detection as an approach to enhance mass-spectral detection.[477-479] A derivatization of maltohexaose with 4-aminobenzoic acid 2-(diethylamino)ethyl ester (ABDEAE), also referred to as procaine, resulted in an up-to-5000-fold increase in the sensitivity over its native analogue and offered a limit-of-detection of 10 fmol using ESI.[477] The improved ion yield was attributed to the basic functional group on the derivatizing reagent that provided a location for proton attachment. Additionally, tandem MS of the derivatized structures resulted in extensive cross-ring fragmentation. While not as extreme, a significant signal enhancement (ca. 50-fold) was also reported for N-linked glycans derivatized with this tag, while carbohydrates derivatized with the analogue *N*-(2-diethylamino)ethyl-4-aminobenzamide[480] (procainamide) produced somewhat lower intensities when analyzed by ESI. This derivative performed better under MALDI conditions. Interestingly, glycans derivatized with this group were observed as doubly-charged species and were ionized as $[M + H + X]^{2+}$, where H is hydrogen and X is an alkali metal. The tandem MS fragmentation patterns of these derivatives were dependent on the metal ion. Generally, the heavier metals tended to shift the fragmentation pathway toward glycosidic bond cleavage, while the lighter metals resulted in a loss of the tagging group.[481] Several other tags were included in this study, as summarized in Figure 18, with varying effects on the ionic signals for MALDI and ESI measurements.

While generally considered as labels to assist in UV- or fluorescence-based detection schemes, 2-AB and 2-AA have been applied to both MALDI and ESI studies of carbohydrates. The benefits to the sensitivity were demonstrated for MALDI using 2-AB-labeled glycans derived from ovalbumin, where signal intensity was increased approximately 3 times over native structures.[482] Under MALDI tandem MS conditions, a series of B- and Y-type ions have been reported,[482] and cross-ring fragmentation across the innermost GlcNAc unit seems to be enhanced by the conjugation of this molecule.[483] Such cleavages allowed a more definitive differentiation between an α1-3- and an α1-6-linked core fucose from the honey bee protein PlA_2. While 2-AB is an effective tag for positive-ion mode studies, the carboxylate group associated with 2-AA is more appropriate for negative-ion mode studies. Glycans modified by this label were shown to have good sensitivities in this mode of operation in MALDI experiments, and the limits-of-detection were approximately 5-fold better than those for 2-AB labeled glycans.[484] However, the gains in sensitivity were countered by an overall loss in tandem MS fragmentation performance. Thus, this tag may find its best applications for previously well-characterized glycans when more highly sensitive measurements are needed.

Alternative chemistries to reductive amination are the hydrazine-based reactions. The long-used arylhydrazines for carbohydrate analysis have been shown to improve the sensitivity of ESI-based measurements by Lattova and Perreault using phenylhydrazine.[485] The labeling reaction with this class of compounds is generally straightforward and requires no additional salts; thus, sample purification steps are simplified and improved measurement sensitivities result. Importantly, the losses of sialic acids are minimized since this reaction is frequently performed at slightly basic pH values. To study the influence of hydrophobicity/hydrophilicity on the ion yield for ESI studies, a series of hydrazine tags has been synthesized.[486,487] While it may seem to be intuitively reasonable that a permanent charge would result in the best overall ion yield, this has not been observed experimentally, with the overall level of hydrophobicity dictating the ionic signal.[487] Seemingly, the more hydrophobic character of this tag led to a better desolvation during the ESI process than for those structures possessing a charged moiety.

Correspondingly, permanent charges have also been incorporated at the reducing terminal through the use of carboxymethyltrimethylammonium chloride hydrazide, also known as Girard's T reagent, and the use of this reagent increased the sensitivity of modified glycans 10-fold for MALDI analyses, similar to that observed with trimethyl(4-aminophenyl)ammonium chloride (TMAPA),[488] another reagent capable of incorporating a positive charge into the glycan. In an extension of this method, sialylated glycans have been first esterified followed by a derivatization with Girard's T reagent[489] to further enhance the sensitivity of the measurement and to stabilize the labile sialic acid monosaccharides. This approach was then used to study differences in sialylation between two different CHO cell lines.

7.4. Quantitation of Oligosaccharides through Stable Isotope Labeling

On the basis of the premise that there are many suspected or proven associations of human disease conditions with aberrant glycosylation, the rapid comparative profiling of structurally known, or at least tentatively identified, glycans could be a significant starting point for more in-depth investigations of these diseases. Comparative glycan profiling can similarly be applied to a number of biological studies of any "normal" or "perturbed" systems, a comparison of glycosylation in different body tissues or organs, chemotaxonomies of different organisms, phylogenetic trees, etc. In all of these situations, high precision and accuracy in measuring glycan abundances for some or all profile constituents becomes essential. Here, the use of isotopic labeling for glycans and MS measurements opens new possibilities. It provides an approach in which multiple samples can be measured simultaneously and directly compared during a single data acquisition. Through the use of methyl iodide with varying deuterium substitutions, direct differential permethylation studies may be performed with up to four samples being simultaneously monitored.[490] Importantly, the linearity of this method was acceptable at nearly 2 orders of magnitude. In an adaptation of this method, a different research group employed a combination of ^{13}C- and deuterium-labeled methyl iodide reagents ($^{13}CH_3I$ and $^{12}CDH_2$) to incorporate stable isotopes into glycan structures through permethylation to achieve similar goals.[491,492] While this may appear to be an "isobaric" method, this approach actually introduces a mass difference of 0.002922 Da for each site of derivatization. While this small

mass difference is difficult to detect by modern MALDI-based instruments, it can be easily measured with a high-resolution mass spectrometer (i.e., an FT-ICR instrument or an orbitrap).

Isotopically-coded tags may further be introduced into the carbohydrate structure through other methods and locations on the glycan structure. The free reducing end of an oligosaccharide provides a convenient site for modification, and several isotopically-coded chromophores can be incorporated at this location, including aniline,[493-495] 2-amino-pyridine,[496] 2-aminobenzoic acid,[497] and 1-phenyl-3-methyl-5-pyrazolone.[498] Additional tags have been synthesized, including ($^{13}C_6$ and $^{12}C_{12}$) 4-phenethyl-benzohydrazide,[499] a hydrophobic tag that may enhance the sensitivity of ESI-based measurements through a more efficient desolvation process,[486,487] and a novel set of tetraplexed tags,[500,501] each separated by 4 Da and analyzed by a direct infusion into an ESI-based q-TOF MS instrument. Alternatively, in a closely related analogue to the stable-isotope labeling by the amino acids in a cell culture (the so-called SILAC method, which is widely employed in the proteomics field), isotopically-labeled glutamine, which is further used as the sole source of nitrogen for the synthesis of *N*-acetylglucosamine, *N*-acetylgalactosamine, and the sialic acids, has been reported[502] and utilized in conjunction with cultured mouse embryonic stem cells.

8. SEPARATIONS OF GLYCAN POOLS

8.1. General Considerations

Glycan pools isolated from large glycoprotein molecules or, alternatively, deglycosylated mixtures of glycoproteins and glycopeptides, can be very complex. Various combinations of monosaccharides can biosynthetically yield different structural configurations with functionally different roles, so that the glycan pool complexities are fully expected. While MS techniques are now readily available to profile the glycan pools for major components with different molecular masses, distinguishing various isomers in such mixtures is still problematic. The use of selective forms of chromatography offers here a great potential for analytical applications as well as future preparative (large-scale) utilizations of unique solute–solvent interactions.

The separation of carbohydrates remains a very active field for other reasons as well. In dealing with complex mixtures, *chromatographic enrichment* of selected glycans can yield optimum amounts and concentrations of glycans to be measured precisely by the MS techniques in the following step. Chromatographic enrichment of the minor components is essential in certain applications, where “fractionation by class” aids in a clear differentiation of major and minor mixture components as based on different substitutions, polarity or degree of sialylation, sulfation, phosphorylation, etc. Even an effective removal of nonglycan impurities through a filled-pipet step, however simple, is based on chromatographic separation principles.

The effectiveness of chromatographic separations is reflected in two sets of physicochemical processes: (1) chromatographic peak band-broadening (diffusionally controlled) phenomena, also expressed in the values known as “chromatographic efficiency” and “theoretical plate

measurements", and (2) thermodynamically based "column selectivity". During the past decade, significant advances were made in both areas that pertain to the design and operation of today's chromatographic columns in carbohydrate analysis. In the kinetically favored HPLC separations, the particle size has been decreased from "traditional" 5 and 3 μm values to much smaller diameters in the range of 1–2 μm. The consequent increases in column efficiency are substantial, as is the increased speed of analysis. The prices to pay for these analytical advantages are the elevated column inlet pressures and a more sophisticated instrumental design in this so-called "ultra-high-pressure liquid chromatography" (UPLC) (see a recent review by Jorgenson[503]). The selectivity advances are being largely reflected in the design of new HILIC and carbonaceous columns and their optimization with the use of "MS-friendly" mobile phases. The separation of glycan isomers and other hard-to-resolve components has often been the motivation for these advances in column technology.

Historically, HPLC of carbohydrates has lagged behind the applications of this method to other biomolecules. Besides a relative lack of suitable columns for the separation of these hydrophilic molecules, the detection problems were initially the main issue in carbohydrate analysis. Due to the absence of a distinct chromophore in their molecules, carbohydrates had to be detected through changes in refractive index, indirect photometry or fluorometry, evaporative light-scattering detection, and other less popular HPLC detection techniques.[504] These approaches seldom provided the required sensitivity, reproducibility, and capability to work under a gradient elution. Introducing a chromophore into the sugar molecules through derivatization has become popular since the 1980s, and these activities continue to this date. The most common approaches include benzoylation of hydroxy groups, formation of hydrazones, and various other modifications at the reducing end of oligosaccharides.[450,505] One particularly popular approach, developed originally by Hase and co-workers[506,507] in Japan is derivatization with 2-aminopyridine, yielding a fluorescent derivative for each analyzed glycan. Chromophoretagging techniques, in general, provide an added advantage to chromatographic procedures in terms of increasing retention on the relatively hydrophobic stationary phases. While the evolution of reliable HPLC methodologies for the chromophore-tagged oligosaccharides initially favored reversed-phase separation systems, the HILIC-based procedures have been increasingly adopted more recently, as detailed below.

The chromatographic separations and measurements of native glycans received a significant boost through the introduction of high-pH anion-exchange chromatography in combination with the pulsed amperometric detection[508,509] and the subsequent commercial development of carbohydrate analyzer instruments. However, the applications of this type have recently declined due to the availability of LC-MS methodologies which can deal with the separation and detection of underivatized glycan mixtures under less drastic mobile-phase conditions, and at the higher sensitivities needed in contemporary glycobiology.

Miniaturization in chromatography has been an ongoing active trend for many years. Besides the above-mentioned benefits of the decreased particle size in terms of separation performance, a concurrent decrease in column diameters leads also to enhancement of mass sensitivity[510-512] of MS and other concentration-sensitive detectors. Consequently, this trend favors the sample-limited applications of today's glycobiology. It also enables the use of

multidimensional separations (LC/LC or LC/CE combinations) which utilize orthogonal separation principles in dealing with the inherent complexity of biologically derived mixtures. Today's small-diameter columns used in glycan analysis are most typically fused-silica capillary columns filled with small chromatographic particles, although other column geometries may include open tubular format[234] or monolithic columns with in situ polymerized materials.[513,514]

8.2. Reversed-phase HPLC

As hydrophilic molecules, natural sugars exhibit only a small retention on typical reversed phases designed for HPLC. Their conversion into more hydrophobic derivatives requires a chemical modification of the hydroxyl groups or a derivatization at the reducing end or, in the case of sialylated structures, an esterification of the carboxylic moieties. With the exception of simple structures, a complete conversion is difficult to accomplish, leading to the adverse appearance of multiple chromatographic peaks due to incomplete derivatization. A time-honored peralkylation of sugars gets the closest to the desired goal of the fully derivatized solutes, with peracetylation being a distant second. Due to steric restrictions, derivatizations introducing a chromophore through the hydroxyl groups have not been particularly successful with the typical glycan structures originated from glycoproteins. Chemical derivatizations at the reducing end of the sugar molecules yield a number of more interesting possibilities leading to better analytical performance through enhanced hydrophobicities and improved detection parameters; these reactions are generally more applicable to *N*-glycans than *O*-glycans, which are most commonly released under reducing alkaline conditions that eliminate the reactive aldehyde used in many tagging procedures (see section 6.1 of this review), unless a nonreductive procedure is employed.[359] Unfortunately, some derivatization procedures lead to the occurrence of double peaks during chromatographic resolution of syn/anti isomers and anomers. This can happen in all forms of chromatography, including RPLC (reversed-phase liquid chromatography).

More recently, some tagging strategies have been explored for their effects on enhanced solute ionization in MS (in both positive- and negative-ion mode) and directing fragmentation processes.[6,482,485,506,507,515-522] This orientation is potentially fruitful, with the increasing use of LC-MS in evaluating glycan profiles and assigning correct structures of isomeric alternatives. It is not surprising that the enhanced analytical capabilities through the derivatization of glycans were primarily exploited for N-linked oligosaccharides possessing a reducing end, unlike the alditols released from O-linked structures. Unless a reactive aldehyde group is generated through an elaborate conversion of alditol structures,[523] peralkylation remains the most practical option for this group of glycans.

Quantitative permethylation, in which all of the polar groups in sugar molecules become fully converted, has become a viable option for RPLC separations of both N-linked and O-linked glycans. As there are distinct advantages of permethylation in MS, such as marked increases in ionization efficiency[524] and more predictable fragmentation patterns[404,525] in tandem MS, it is reasonable to combine these favorable MS attributes with a chromatographic separation of predictable retention characteristics of the hydrophobized solutes.

Delaney and Vouros[526] were among the first to explore the merits of RPLC coupled to an ion-trap instrument through ESI. They used a 2.1 mm i.d. C_{18} (octadecyl silane-derivatized) column to separate doubly-derivatized (2-AB and then permethylated) oligosaccharides and recorded their spectra in the MS^2 and MS^3 tandem MS modes during an elution of a chromatographic peak. The recognizable spectra and structurally indicative fragments were observed even in the absence of the labeling 2-AB group, so permethylation alone appears to be sufficient in effective LC-MS structural investigations. Using a very different format (microchip RPLC) and MS detection, this separation mode, based on the hydrophobicity of fully methylated glycans, was further suggested for oligosaccharide profiling by Novotny and Mechref.[527] To avoid peak splitting for each chromatographed component into α- and β-anomers, it is necessary to reduce cleaved oligosaccharides to alditols prior to their LC-tandem MS analyses.[458,528]

While permethylation of glycans followed by LC-ESI-tandem MS could potentially lead to a generally acceptable analytical platform for glycomics,[528] it is first essential to resolve the numerous structurally related mixture components, including structural and compositional isomers, into the distinct entities that are easily quantifiable by MS and verifiable by the tandem MS modes. While RPLC is still the most facile and easily understandable separation mode in terms of regular retention increments, it performs poorly in resolving structural isomers (with a few exceptions[529]). As discussed below, isomeric separations can be accomplished through some other modes of chromatography and CE.

RPLC of permethylated glycan alditols exhibit a predictable retention of solutes according to their increasing hydrophobicities and greater molecular size. This is shown in the retention plots (Figure 19) due to different subclasses of oligosaccharides (high-mannose, complex, and fucosylated-complex) following the separate trends with a high degree of correlation.[458] These or similar plots may be useful for assigning structures of the components in unknown mixtures. As with any derivatization approach, permethylation is prone to forming reaction byproducts and impurities, so that it has been critical to minimize these through a careful optimization of the reaction conditions and glycan purification steps.[456,458]

Attaching a chromophore or a fluorophore to a sugar through reductive amination with an aromatic amine first became popular at roughly the same time when the notion of HPLC was largely synonymous with the LC using the reversed-phase mode. The now classical papers by Hase and co-workers[506,507] introduced and further developed 2-amino-pyridine (2-AP) as a fluorescence-labeling agent. The reductive pyridylamination approach was tested extensively on a variety of glycoproteins[506,507] and later extended into a two-dimensional sugar mapping analytical system.[516,530] Through comparing the retention of numerous oligosaccharide 2-AP derivatives on two different stationary phases (RPLC and HILIC), some structural predictions can be made for the elution of unknown glycans. The development of a two-dimensional system implicitly recognizes the limitations of RP-LC in resolving certain glycan types from each other. Careful referencing to the retention values of standard oligosaccharide mixtures is needed in such sugar mapping studies. Using comparative chromatography together with sequential exogly-cosidase treatments can be very useful in structural studies (sequence determination). However, extended reactions,

which may increase the level of sample loss, associated with each exoglycosidase treatment step become the limiting factor of such HPLC-based methods.

Labeling oligosaccharides with 2-AB, for the sake of fluorescence detection in HPLC, has been reported[517,531] a decade ago. The approach has since been adapted to HILIC conditions and applied in the search for glycan disease biomarkers.[18,532-534] The number of fluorescence-tagging techniques and reagents has continued to increase substantially during the last 10-15 years. The reviews of Anumula[535,536] account for a number of aromatic amines which were developed by different groups as fluorescent tags for glycoconjugates. The fluorescence derivatization has safely pushed analytical glycobiology to the low picomole range.

Representative structures of fluorescence-tagging reagents are shown in Table 3. The variations in the structures of these reagents undoubtedly reflect different efforts to enhance the sensitivity, the resolution of glycans that are important to a particular application, and the convenience to reduce the interfering effects of the excess reagents and reaction byproducts. It is noteworthy that some of these reagents find today their applicability in more than one chromatography mode,[536] that is reversed-phase, normal phase, and ion-exchange chromatography, as well as CE. Besides the very commonly used 2-AP and 2-AB derivatization reagents, there are acridine- and acridone-based tagging schemes,[537,538] and 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) used in an ion-pairing mode of chromatography.[539] While attaching a fluorescent tag to hydrophilic glycans makes the derivatives sufficiently amenable to RPLC, with the usual mobile phases, the choice of a particular tagging reagent seems less beneficial in resolving the structural nuances in glycans, rather than shifting their overall retention due to the tag-imparted hydrophobicity in a given RPLC system. It is not surprising that a number of derivatizing methodologies were more recently adapted to HILIC separations which permit the hydrophilic nature of glycan molecules to be expressed in the more selective solute–solvent interactions.

Some interesting applications of RPLC using carbohydrate derivatization aim at an improved detection/identification through MS and its tandem modes. Compared with the conventional fluorescence detection in LC performed in the picomole range, the most advanced forms of MS detection can push sensitivities down to low femtomole levels and, potentially, below. Aiming at improved MS detection, Perreault and co-workers utilized derivatization with 1-phenyl-3-methyl-5-pyrazolone[519,520] and phenylhydrazine.[485,521,522] The used RPLC phase systems typically favor easier desolvation in an ESI interface for MS than other chromatographic mobile phases.

8.3. Hydrophilic Interaction Chromatography (HILIC)

As outlined in section 4.5, this versatile type of chromatography has its historical connection to the classical normal-phase systems in which the relatively nonpolar mobile phase (albeit readily miscible with water) partitions the polar analytes during their transport though a column, containing a highly polar stationary phase, which retains chromatographed solutes with a distinctly polar nature. The water content of this phase system is essential, as the water molecules can readily associate with the polar structures of the stationary phase, producing a “mixed mode” situation, which is further conducive to the retention of polar

molecules such as the multiply-hydroxylated sugar molecules. The mobile-phase aqueous components can gradually be increased from a typical equilibrium state of sample injection during the gradient elution, in which the highly polar molecules can gradually be eluted through added water and buffer content. Acetonitrile–water mixtures appear to be the most favored mobile-phase components, while the designed stationary-phase polymers have undergone an enormous evolution in structures since HILIC introduction[225] and its early application to complex carbohydrates:[540] from relatively simple hydroxy-, amino-, and amido-functionalized polymers to cationic and ionic, and even zwitterionic (the so-called ZIC-HILIC) structures, although, according to a recent review summary,[253] the amide polymeric HILIC column materials are predominantly used for glycoconjugate analyses of different kinds.

The great popularity of HILIC in both glycan analysis enrichment schemes for glycosylated peptides and other applications in glycobiology during the past decade is largely due to the availability of more rugged chromatographic packings, which can be either cross-linked polymer-based or siliceous. A controlled retention of water in such phase systems is likely a key factor in the recent commercial success of the HILIC column technologies.

HILIC becomes a very convenient choice in retention comparisons in both LC-fluorescence detection and LC-MS. Retention in HILIC is predominantly controlled by the number of polar groups in a sugar molecule and, hence, the molecular size. Regular and predictable retention increments are observed with the increasing size of glycan molecules, with monosaccharide compositional changes playing some role.[505,541-543] Carbohydrates labeled with typical fluorescent tags, such as 2-AP,[506,507] 2-AB,[474,544] 2-AA,[474,505,545] or acridine derivatives[537] are easily amenable to HILIC conditions, albeit with a different retention from the unmodified sugar structures. Some glycan isomer separations due to HILIC have already been observed,[543,546] and with a further optimization of the phase systems and improvement in column technology, additional cases of isomer resolution are expected.

The established fluorescence tags largely control retention in RPLC due to their hydrophobicity, but not in the HILIC applications where they primarily serve for the detection purpose. A very substantial increase in the number of HILIC/fluorescence applications during the past decade has been evident throughout the literature. While fluorescence labeling is not without problems in terms of quantification, this approach provides the needed simplicity for routine pharmaceutical and biotechnological applications. HILIC-based measurement techniques have been recommended as reference methodologies for the quantification of glycans in the biopharmaceutical industry in Europe.[547,548] The more recent method[548] demonstrated its reproducibility for analyzing 2-AB-labeled glycans from monoclonal antibody samples from six different laboratories; HILIC was performed using an amide commercial column (3 μm packing material) using an aqueous/acetonitrile gradient elution, with ammonium formate as a mobile-phase additive.

The current popularity of the HILIC/fluorescence approach has also been reflected in the recent applications in the disease biomarker discovery area and large-scope screening of clinical samples. On the basis of the derivatization strategy pioneered by Bigge et al.,[474] with the use of 2-AB, a complete HPLC-based analytical platform was developed for the

analysis of *N*-glycans in human blood serum.[549] Using only microliter volumes of this biological fluid in a multiplex arrangement (96-well plate), which involves sample immobilization, enzymatic glycan release, and fluorescent labeling, an automated system can repeatedly profile major *N*-glycans in serum of all samples in a few days. While the initial application was described for rheumatoid arthritis, methodologically similar protocols were also developed for ovarian cancer,[532] breast cancer,[550,551] and lung cancer.[534] As methodological improvements are gradually achieved in sample preparation (glycan extraction and purification) and HPLC column technology (e.g., the use of UPLC with very small particles), the information content of glycan profiles will undoubtedly improve for the sake of clinical diagnosis, prognostic measurements, and our understanding of the heritability in different human populations. These trends have recently been demonstrated through extensive profiling of different isolated populations of humans (more than 1,000 island inhabitants) and correlations with genome-wide associations and environmental influences.[552-554] In a study pursuing the glycan biomarkers of the aging process, another group has also utilized the HILIC-based separations and compared the *N*-glycan profiles in plasma samples of 2396 study participants,[555] although their fluorescent labeling and other ancillary techniques were different from those by Knezevic et al.

HILIC/fluorescence detection represents a valuable approach to sensitive and routine measurements of the major *N*-glycans in physiological fluids, such as blood serum and plasma. These glycans mainly originate from immunoglobulins and certain so-called acute-phase proteins. Verification of the structural identity of these glycans in the above-referenced applications has mainly been accomplished, to the first approximation, through referencing to the elution of an appropriately labeled glucose ladder (a mixture of oligosaccharides), wherein the glucose units (GU) values are assigned to individual chromatographic peaks. A representation of this system is depicted in Figure 20 with the example of human serum IgG (heavy chain).[542] A more detailed structural analysis/verification for the individual glycans can further be accomplished through a series of specific exoglycosidase enzymes used as reagents. An example of this approach, Figure 21, demonstrates the exoglycosidase sequencing with the analysis of human IgG.[542] Sequential exoglycosidase treatments, aimed at specific cleavages of the original glycan structures, now result in different chromatographic peaks with a recognizable pattern (shifts in GU values) after additional chromatographic runs on the same column. Database matching is further necessary to assign correct glycan structures. While the application of exoglycosidase sequencing represents an elegant approach to glycan structural analysis, sample dilution in each step is likely to decrease the scope for analysis of minor components.

The versatility and structural variation in the design of HILIC stationary phases (for a review, see Boersema et al.[556]) still show considerable promise for further optimized retention in different applications. Disregarding tagging technologies, the hydrophilic nature of glycan molecules can be reflected in different solute-solvent interactions, including the applications to glycan isomeric separations. In this regard, the uses of zwitterionic media, as shown by Takegawa and co-workers,[223,246] are particularly interesting. ZIC-HILIC columns featuring sulfobetaine functional zwitterionic groups can selectively interact with certain neutral and differently sialylated triantennary *N*-glycan isomers as based on the hydrophilic

interactions with the water-rich layer in these stationary phases. A proposed retention model[249] takes into account the role of mobile-phase additive (ammonium acetate) in electrostatically shielding differently sialylated glycans and a different conformational flexibility of α2–6 vs α2–3-linked isomers in complexation. Figure 22 demonstrates isomeric separations for both neutral and sialylated triantennary glycans originated from α-1-acid glycoprotein (AGP).[226]

A major strength of HILIC separations is that no tagging is actually needed when using MS as a means of detection. This strength translates into procedural simplicity. Coupling to MS is best accomplished with columns of reduced diameters (capillaries), with the corresponding enhancement of mass sensitivity in such nanoflow LC columns, as demonstrated by Wuhrer et al.[557] in the application to keyhole limpet hemocyanin, a model glycoprotein. The achieved sensitivity with unlabeled glycans was approximately 1 fmol with an ion-trap mass spectrometer. Additional applications and analytical attributes of HILIC-MS and tandem MS were more recently reviewed by Wuhrer and co-workers.[253,558,559] Capillary HILIC/tandem MS is increasingly seen in the applications to glycomic measurements in complex biological samples, such as mixtures of glycoproteins in blood serum and plasma. Zhao et al.[160] used this method to profile *N*-glycans in serum samples of pancreatic cancer patients, identifying 44 oligosaccharides as distinctly different in different disease states. Plasma samples of ovarian cancer patients were compared by Bereman et al.,[560] who used LTQ Orbitrap mass spectrometer to assess glycan profiles.

8.4. LC on Porous Graphitized Carbon (PGC) Columns

Porous carbon packings were suggested for carbohydrate separations already a long time ago,[561-563] in the studies exploring the potential applications of the first commercially available HPLC columns packed with this “less usual” chromatographic material. These first chromatographic retention studies explored different mobile phases and their additives, such as small percentages of trifluoroacetic acid, noting separations based on size (for different oligosaccharide series) and anomeric resolution for most solutes. Oligosaccharide alditols were next considered as more appropriate solutes[562] in the glycoprotein glycopeptides and *N*-glycan investigations, which, in turn, needed MS for detection.[563] Column miniaturization, accomplished commercially during the more recent years, has significantly extended the scope for LC-MS applications to glycoprotein analysis, actually to the point where many of today’s investigators not only consider PGC-LC a useful addition to the LC-based techniques but also endorse it as the major component of future analytical platforms.

The capability of PGC-LC to resolve different types of carbohydrate isomerism is the major positive analytical attribute of this separation methodology, making it perhaps far more orthogonal to MS than the remaining chromatographic modes (size vs isomer separations). While the potential for isomer separation was already noted in most initial studies on PGC columns during the early 1990s, the effectiveness of this approach was clearly recognized with the resolution of oligosaccharide branching isomers[564] and biantennary glycans featuring differently linked galactosyl and sialyl residues.[565]

While a propensity to recognize carbohydrates on the basis of their shape as well as their size has now been widely recognized in many practical separations throughout the current

literature, the retention mechanisms leading to these desirable characteristics can be summarized as “mixed mode”: graphitized carbon has long been recognized as an effective adsorbent in different forms of chromatography (as far as its surface recognition of molecular geometries is concerned[566,567]), but some hydrophobicity effects are also expected. Besides their chromatographic selectivity, PGC columns offer additional unique features: (a) they are stable across a very extensive pH range, unlike the silica-based packings,[568] and (b) they appear to maintain their integrity at elevated temperatures used for kinetically favored separations. These attributes extend significantly the scope for using different elution solvents, mobile-phase additives, and concentrations (so long as these do not interfere with MS detection). The variation in solvents, temperatures, and ion polarity dependence on column performance in PGC-LC/ESI-MS was systematically explored by Pabst and Altmann,[567] with a particular emphasis on the multiply sialylated (acidic) glycans. Longer retention times were observed in association with lower pH-values, while neutral oligosaccharides were mostly unaffected. The retention of carbohydrates in these systems generally increases with temperature, as opposed to the mechanisms associated with the other chromatographic modes. A low mobile-phase ionic strength was found to be undesirable with PGC[567] and a preference was expressed for using ammonium carbonate over ammonium acetate or formate, which were found adequate for elution of tetrasialylated glycans by other research groups[569-571] or operating conditions during LC-ESI/MS.[572]

Owing to a wide range of possible operating conditions in PGC-LC, there are analytical options on whether to derivatize oligosaccharides with fluorescence tags and permethylation agents or, as currently preferred by a number of investigators, separate them in their native forms without derivatization. Since analyzing cleaved reducing oligosaccharides directly can lead to added complexity due to the separation of anomeric species, conversion to alditols is preferable. Alternatively, Fan et al.[568] achieved on-line conversion through addition of 10 mM ammonia to the mobile phase. Kawasaki et al. have systematically developed PGC-LC coupled with MS,[569,573-576] decreasing gradually the column inner diameters down to capillary LC conditions.[569] Using small column diameters is conducive to greater sensitivities in LC-MS, as was also demonstrated by the Packer group following a series of communications and different applications.[577-579] It appears that PGC columns can be interfaced to a variety of mass analyzers via ESI miniaturized sources while native glycans and their derivatized forms can be separated and analyzed in either negative-or positive-ion mode down to femtomole levels.[580] The applications emphasizing extremely high sensitivity of measurements on both *N*- and *O*-glycans using PGC-LC/MS techniques demonstrate excellent results, with the biological extracts corresponding to roughly 10^6 cells,[581,582] and both N- and O-linked glycans derived from low femtomole amounts of erythropoietin have been analyzed using this medium.[465]

The gradually rising popularity of graphitized carbon columns is documented by numerous practical applications to glycoprotein structural determinations and analyses of complex biological materials. The following list is representative of these efforts, albeit not entirely comprehensive: very early application to sulfated glycans isolated from mucous materials,[583] immunoprecipitated proteins from tissue extracts,[581,582] human bronchial epithelial cell cultures,[584] keyhole limpet hemocyanin,[585] human and bovine milk

oligosaccharides,[586-588] frog eggs,[589] fibrin and fibrinogen isolated from different animal species,[565] tear fluid,[590] human blood plasma,[560,579] and blood serum.[591,592] In a number of these investigations, both N- and O-linked oligosaccharide profiles were satisfactorily analyzed on PGC columns in both derivatized and native forms. The profiling capabilities of PGC-LC/MS and achieved measurement sensitivities appear to be adequate for characterization of glycoproteins isolated through SDS-PAGE[578,579] in gel spots; SDS-PAGE is still one of the time-honored research tools in protein chemistry and glycobiology.

References to “ruggedness” (or a lack of) with respect to PGC columns are repeatedly seen throughout the recent literature. Several authors have compared different chromatographic modes, i.e., RPLC, HILIC, PGC-LC, and ion-exchange chromatography in terms of their analytical figures of merit.[77,546,560,566] While PGC-LC has been universally praised in terms of its unusual selectivity for isomers and its potential for developing a general glycomic platform, the comparative studies tend to give higher marks to HILIC in terms of reproducibility of retention and analytical validation aspects. These attributes are particularly important to the measurements performed in biotech and modern pharmaceutical industries where recombinant antibodies and other glycoproteins are being increasingly assessed through efficient analytical techniques. The unique isomer selectivity of PGC, in comparison to the other chromatographic modes, will further be discussed below in relation to multidimensional separation approaches.

8.5. Multidimensional Glycan Separations and Development of Platforms and Strategies for Structural Assignment

Because glycan mixtures that originate from different biological materials can, in principle, be extremely complex and variable, it may be unreasonable to assume that any single analytical technique will ever suffice to measure entire glycomes. The need for a multimethodological, or perhaps multimodal, approach to oligosaccharide analysis may not be appealing to some investigators due to the procedural complications, but combining the best of the current MS procedures with the most advanced chromatographic techniques could lead to very powerful automated procedures to meet the goals of a comprehensive glycan analysis, including isomeric determinations. Besides oligosaccharide mixture complexities, the dynamic concentration range is yet an additional reason for combining the best features of LC separations and MS technologies. The developments of the past decade seem to endorse these analytical trends. As pointed out appropriately in a recent instrumentation review by Pabst and Altmann,[543] a true structural elucidation system requires that (a) all glycans be detected *and* quantified; (b) isomers become resolved; and (c) all sample components can be assigned their respective structures, including the overall topology of the molecule and all linkages. Different directions leading to these goals will be briefly reviewed below.

Starting with the chromatographic glycan methodologies, the interesting developments in the efficiency directions involve a decrease of particle size for HILIC-based materials below 2 μm dimensions. Just how dramatically this affects the component resolution is seen in Figure 23 through the comparison of fetuin 2-AB-labeled glycans (fluorescence detection).[593] Small-particle technologies could be developed for other column materials as

well. Yet another interesting route to high-efficiency glycan separations may potentially be the use of an open tubular column format[234] when a HILIC phase layer is deposited on the wall of a 10 μm i.d. capillary (25 cm length) operated at 20 nL/min flow for multiple tandem MS detections of oligosaccharides at femtomole levels. Interestingly, low-nanogram initial quantities of model glycoproteins were sufficient to identify numerous glycans with confidence.

Combining glycomic retention data from different chromatographic columns to yield positive identification and structural assignments has been pursued in a number of studies using either derivatized or native glycans. Tomiya et al.[530] introduced a two-dimensional sugar mapping procedure, in which they labeled *N*-glycans with 2-AP, while the mixture separations were accomplished using a HILIC phase as well as an octadecyl RPLC column. Using over 100 standard oligosaccharides, they constructed an “oligosaccharide map”, in which the glucose units (GUs), obtained through the reference oligosaccharide ladder, were recorded for the RP column and plotted against those obtained with the HILIC column. The overall procedure is further aided by the extensive use of sequential digestion with exoglycosidase enzymes, whereby any shifts in retention (GU values) are carefully examined, correlated with the known glycoprotein hydrolysates, and potentially used in predicting structures of unidentified glycans. The Hase group[516] has further extended the sugar mapping approach through a controlled partial hydrolysis of oligosaccharides to yield their fragments and determine their chromatographic retention data.

As shown in the above-referenced studies and numerous investigations by others, through using either a single chromatographic column or a 2-D system, aliquoting the glycan sample for different exoglycosidase digestions (followed by measuring glycan retention shifts) can be valuable in identification studies. Naturally, it is of the utmost benefit if the retention is measured in the columns of a dissimilar mode of separation, so that highly orthogonal data can be generated. The elaborate systems involving 2-D, or even 3-D, glycan separations[594,595] provide a wealth of useful data to glycoprotein researchers; however, the necessary off-line collection of glycan fractions between different chromatographic steps is somewhat tedious and time-consuming. On the basis of the different orthogonalities of certain chromatographic modes in glycan separations,[546] some combinations are preferable to others to attain maximum peak capacity in 2-D separations. Additional considerations involve compatibility of the mobile phases between two chromatographic modes for a solute transfer/peak compression for rechromatography, but also for the sake of MS detection (a need for “MS-friendly” buffers and solvents). According to the conclusions of Melmer et al.,[546] new (small-particle) HILIC columns are eminently suited for detailed analysis of complex glycan mixtures, which explains their increasing popularity, but coupling RPLC with HILIC on-line is technically difficult. With respect to sialylated isomers, ion-pairing RPLC appears directly compatible with PGC-LC in terms of reinjection into the second dimension, while the high selectivity of the latter mode would be a significant asset of this 2-D approach.

An interesting alternative to 2-D glycan chromatography has been described by Deguchi et al.[596] in coupling an anionexchange column to a HILIC or ZIC-HILIC column and resolving various sialylated *N*-glycans as 2-AP derivatives. This approach is somewhat similar to the

widely used proteomic methodology known as MudPIT,[597] albeit with very different phase systems used on-line. In the study of Deguchi et al., the properly chosen gradients of aqueous acetonitrile and an ammonium acetate additive were used to sequentially elute sialylated glycans, from biantennary to tetra-antennary structures, in an automated program run. A miniaturized column-switching system combining RPLC and HILIC was described by Lam and co-workers,[247] who have overcome the above-mentioned solvent strength incompatibility through a valve-assisted on-line solvent mixing system, although the paper primarily dealt with glycopeptides and showed only a brief application to the ribonuclease B glycans. In general, on-line 2-D separations of glycans still appear in a very preliminary stage of development.

With the well-known uncertainties in the natural encoding of the glycan structures, the analytical capabilities for structural elucidation should be made superior to those for genomic and proteomic studies. While the theoretical prediction of all possible isomeric structures[1] seems exceedingly high and certain biosynthetic restrictions seem plausible,[543] there has been substantial interest in the analytical solutions to isomer resolution, which thus far seem most favorable to PGC-LC used in conjunction with various forms of tandem MS.

The work with capillary PGC-LC[577] has established their potential for sensitive MS detection at low femtomole levels. Incorporating the PGC materials into the form of a chip-based procedure and its commercial product (presented as a “specialized inlet” to mass spectrometers) seems to present a further important step toward the standardization of this approach at the nanoscale level. In the profiling investigations of N-linked glycans in human serum,[591] the nano-LC chips with varying column lengths were successfully tested. The extracts of oligosaccharides from human serum are displayed as a series of profiles (Figure 24) or base-peak chromatograms, with each of them previously enriched through solid-phase extraction (SPE) into (a) neutral oligosaccharides, (b) neutral and some anionic oligosaccharides, and (c) anionic oligosaccharides. The loading capacity of these chips was sufficient to collect spectra through TOF/MS and interpret a number of structures. This has further led to annotation of a serum *N*-glycan library,[592] representing an important stage in building a potential analytical platform using native, underivatized glycan samples to be determined in complex mixtures through LC/MS. Similar efforts were extended by the same group to profile human and bovine milk oligosaccharides[588] and develop their annotated libraries.[586,587]

Another glycomic platform utilizing PGC-LC has been proposed by Costello and co-workers.[528] This approach takes advantage of the superior ionization and fragmentation properties of permethylated glycans together with the separating compatibility of these derivatives with PGC columns. The separation of isomers, together with their MS fragmentation data, is shown in Figure 25 for *N*-glycans extracted from anticular cartilage decorin. While the proposed use of up to MS^3 fragmentation of permethylated glycans is appealing in its information content and structural assignments, it was also observed that highly sialylated permethylated glycans featured broad peaks during PGC-LC. Other problems pertaining to sample preparation with high-polarity glycans, such as phosphorylated and sulfated structures, will also need to be solved following this approach. The recent review by Pabst and Altmann[543] indirectly endorses both permethylation (for the

sake of MS fragmentation) and the potential of PGC-LC (for the sake of sugar isomeric resolution).

The once-perceived idea that many glycan mixtures can be sorted out directly by tandem MS through interpretation of fragmentation data and bioinformatics is increasingly seen as unrealistic.[543] Identifying isomers in complex mixtures will clearly benefit from the future analytical platforms combining appropriate derivatization and preconcentration techniques with the best that contemporary LC and MS can offer. These efforts can undoubtedly be aided and accelerated by the availability of reference glycan standards. Reference glycans may be available synthetically or isolated/purified from natural sources.

8.6. Capillary Electrophoresis

When modern biological mass spectrometry was in its infancy and undergoing a rapid growth, the developments in capillary electrophoresis/laser-induced fluorescence (CE-LIF) for derivatized glycans in the early 1990s[598-600] allowed glycomic maps to be displayed at a high sensitivity. Even as MS instrumentation and techniques have matured and dramatically increased in their numbers and applications, CE-LIF still continues to be a key technique, and the area continues to evolve. This is driven by the ability of CE-LIF to reproducibly record very complex glycan profiles from biological samples at a very high sensitivity. Additionally, its ability to resolve the potentially biomedically important isomeric glycans, which is, at best, extremely difficult using current MS methods, ensures that CE-LIF will continue to play a role in glycoanalytical investigations, even if it may lack the identification/characterization capabilities easily offered by MS and tandem MS. The ability for CE to resolve structurally similar glycans is exemplified with the glycan profile of a monoclonal antibody compared to several glycan standards.[87] With new applications, developmental trends, hardware innovations, and miniaturization that are mainly due to the instrumental simplicity of CE-LIF, the technique will surely continue to make important contributions to the field.

Similarly to other analytical approaches, there has long been an interest in CE-LIF to improve the overall performance aspects of the technique in terms of the speed of analysis and the limits of detection, with the selection of an appropriate fluorescent tag still being the subject of different communications in the current literature. While different fluorescence-labeling reagents were developed and explored in the early work on CE-LIF of carbohydrates,[598] the introduction of the 8-aminopyrene-1,3,6-trisulfonic acid (APTS) tag by Guttman and co-workers[601,602] has been widely accepted and is still the most popular label. This molecule modifies the reducing end of a glycan through a reductive amination mechanism. Other more recent examples of fluorescence-labeling approaches include 4-fluoro-7-nitro-2,1,3-benzoxadiazole[365] and rhodamine 110 dye with its large fluorescence quantum yield.[603] Since each derivatization results in slightly different derivatized glycan structures, the buffer composition and separation conditions need to be modified for each derivatization procedure to ensure optimal solute resolution.

Many new biopharmaceutical products, including monoclonal antibody-based therapeutic agents and vaccines, are glycoproteins, and CE-LIF is one of the methods of choice to provide quantitative glycomic profiles to demonstrate product efficacy and minimize

immunogenicity effects[75,604] in the biotechnology industry. In contrast to profiling glycans in the biomarker discovery area, where complicated pools of glycans derived from complex biological mixtures are analyzed and highly sensitive measures are a requirement, most biotechnologically oriented applications place less stringent demands on sensitivity and the identification of the unknown components. However, an industrial setting, and similarly a clinical one, demands a high throughput, since thousands of samples may need to be analyzed, as demonstrated by a CE-based study to evaluate glycan profiles for possible indicators of different liver diseases.[605,606] As seen in Figure 26, an optimized protocol of sample treatments, beginning with a 3-μL aliquot of serum, leading to APTS labeling and, ultimately to CE-fluorescence glycan profiling, can be performed for clinical applications using a DNA sequencer.

While CE-based glycan separations of biomedical interest and other applications have been routinely performed in a capillary, there has recently been a trend toward the use of chipbased systems.[607-611] This format offers significant gains in the measurement speed, with a single run for a complex carbohydrate mixture requiring just a few minutes (typically less than 5), along with more reproducible separations and signal recordings. As one of the early examples of this trend,[608] Figure 27 depicts the separation of glycans derived from glycoproteins from a serum sample provided by a breast cancer patient. This figure highlights the attractive features of the chipbased approach in terms of the analysis time. Whereas the separation in a fused-silica capillary required over 30 min, the chip-based run using a spiral channel design was completed in only 2.8 min at a comparable, or better, separation efficiency. More recently, a chip with an advanced serpentine design was developed that resulted in even more efficient separations for similar biological samples.[612]

A definitive structural assignment of peaks present in an electrophoretogram may be difficult, partly due to a lack of many key oligosaccharide standards which could be used for migration time comparisons. For structural determinations, oftentimes additional experiments are required. One possible method to reveal a glycan's identity is through the use of sequential digestions with exoglycosidases to remove specific monosaccharide components, followed by a CE analysis.[613,614] While such a method can be useful in structural assignments and glycan mapping with relatively simple glycoproteins, it is generally less effective with biologically complex systems. Additionally, exoglycosidases are relatively expensive reagents. A recent innovative extension of this approach, decreasing consumption of the exoglycosidases and enhancement of theoverall analytical performance, appears to be the phospholipid-assisted CE.[76,615] In this technique, the phospholipid additives were used in a segmentation process to incorporate the various exoglycosidases, while multiple enzymes were added sequentially for specific incubation times to cleave selected monosaccharides from the APTS-labeled glycans from a recombinant glycoprotein cancer drug inside the CE capillary.[76]

The main limitation of CE, preventing its widespread application in the glycoanalytical direction, is still its somewhat limited compatibility with MS. To achieve the highest possible separation efficiencies using capillary zone electrophoresis (CZE), only minute quantities of the analytes may be introduced to the inlet of the separation capillary, and the same holds true for the chip-based analogues. Consequently, most on-column

preconcentration approaches (for example, stacking or solute trapping) offer only a little assistance to enhance the signals in different CE-MS combinations. However, work in coupling these two complementary powerful techniques continues, as recently demonstrated with a CE-LIF-negative-ion-mode-MS setup that was used to successfully analyze recombinant monoclonal antibody glycans as APTS-labeled analytes.[82]

Presently, additional advances in CE-MS of glycoconjugates are clearly desirable to further the development of analytical glycobiology. Ironically, the best up-to-date CE separations have been achieved with the buffer media and polymeric additives, which are largely incompatible with typical MS conditions, and it seems that more “MS friendly” buffer systems will need to be designed to make this coupling more feasible. The designing of different derivatization schemes and optimized separation conditions, together with any break-through developments in the CE-MS interfacing technologies, MS designs, or combined incremental improvements in all these areas may also assist in this endeavor. Various advances in CE-MS of glycoconjugates have been the subject of recent reviews.[382,611,616]

9. CONCLUDING REMARKS

Since our last review of the area a decade ago, the field of analytical glycobiology has evolved substantially and grown at a considerable pace. The major advancements for this methodological progress have been initiated by the increased awareness of the importance of the connection of different glycoconjugates to some of the most important fields of human activities and scientific endeavors, including (i) the search for disease biomarkers; (ii) recombinant glycoprotein pharmaceuticals; (iii) developmental biology and microbiology; (iv) immunology; (v) plant biology; and (vi) biofuels, among others. This review has summarized a representative cross section of the new key analytical techniques and instrumentation in the field that has expanded in its breadth significantly in the past decade.

Glycoproteins are methodologically unique and among the most analytically challenging from the different classes of glycoconjugates. While inherently connected, the areas of glycomics and glycoproteomics necessitate a different emphasis on how the biological samples are fractionated, enzymatically or chemically treated, and analyzed. The emphasis on very highsensitivity measurements, which is clearly dictated by the exceedingly large dynamic concentration range in which different glycoproteins occur in biological samples, favors the most technologically advanced forms of instrumental methods such as MS, miniaturized LC, and CE-LIF. The complementary nature of these analytical approaches in glycomic and glycoproteomic measurements, and their different couplings together (in the so-called “hyphenated” techniques), will be essential in future investigations. Substantial advances in bioinformatics to facilitate computer-assisted data analysis and processing in the evaluations of the highly complex analytical data add to the unprecedented opportunities for future explorations of the mysterious problems of different glycomes and glycoproteomes.

Acknowledgments

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Biographies

William R. Alley, Jr., earned his bachelor's of science degrees in chemistry and chemistry education from Dickinson State University in Dickinson, ND. He then pursued a Masters degree at Oklahoma State University in Stillwater, OK, under the guidance of Prof. Thomas Burgoyne. In 2002, he joined Prof. Novotny's laboratory to pursue his Ph.D. After graduating, he remained in Prof. Novotny's laboratory as a postdoctoral fellow. His research interests include developing highly sensitive MS glycomic methods and comprehensive characterization of glycopeptides.

Benjamin F. Mann received his bachelor's of science in chemistry from Butler University (Indianapolis, IN) in 2004. After working briefly in the pharmaceutical industry, he moved to Bloomington, IN, in 2006, to pursue his Ph.D. in analytical chemistry at Indiana University under Professor Milos Novotny, graduating in 2012. His research focuses on the development of separation strategies for enhanced detection of glycoproteins in biological mixtures, utilizing high-sensitivity mass spectrometry for quantitative comparative analyses.

Milos V. Novotny is a Distinguished Professor at Indiana University, where he directs the National Center for Glycomics and Glycoproteomics and the Institute for Pheromone Research. He received his Doctorate in Biochemistry at the University of Brno, Czechoslovakia (1965). His postdoctoral training in separation science and mass spectrometry was conducted at the Institute of Analytical Chemistry, Czechoslovak Academy of Sciences (1965–67); Royal Karolinska Institute, Sweden (1968); and the University of Houston (1969–1970). Dr. Novotny joined the Department of Chemistry at

Indiana University as a faculty member in 1971, where he has taught analytical chemistry and biochemistry. He has trained numerous graduate students and postdoctoral fellows for positions in academia and industry and published over 500 scientific publications and patents. He was a pioneer in several areas of separation science (capillary gas chromatography/mass spectrometry, supercritical fluid chromatography, microcolumn liquid chromatography, and capillary electromigration techniques). Dr. Novotny and his group were also responsible for identification of the first definitive mammalian pheromones. He has been a recipient of numerous awards, medals, and other distinctions, including four national ACS awards and three honorary doctorates. He has consulted widely for industrial and government institutions, taught numerous short courses, and lectured worldwide. Dr. Novotny's current research interests center on the development of new methodologies for glycoproteomics and glycomics analysis. He also continues his involvement in structural identification of mammalian pheromones and the biochemical aspects of chemical communication.

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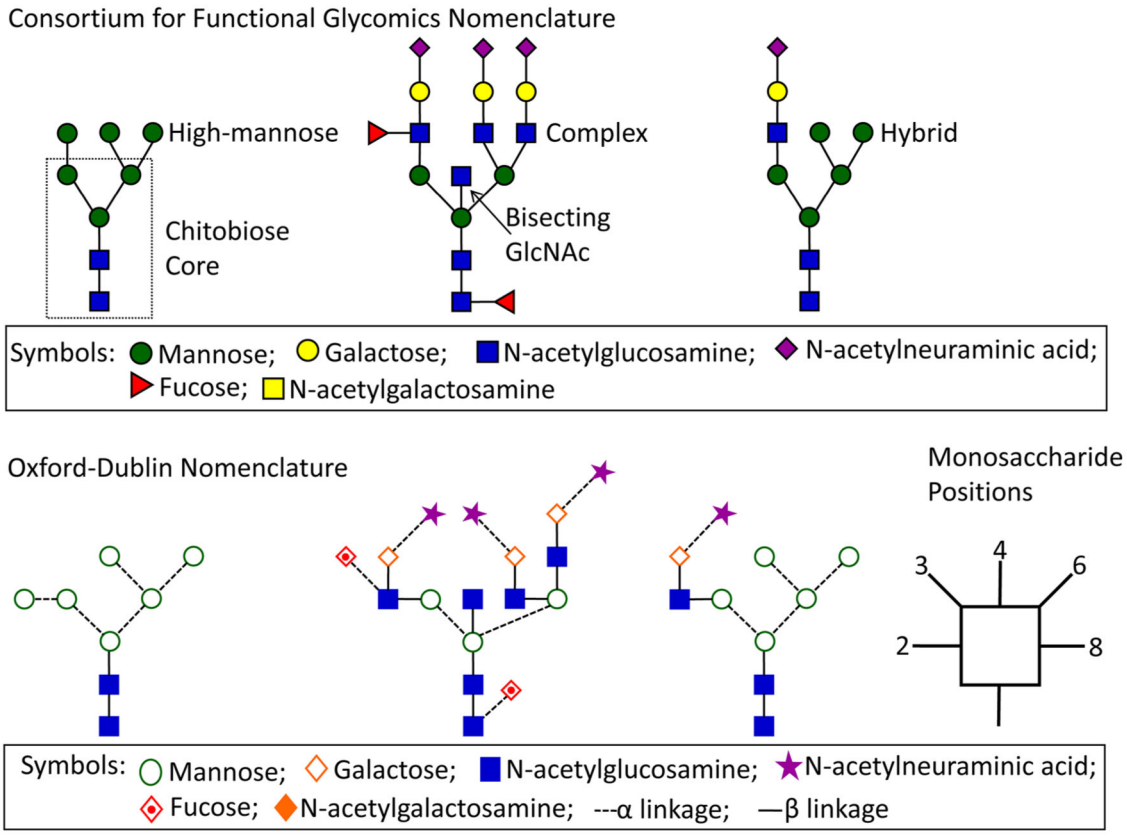


Figure 1.

Representations of the high-mannose-type glycans, complex oligosaccharides, and hybrid structures depicted using the Consortium for Functional Glycomics system and the Oxford-Dublin style.

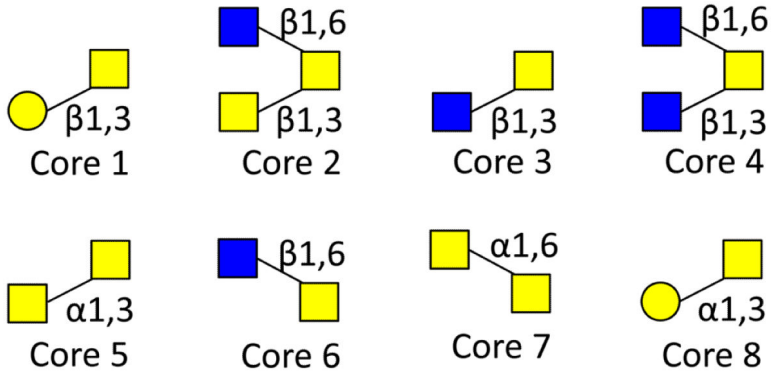


Figure 2.
Eight common O-linked glycan cores.

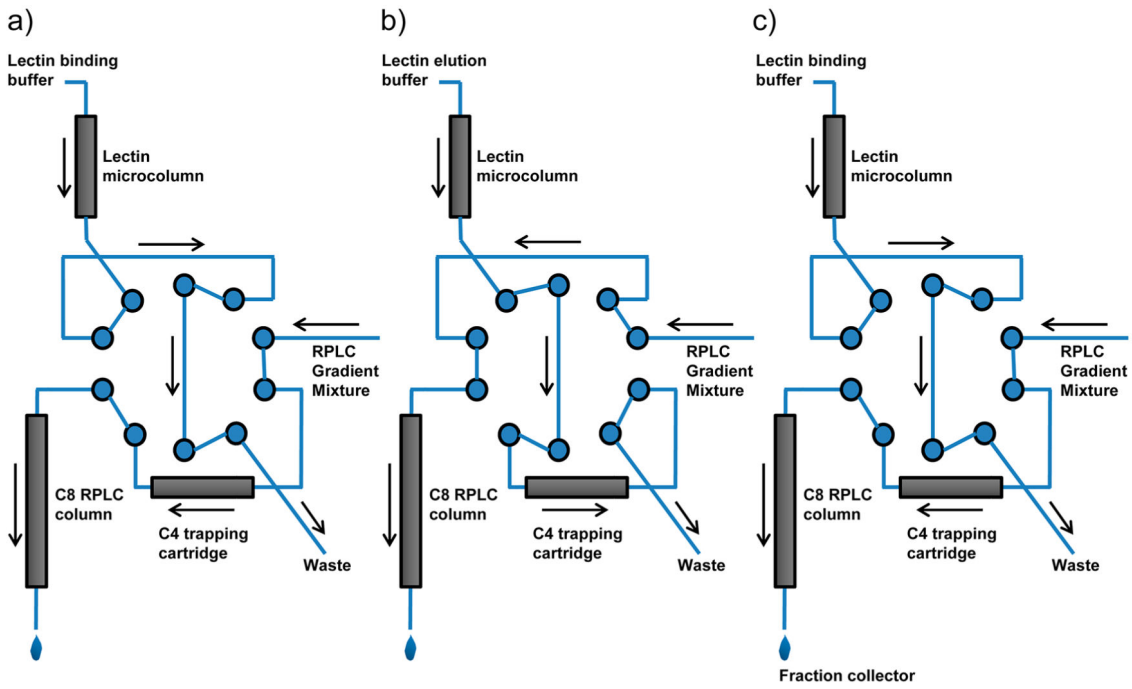


Figure 3.

Instrumental setup of an automated system for the on-line coupling of a lectin affinity silica-based microcolumn with RPLC glycoprotein fractionation. (a) Sample loading, affinity capture of select glycoproteins, and washing out unbound proteins; (b) elution of bound glycoproteins with an elution buffer, desalting on C_4 trap; (c) elution of the glycoproteins from the C_4 trap followed by gradient-based RPLC fractionation of enriched glycoproteins. (Reprinted with permission from ref 143. Copyright 2006 American Chemical Society.)

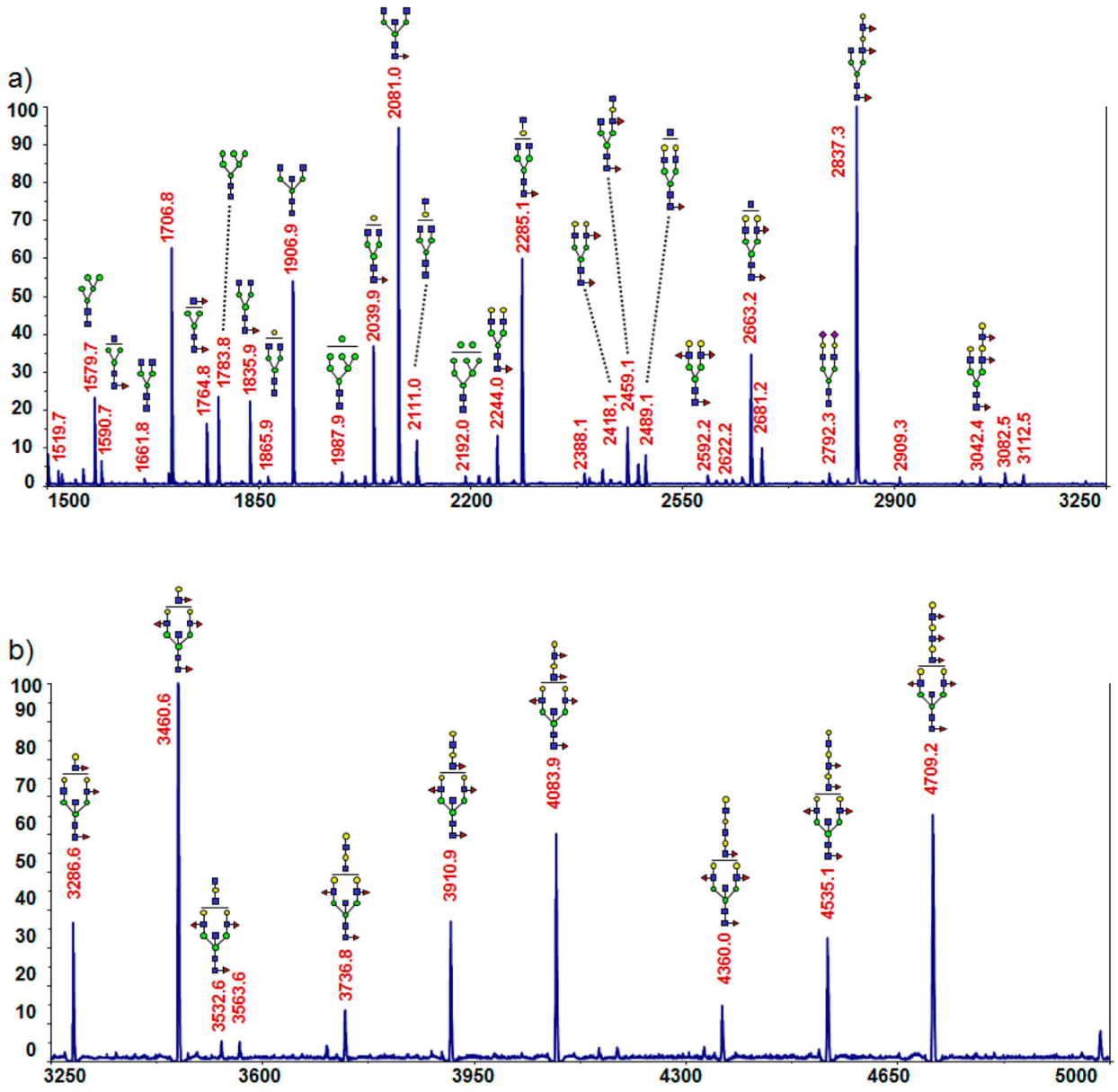


Figure 4.

Glycomic and glycoproteomic analysis of multiply fucosylated glycoproteins in pancreatic cyst fluids; MALDI-TOF-MS of permethylated *N*-glycans (a) from *m*/*z* 1500–3250 and (b) from *m*/*z* 3250–5000. (Reprinted with permission from ref 161. Copyright 2012 American Society for Biochemistry and Molecular Biology.)

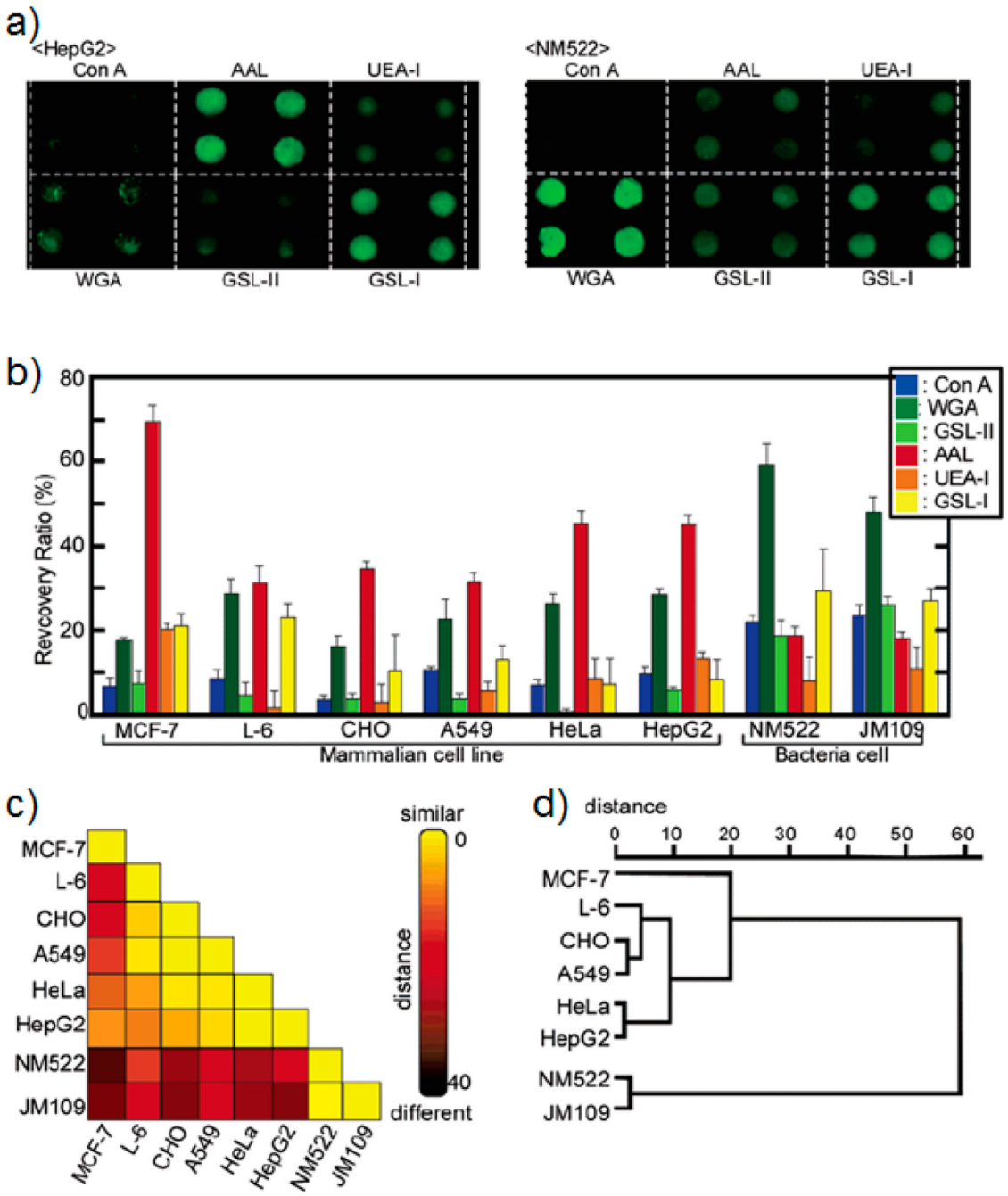


Figure 5.

Pattern detection of carbohydrates in cell lysates by lectin microarray chip: (a) fluorescent images for a lectin chip analyzing the human cell line (HepG2) and bacterial cells (NM522); (b) bar graph of the fluorescent recovery ratio by the addition of the mammalian cell lines (MCF-7, L-6, CHO, A549, HeLa, HepG2: 10,000 cells/μL) and bacteria cells (NM522, JM109: 10 μg/μL by wet weight); bar heights and error bars were estimated by the average and standard deviation, respectively, of four spots on the same plate; (c) the Euclidian distance matrix between the patterns of the different cell lines obtained by the lectin microarray was represented by color coding (yellow for highest, black for lowest similarity); (d) the dendrogram of the response patterns for the eight cell lines generated by the analysis of Euclidean distances; the horizontal axis represents the distances among the

normalized lectin chip patterns (left for patterns with the highest similarity and right for patterns with lowest similarity). (Reprinted with permission from ref 170. Copyright 2006 American Chemical Society.)

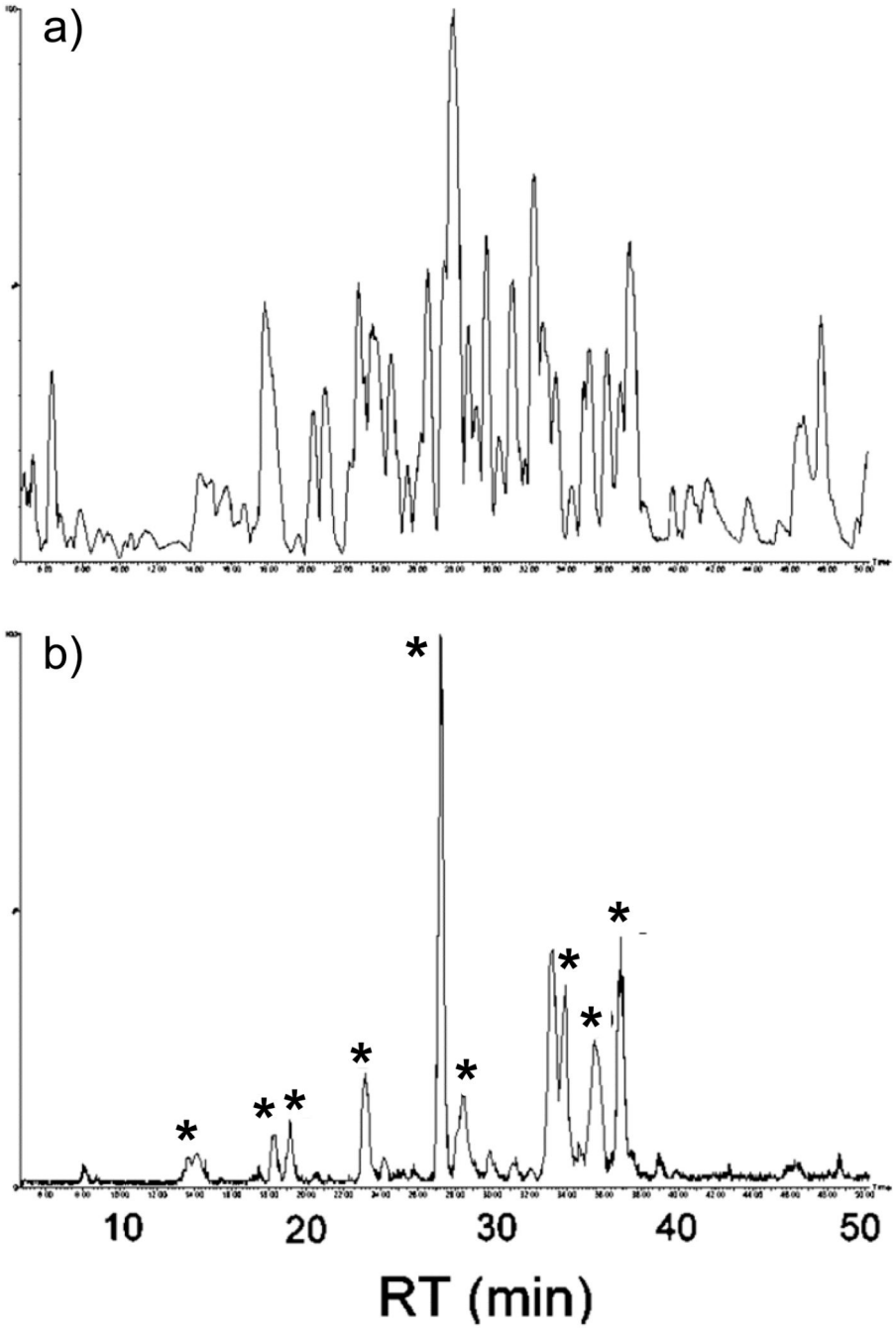


Figure 6.

(a) Base-peak chromatogram of a tryptic digest of the A/Vietnam/1203/2004 H5N1 whole inactivated virus reagent without any glycopeptide enrichment prior to an LC-MS analysis; (b) base-peak chromatogram of a tryptic digest from the same virus strain after an isolation of the tryptic glycopeptides by hydrazide SPE. Ions observed were the deglycosylated forms of the captured glycopeptides. The asterisks correspond to glycopeptides where the predicted glycosylation sites were identified as occupied by other modes of characterization. (Reprinted with permission from ref 202. Copyright 2009 American Chemical Society.)

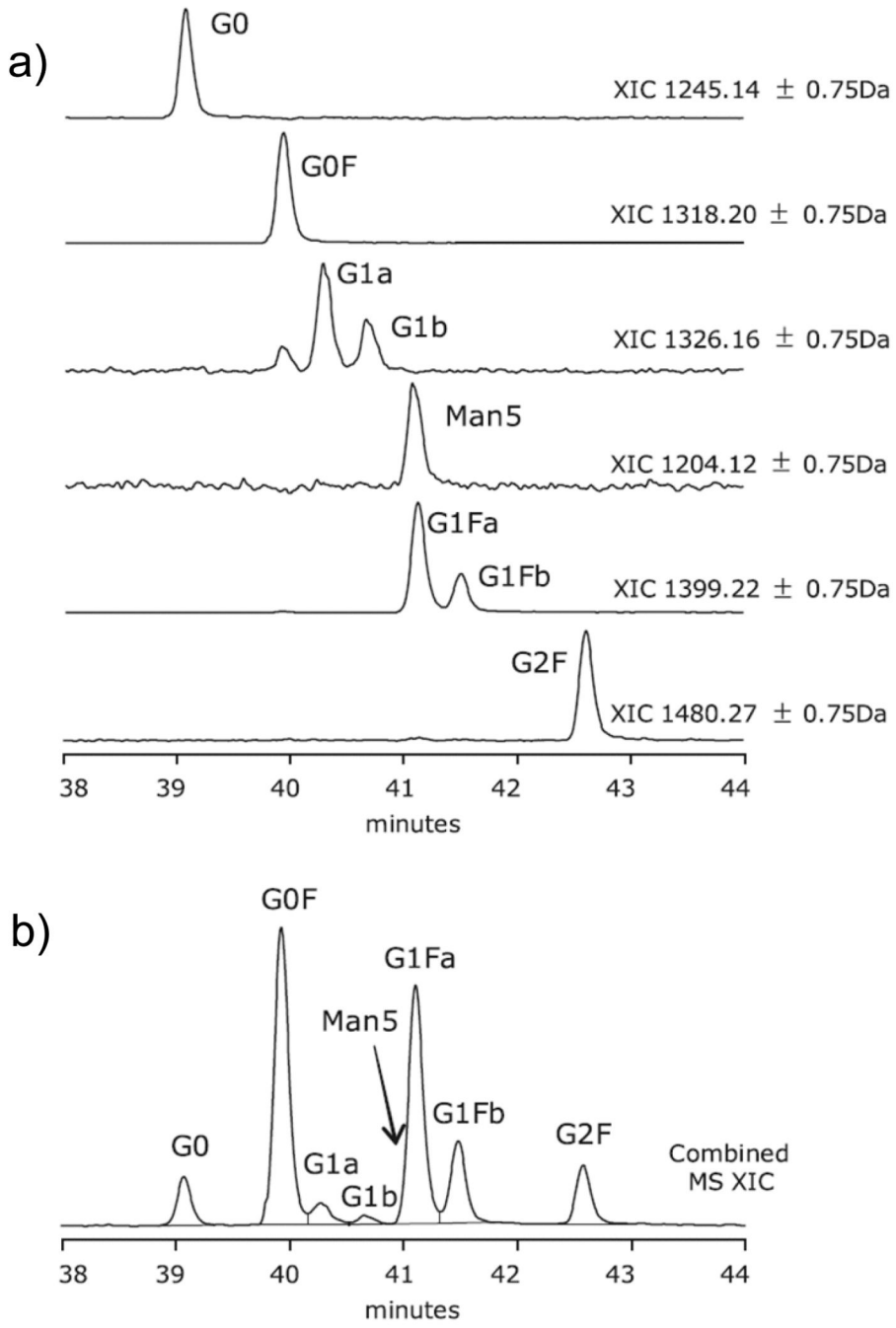


Figure 7.

Quantitation of mAb EEQYNSTYR glycoforms: (a) extracted-ion chromatograms (XICs) for different glycoforms from HILIC-ESI-MS analysis; (b) combined XICs. (Reprinted with permission from ref 248. Copyright 2011 Elsevier.)

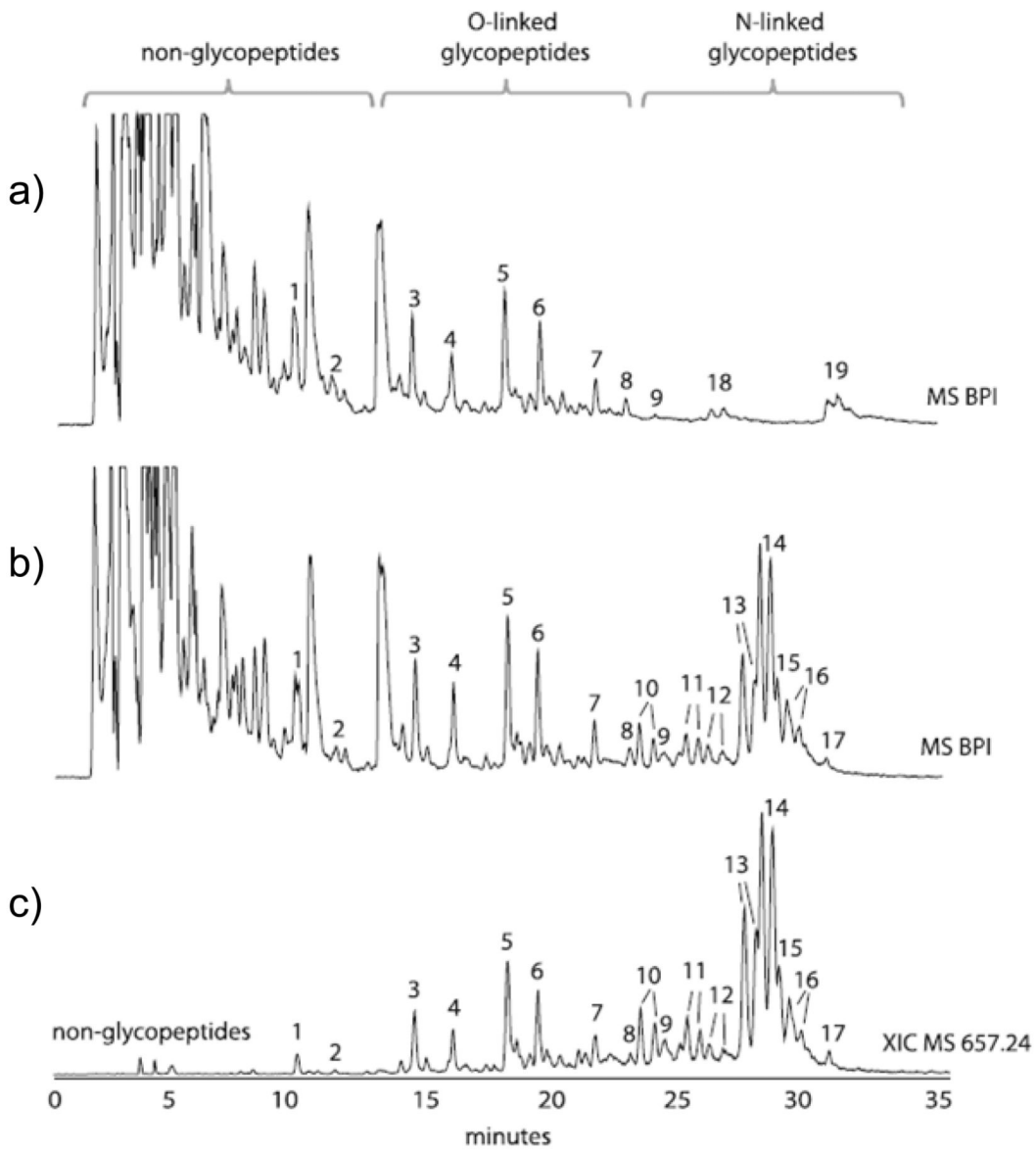


Figure 8.

HILIC-ESI-MS analysis of bovine fetuin tryptic digest: (a) Fetuin tryptic digest exposed to PNGase F, N-linked glycans was removed (only O-linked glycopeptides are present); (b) fetuin tryptic digest with no PNGase F treatment (both N- and O-linked glycopeptides are present); (c) XIC for glycan oxonium ion fragment 657.24 Da, revealing the positions of glycopeptides. (Reprinted with permission from ref 248. Copyright 2011 Elsevier.)

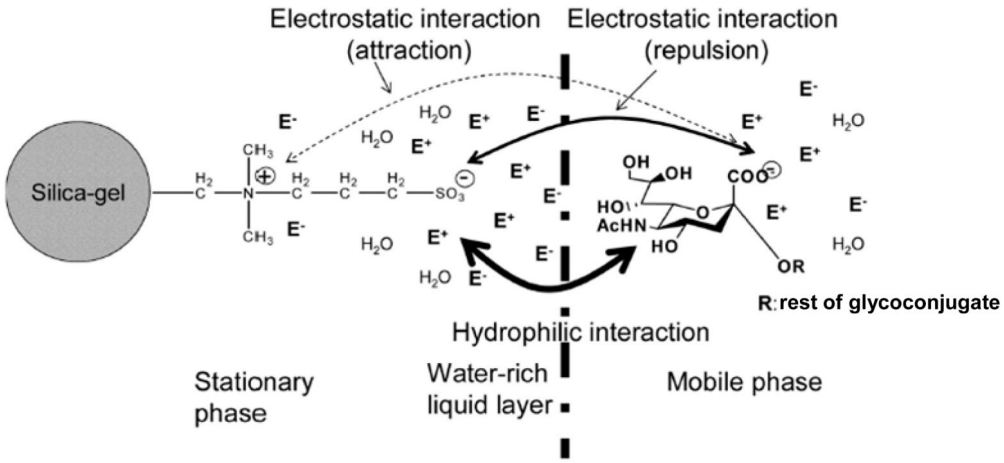


Figure 9.

Schematic diagram of ZIC-HILIC interactions of a sialylated *N*-glycan. Electrostatic (attraction and repulsion) and hydrophilic interactions between the sialic acid and the sulfobetaine group on the surface of the column are schematically shown. E^+ and E^- are positive- and negative-electrolyte ions in the eluent, respectively. (Reprinted with permission from ref 226. Copyright 2006 Wiley.)

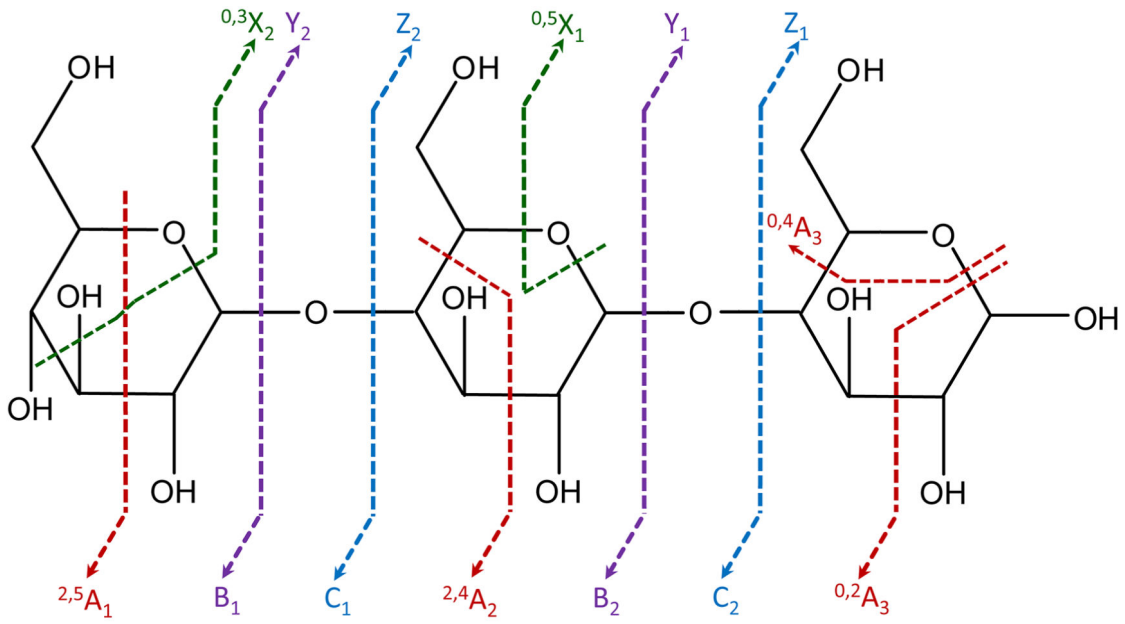


Figure 10.

Schematic representation of several common cleavages observed in the tandem MS analyses of carbohydrates. (Reprinted with permission from ref 269. Copyright 1988 Springer.)

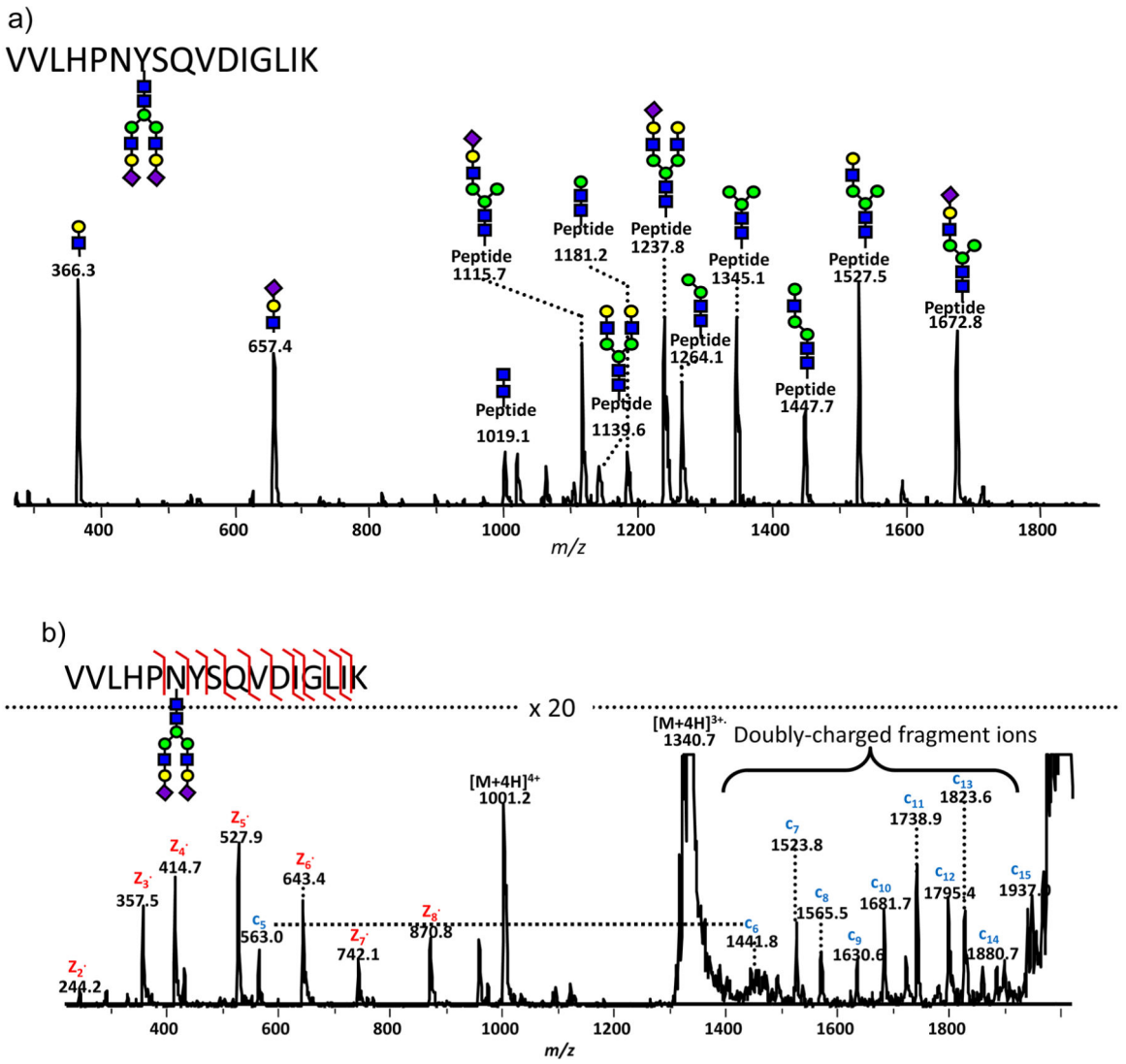


Figure 11.

Tandem MS analyses of a haptoglobin tryptic peptide fragmented by (a) CID, showing mainly carbohydrate glycosidic bond cleavages, and (b) ETD, resulting in peptide bond fragmentations. (Reprinted with permission from ref 271. Copyright 2009 Wiley.)

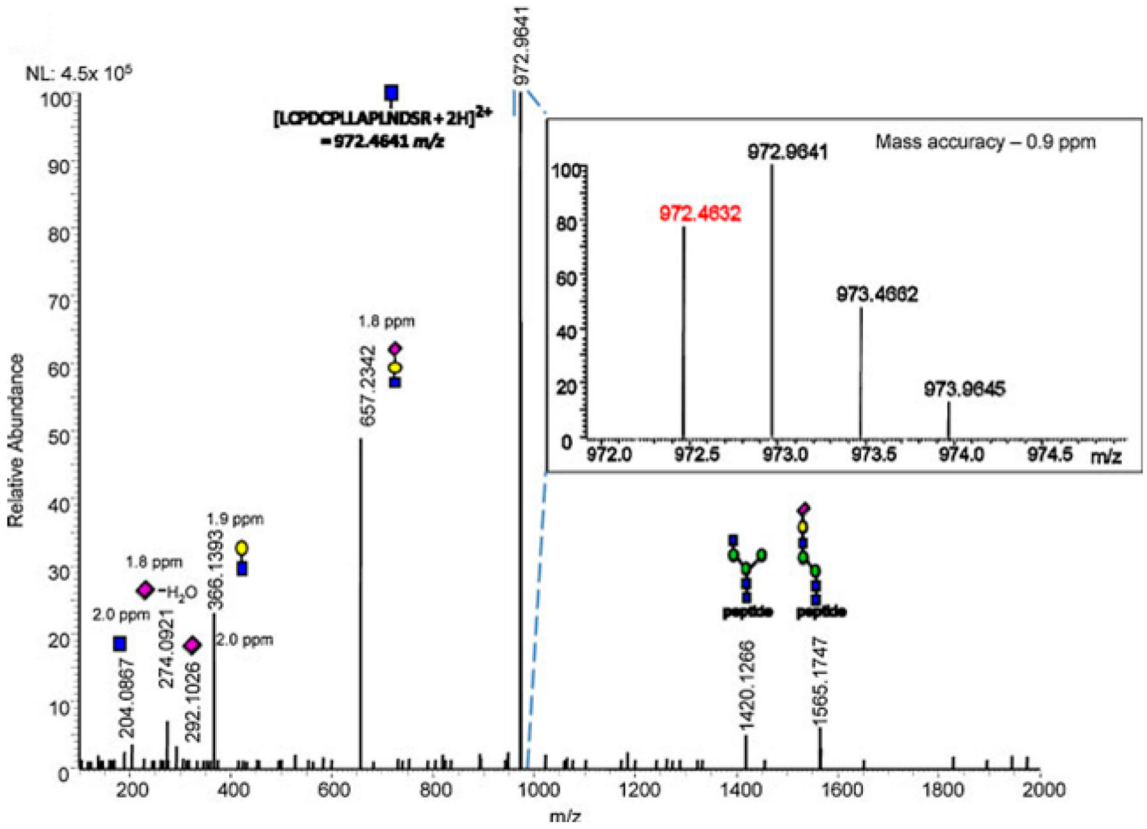


Figure 12.

HCD spectra of a glycopeptide showing the formation of the Y_1-type ion (peptide + GlcNAc) (Reprinted with permission from ref 315. Copyright 2010 Wiley.)

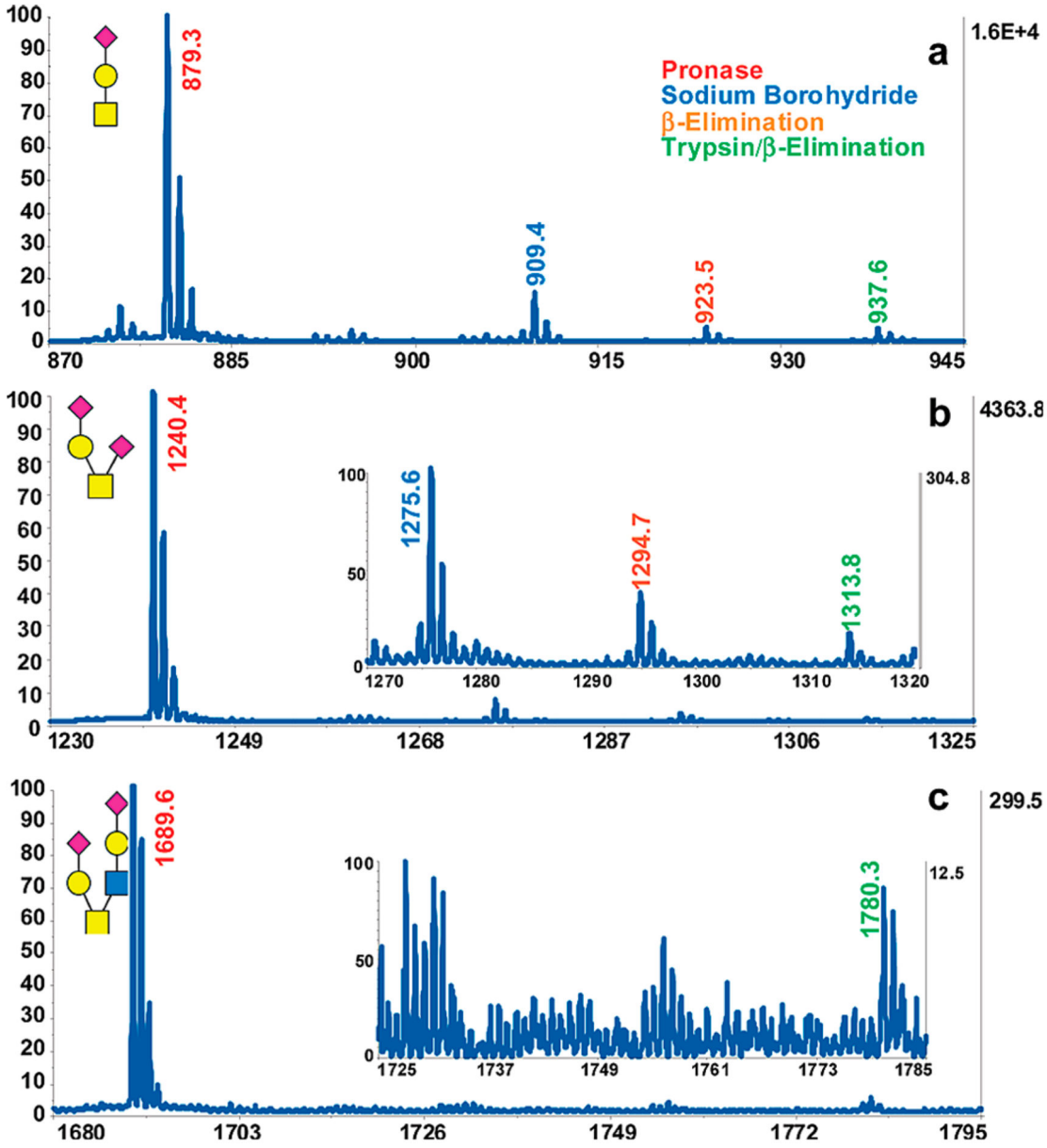


Figure 13.

Direct comparison of different O-glycan release methods for bovine fetuin O-glycans permethylated with different isotopically-labeled methyl iodide reagents. For the different carbohydrates shown in (a), (b), and (c), and Pronase/chemical procedure resulted in the most sensitive MALDI MS measurements. (Reprinted with permission from ref 378. Copyright 2009 American Chemical Society.)

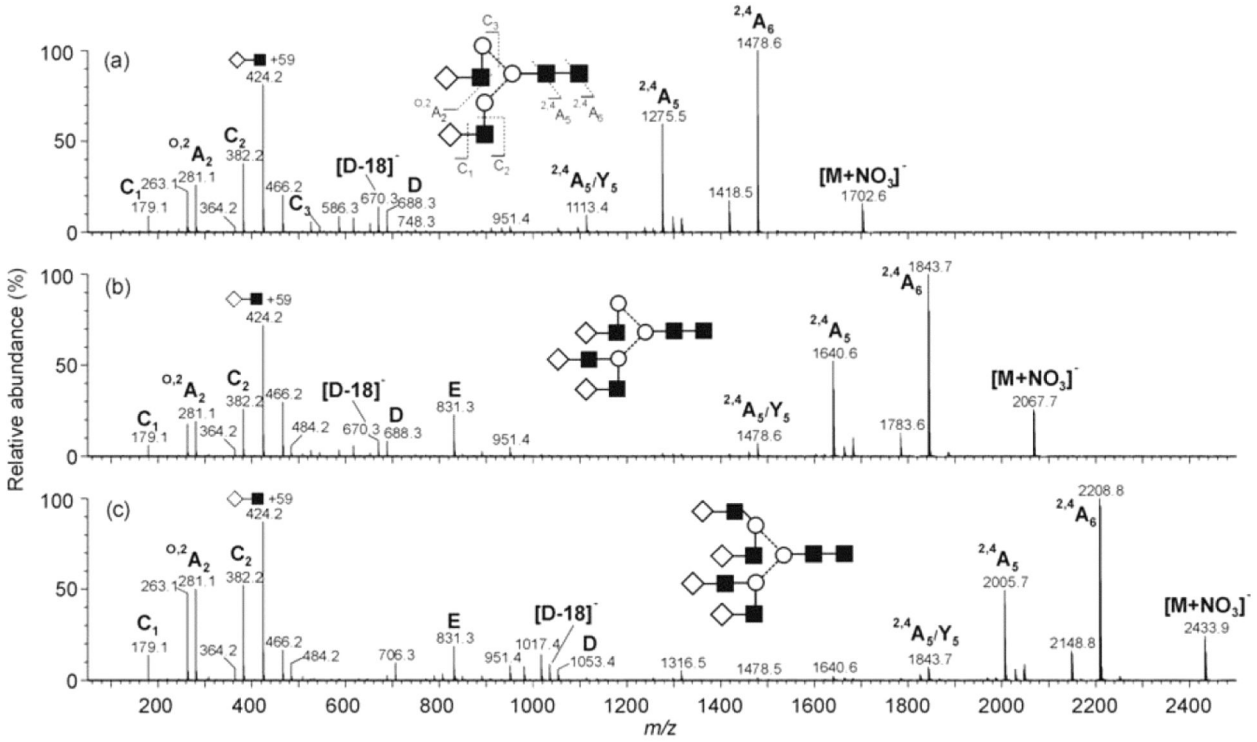


Figure 14.

(a) Bi-, (b) tri-, and (c) tetra-antennary glycans fragmented in the negative-ion mode, resulting in the formation of many cross-ring fragments. (Reprinted with permission from ref 412. Copyright 2005 American Society for Mass Spectrometry.)

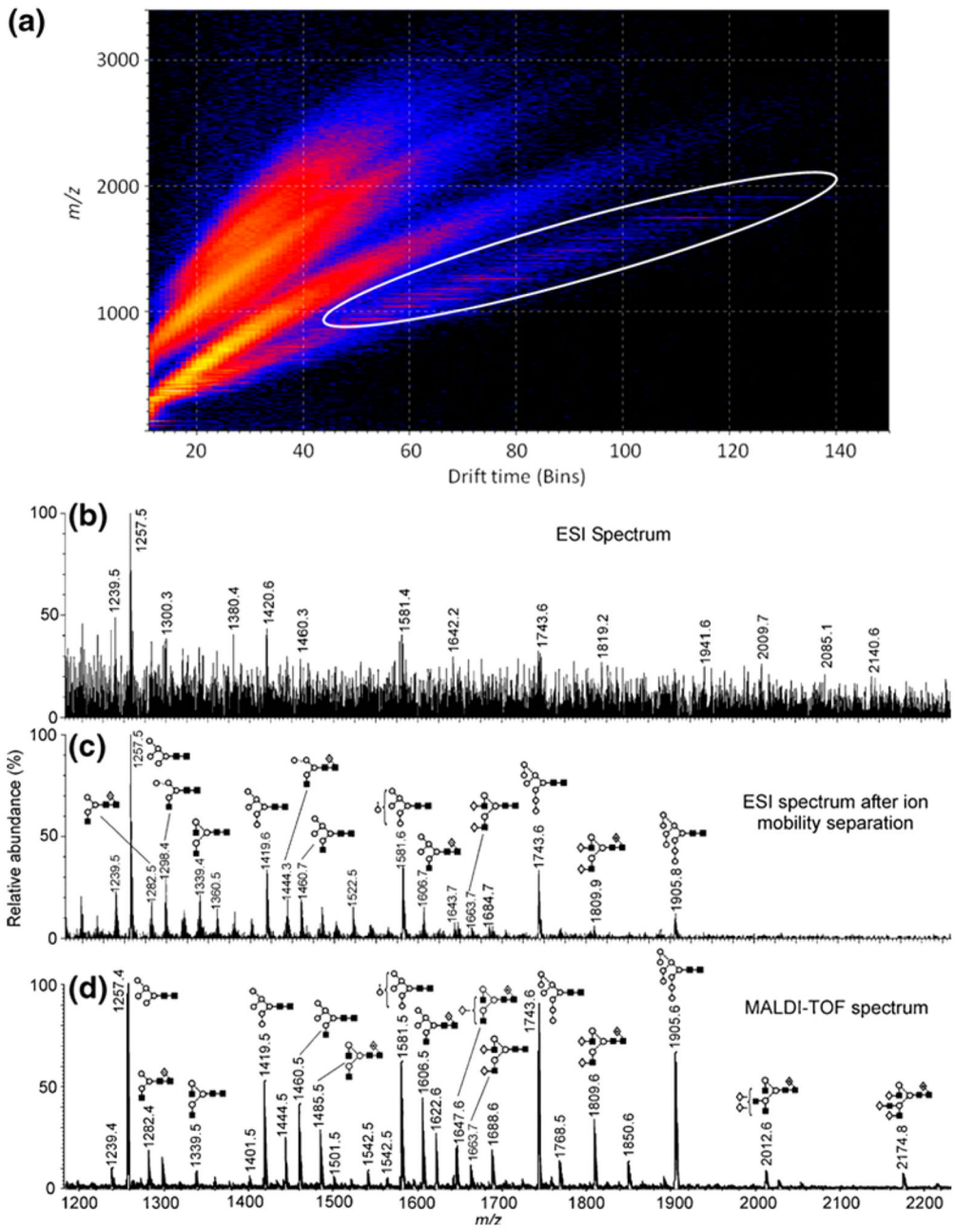


Figure 15.

IMS analysis of a crude glycan digest (still containing peptides, proteins, etc.) of the human immunodeficiency virus protein gp120. The total drift scope is shown as part a. An ESI MS spectrum is presented as part b, and part c depicts the glycans after an IMS separations, while part d is a MALDI profile of this protein. (Reprinted with permission from ref 447. Copyright 2011 American Society for Mass Spectrometry.)

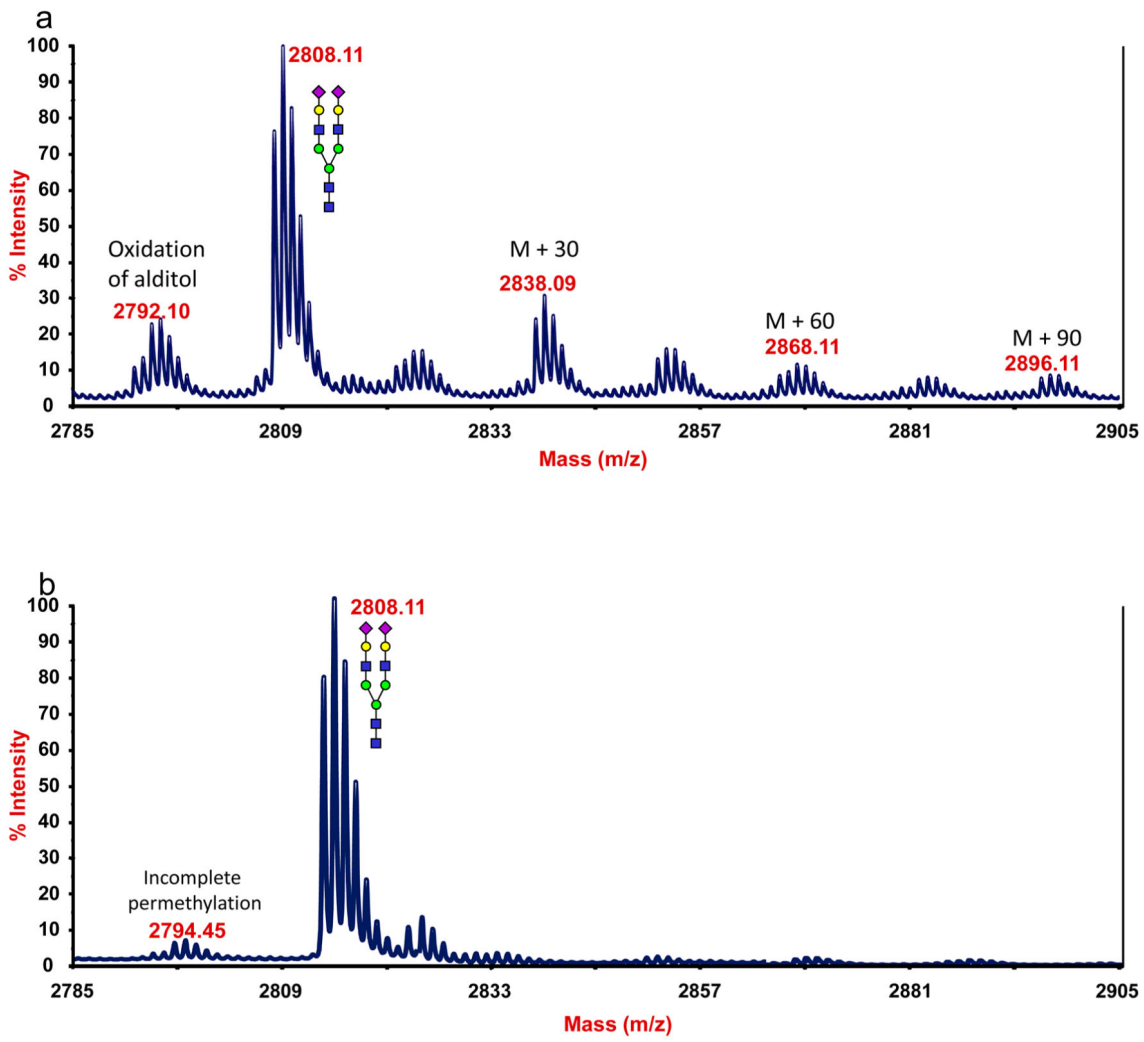


Figure 16.

MALDI profile a glycan alditol permethylated (a) in dimethylsulfoxide (DMSO), which shows a regeneration of the closed-ring structure and several +30-Da artifacts, and (b) in dimethylformamide, where these extraneous products are significantly reduced. (Reprinted with permission from ref 458. Copyright 2010 American Chemical Society.)

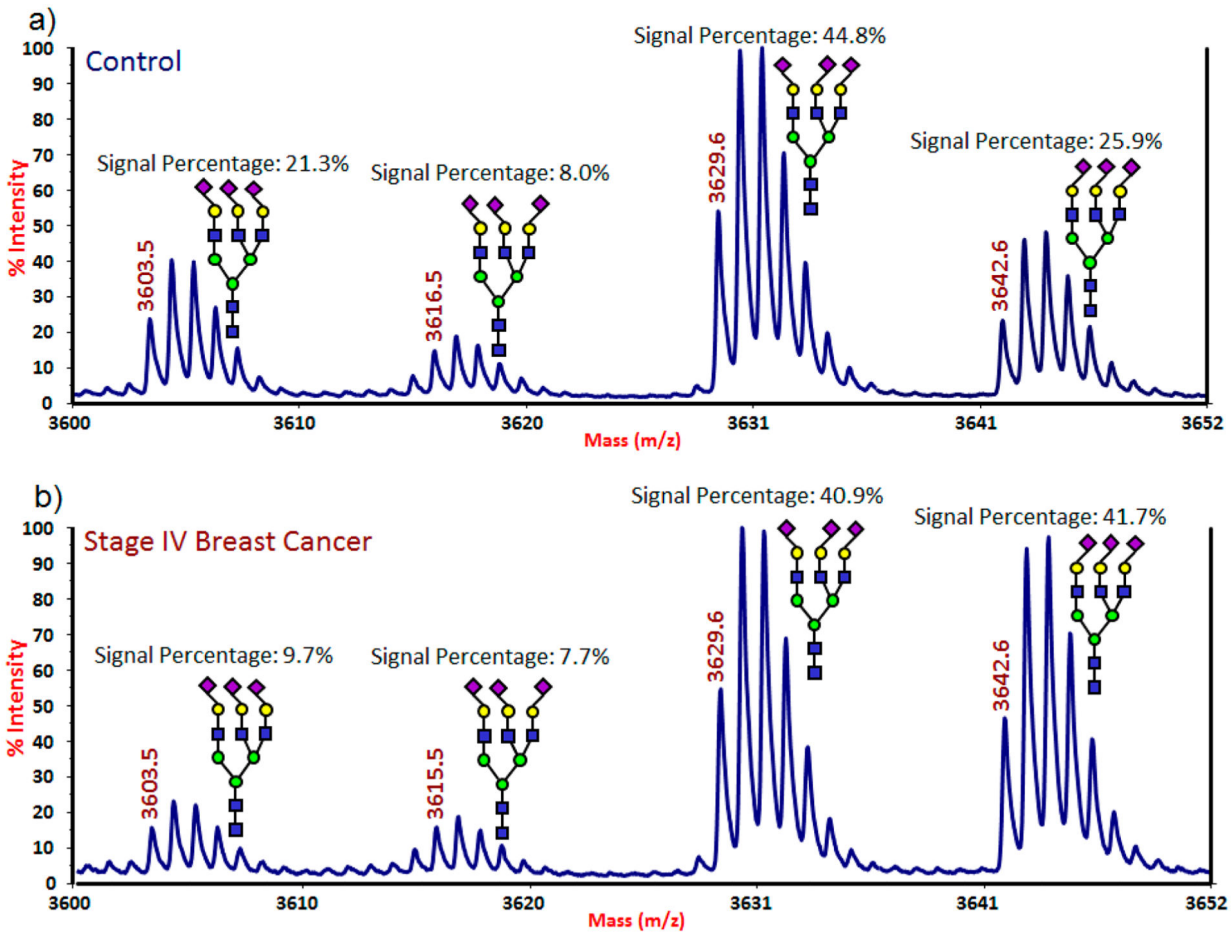


Figure 17

. MALDI MS recordings of an amidated- and-permethylated glycan derived from blood serum glycoproteins showing the differences in sialic acid linkages in (a) a control individual and (b) a woman diagnosed with late-stage breast cancer. In this figure, diamonds pointing to the left indicated α2-3-linked sialic acids, while those pointing to the right are α2-6-linked. (Reprinted with permission from ref 471. Copyright 2010 American Chemical Society.)

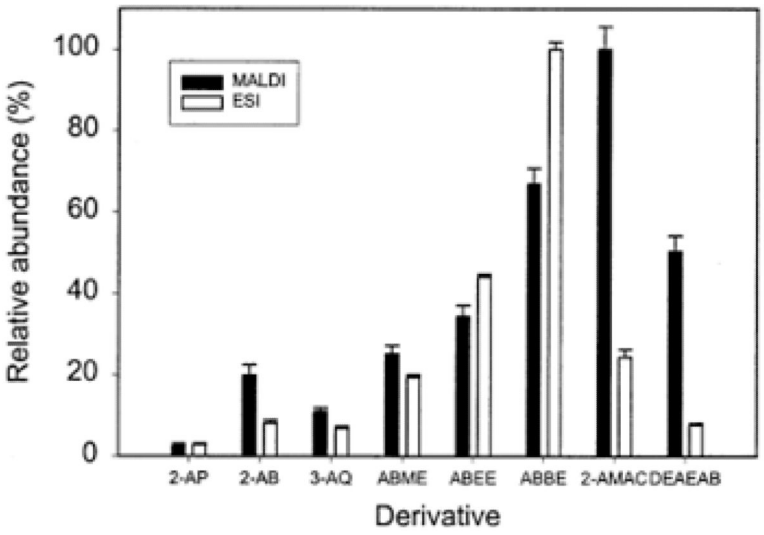


Figure 18.

Comparison of several reducing-end tags to enhance MALDI and ESI signal intensities. (Reprinted with permission from ref 480. Copyright 2000 American Society for Mass Spectrometry.)

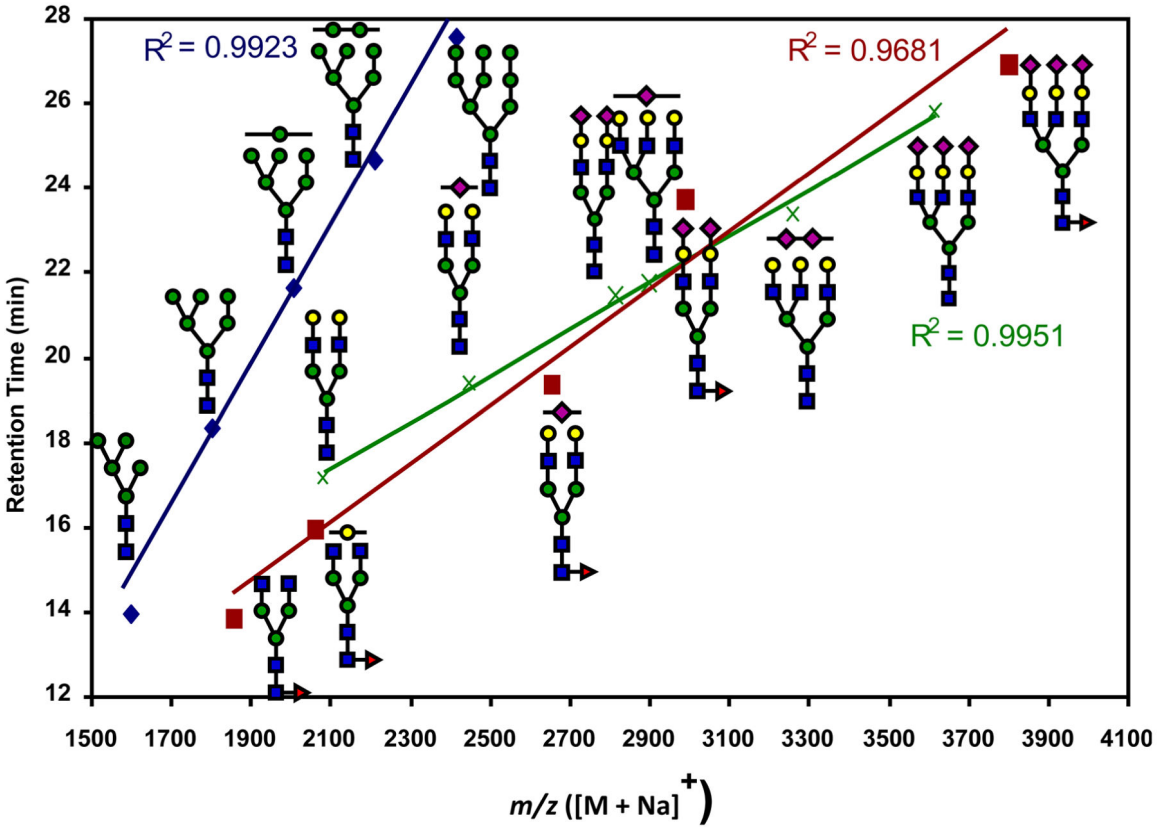


Figure 19.

Relationship between chip-RPLC (using C_{18}) retention time and the *m*/*z* value for high-mannose (blue trace), complex (green line), and sialylated- and-fucosylated (red trace) permethylated glycans. (Reprinted with permission from ref 458. Copyright 2000 American Chemical Society.)

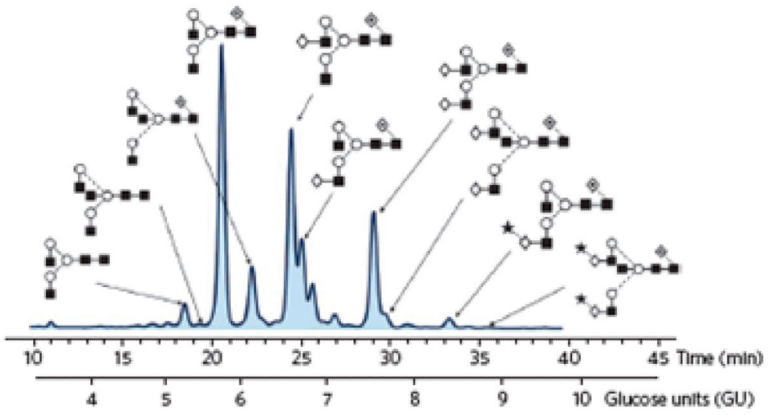


Figure 20.

HILIC-based HPLC separation of glycans derived from the heavy chain of IgG (from ref 533.) The column used in the study was a TSKgel Amide-80 column (250 mm × 4.6 mm) packed with 5 μm particles. The glycans, fluorescently labeled with 2-AB and detected at 330 and 420 nm (excitation and emission, respectively) were separated using a gradient from high organic solvent (acetonitrile) to high aqueous solvent (50 mM formate buffer, pH 4.4) at 30 °C. (Reprinted with permission from ref 542. Copyright 2010 Nature.)

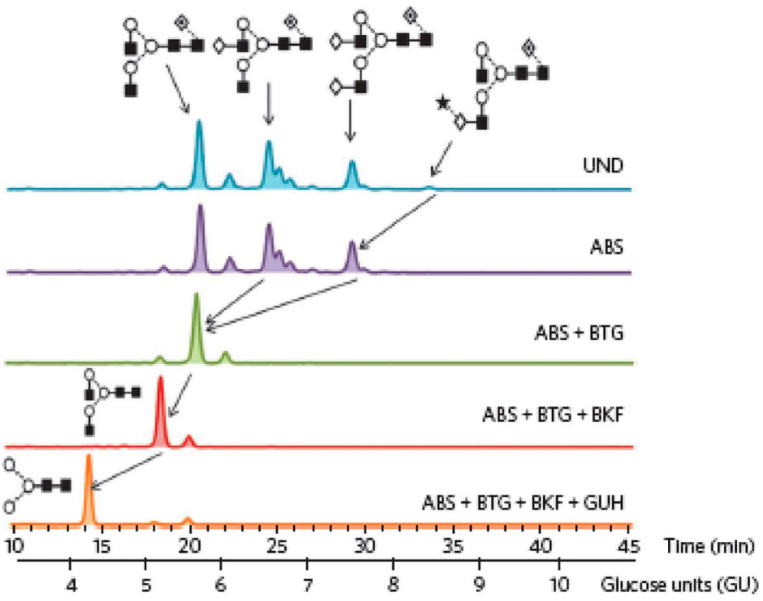


Figure 21.

HILIC-based LC separation of IgG glycans after being subjected to various exoglycosidases for their structural characterization. The enzymes used were *Arthrobacter ureafaciens* sialidase (ABS); bovine testes β-galactosidase (BTG); bovine kidney α-fucosidase (BFK); and β-*N*-acetylglucosaminidase (GUH). (Reprinted with permission from ref 542. Copyright 2010 Nature.)

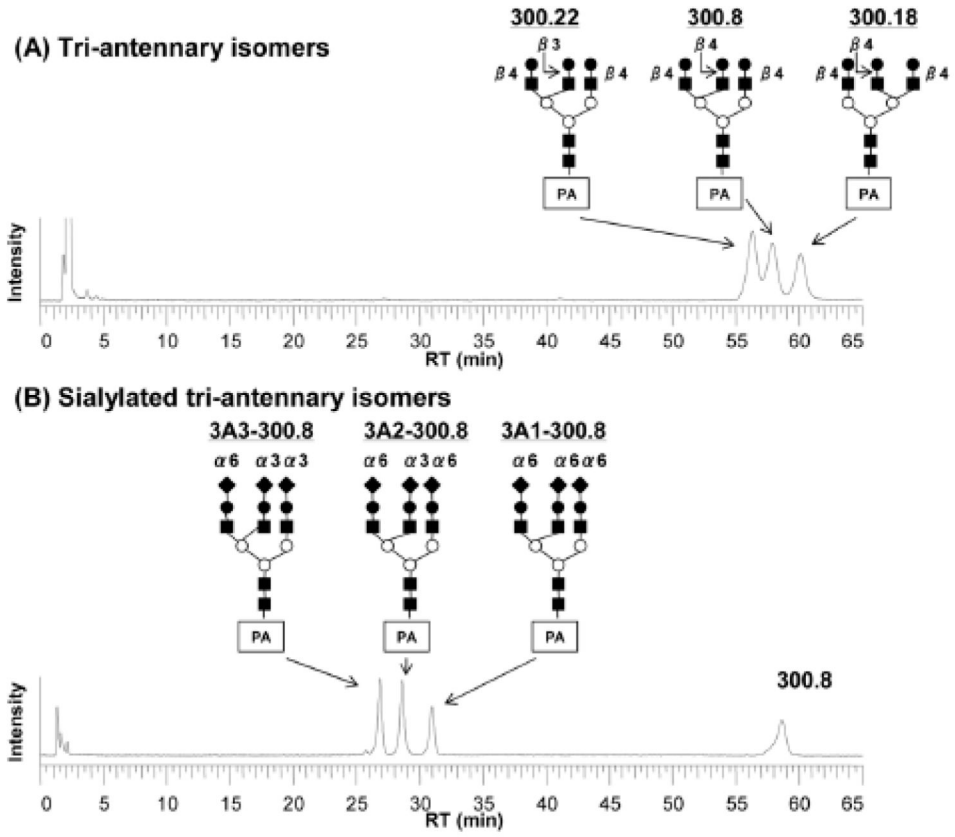


Figure 22.

Chromatograms for the ZIC-HILIC separation of (a) neutral and (b) sialylated triantennary glycans. (Reprinted with permission from ref 226. Copyright 2006 Wiley.)

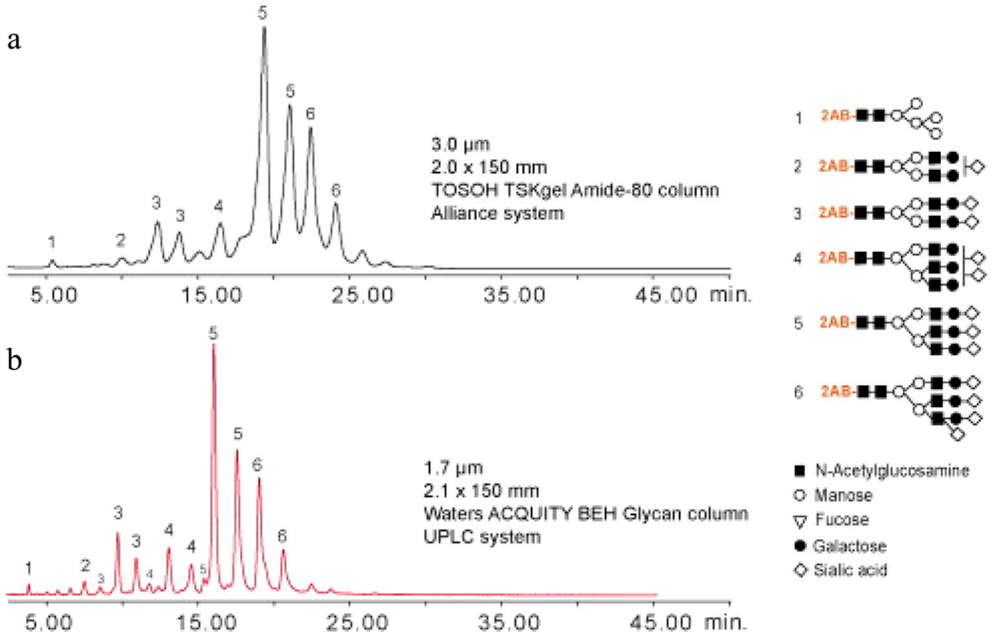


Figure 23.

Glycans derived from bovine fetuin separated by (a) an LC column packed with 3 μm TSKgel Amide-80 particles packed in a 2.0 mm × 150 mm column and (b) a UPLC column using 1.7 μm BEH (from Waters) sorbents packed in a 2.1 mm × 150 mm column. (Reprinted with permission from ref 593. Copyright 2010 Elsevier.)

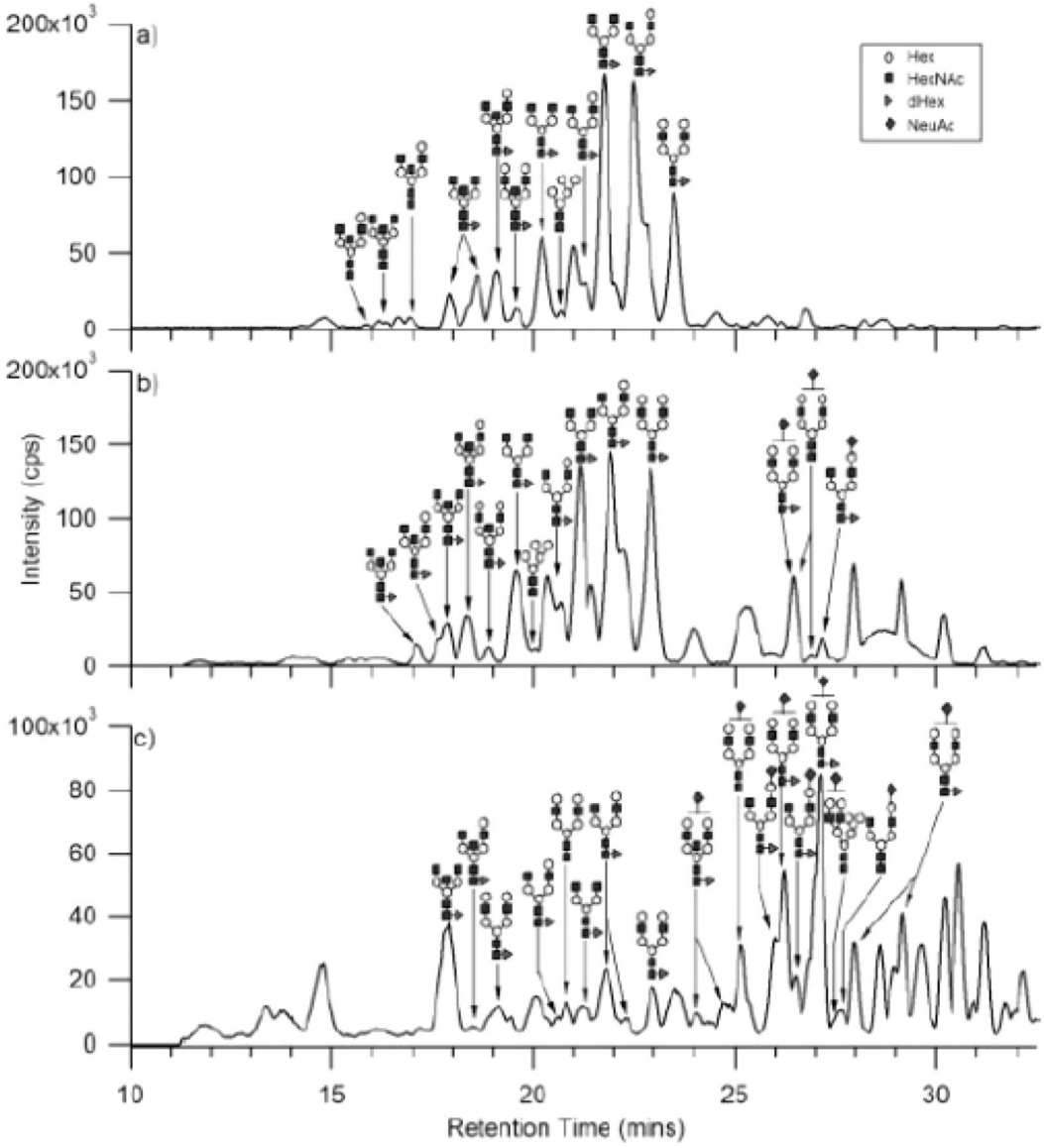


Figure 24.

Base-peak chromatograms of (a) the neutral glycans, (b) the neutral and sialylated oligosaccharides, and (c) the sialylated carbohydrates derived from human serum glycoproteins. An HPLC-chip (75 μm × 150 mm) packed with porous graphitic carbon was used in these separations, using a gradient from high aqueous solvent (3% acetonitril/0.1% formic acid) to high organic solvent (90% acetontrile/0.1% formic acid). (Reprinted with permission from ref 591. Copyright 2009 Elsevier.)

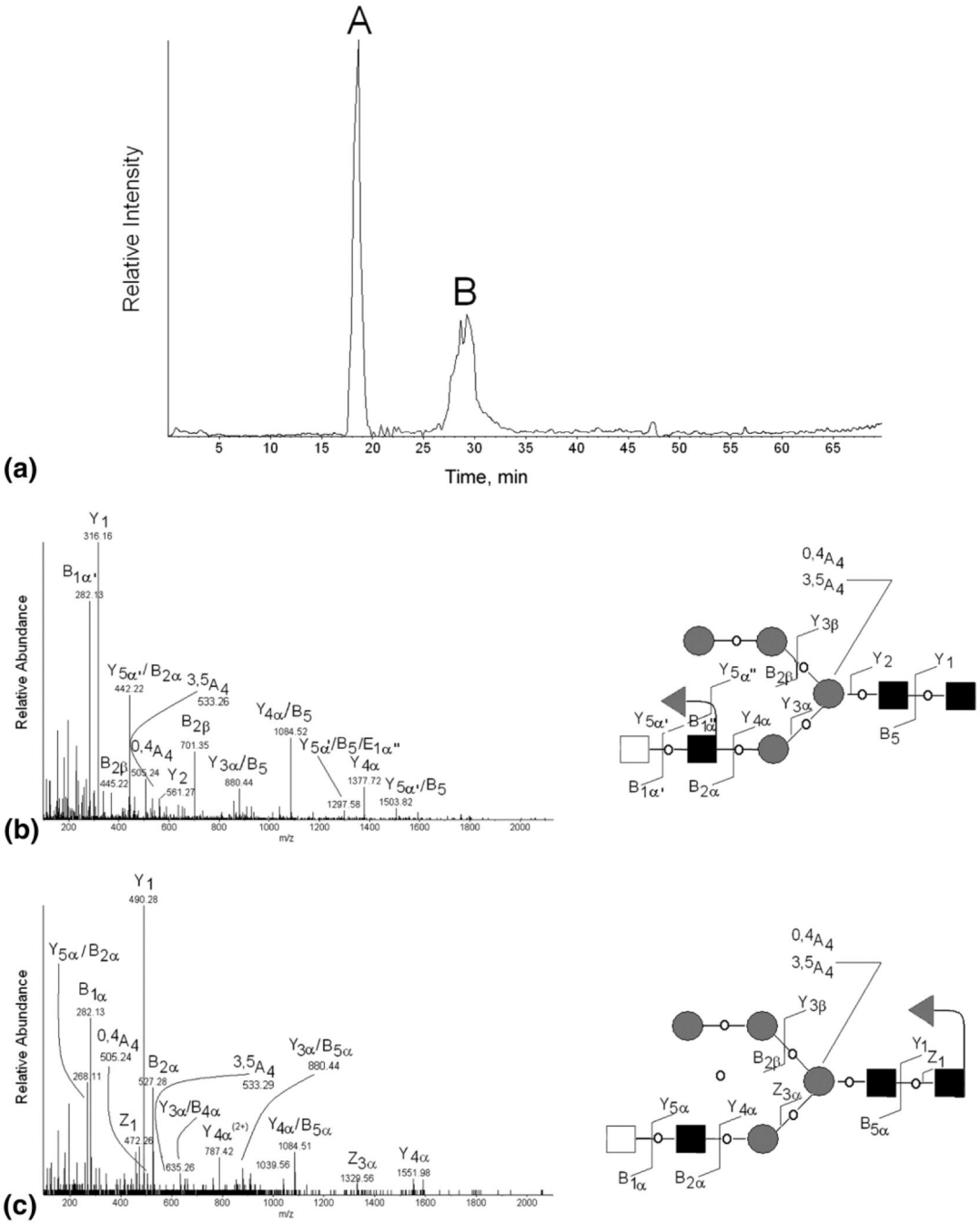


Figure 25.

LC/MS analysis using porous graphitic carbon as the stationary phase of articular cartilage decorin permethylated *N*-glycans. (a) Two well-resolved peaks were observed for a permethylated glycan with a sequence of dHex1Hex$_4$HexNAc$_4$. Parts b and c present the CID QoTOF MS/MS for these LC peaks and show different fragmentation patterns for each isomer. (Reprinted with permission from ref 528. Copyright 2007 American Society Mass Spectrometry.)

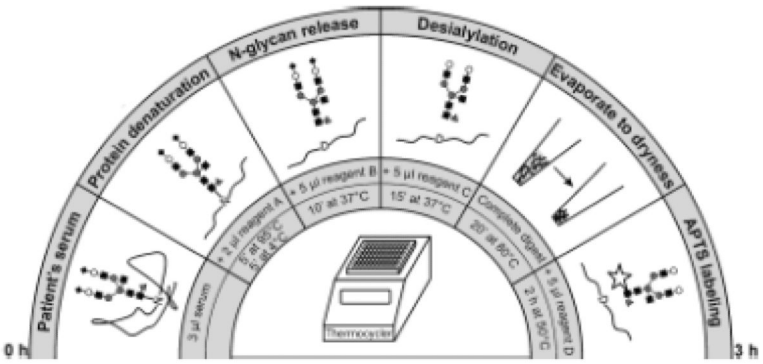


Figure 26.

Optimized workflow for the preparation of glycans prior to a capillary electrophoretic analysis. (Reprinted with permission from ref 606. Copyright 2010 American Chemical Society.)

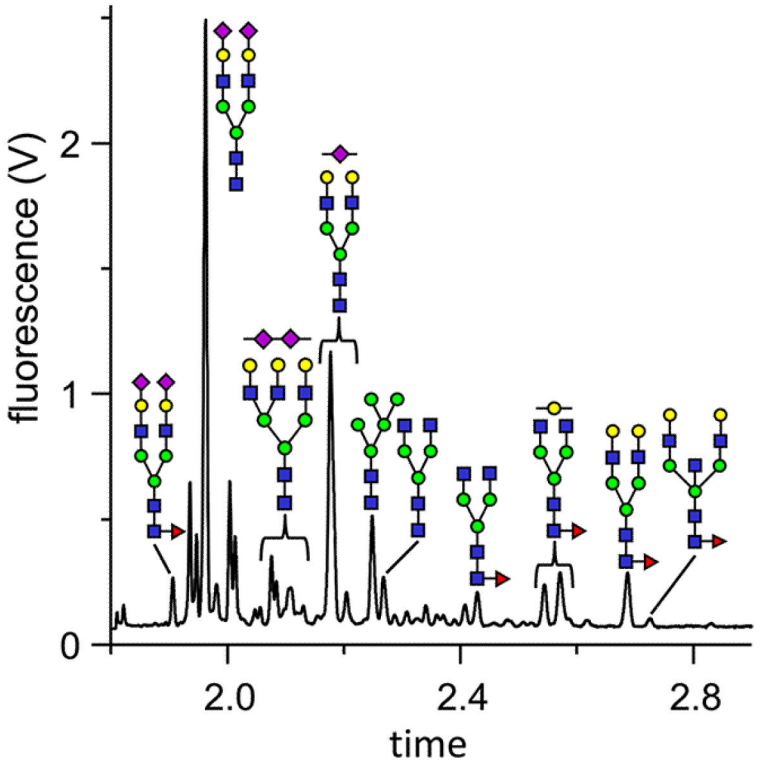


Figure 27.

Chip-based electrophoretogram of N-linked glycans from the serum of a patient with late-stage breast cancer. (Adapted with permission from ref 608. Copyright 2007 American Chemical Society.)

Table 1
Common Lectins Exploited for Glycoconjugate Investigations

lectin	specificity	ref
Aleuria aurantia lectin (AAL)	Fuc: α1-6 >> α1-2[a] > α1-3 > α1-4	617-621
Aspergillus oryzae lectin (AOL)	Fuc: α1-6 >> α1-2[a] > α1-4 > α1-3	619, 620
Conavalia ensiformis agglutinin (Con A)	tri-Man (as in the chitobiose core); high-mannose/biantennary complex> bisecting and tri-/tetraantennary complex N-glycans; α-Man >> α-Glc and select amino acid sequences[b]	132, 622-624
Datura stramonium lectin (DSL)	GlcNAc: chitotriose > chitobiose >> single GlcNAc; Galβ1-4GlcNAc	625-627
Galanthus nivalis lectin (GNL)	Manα1-3Man >> single α-linked Man	628, 629
Jacalin	Galβ1-3GalNAc	630, 631
Lens culinaris agglutinin (LCA or LCH)	α-linked Man; chitobiose core	632, 633
Lotus tetragonolobus agglutinin (LTA/LTL)	α-linked Fuc	634, 635
Lycopersicon esculentum lectin (LEL)	LacNAc, polyLacNAc	636
Maackia amurensis lectin I (MAL I/MAL)	Siaα2-3Galβ1-4GlcNAc > Galβ1-4GlcNAc; SO_4-3-Galβ1-4GlcNAc > SO_4-3-Galβ	ref 637 and refs therein[638]
Maackia amurensis lectin II (MAL II/MAH)	Siaα2-3Galβ1-3(±Siaα2-6)GalNAc; SO_4-3-Galβ1-3(±Siaα2-6)GalNAc	ref 637 and refs therein
erythroagglutinating phytohemagglutinin (E-PHA), leukoagglutinating phytohemagglutinin (L-PHA)	bisecting GlcNAcβ1-4; β1-6 branch of tri-/tetrantennary complex *N*-glycans[d]	126, 639, 640
peanut agglutinin (PNA)	Galβ1-3GalNAc; β-linked Gal; binding prevented by sialylation	641, 642, 127
Pisum sativum (pea) lectin	α-linked Man	643, 644
Sambucus nigra agglutinin (SNA)	Sia: α2-6 >> α2-3	638, 645, 646
soybean agglutinin (SBA)	α/β-linked GalNAc	642, 647
Ulex europaeus agglutinin I (UEA-I)	α-linked Fuc (conflicting linkage specificity reported); binds well to O-blood group antigen (i.e., terminal Fucα1-2)	635, 648, 649
wheat germ agglutinin (WGA)[c]	GlcNAc (sugar w/multiple GlcNAc residues >> GlcNAc monosaccharide); α-linked Sia	650-653

[a] Binding of Fucα1-2 may be severely sterically hindered by adjacent residues.

[b] See ref 615.

[c] A succinylated form is commonly available that efficiently binds GlcNAc, but does not bind Sia as well as the native lectin.[654]

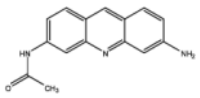
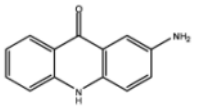
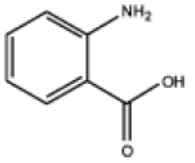
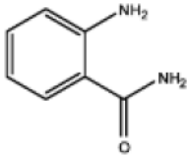
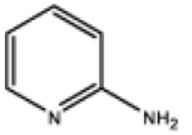
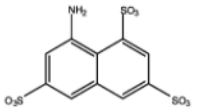
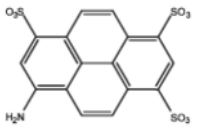
[d] Reference 126 reported E-PHA was specific for glycopeptides with bisecting GlcNAc, and L-PHA was specific for tri-/tetraantennary structures; ref 639 reported both types bound to E-PHA and L-PHA from studies of free glycans, while results from ref 640 utilizing free glycans were in close agreement with ref 126.

Table 2

Proteins Overexpressed in Multiply-fucosylated Pancreatic Cyst Fluids

		comparative proteomics			
		no enrichment		AAL-enriched	
accession	protein name (alternate name)	(G1/G2)*	*P*-value	(G1/G2)*	*P*-value
P00995	pancreatic secretory trypsin inhibitor (tumor-associated trypsin	8.4	0.0040	50.1	0.0101
P04746	pancreatic α-amylase	1.7	0.0476	22.4	0.0017
P16233	pancreatic triacylglycerol lipase	2.9	0.0001	20.2	0.0009
P07478	trypsin-2	6.3	0.0010	15.6	0.0048
P09093	chymotrypsin-like elastase family member 3A (elastase-3A)	2.5	0.0157	11.2	0.0158
P19835	bile salt-activated lipase	3.5	0.0001	9.8	0.0067
P05451	lithostathine-1-α (pancreatic stone protein)	5.9	0.0012	5.7	0.0030
P08217	chymotrypsin-like elastase family member 2A (elastase-2A)	3.0	0.0022	4.8	0.0010
Q99895	chymotrypsin-C (caldecrin)	4.0	0.0013	2.7	0.0017

Table 3
Fluorescent Tags Commonly Used in Carbohydrate Analyses

Tag	Structure	Fluorescence Excitation wavelength (nm)	Fluorescence Emission Wavelength (nm)	Mass Added to Carbohydrate (Da)
3-(Acetylamino)-6-aminoacridine (AA-Ac)		442	525	235.11
2-Aminoacridone (AMAC)		428	525	194.08
2-AminobenzoicAcid (Anthranilic Acid, 2-AA)		230/360	425	121.05
2-Aminobenzamide (2-AB)		330	420	120.07
2-AminopyridIne (2-AP)		310/320	380/400	78.06
8-Aminonaphthalene-l,3,6-trisulfonic Acid (ANTS)		353	535	366.95
8-Aminopyrene-l,3,6,trisulfonic Acid (APTS)		488	520	440.96