Structural Investigations of Glycoconjugates at High Sensitivity

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I. Introduction

A. General Considerations

The structural uniqueness of carbohydrates has fascinated many generations of scientists. Through linking carbohydrates together and with other structural entities such as peptides and lipids, Nature presents us with an enormous set of highly versatile biomolecules: from the relatively small glycolipids to highly structurally variable glycoproteins, to the giant extracellular molecules called proteoglycans. The central biological roles of glycoconjugates derive in no small part from the basic propensity of these molecules to exist as different isomeric forms and, in contrast to the other biopolymers (nucleic acids and polypeptides), to yield various branching forms. The resulting three-dimensional intricacies of glycoconjugate molecules are clearly at the heart of their unusual biological selectivities.

At present, deep structural insight to understand the rapidly advancing discoveries of new and varied functions of glycoconjugates in biological systems is often missing. It is often said that glycobiology (i.e., the science of glycoconjugates) is 2−3 decades behind the current understanding of nucleic acids and proteins. While the earlier tendencies of scientists to de-emphasize the role of carbohydrates in biological regulation and biochemical processes can largely be blamed for this situation, the chemical tasks of glycobiology have been exceedingly arduous as well. This is true for both glycoconjugate synthesis and structural analysis. However, the recent advances in oligosaccharide synthesis[1−4] and the means to intercept carbohydrate biosynthetic pathways[5] provide considerable rationale for an accelerated development of new therapeutics and vaccines based on sugar−sugar and sugar−lectin interactions. Simultaneously, the field is now being rapidly transformed through the availability of new structural tools and analytical

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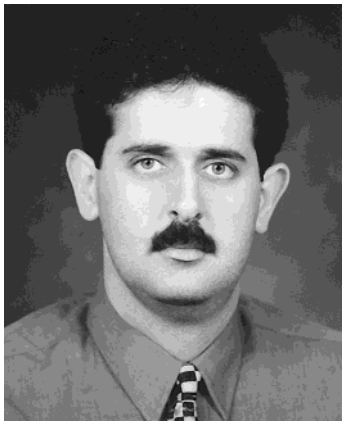


Yehia Mechref received his bachelor degree in chemistry (1991) from the American University of Beirut (Beirut, Lebanon), where he also worked as an assistant instructor for a year prior to moving to the States to pursue his Ph.D. He received his Ph.D. in analytical chemistry with honorable mention (1996) from Oklahoma State University (Stillwater, Oklahoma). In June 1996, he joined Professor Milos Novotny as a postdoctoral researcher and worked on the development of sensitive methodologies for the characterization of glycoproteins. From September 1997 through December 2000, Dr. Mechref assumed a faculty position at the department of chemistry of the United Arab Emirates University (Al-Ain, United Arab Emirates). Dr. Mechref is currently an assistant scientist at Indiana University working with Professor Novotny on the development of highly sensitive methodologies in glycobiology and proteomics.

Milos V. Novotny is Distinguished Professor and the Lilly Chemistry Alumni Chair at Indiana University, where he also directs two scientific centers: the Proteomics Research Development Facility 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 done at the Institute of Analytical Chemistry, Czechoslovak Academy of Sciences (1965–67); Karolinska Institute, Sweden (1968); and University of Houston (1969–70). Dr. Novotny accepted a faculty position in the Department of Chemistry, Indiana University, in 1971, where he has taught analytical chemistry and biochemistry, while developing an extensive research program in separation science and bioanalytical chemistry. He has trained numerous graduate students and postdoctorals for positions in academia and industry and published close to 400 scientific publications and patents. He was a pioneer in several important 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 three national ACS awards and two 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 around the development of new methodologies for proteomics and glycoconjugate analysis. He also continues his involvement in structural identification of mammalian pheromones and the biochemical aspects of chemical communication.

methodologies. While the challenges of analytical glycobiology are far greater than those encountered in genomics and proteomics, the new forms of mass spectrometry (MS), their combination with modern separation methodologies, and the increasingly more sensitive techniques of powerful Nuclear Magnetic Resonance (NMR) spectrometry are particularly conducive to future progress in structural analysis.

The multilateral importance of glycosylated structures ranges from issues as important as developmental biology, immune response, pathogens homing on their host tissues, cell division processes, cancer cells' camouflaging to escape detection by the immune system, injury and inflammation, and prion diseases, among others. Glycosylation is an abundant post-translational modification that assumes a particular degree of sophistication in mammalian systems. The relevant biochemical and biomedical literature of the past decade[6−13] underscores the future importance of glycobiology research and its expected growth. However, given the immense structural variation of glycan structures and their frequently low abundance, glycobiology will continue demanding the best out of analytical tools and methodologies.

B. Recent Evolution of Analytical Glycobiology

Today's accumulated knowledge on the structure and function of glycoconjugates already gives much credit to the analytical methodologies developed during the last 10−15 years. Some of the instrumental advances in this area are being currently shared with the burgeoning field of proteomics: the isolation and purification methodologies and MS,[14,15] in particular. It is thus likely that the current transition from the "successful beginning" of the glycobiology field toward "one of the last great frontiers of biochemistry"[6] will necessitate even greater gains in sensitivity and compound resolution than currently practiced.

Due to the enormous complexity of biochemical reactions in eukaryotic systems, in general, and the structural intricacies of glycans, in particular, it is often necessary to use a full range of various analytical methodologies to achieve complete structural assignments. The task of isolating a glycoprotein from various biological materials somewhat parallels the situations encountered with other proteins, where a variety of modern chromatographic and electrophoretic methodologies have been particularly helpful. The availability of immobilized lectins[16] and lectin chromatography has been an added bonus to the field of glycobiology; many previously studied glycoproteins could not be easily isolated without lectins. The isolated glycoproteins, however, can seldom be studied structurally in their intact forms because (a) they seldom are amenable to X-ray crystallographic studies; (b) a glycoprotein may represent a convoluted mixture of different glycoforms, and with the exception of certain simple cases, direct resolution is not easily achievable; and (c) the amounts isolated from biological materials may be too small for the more informative, albeit less sensitive, structural tools. It is thus most often necessary to cleave

the biomolecules of interest into smaller fragments (glycopeptides or glycans) for further structural determination.

Controlled cleavages of glycoproteins can often yield highly complex pools of glycopeptides or glycans. While the less complex mixtures can sometimes be investigated directly by MS, the enormous complexity of different pools has been a constant challenge to even the best available separation methodologies such as high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE). However, the individually separated oligosaccharides provide the best opportunity for subsequent sequencing and determination of linkage forms. Peptide mapping procedures, when combined with MS, can also provide the best means for securing the structural information on a glycan substitution within the polypeptide chain. "Hyphenation" of modern separation techniques with MS, i.e., the on-line, direct coupling of such methodologies, is now becoming fundamental to proteomics and glycobiology alike.

During the last 2 decades, chromatographic separation of complex carbohydrate mixtures has undergone a remarkable development. The earlier strategies involved the use of gas chromatography (GC) and GC/MS[17] for quantification of monosaccharides and linkage determinations. To increase the volatility and hydrophobicity of sugar molecules, permethylation was utilized in the gas-phase analyses. While permethylation is still practiced to this date to enhance certain analytical objectives, the low volatility of larger oligosaccharides made the GC approach somewhat unattractive. A search for optimum separation media in the liquid-phase separations of glycans has been evident for a number of years. Additionally, carbohydrates in their native state yield poor detection in chromatography, because of the lack of chromophores in their molecules. A derivatization (typically occurring at the reducing end of oligosaccharide chains) can introduce a chromophore or a fluorophore for the sake of detection and simultaneously enhance the solutes' hydrophobicity for an appropriate separation. If polar interactions between native carbohydrates and certain chromatographic columns are utilized in separation, MS becomes a choice for detection.

A significant breakthrough in carbohydrate chromatographic analysis was achieved through combining anion-exchange chromatography of glycans with pulsed-amperometric detection (PAD).[18−20] Under highly alkaline pH conditions, carbohydrates become charged, interactive with the ion-exchange resins, and detectable electrochemically. Following a series of incremental improvements, this form of chromatography was commercialized, becoming a standard tool of glycobiology. While this combination allows fairly effective separations of unmodified carbohydrates and quantification in the picomole range, further structural investigations of the separated glycans are complicated by their recovery from high-salt media.

High-performance capillary electrophoresis (HPCE) now represents a set of powerful electromigration techniques whose impact has been felt in virtually all areas of biochemical analysis, including carbohydrate separations.[21−24] While the first applications of HPCE to sugar analysis were described[25−27] more than a decade ago, high interest in HPCE methodologies remains to this date. Unprecedented separations of highly complex oligosaccharide mixtures were demonstrated,[28,29] as was the resolution of sugar optical isomers[30] and extremely sensitive detection of fluorescently labeled glycans from single biological cells[31] through laser-induced fluorescence (LIF) detection. To comply with the sensitivity requirements, labeling with a fluorophoric group is often the requirement for HPCE carbohydrate applications. Coupling HPCE with MS is currently tedious, albeit feasible, because of the buffer composition issues. However, some recent advances in capillary electrochromatography (CEC) are promising in combining highly effective separation with the use of " MS-friendly" mobile phases.[32,33]

The enormous advances in MS technologies and instrumentation made during the past decade are of paramount importance to nearly all structural aspects of glycobiology. Undoubtedly, the most important innovations came through the introduction of electrospray ionization (ESI) and matrix-assisted laser desorption−ionization (MALDI) sources that gradually displaced the previously dominant fast atom bombardment (FAB) techniques in the area. Accurate mass values obtained with new types of mass analyzers and the capabilities of tandem mass spectrometry (MS/MS or MS^n) provide unprecedented capabilities for sequencing and fine structural assignments at very high sensitivity.[34,35] The coincidental availability of exoglycosidase enzymes as reagents, in conjunction with MS, now permits sequencing and linkage form determinations from small quantities of analyzed glycans.[36]

NMR spectrometry has been among the standard tools in carbohydrate structural work for a long time.[37] It has the unmatched capability of unambiguous structural assignment in cases where MS may not be effective: chemical shifts, integrated areas of signal peaks, signal multiplicity, and line-widths are all very sensitive to structural changes and spatial arrangements within the oligosaccharide antennas.

The gains in measurement sensitivity achieved with carbohydrate molecules during the past decade have been highly significant. Earlier, it was quite ordinary to require gram to milligram quantities of a glycoprotein for analysis, and nearly heroic effort was often extended toward isolating such quantities from biological materials. While the new, more sensitive methodologies of capillary separations and MS had to be first validated on the previously explored "standard" glycoproteins, the impact of new methodologies on glycobiology has already been felt during the last several years. Routine determinations at the femtomole levels now appear to be a reasonable goal.

This review summarizes recent methodological advances in the analysis of glycoproteins and their glycan structures. Glycolipids and proteoglycans, while sharing some methodological aspects with

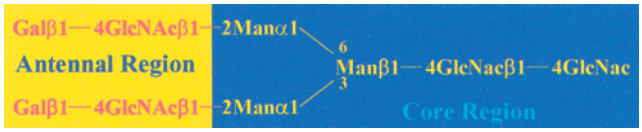


Figure 1. Structure of a typical biantennary *N*-glycan.

glycoproteins, are beyond the scope of our discussion. The glycoprotein structural analysis has clearly become a multimethodological task in which MS and separation methodologies are particularly eminent. We wish to emphasize the overall, integrated analytical strategies toward the goal of a complete structural analysis at high sensitivity.

II. Basic Structures and Nomenclature for Glycoprotein Glycans

Proteins conjugate with various sugar chains through selected amino acid residues: through asparagine (N-linked glycans), serine, or threonine (O-linked glycans) and, very seldom, other residues. Among notable exceptions are the linkages through hydroxylysine, which are confined to collagens,[6] or glycation of proteins through lysine residues, which are observable in diabetes.[38] N-Linked glycans contain *N*-acetylglucosamine (GlcNAc) linked via an amide bond to asparagine residues of a protein. For *O*-glycans, *N*-acetylgalactosamine (GalNAc) is ubiquitous, linking an oligosaccharide's reducing end to the hydroxyl groups of either serine or threonine residue within a protein. Other monosaccharide units have also been described to be involved in an O-glycosidic linkage to hydroxy amino acids; most are rare, but some, such as the *O*-GlcNAc in cytosolic and nuclear proteins,[39] are quite ubiquitous. There are fucose residues linked to serine in the epidermal growth factor domains[40] and Chinese hamster ovary cells[41] or the mannose linked to serine in sheep dystroglycan[42] and brain glycoproteins.[43] Proteins in *Saccharomyces cerevisiae*[44] and *Pichia pastoris*[45] yeast cells also contain O-linked mannose. Other cases include xylose−serine O-linked structures in proteoglycans,[46] galactose−hydroxylysine in collagens,[47] and arabinose−hydroxyproline in the hydroxyproline-rich cellular walls of plants.[48]

The variations in sequence and linkage between the individual monomers that constitute a given oligosaccharide chain, as well as the general tendency of both the N-linked and O-linked structures to branch (i.e., to form antennas), are the structural features that our analytical tools must recognize, as does Nature in the relevant biological processes. An occasional addition of modified residues through sulfation or other modifications, as perhaps best exemplified by the heparin-like attachments with the mind-boggling variability of substitution,[46] can further complicate the overall analytical task.

The complexity of N-linked oligosaccharide structures is reduced by the existence of their core region but enhanced significantly through the variations in the antennal region (Figure 1). *N*-Glycans are composed of the common trimannosyl chitobiose core (consensus sequence) with two or more antennas extending from it.

They can be classified into three subgroups on the basis of nature and location of sugar residues added to the core. In building the final type of glycan structures that meet their biological destinations, the cellular processes utilize both α- and *β*-linkages for different sugar residues: GlcNAc, GalNAc, galactose (Gal), glucose (Glc), mannose (Man), fucose (Fuc), and *N*-acetylneuraminic acid (NeuAc). The glycans that contain only *N*-acetyllactosamine (Gal*β*1−3/4 GlcNAc) in their antennal region are classified as "complex type" (Figure 2a), while those containing just mannose residues attached to the core structure are called "high-mannose type" (Figure 2b). The third class of *N*-glycans (Figure 2c), the so-called "hybrid type", contains both mannose residues and *N*-acetyllactosamine attached to the trimannosyl chitobiose core. Moreover, the presence or absence of an α-fucosyl residue and a GlcNAc residue attached in C-4 position of the core mannose, known as "bisecting GlcNAc", further adds to the structural variability of oligosaccharides in each subgroup. "Decorating residues", such as Fuc or NeuAc added in the final biosynthetic stages, are most often α-linked. L-fucose is the exceptional residue configuration, as all other monosaccharides occur in their D-forms. In some nonmammalian eukaryotic systems, D-xylose (Xyl) appears with some frequency.

Unlike N-linked oligosaccharides, *O*-glycans do not share a common core structure. Instead, they comprise a number of different regions with some common motifs. The six commonly occurring structural types are illustrated in Figure 3. The structural diversity introduced through elongation of these basic structures with residues such as Gal, GlcNAc, Fuc, and NeuAc can lead to a large number of structural variants. This complexity is further augmented by the presence of different substitution sites and anomeric sugars. For example, for a hexasaccharide structure, a theoretical number of over 1.05×10^{12} possible *O*-glycan structures can be calculated, if we consider that each monosaccharide residue has six substitution sites and assuming that five α-anomeric monosaccharides could be linked to these positions.[49]

The branched structures in both *N*- and *O*-glycans, with variations in the linkage forms, are among the most spacially complex biomolecular entities imaginable. Their oligosaccharide chains are often terminated by sialic acid residues. Although *N*-acetylneuraminic acid (NeuAc) is largely viewed as the key acidic residue, there are more than 30 sialic acid structural types that could have additional significance in recognition processes and different biological systems.

The extent of glycosylation varies with the type of modified protein, such as its size and abundance of asparagine, serine, and threonine residues within the polypeptide backbone. Additionally, it will depend on how close the glycosylation sites are to each other. Any potential N- or O-glycosylation site in a glycoprotein may or may not be occupied in each molecule. Moreover, an entire range of glycans can exist at a particular glycosylation site. As evidenced by the modern analytical techniques, any glycoprotein must be viewed as a mixture of molecules with a tightly

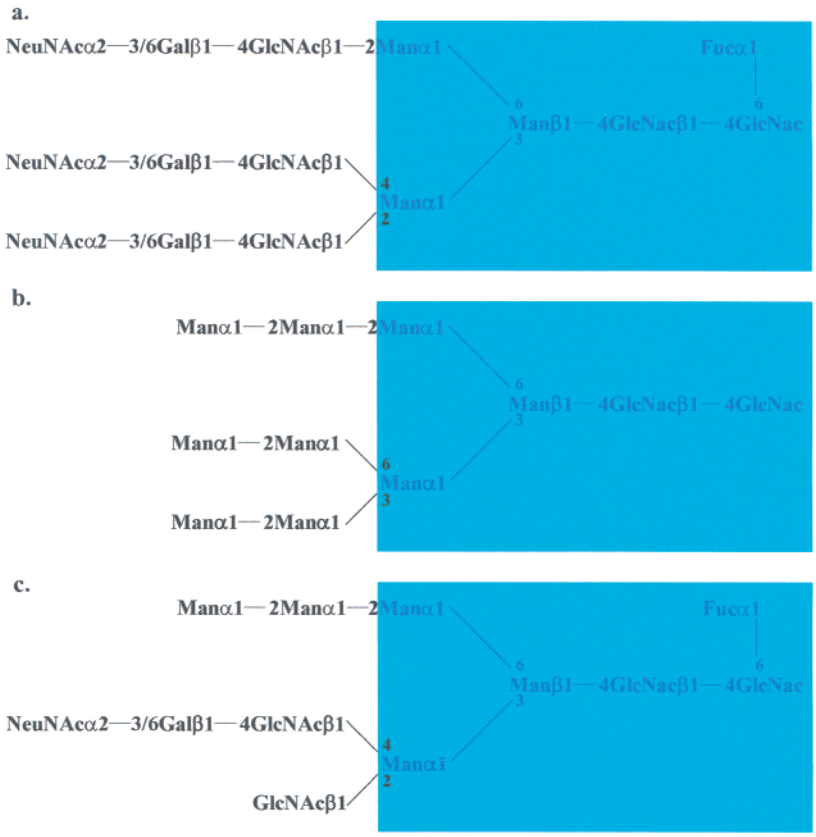


Figure 2. Representative structures of (a) complex type, (b) high-mannose type, and (c) hybrid-type *N*-glycans.

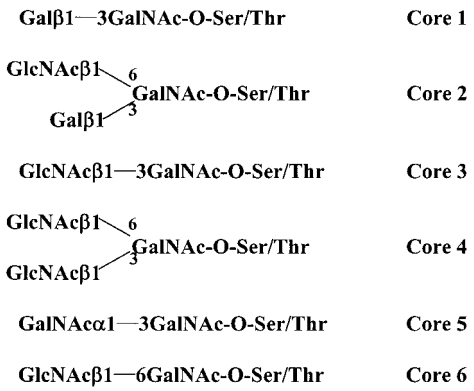


Figure 3. Representatives of the typical core structures of *O*-glycans.

controlled number of amino acid residues, but a fairly variable substitution of glycans. These glycosylated protein variants are generally known as "glycoforms".

Among the crucial methodologies in glycoprotein analysis have traditionally been various approaches to cleave glycans from the polypeptide backbone through both enzymatic and chemical procedures. In fact, the success of particular cleavage techniques with very small quantities of an isolated glycoprotein has been central to many new investigations. The recent years' availability of *N*-glycanases,[50] the enzymes providing a facile cleavage of asparagine-linked oligosaccharides, has revolutionized the analysis of *N*-glycans as compared to *O*-glycans, which still need to be released from their corresponding glycoproteins chemically.[51−54] Small-scale digestion with proteases is also essential to yield the mixtures of peptides that can later be studied to reconstruct the information concerning the sites of glycosylation and the abundance of glycoforms at a particular substitution site.

The most pertinent structural intricacies of isolated glycoproteins that are currently revealed by modern analytical methods involve (a) a display of all glycosylated structures into an "oligosaccharide map", (b) carbohydrate sequence, (c) branching and sugar linkage types, and (d) information about the sites of glycosylation and the presence of different oligosaccharides at each substitution site. This type of information will be essential to future considerations of structure−function relationships and three-dimensional modeling of biomolecular events. The goals of analytical glycobiology are somewhat parallel to the current efforts of proteomics field, where the most advanced analytical methodologies are being brought to the tasks of mapping cellular processes and explaining the function of proteins from analytical data.

III. Biological Roles of Glycosylated Structures

Glycosylation is a very widespread posttranslational modification of proteins in eukaryotic systems. Beginning with the relatively simple glycosylation in yeast, the glycosylated proteins are becoming increasingly more sophisticated structures in higher organisms through addition of the enzyme-catalyzed biosynthetic pathways, as clearly evidenced in vertebrates. The known gene products participating in the oligosaccharide biosynthesis in vertebrates are estimated to be up to 1% of the translated genome,[55] representing a major commitment of these systems

to glycosylation. Phosphorylation is the only other known posttranslational modification of a similar magnitude. The process of assembling and trimming various oligosaccharide structures in the endoplasmic reticulum (ER) and the Golgi apparatus (GA) of eukaryotic cells is quite complex. Through assistance of the membrane-bound glycosyltransferases and glycosidases in ER and GA, various specialized cells can produce glycoproteins of highly targeted structures. This elaborate and energetically costly system of adding enzymatically different sugars and trimming the structures to the "right dimensions" appears vital to cellular specialization: sites of substitution and sugar population types within a glycoprotein are now known to distinguish various cell types. For example, normal and pathological cells often feature different glycosylation patterns, while cancerous cells use abnormal glycosylation to evade detection by the immune system.[56,57]

Formation of the elaborate hybrid molecules between polypeptides and glycans has its functional justifications. Although there are seemingly endless variations in which the individual amino acids can be arranged in a polypeptide chain, the added glycan moieties provide even greater scope in dimensionality through their isomerism and branching, i.e., the propensity that peptides themselves lack. Not only can a glycan chain reach further away from the surface of a globular protein for a specific interaction,[58] but quite importantly, many vital proteins would not be able to fold properly [59] without the glycans attached to the backbone at their strategic residues. Glycosylation pathways can thus be quite specific for different tissues and cells, allowing very precise tuning of their proteins' functions that neither variations in the amino acid sequence nor the more simple protein modification could easily accomplish. Thus, glycans with their highly tuned structures and sites of glycosylation are widely viewed as the "zip codes" of a complex biological system. A loss of this fine-tuning capability can result in chemically measurable attributes of disease states.[12]

A repertoire of structures, commonly referred to as "microheterogeneity," is often associated with a particular modification site. It is not currently clear as to what biological significance is associated with different quantitative proportions of glycans at a particular substitution. Both membrane-bound and secretory glycoproteins feature a great abundance of both N-linked and O-linked structures that serve for intercellular as well as intermolecular interactions. Understanding cell−cell and cell−matrix recognition events at the molecular level is important in the multicellular contex, thus becoming a central issue of modern medicine.

From all glycosylated structures, *N*-glycans and their numerous functions have been most extensively studied. N-Glycosylation has been highly conserved in evolution of all eukaryotic systems. Distinct genes for the glycosidases and glycosyltransferases that are responsible for the assembly and modification of *N*-glycans in ER and Golgi have now been identified.[11] Since the pioneering work of Kornfeld and Kornfeld[60] explaining the core structure of *N*-glycans, the availability of new model systems (ranging from the yeast mutants to transgenic mice) has helped in elucidating many relations between N-glycosylation and important cellular functions, such as morphogenesis, proliferation, cellular differentiation, and programmed cell death. N-Glycosylation is widespread among nearly all surface and secreted proteins. As recently reviewed by Parodi,[61] substantial progress has been made in understanding of how *N*-glycans are assembled on the surface of a new glycoprotein and folded into their functional forms inside ER and GA. Likewise, it is becoming increasingly known how a misfolded glycoprotein is being recognized and recycled by the complex cellular machinery. For the additional class of proteins linked to membranes through glycosylphosphatidylinositol (GPI) anchor, the key steps in biosynthesis have also been elucidated.[62]

While the core structures (consensus sequence) of *N*-glycans are well preserved in all different eukaryotic organisms, the astonishing propensity of the glycans toward branching and formation of multiantennary structures is a clear attribute of the metabolic responsiveness of the more advanced cellular types, created presumably through the necessities of intercellular communication in multicellular systems, evolution of the immune system, and a response to parasites. The occurrence of *N*-acetylglucosaminyl transferases, the enzymes responsible for branching,[63,64] is now viewed as an important developmental marker. The relative ease with which the N-glycosylated structures could be studied during the recent years (compared to the O-linked structures, as discussed below), has undoubtedly contributed to discovery of the amazing array of biological activities and attributes of these oligosaccharides in cell development and disease states. The pertinence of complex *N*-glycans to certain functions of the immune system (for a review, see ref 13), protein misfolding in neurodegenerative diseases,[65,66] susceptibility to infection,[67] and their differential expression in tumor cells[11] is the major reason for the continuous interest of the pharmaceutical community. Analytically, N-linked oligosaccharides represent a more easily approachable class of compounds than O-linked structures in glycoproteins or proteoglycans.

Compared to the abundant studies on *N*-glycans, considerably less has been known about the O-linked structures and their biological roles. The studies in this area have been complicated by (a) the apparent lack of consenses sequence for O-glycosylation, (b) the frequency at which many serine and threonine resides are located together in patches, and (c) the lack of the enzymes that could be used to remove such glycans from their respective glycoproteins.

The simplest types of *O*-glycans, containing just one sugar residue such as the ubiquitous *N*-acetylglucosamine (*O*-GlcNAc)[39,68] are very abundant in eukaryotic cells. Most O-linked structures, however, involve GalNAc in the linkage to protein. Their biosynthesis is known to be initiated in the Golgi through the addition of GalNAc to serine and/or threonine residues. Although biosynthetic details of further structural attachments remain largely un-

explored, at least six groups of attachments altering GalNAc, GlcNAc, and Gal in different linkage modes are currently known.

Although most *O*-glycan structures are largely viewed as short chains that are located in a close proximity to each other within the polypeptide entities (the so-called "mucin-type" glycans), larger oligomers are probably quite abundant among the common secreted glycoproteins. The glycoproteins featuring a large surface area covered by carbohydrate moieties (sometimes more than 50% of their total weight) can play significant roles in protecting various epithelial cells and mediating cell adhesion in specialized tissues. Patches of small glycosylated structures are often found in membrane-bound glycoproteins. Alternatively, the rigid rods of glycosylated domains can serve important functions in recognition processes. The overwhelming importance of O-linked structures has been featured in masking surface recognition molecules in cancer,[8] in cystic fibrosis,[69] for immunomodulary effects,[70] etc.

Both N-linked and O-linked glycan structures can also be divided into neutral oligosaccharides and acidic oligosaccharides, depending on whether they feature terminal sialic acid residues. Less commonly, the acidic character is imparted to an oligosaccharide through sulfation of selected residues. The functional importance of terminal sialic residues has widely been appreciated in the experiments demonstrating a partial or a complete loss of biological activity after treatment of a glycoprotein with sialidase enzymes. For example, the correct sialylation of the N-linked branched structures seems essential to the activities of tissue plasminogen activator and erythropoietin,[71] both glycoproteins with pharmaceutical use. Another type of acidic structural entity receiving considerable attention is sialyl Lewis X oligosaccharides,[72] which are involved in leukocyte homing to the sites of inflammation as a part of selectin molecules.[73] Cellular adhesion phenomena, mediated largely through the presence of sialic acid residues, are among the most intriguing aspects of modern biology. Sialic acids are extremely versatile molecules. Besides their termination of the oligosaccharide chains, they can also be present with a large degree of oligomerization in an attachment to the ordinary N-linked or O-linked structures.[74] Such structures, know as polysialic acids, have been shown to be important to oncology and neurochemistry.[74] Additionally, as pointed out by Hart,[6] the structural diversity of sialic acids (and their potential functions) may currently be underestimated: while more than 30 different sialic acids may be encountered in Nature, only *N*-acetylneuraminic and *N*-glycolylneuraminic acids have been investigated extensively. Yet another important variation may reside with the linkages of the sialic acid residues that are specially accessible and thus available for various recognition and binding phenomena. For example, the difference of $\alpha(2\rightarrow3)$ vs $\alpha(2\rightarrow6)$ sialyl−galactosyl linkages in cell−cell interactions has been demonstrated.[75−77]

Whereas glycoproteins are traditionally thought as being confined to lumenal and extracellular media, simple glycosylated structures are also widely found in cytoplasm and nucleoplasm.[6] The most simple O-linked structure, *O*-GlcNAc, is now being investigated for its role in signal transduction and a possible involvement in disease states.[68]

IV. Isolation and Direct Analysis of Intact Glycoproteins

A. General Aspects

Glycosylation occurs with proteins small and large. With an increase of molecular size, the occurrence of multiple glycosylation sites typically increases, adding to the complexity of glycosylation patterns and the usual difficulty of resolving fine structural differences in a large biopolymer. The successful attempts to resolve partially or completely different glycoforms of native glycoproteins are thus generally confined to relatively small proteins or glycopeptides. Following the first successful results on transferrin by Kilar and Hjerten[78] and different recombinant variants of human recombinant tissue plasminogen activator (rtPA) by Yim[79] through HPCE, similar approaches have often been attempted by others. MS techniques have also been applied for the same purpose.

In biological materials such as cellular extracts and physiological fluids, glycoproteins are often encountered in minute quantities, placing high demands on both the measurement sensitivity and proper isolation procedures. A combination of orthogonal separation techniques and the use of affinity principles are the most commonly practiced isolation/fractionation strategies. Miniaturization of these separation methodologies represents a general trend in bioanalysis[23] and is thus applicable to all stages of modern glycoanalysis.

B. Isolation Methodologies

Glycoproteins of interest may be encountered as either soluble or membrane-bound molecules. The isolation strategies will vary, depending on whether such glycoproteins occur in cytosolic space, nucleus, extracellular space, cellular, or subcellular membranes, etc. Also, any isolation protocol has to be adjusted to take into account the physicochemical properties of a given glycoprotein or a glycoprotein class. For example, detergents must be included in the extraction buffer to yield membrane-bound glycoproteins. For the extraction and fractionation of soluble glycoproteins, detergents are usually not needed. However, the addition of certain detergents can occasionally increase extraction yields and reduce contamination during purification.

The methods used in any glycoprotein isolation protocol are very dependent on whether a biological activity must be retained for further studies. Many glycoproteins, including a number of membrane-bound molecules, can be easily denatured on contact with the surface of glassware or chromatographic packing. If the sample is to be subjected to a protease cleavage or a release of glycans, isolation or purification under harsher conditions is unlikely to affect adversely the final analysis. Generally, glycoproteins

can be purified by most conventional protein separation methodologies, including gel electrophoresis and various forms of HPLC (ion-exchange, size exclusion, reversed phase using C_{18}, C_8, or C_4 columns, hydrophobic interaction, and affinity). A most useful, specific isolation principle is the use of lectins that are immobilized on chromatographic resins (see below). Selected procedures for the use of liquid chromatography in glycoprotein analysis have recently been summarized.[80,81]

Electrophoretic separations in gels are now widely applied to a variety of problems in the isolation and analysis of proteins. Simplicity of the apparatus and robustness are the chief selling points of both sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) and two-dimensional electrophoresis (2-DE) in gels, which combine the separation mechanisms based on molecular size and isoelectric points. Both techniques have found their wide application in glycoprotein isolation/analysis. Due to the recently advanced sensitivity of MS measurements, it has become feasible to analyze the contents of individual spots on a gel.

SDS−PAGE has frequently adequate resolving power to migrate proteins according to their size and to provide a rough estimate of their molecular weights. It is particularly informative to compare the "profiles" of proteins in a complex sample with those processed through a lectin chromatographic column, indicating which components of a mixture are glycosylated. Typical gel/buffer systems used in this procedure are essentially those described in the pioneering work by Laemmli.[82]

When applying SDS−PAGE to the separation of glycoproteins, one must be aware of certain pitfalls. A positive bias in the estimation of molecular weights can be observed due to a lower SDS binding in the presence of carbohydrate structures. Conversely, negative deviations are experienced due to the presence of charged sialic acids, which contribute to the overall electrophoretic mobilities of glycoproteins. The glycoprotein bands observed on gels are often broad due to the tendency to resolve microheterogeneities.

In 2-DE separations, glycoproteins tend to be translocated into "trains" of spots, reflecting their differences in both the molecular mass and isoelectric points. In various proteomics applications, the in-gel digestion of the isolated spots and a subsequent analysis by MALDI/MS or ESI/MS have become common in providing peptide fingerprints that can be matched with database entries. Although this appears relatively straightforward, one shortcoming with glycoproteins is an inadequate separation of glycovariants. Thus far, only a few publications appeared on the MS analysis of the glycoproteins separated through 2-DE.[83−87]

In the ultrasensitive measurements of glycobiology, extreme caution must be exercised in manipulating the minute quantities of biomolecules. Glycoproteins at the low-microgram scale, while becoming measurable with modern instrumental techniques, can easily be adsorbed on the surface of glassware before such measurements. Sample loss during ultrafiltration, dialysis, lyophilization, etc. can easily become a bottleneck of the entire analysis. Another problem with working at such a reduced scale is contamination (dust, solvent, reagent impurities, etc.). It is thus crucial to minimize the number of handling and transfer steps during the analysis. Miniaturized forms of separation, in terms of reduced column diameters, solvent flow-rates, and the overall surface area that a glycoprotein sample may encounter during analysis, are becoming significant in high-sensitivity work.

C. Lectin Affinity Chromatography

For a number of years, lectins isolated from plants and animal sources have been employed to study their interactions with glycoconjugates.[88,89] As recognition for their unique ability to bind sugars and to agglutinate cells, these proteins are now used to identify and separate cells; to assay for activities of glycosyltransferases; and to isolate, purify, and characterize glycoproteins and glycolipids in affinity chromatography.[88] Lectin affinity chromatography has now been developed into a powerful technique for purification of glycoproteins.[90] The lectins with high specificity toward oligosaccharides have been immobilized to agarose and other separation matrixes. Some of these materials have been commercially available to isolate glycoproteins on the basis of their different glycan structures.[88,89,91,92] Due to the method's high specificity, it is often advantageous to use lectin affinity chromatography in the early stages of a glycoprotein isolation. While glycoproteins can be separated on the basis of their different glycan moieties[88,89,91,92] through lectin specificity, most cases of lectin affinity chromatography use either wheat germ agglutinin or concanavalin A, both lectins with a broad specificity,[93,94] to isolate the entire pools of glycoproteins rather than specific structural types. No systematic studies currently exist to address whether some glycoproteins are weakly retained by these materials and thus escape detection. It is assumed that different glycoproteins produced by a certain cell type produce similar types of glycans, and typically observed microheterogeneities display slight differences in structure. Because of the gel nature of typical commercial packings, the most popular technique of lectin affinity chromatography involves preparation of a small column containing 1−5 mL of the packing. Employing a gravity-flow mode, glycoproteins are displaced from a column with haptene saccharides.[88]

Different types of lectins can still be explored to optimize the yields of retained glycoproteins. Until recently, the widespread use of lectin chromatography has been hindered by the absence of a simple, rapid, and cost-effective procedure for screening different types of lectins. Wiener and van Hoek[95] devised a method that allows a rapid screening of more than 20 commercially available fluorescent lectins using less than 200 μg of glycoproteins.

To address the effective isolation of trace glycoproteins from biological samples, future efforts will undoubtedly be directed toward miniaturization of lectin-based procedures. Bundy and Fenselau[96] re-

cently demonstrated the use of lectin-derivatized surfaces to capture and concentrate glycoconjugates from bacteria and enveloped viruses prior to a direct analysis by MALDI/MS. In their approach, a lectin is first immobilized to a membrane surface via a primary amine group. Subsequently, the samples containing microorganisms are permitted to equilibrate with the membrane. Next, a series of washes are performed to remove weakly bound materials. Finally, acidic solution is added to facilitate a release of the proteins to be analyzed. This procedure could be expanded to a submicroscale isolation of glycoproteins from different biological samples. Recently, the potential of this approach was demonstrated in determining the oligosaccharides that bind preferentially to cortical granule lectin.[97] The use of this bioaffinity probe in conjunction with collision-induced dissociation MS revealed that oligosaccharides with sulfate esters at the nonreducing ends preferentially bind to this lectin.

D. Glycoform Separations by Capillary Electrophoresis

Due to the high resolving power of HPCE, numerous attempts have been made to use capillary electromigration techniques for both the qualitative and quantitative assessment of glycoprotein heterogeneities. While the capability of this methodology to work at microscale is generally appealing for most biochemical investigations, the speed of analysis achievable with HPCE and its quantitative capability are particularly interesting for an ever-increasing number of applications in biotechnology and pharmaceutical industry. Several recent reviews[22,98,98−103] have dealt with this area.

Kilar and Hjertén[78] were first to report on the effectiveness of capillary zone electrophoresis (CZE) and capillary isoelectric focusing (CIEF) in glycoform analysis with the example of transferrin. Since then, the number of reports on other glycoproteins, using similar approaches, has increased remarkably. Table 1 lists various applications in the area, summarizing the types of glycoproteins along with the employed mode of HPCE. To optimize resolution of glycoforms, operational parameters can be changed, including the mode of separation (e.g., CZE vs CIEF), buffer type, pH of the background electrolyte, and buffer additives, such as organic cations, detergents, and water-soluble polymers. While the earlier reports emphasized the methodological aspects and optimization toward the maximum resolution of glycoforms, the most recent reports are clearly more application-oriented (efficacy in clinical diagnosis, quality control, monitoring of glycoproteins in formulations or body fluids, etc.).

Partial or a nearly complete resolution of glycoforms through HPCE has now been demonstrated with a variety of natural glycoproteins: ribonuclease B,[104−108] bovine fetuin,[109] ovalbumin,[108,110−112] α_1-acid glycoprotein,[109,110,113] horseradish peroxidase,[107,108] pepsin,[111] human chorionic gonadotrophin,[114] and hirudin.[115] In addition, several recombinant glycoproteins of medical importance, including the recombinant human blood coagulation factor VII,[116] soluble T4 receptor protein,[117] human bone morphogenic protein-2,[118] human granulocyte-macrophage colony stimulating factor,[119] and both natural and recombinant interleukin-2,[120] were also analyzed by CE. While some of these biomolecules were used as "model glycoproteins" to demonstrate the technique's effectiveness and establish the critical separation parameters, it is apparently feasible to study unknown glycoproteins so long as their complexity is not extraordinarily high. Most of these studies paid a due attention to the needs of using a correct modification of the capillary wall and buffer composition toward optimum resolution. Apparently, some good separations were achieved even with unmodified capillaries, but most cases required additives to be included to the background electrolytes in minimizing or eliminating solute adsorption in the system. Such additives include Triton X-100,[79] ϵ-aminocaproic acid,[79] diaminopropane,[114] diaminobutane,[116] or ω-amino acids.[121] Alternatives to the use of additives are to either conduct a separation under very acidic conditions, eliminating the negatively charged groups on the capillary wall,[117,120] or use covalently modified capillaries.[108,118,120,122]

Transferrin, a marker for glycosylation abnormalities associated with a number of pathological conditions, was initially analyzed by CZE and CIEF using an untreated glass capillary.[78] More recently, Landers and co-workers[123] devised a simple method utilizing a covalently modified fused silica capillary and a sieving buffer containing hydroxyethylcellulose, allowing the resolution of the lower sialoforms in less than 8 min. This method demonstrated the applicability of CE for a facile diagnosis of the carbohydrate-deficiency glycoprotein syndrome.

As seen in Table 1, HPCE is increasingly being used for monitoring recombinant glycoproteins for clinical use. Although different CE modes can be utilized for this type of analysis, including CZE, CIEF, capillary gel electrophoresis (CGE), and micellar electrokinetic chromatography (MEKC), "straight" CZE has been most commonly employed in the routine analysis of the glycoform populations of intact glycoproteins because of its simplicity and speed. CZE has been increasingly employed to monitor the bioproduction of pharmaceutical glycoproteins at different stages of the overall process, such as the cultivation step, downstream processes, and characterization.

The applications of HPCE reported to date on recombinant glycoproteins are not confined to just resolving glycoforms. Some reports also deal with separating glycoproteins from the excipients or process impurities. A good example of this situation is erythropoietin (EPO), a key protein that is primarily produced by the kidney to regulate production of red blood cells.[124] Recombinant human EPO products, which are commercially available and clinically used, have been separated into distinct glycoforms.[109,125−127] Moreover, Bietlot and Girard[128] developed a CZE method for the analysis of recombinant human EPO in the final drug preparation that differs from the other reported methods. Since the final products contain certain amounts of human serum albumin as the excipient, nickel chloride was added to the

Table 1: Glycoproteins Separated by HPCE

glycoprotein	HPCE mode[a]	capillary type	ref
human transferrin	CZE	glass pretreated with polyacrylamide	78
	CZE	fused silica	123
	CZE	fused silica coated with a covalently bonded polymer	123
	CIEF	glass pretreated with polyacrylamide	78
ribonuclease A and B	CZE	fused silica	104−106
	CZE	fused silica coated with a covalently bonded polymer	108
	MEKC	fused silica	107
pepsin	CZE	fused silica	111
ovalbumin	CZE	fused silica	110−112
	CZE	fused silica coated with a covalently bonded polymer	108
bovine fetuin	CZE	fused silica coated with a covalently bonded polymer	109
α_1-acid glycoprotein	CZE	fused silica coated with a covalently bonded polymer	109
	CZE	fused silica	110
	CZE	fused silica coated with a covalently bonded polymer	113
horseradish peroxidase	MEKC	fused silica	107
	CZE	fused silica coated with a covalently bonded polymer	108
hirudin	CZE	fused silica	115
glycoprotein factor associated with cancer cachexia	CZE	fused silica	139
human chorionic gonadotropin (hCG)	CZE	fused silica	114
recombinant soluble T4 receptor protein (rCD4)	CZE	fused silica coated with a covalently bonded polymer	117
recombinant tissue plasminogen activator (rtPA)	CZE	fused silica coated with a covalently bonded polymer	79, 125
	CZE	fused silica	133
	CZE	fused silica coated with a covalently bonded hydrophilic polymer	121
	CIEF	fused silica coated with a covalently bonded polymer	79
	CIEF	fused silica coated with a covalently bonded polymer, in the presence of hydroxypropylmethylcellulose	134−136
	CIEF	fused silica coated dynamically with either poly(ethylene glycol) or hydroxypropylmethylcellulose	137
	CGE	fused silica coated with a covalently bonded hydrophilic polymer	121
recombinant human erythropoietin (rhEPO)	CZE	fused silica	125, 126
	CZE	fused silica coated with a covalently bonded polymer	128
	CZE	fused silica	127
	CZE	fused silica coated with a covalently bonded polymer	109
	CIEF	fused silica coated dynamically with either poly(ethylene glycol) or hydroxypropyl methylcellulose	137
recombinant human granulocyte-macrophage colony stimulating factor (rhGM-CSF)	CZE	fused silica	119
recombinant human blood coagulation factor VII (rFVIIa)	CZE	fused silica	116
recombinant basic chimeric glycoprotein (FG)	CZE	fused silica dynamically coated with an amphipathic polymer	122
recombinant human bone morphogenic protein-2 (rhBMP-2)	CZE	fused silica coated with a covalently bonded polymer	118
natural interleukin-2 (nIL-2)	CZE	fused silica coated with a covalently bonded polymer	120
recombinant interleukin-2 (rIL-2)	CZE	fused silica coated with a covalently bonded polymer	120
Desmodus salivary plasminogen activator (DSPAα1)	CZE	fused silica	129
recombinant proteinase A	CZE	fused silica	130
recombinant antithrombin III (rATIII)	CZE	fused silica dynamically coated with hydroxypropyl-methylcellulose	131
	CIEF	fused silica coated with a covalently bonded polymer	131
	CGE	fused silica coated with a covalently bonded polymer	131
recombinant humanized monoclonal antibody HER2 (rhuMAbHER2)	CIEF	fused silica coated with a covalently bonded polymer	132
	CGE	fused silica coated with a covalently bonded polymer	132
recombinant human interferon-γ (rIFN-γ)	MEKC	fused silica	107

[a] Abbreviations: CZE, capillary zone electrophoresis; cIEF, capillary isoelectric focusing; CGE, capillary gel electrophoresis; MEKC, micellar electrokinetic chromatography.

background electrolyte to permit a complete resolution of the two proteins, now including a separation of the different glycoforms.

CZE was used for a purity control of the sample preparation of *Desmodus* salivary plasminogen activator (DSPAα1), a serine protease that has potential applications in different cardiovascular diseases.[129] The separation was conducted in an unmodified fused silica capillary under acidic conditions (pH 2.4) to prevent sample adsorption. Although no resolution of the glycoforms was achieved, the method was chiefly developed to monitor purity of the final product rather than characterizing its glycovariants.

The production of recombinant proteinase A and antithrombin III (rAT III) was monitored by CZE.[130,131] While the purity of proteinase A was measured using a CZE technique with an unmodified capillary,[130] a dynamically coated capillary using hydroxypropyl-

methylcellulose was used in the case of rAT III.[131] In both cases, analyte adsorption was eliminated by performing the separation under acidic conditions. An off-line MALDI/MS method was additionally employed[130] in characterization of proteinase A.

Recombinant humanized monoclonal antibody HER2 (rhuMAbHER2) is an antibody directed against the human epidermal growth p185 gene product $p185^{HER2}$. Hunt et al.[132] demonstrated the feasibility of using CE for the analysis of rhuMAbHER2 in a quality-control environment. They employed both CIEF and capillary gel electrophoresis to monitor and characterize the five glycoforms of this antibody. The data exhibited high correlation with the standard, but more time-consuming and labor-intensive slab gel techniques were involved. The authors suggested a possible replacement of these conventional techniques with CE.

Another recombinant glycoprotein that has been extensively studied by CE is the recombinant tissue plasminogen activator (rtPA), a fibrin-specific protein approved for treatment of myocardial infarction. Although several communications addressed the feasibility of using CZE for the separation of rtPA glycoforms,[79,117,121,133] the resolution in this case did not approach what had been attained by CIEF.[79,134−137] The first report[79] on the application of CIEF to separate rtPA glycoforms illustrated the potential of CIEF to discriminate subtle differences between two variants of this glycoprotein. However, the method suffered from poor reproducibility in migration time, making the correlation of pI against migration time very difficult to assess. Later, several groups directed their efforts to improve CIEF reproducibility. A rapid, one-step CIEF method was developed, taking advantage of improved performance in coated capillaries and the presence of hydroxypropylmethylcellulose.[134] The best resolution was attained by mixing a wide-range ampholyte (pH 3−10) with another covering a narrower range. The addition of urea was required to maintain protein solubility during the focusing. The same group validated the CIEF method by demonstrating its accuracy, precision, specificity, and ruggedness.[135] Other reports investigated the effect of ampholytes[136] and the addition of urea[137] on the resolution of rtPA glycoforms in CIEF.

Recently, the potential of CE has been demonstrated in the examination of a glycoprotein factor associated with cancer cachexia.[138,139] A comparison of the CE profiles of a healthy volunteer and a cancer patient showed the presence of additional peaks in the electropherogram of the latter that could be associated with cachexia. In this study, micropreparative CE was performed with a 180 μm, i.d., fused silica capillary with tapered ends to collect CE fractions for further identification by MALDI/MS. The urine samples analyzed by CE and off-line MALDI/MS exhibited a characteristic ion at ca. 24 kDa that could be identified as the cachectic factor, a glycoprotein. The combined use of CE and MALDI/MS was successful in detecting cachexia in all remaining patients, including one patient that was in an early stage of the disease.

E. Mass Spectrometry

Over the past decade, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI/TOF-MS) and electrospray mass spectrometry (ESI/MS) have emerged as powerful tools for the analysis of large biomolecules, contributing extensively to progress in life sciences. To a variable extent, the intact glycoproteins can be resolved to their individual glycoforms by both methodologies, depending on their mass and the extent of glycosylation.

1. MALDI/MS

MALDI/TOF instruments now often furnish sufficient resolution to detect glycoforms of small glycoproteins with a single glycosylation site and a limited number of glycans.[140] However, broad and unresolved peaks are observed in the case of large glycoproteins with multiple glycosylation sites.[141] It was recently demonstrated that the appropriate choice of parameters, which determine the desorption/ionization of glycoproteins, can improve significantly mass measurement reproducibility and resolution.[142] These parameters included selection of a desorption matrix, a sample-matrix preparation technique, pH, and instrumental conditions. Although some of the matrixes with a general applicability to proteins are also capable of ionizing glycoproteins, most large proteins require different media. In addition, no single matrix ionizes glycoproteins to the same extent, so several matrixes had to be tested for each structural type. Table 2 lists the matrixes that have been successfully utilized for the analysis of glycoproteins.

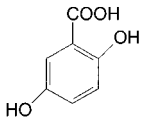
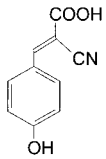
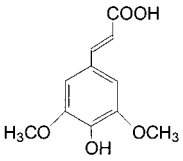
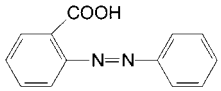
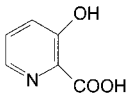
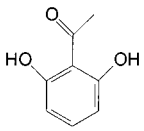
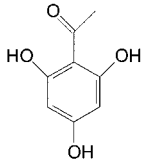
Although an improvement in resolution was accomplished by optimizing the aforementioned parameters, this did not even remotely match the resolution attained using the instruments with the delayed ion extraction.[143,144] This is because the delayed ion extraction minimizes the observed metastable fragmentation, which is known to adversely influence a MALDI analysis of glycoproteins, particularly in the reflectron instruments.

2. ESI/MS

At first glance, there is no reason ESI could not be as successful as MALDI in the analysis of intact glycoproteins. However, the dearth of data in the area suggests difficulties that may be, to begin with, due to the data complexity with multiply charged ions and the formation of adducts due to the binding of salts to the oligosaccharide moieties. Moreover, the casual observations that the enzymatically deglycosylated proteins "spray" better than their corresponding glycoconjugates point to additional sources of difficulties: (a) numerous potential protonation sites may be covered through substitution with glycans, and (b) under typical ESI conditions, the desolvation phenomena could be less effective with the carbohydrates attached.

The importance of solvation phenomena in ESI analyses of glycoproteins is further underscored by the differences between a normal ESI and the so-called "nanospray." Wilm and Mann[145] were the first

Table 2: Matrixes Utilized in Analysis of Glycoproteins

matrix	abbreviation	structure
2,5-dihydroxybenzoic acid	2,5-DHB	
α-cyano-4-hydroxycinnamic acid	4-HCCA	
sinapinic Acid	—	
2(*p*-hydroxyphenylazo)benzoic acid	HABA	
3-hydroxypicolinic acid	—	
2,6-dihydroxyacetophenone	—	
2,4,6-trihydroxyacetophenone	2,4,6-THAP	

to report success in using nano-ESI/MS to analyze intact glycoproteins. The results they have shown with ovalbumin glycoforms were vastly superior to the previous recording using a conventional ESI source.[146] Additionally, a recent combination of nano-ESI with a very low nozzle/skimmer voltage on an orthogonal TOF mass spectrometer[147] was conducive to a very good resolution of the bovine α_1-acid glycoprotein glycoforms. Clearly, the use of nanospray permits the use of solvents that are unfeasible with normal ESI (e.g., water). Nanospray also tolerates high salt and buffer concentrations, thus enhancing the scope of applications and a coupling with electromigration techniques. Its very low sample consumption shows considerable potential in ultrasensitive measurements, as needed in glycobiology.

V. Analysis of Glycopeptides and Determination of the Sites of Glycosylation

It is most common that glycosylation of a particular protein is investigated through a chemical or enzymatic release of glycans and their subsequent characterization such as sequencing and linkage analysis. While this information may be highly significant (see below), there are additional structural aspects that must be addressed. For each site of glycosylation, there are possible structural variations (extent of substitution, site-specific microheterogeneities, a site-specific accessibility for particular glycosyltransferases, etc.) that can all have important biochemical consequences. Investigating protein glycosylation at the level of glycopeptide is at least as important as the investigation of released glycan structures. Certain methodologies of this area overlap with those encountered in the current proteomics efforts.

In 1996, Mann and co-workers[148] devised a strategy based exclusively on MS to identify reliably and sensitively the proteins separated by 2-DE. The strategy, as a first screen, used MALDI/MS of the peptide mixture produced by an in-gel tryptic digestion of a protein. Next, the peptide masses obtained by MALDI/MS were searched against sequence da-

tabases. This allowed about 90% of the proteins isolated by the 2-DE to be identified with high accuracy. The remaining proteins were identified by partially sequencing several peptides of the unseparated mixture by nano-ESI tandem MS. This approach generates a partial or a complete amino acid sequence that, with the associated masses of peptides, is assembled into a "peptide sequence tag". This consists of a short sequence combined with the distance, in mass units, to the N- and C-termini of the peptide,[149] constituting a highly specific probe for the sequence database searches. The strategy was successful in characterizing a total of 150 gel spots, many of them at subpicomole amounts, thus enlarging a yeast 2-D gel database. Since then, the number of reports utilizing this strategy, or its modified versions for mapping of total cellular proteins, subcellular complexes, and organelles and protein phenotypes and functions, has increased substantially.[14] In principle, the same strategy could be extended to study site-specific glycosylation of proteins at the glycopeptide level, with the overall goal of generating more elaborate information on glycoprotein heterogeneity.

It is difficult to directly identify glycopeptides in a complex protein digest by MS. This is partly due to the low sensitivity of the detection of glycopeptides caused by site heterogeneity and/or ion adduct formation. Glycopeptide signals are often suppressed in the presence of other peptides, especially if the glycans are terminated with the negatively charged sialic acid moiety. Due to the glycan heterogeneity and a frequent multiple adduct formation, the overall glycopeptide signal distributes into several peaks, resulting in weak signals detected by MS. Consequently, it appears necessary to use multiple analytical techniques, either in parallel or sequentially. Interfacing MS with different separation techniques is particularly appealing, as this strategy offers the combination of orthogonal principles. In this context, CE and microcolumn liquid chromatography (μLC) are exceptionally attractive, due to their low sample loading, different selectivity modes, and high separation efficiency.

The next section focuses on reviewing the methods which have been recently developed to probe the glycosylation sites of glycoproteins. Both a direct MS and the coupled methods will be evaluated.

A. Mass Spectrometry

1. MALDI/MS

In two separate reports, Mortz et al.[150] and Harmon et al.[151] employed MALDI/MS for characterization of a site-specific glycosylation microheterogeneity of recombinant human γ-interferon. Both groups used microbore chromatographic techniques with off-line MALDI/MS to determine the sites of glycosylation and the glycosylation extent. Mortz et al. determined the presence of nonglycosylated and mono-and diglycosylated forms of the glycoprotein by MALDI/MS after electroelution of the proteins from electrophoretic gel. A direct MS analysis of the peptide mixture generated through in-gel digestion allowed only the detection of one glycopeptide. The glycan structures at this site were determined by a sequential treatment of the peptide mixtures with different exo- and endoglycosidases while mass shifts were monitored. The second glycopeptide was not directly observable by MS, so microbore HPLC separation of the mixture was needed prior to its identification. The glycan structures on this second glycopeptide were determined by employing the same strategy as used for the first glycopeptide. This report demonstrated clearly the drawback of a direct MS in characterization of a glycoprotein microheterogeneity at the glycopeptide level.

In the second report,[151] an automated analytical system with minimum sample handling was devised for the rapid assessment of the site-specific microheterogeneity of the two potential N-linked glycosylation sites on the same glycoprotein. First, the target glycoprotein was purified from the culture supernatant by immunoaffinity chromatography, while the acidic eluent was neutralized through an in-line mixing tee. On-line proteolysis was performed in an immobilized trypsin cartridge, followed by microbore reversed-phase LC, which isolated the two pools of glycopeptides representing the glycosylation sites. Using MALDI/MS, site-specific oligosaccharide structures were deduced from the mass shifts of the glycopeptides relative to the known masses of their constituent amino acids. Just as in the other report, the glycan structures were determined through the use of endo- and exoglycosidases. The procedure automation allowed the evaluation of glycoprotein microheterogeneity in a relatively short time (ca. 2 h) while using submicrogram quantities.

Küster and Mann[152] devised another approach for the unambiguous identification of the N-glycosylation sites at high sensitivity. Their approach involved an enzymatic removal of N-linked glycans with a simultaneous partial (50%) ^{18}O-labeling of glycosylated asparagine residues prior to proteolysis and MALDI peptide mass mapping. Accordingly, more peptides were observed in the MALDI spectra, thus increasing the specificity of subsequent database searches. This increase in specificity allowed the unambiguous identification of glycoproteins that could not be identified through a mere comparison with a list of mass values. The presence of a consensus glycosylation sequon easily allowed the determination of the actual position of glycan attachment.

MALDI/MS, in conjunction with proteolytic and glycolytic digestions, was applied to determine the extent and distribution of N-glycosylation and the nature of most disulfide linkages in bovine lactoperoxidase.[153] The five present asparagines were found to be glycosylated, predominantly by high-mannose and complex structures. The methodology also allowed the assignment of six disulfide bond linkages. In addition, 98% of the primary amino acid sequence of the protein was confirmed. A similar strategy has also been successfully utilized for determination of the glycosylation sites in human serum transferrin, α_1-antitrypsin, and β-glucosylceramidase,[40] as well as those in human γ-interferon isolated from Chinese hamster ovary cell culture.[154]

The application of MALDI/MS to the characterization of glycosylation sites was recently extended to include glycoproteins in diseases. The method was utilized to assign the glycosylation sites on HIV-1_{SF2} gp 120 expressed in Chinese hamster.[155] Also, glycosylation sites and heterogeneity of glycosylated sites were determined for cyclooxygenase-2, an enzyme involved in the biosynthesis and metabolism of prostaglandins.[156] Another interesting example is the use of MALDI/MS for determination of the sites of glycosylation and disulfide bond assignments in the human gastrointestinal carcinoma antigen GA733-2, which is a cell surface glycoprotein with high expression in most human gastrointestinal carcinomas and, at a lower level, in most normal epithelia.[157]

MALDI/MS was recently employed to identify and localize O-glycosylation sites.[158] The method is based on the addition of alkylamine as a label of the previously occupied O-glycosylation sites to the unsaturated derivative of serine and threonine, which are the elimination products. The alkylaminylated products principally allow not only the proteolytic cleavage but also the identification of the O-glycosylation site. This approach was first introduced by Rademaker et al.;[159] in that case, ammonia was used as the label. However, the mass difference of only one mass unit between the original OH and NH_2 groups renders a determination of the glycosylation site quite challenging. Conversely, alkylaminylation products are easier to determine, since the mass difference becomes noticeable and the aminylation products are stable under MALDI postsource-decay analysis as well as in the collision-induced dissociation experiments. This permits sequencing and, subsequently, peptide localization of the O-glycosylation sites. Nevertheless, determination of a site microheterogeneity is not possible through this procedure.

The number of O-linked sugar chains per heavy chain of IgA_1 could be estimated by MALDI/MS using the hinge glycopeptide.[160] A sequential enzymatic digestion of the glycopeptide allowed determination of the number of attached sugar chains as well as their identity. In a separate study,[161] the number of nonsubstituted *N*-acetylgalactosamines and the core *O*-glycans per a heavy chain of the normal human serum IgA_1 were estimated through digestion of the asialo-hinge glycopeptide with α-*N*-acetylgalactosaminidase or *endo*-α-*N*-acetylgalactosaminidase. The study concluded that the asialo-hinge glycopeptide had three glycoforms. The first had four repeating core-1 structures and one GalNAc. The second was composed of only four repeating core-1 structures, while the third has three repeating core-1 structures and one GalNAc.

Recently, Udiavar et al.[162] devised a strategy employing CE, LC, ESI/MS, and MALDI/MS for characterization of the complex peptide mixture resulting from a proteolytic digestion of the single-chain plasminogen activator. Earlier, an unfractionated peptide mixture of DSPAα1 was analyzed by MALDI/MS; however, not all peptides were observed for the reasons discussed previously.[129] Fractionation of the peptide pool by either HPLC or CE substantially improved MALDI/MS performance.[163] A combination of LC, CE, and an off-line MALDI/MS simplified the degree of complexity of the individual samples to allow tentative identification of about 30 glycoforms present at a single site in DSPAα1.[162]

More recently, identification of glycoproteins in complex mixtures derived from either human blood serum or a cancer cell line was achieved by employing an off-line MALDI/MS of the chromatographed proteolytic digests of glycoproteins.[164] The process involved reduction, alkylation, and proteolysis of all proteins in the mixture, a lectin affinity chromatographic selection of the glycopeptides, fractionation by reversed-phase liquid chromatography (RPLC), MALDI/MS of the individual RPLC fractions, and peptide identification based on a database search. Although this approach is fast, while targeting specific molecular species or classes of glycoproteins for identification, it does not discriminate between the glycoforms, nor does it permit the determination of heterogeneity. The data obtained from a digestion of several glycoproteins could be very complex and difficult to interpret.

The aforementioned methods clearly exemplify the effectiveness of MALDI/MS in the characterization of glycosylation sites of glycoproteins. However, the observed sample complexity and the inherent difficulties associated with MALDI detection of glycopeptides call for a wider use of microseparation methodologies (single or even multidimensional) prior to MALDI analysis.

2. ESI/MS

The advantages of using electrospray ionization for tryptic mapping of glycoproteins was first illustrated by Ling et al.[165] in the application using the recombinant human tissue plasminogen activator as a model glycoprotein. ESI/MS was simply utilized as an on-line HPLC detector for tryptic mapping of this glycoprotein. Here, MS significantly enhances the high resolving power of chromatography by allowing mass resolution of coeluting components. Since then, the glycosylation sites of several glycoproteins have been characterized by LC/ESI/MS and LC/ESI/MS/MS, including mouse monocolonal immunoglobulin (IgG2b),[166] murine scrapie prion protein (PrP^{sc}),[167] cyclooxygenase-2,[156] lutropin receptor glycoprotein,[168] endopolygalacturonase I,[169] and gelatinase B.[170] Also, several determinations of the glycosylation sites of recombinant glycoproteins have been communicated, including recombinant *endo*-polygalacturonase I from *Aspergillus niger*,[171] pectate lyase,[172] human coagulation factor VIIa,[173] human erythropoietin (rhEPO),[174,175] and corticotropin-releasing factor binding protein.[176]

In 1993, Carr and co-workers devised an MS method for the selective detection of glycopeptides at the low picomole level during the chromatography of glycoprotein digests.[177,178] This procedure allowed differentiation between *O*- and *N*-glycans of glycopeptides. The method involves observation of the diagnostic sugar oxonium ion fragments, particularly the $HexNAc^+$ fragment at *m*/*z* 204, originating from collisionally excited glycopeptides (Hex = hexose).

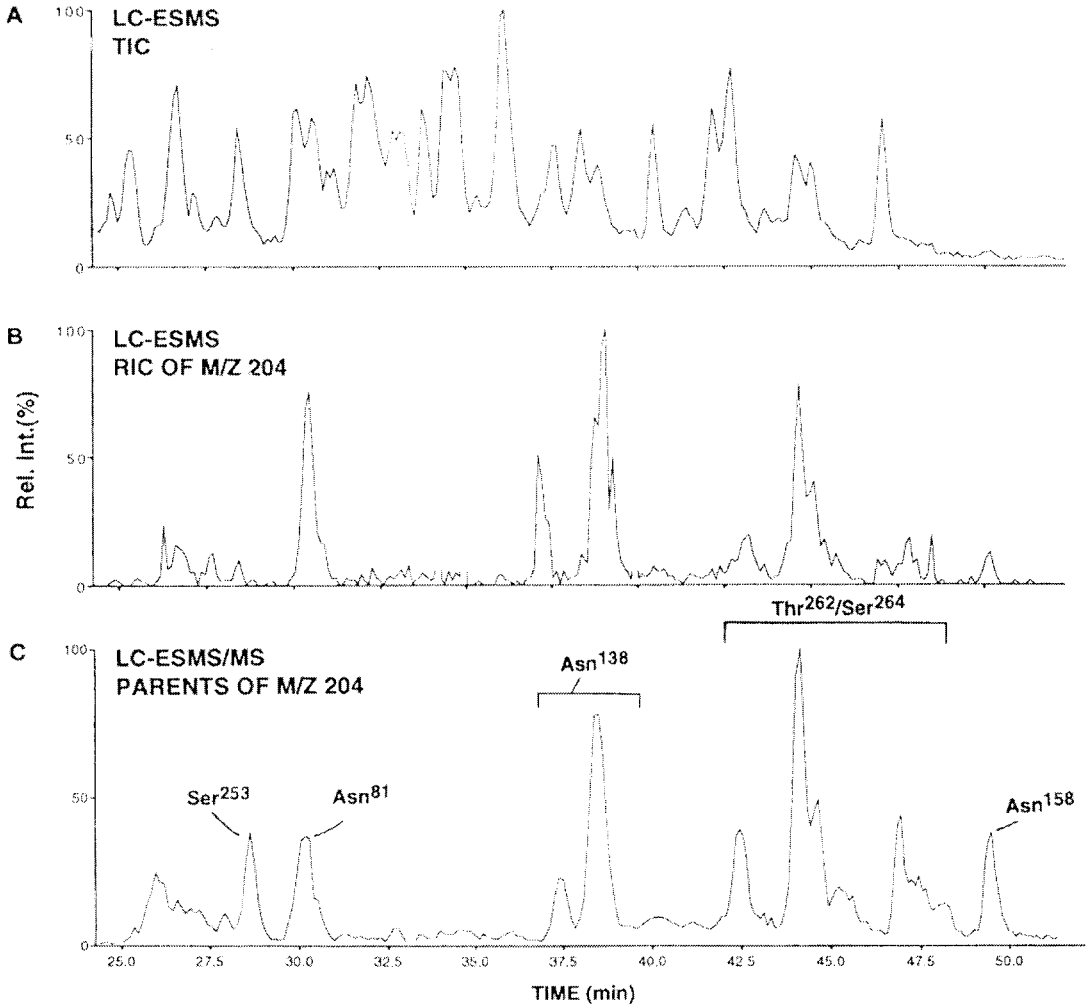


Figure 4. (A) LC-ESI/MS total ion current (TIC) trace with the orifice voltage ramped from 120 V at *m/z* 150 to 65 V at *m/z* 500; from *m/z* 500 to *m/z* 2000 the orifice voltage was held constant at 65 V. (B) Reconstructed ion chromatogram (RIC) of *m/z* 204 ($HexNAc^+$) from the ramped orifice LC-ESI/MS data. (C) LC-ESI/MS, parent ion scan of *m/z* 204 ($HexNAc^+$), TIC trace. (Reprinted with permission from ref 177. Copyright 1993 Publisher.)

Such determinations can be performed on a single or a triple quadrupole mass spectrometer equipped with an ESI source. In the case of the single quadrupole instrument, it is possible to enhance formation of the carbohydrate-related fragment ions without distorting the distribution of peptide and glycopeptide signals by increasing the collisional excitation potential during that portion of the scan in which the low-mass, carbohydrate-related ions are being detected. This procedure identifies putative glycopeptide-containing fractions in a chromatogram, but suffers from a lack of specificity in the case of other coeluting peptides. A triple quadrupole instrument furnishes higher selectivity, as it selectively detects only those parent ions that fragment in the second collision region of the triple quadrupole to produce an ion at *m/z* 204 (see Figure 4). Accordingly, only $(M + H)^+$ ions of glycopeptides are observed in such LC/MS/MS "parent-scan" spectra. Since both *N*- and *O*-glycans generate different ions, albeit with the same *m/z* value, N-glycosylated peptides are differentiated from O-glycosylated species by LC/MS/MS analysis of the digested glycoprotein prior to and after treatment with peptide *N*-glycosidase F. The effectiveness of this method was demonstrated for bovine fetuin[177] and soluble complement receptor glycoprotein.[178] Later, this method was successfully employed in the determination of a site-specific *O*-GlcNAc glycosylation of the human cytomegalovirus tegument basic phosphoprotein.[179]

O-GlcNAc glycosylation of peptides was also determined by a different approach that utilized a conventional β-elimination of *O*-glycans in conjunction with ESI/MS/MS.[180] The method is based on conversion of glycosylserine to 2-aminopropenoic acid (2-ap) by β-elimination. This treatment decreases the mass of a glycopeptide by *m/z* 222, resulting in a CID (collision-induced dissociation) fragment ion that represents the loss of *m/z* 69 (that of 2-ap) instead of *m/z* 87 (that of serine) at the position of glycosylation. This method allowed a rapid identification of *O*-GlcNAc-modified peptides in a complex mixture as well as the site of glycosylation at the low picomole level.

A simultaneous detection of *O*-GlcNAc-modified peptides in a sample and identification of the modified protein was achieved through development of an MS/MS-based protocol. It specifically detects peptides carrying the *O*-GlcNAc modification by monitoring a specific reporter ion and identifying the protein from which the labeled peptide originated through CID and database search.[181] The labeling of an

O-GlcNAc with galactose using galactosyl tranferase produces a disaccharide substituent with a diagnostic *m*/*z* 366. This disaccharide can be removed from the glycopeptide by CID at much less energy than needed for the fragmentation of the peptide backbone. The energy difference is utilized to set up a multistage experiment using a triple quadrupole mass spectrometer. The approach involves precursor ion scanning at a relatively low collision energy level to detect those species that produce the *m*/*z* 366 fragment, followed by CID at higher energy to fragment the peptide backbone and to identify the underlying peptides. Sensitivities at the femtomole level were achieved.

Recently, ESI/MS was applied to the determination of the sites of *O*-GalNAc glycosylation[159] in a manner similar to that used for the determination of the sites of *O*-GlcNAc attachment. As mentioned earlier, the method was based on β-elimination of the glycan chains using ammonium hydroxide. Ammonia replaces the glycans on the amino acid residue, thus yielding a modified amino acid residue having a distinct mass. ESI/CID tandem MS then allows the modified peptides to be sequenced and the site of the modified amino acid residues to be identified. Although it was demonstrated that the site determination can be achieved at low picomole sensitivity, the method suffers from two major shortcomings. First, the difference between modified and unmodified amino acid residues is only one mass unit, as mentioned earlier, thus making it a challenge to determine such minor differences. Second, the method does not allow the determination of a site-specific microheterogeneity.

The advantages of nanoelectrospray have been realized in the characterization of O-glycosylation sites in MUC2 and MUC4 glycopeptides.[154,182] The use of nano-ESI Q-TOF (quadrupole/time-of-flight) MS/MS allowed the determination of both the carbohydrate and the peptide/glycopeptide sequence data in a single experiment. Isobaric peptide structures (either with the same number of attachment sites in different positions within the peptide backbone or with the same number of sugar moieties distributed on different attachment sites) demonstrated the feasibility of this highly sensitive and accurate approach. This characterization was performed using no more than 1 pmol of each glycopeptide substrate, which allowed spraying for at least 30 min. In some cases, the relevant glycopeptide fragment ions were obtained with even 50 fmol. The method was further extended to the determination of glycosylation sites in O-fucosylated glycopeptides.[183]

The on-line coupling of CE to ESI/MS has been technically challenging, due to the extremely low flow rates associated with CE and the need for adequate electrical contact for the simultaneously used electrospray ionization and electrophoretic separation. The three principal interface designs for CE−ESI/MS are the coaxial sheath flow,[184] the liquid junction,[185,186] and the sheathless configuration.[187] Although capillary electrophoresis has been successfully coupled to mass spectrometry for over a decade and utilized for the analysis of proteins and peptides, CE−MS for the analysis of glycopeptides was first reported in 1997.[188] A proteolytic digest of ovalbumin was employed as a model mixture for the characterization of coated capillaries for CE−ESI/MS at submicroliter flow rates.[188] The study demonstrated a substantial enhancement of sensitivity using the introduced microsprayer sheathless CE−ESI/MS interface. The sheathless configuration allowed detection at low picomole injections, while the coaxial interface required 20 times higher sample loads. The interface was also successful in the analysis of ovalbumin proteolytic digest by CE−MS/MS. The same group also employed CE−ESI/MS and MS/MS with "parent scan" on a triple quadrupole mass spectrometer for the characterization of O- and N-glycosylation sites of α-amylase, a lectin from *Lotus tetragonolobus*, κ-casein,[189] as well as a lectin from *Phaseolus vulgaris*.[190]

Boss et al.[191] and Udiavar et al.[162] advocated the need for multidimensional separations in conjunction with mass spectrometry to allow a comprehensive characterization of glycosylation in glycoproteins. Both reports demonstrated the limitations of MS alone, or in conjunction with unidimensional separation, to completely characterize glycoproteins.

3. FAB-MS

Prior to the development of MALDI/MS and ESI/MS, fast atom bombardment mass spectrometry (FAB-MS) was employed extensively for the mapping of glycopeptides. FAB-MS was used to identify the N-glycosylation sites of glycopeptide antibiotics bleomycin A2 and B2,[192] and teicoplanin.[193] N-Glycosylation sites of tissue plaminogen activator expressed in Chinese hamster ovary cells[194,195] and a *Drosophila* insect cell line[196] were also characterized by FAB-MS, as were the N-glycosylation sites of the pre-S2 region of the hepatitis B surface antigen,[197] a spermine-binding protein from rat prostate,[198] human serotransferrin,[199] and a recombinant soluble form of the CD4 receptor glycoprotein.[200,201]

Generally, FAB-MS of glycopeptides suffers from a poor response relative to the corresponding peptides, which is attributable to the heterogeneity of the carbohydrate, resulting from the distribution of the molecular ion signals between a number of species. The hydrophilic character of the carbohydrate moiety decreases detectability further through lowering the surface activity of a glycopeptide in the liquid matrix.[200] Therefore, glycopeptides are only detected after a separation of the peptide mixture through HPLC.[193,199,201] Even then, they are difficult to measure unless the peptide backbone is hydrophobic and the carbohydrate moiety is of a relatively low molecular weight.

The last communication on the use of FAB-MS for the mapping of glycosylation sites appeared in 1993,[202] when it was compared to the utility of MALDI/MS. A greater operational simplicity and a higher sensitivity of MALDI relative to FAB were clearly illustrated. MALDI is 10−100 times more sensitive than FAB for the detection of underivatized glyco-

peptides. Moreover, MALDI allows qualitative determination of the site-specific information without isolation and derivatization. Today, MALDI and ESI replace FAB for most such determinations.

VI. Release of Glycans from Glycoproteins

A. Release of *N*-Glycans

1. Chemical Release

Using a classical method of carbohydrate chemistry[51] or its more recently improved versions,[52] *N*- And *O*-glycans can be chemically cleaved from glycoproteins with hydrazine. *O*-Glycans are claimed to be specifically released at 60 °C, while 95 °C is needed to release N-linked oligosaccharides.[52] This chemical release approach suffers from several major disadvantages. Since the reagent cleaves amidic bonds, including the linkage between the *N*-glycans and asparagine, the samples are destroyed. Consequently, any information is lost pertaining to the site of glycosylation and the extent to which it occurs. Second, under the used reaction conditions, the acyl groups of *N*-acetylamino sugars and sialic acids are hydrolyzed, calling for a reacetylation step, assuming that a sialic acid possessed originally an acetyl group rather than any other substitution. Third, the residual hydrazide or amino groups are often incorporated to the reducing terminus of some glycans. Additionally, conducting the reaction at high temperatures may result in a loss of the reducing terminal GlcNAc. And, finally, it is essential to maintain strictly anhydrous conditions, which may not always be feasible. Therefore, hydrazinolysis has now become secondary to the more favored enzymatic release.

2. Enzymatic Release

N-Glycans can be released enzymatically from glycoproteins by several commercially available enzymes. Peptide *N*-glycosidase F (PNGase F) is the most widely used. The enzyme cleaves off the intact glycans as glycosylamines, which are readily converted to regular glycans. The enzymatic release of glycans also results in the conversion of asparagine to aspartic acid at the N-glycosylation site of the protein. PNGase F has a very wide specificity, cleaving all *N*-glycans except those having α(1–3) linkages to the reducing-terminal GlcNAc. Their corresponding glycoproteins are commonly found in plants and can be enzymatically cleaved using PNGase A.[50]

Other endoglycosidases can be more specific, such as endoglycosidase H (Endo H), known to cleave high-mannose structures and most hybrids. Moreover, Endo H hydrolyses the bond between the two GlcNAc residues of the chitobiose core, leaving a GlcNAc attached to the protein. This is disadvantageous, since the information related to the presence of fucose residues at the reducing end of the glycans is lost. As discussed in a recent review,[50] other endoglycosidases with variable degrees of specificity are available.

B. Release of *O*-Glycans

1. Chemical Release

Together with hydrazinolysis,[51,52] alkaline β-elimination[53] is the most commonly used chemical cleavage method for the release of *O*-glycan. Alkaline β-elimination is widely viewed as the most reliable and universal method for the purpose. However, the presence of a strong reducing agent, which converts glycans to their respective alditols, is here necessary to minimize the "peeling reactions"[203] caused by the alkaline medium. The conversion to alditols prevents reductive amination, which may often be needed for the attachment of a fluorophore, or a polyvalent coupling to a lipid or protein for a subsequent immunoassay.[204] Alkaline β-elimination, embodied in the widely practiced Carlson procedure,[53] is also difficult to carry out at microscale, as the minute quantities of released glycans are overwhelmed by the excessive amounts of salts.

While hydrazinolysis[52] is the most widely used chemical approach to yield reducing glycans, it constitutes a tedious procedure with many needed precautions. As detailed previously, it suffers from several disadvantages. Improvements were recently sought,[158,205] through the use of 70% (w/v) aqueous ethylamine to release nonreductively the O-linked oligosaccharides from glycoproteins. Regrettably, the overall reaction yields were low, as the oligosaccharides underwent significant peeling reaction and/or other forms of degradation.

Whereas β-elimination is generally effective, the formation of alditols makes it difficult to investigate released glycans in complex mixtures. The lack of a chromophore in these structures during liquid-phase separations makes their detection difficult: UV absorbance measurements below 200 nm are relatively insensitive, as is MS detection[206,207] due to the low ionization efficiency with intact glycans. In contrast, the cleavage procedures yielding the reducing end make it relatively easy to attach a chromophore or a fluorophore through reductive amination with aromatic amines prior to a chromatographic or electrophoretic separation.[208,209] A derivatization featuring appropriate structural moieties can also be beneficial in enhancing the sensitivity of MS investigations.[206,207]

Recently, Novotny and co-workers introduced a modified β-elimination procedure that cleaves the *N*- and *O*-glycans with the reducing ends intact and, under very mild conditions, makes them amenable to MS analysis.[54] This so-called ammonia-based β-elimination procedure provides a viable alternative to the hitherto used hydrazinolysis and the Carlson β-elimination methods. Its simplicity, the lack of peeling reactions, and deacetylation byproducts supplement its effectiveness at microscale. Minimum sample handling and easy removal of reactants make it possible to cleave effectively the oligosaccharide chains from only low-microgram quantities of glycoproteins. This recently developed procedure is particularly effective in conjunction with the highly sensitive techniques of MS and capillary separation methodologies. The methodology now brings the analysis of *O*-glycans on par with the use of *N*-

glycanases and sensitive measurements on *N*-glycans developed during the recent years. Conversion of O-linked oligosaccharides to the glycans with reducing ends allows their subsequent labeling with a fluorophore or any other structural entity that may facilitate better separation or detection capabilities.

2. Enzymatic Release

Unlike *N*-glycans, no endoglycosidases are reliably available for the release of O-linked oligosaccharides, with the partial exception of endo-α-*N*-acetylgalactosaminidase, permitting the release of unsubstituted core-1 *O*-glycans.[210−212] However, this highly specific enzyme has very limited use, as it does not cover the other core structures. At this time, chemical release methods provide the only universal means for O-linked glycans.

VII. Analysis of Glycan Mixtures

A. Chromatography

Separation of glycans in time and space appears essential to investigating complex glycan pools released from glycoproteins. Chromatography, electrophoresis, and MS are both complementary and competitive in this regard. MS is inherently faster in displaying complex oligosaccharide maps, but chromatographic and certain electromigration techniques can more readily distinguish various isomeric structures. Both the classical forms of chromatography and HPLC have been used extensively in isolation and fractionation of glycans. The gradual development of HPLC over the last 3 decades has enabled higher resolution through both the column efficiency and the ever-expanding range of new sorption materials. The different HPLC modes for glycan analyses include (1) hydrophilic interaction chromatography (HILIC),[213] (2) reversed-phase liquid chromatography (RPLC),[214] (3) size-exclusion chromatography (SEC),[215] (4) high-performance anion-exchange chromatography (HPAEC),[20] (5) lectin-affinity column chromatography,[216] and (6) HPLC with graphitized carbon columns.[217]

1. Hydrophilic-Interaction Chromatography

The mode of liquid chromatography in which a polar stationary phase is used in combination with a less polar mobile phase has been known as "normal-phase" or "straight-phase" LC. Perhaps, a more appropriate term for it is "hydrophilic-interaction chromatography", which was coined by Alpert,[218] with the acronym HILIC. This mode of HPLC has long been popular in carbohydrate analysis. The stationary phases used in HILIC (which are polar by definition) include silica gel, sorbents in which a polar phase is chemically bonded to silica gel, and polymers bearing polar functional groups.

Although LC of carbohydrates on unmodified silica was reported,[213] it is more effective to use silica modified through chemical bonding of suitable functional groups. Of these, bonded aminopropyl silica packings have been used widely. However, this type of packing suffers from a continuous loss of the analyte as a result of the forming of glycosylamines by interaction of the amino groups on the stationary phase with the reducing end of the analyzed carbohydrate. The column lifetimes are also affected adversely. To overcome the problem, other bonded-phase packings have been introduced, including materials bonded with amide, cyano, diol, and polyol moieties.

Due to its solubility at high or low pH, silica-based packings are not suitable for HPLC at pH greater than 8, or under strongly acidic conditions (pH below 2). Therefore, polymeric packings now appear more stable under such conditions. Highly cross-linked, sulfonated polystyrene cation-exchange resins and vinyl polymers have been used in HILIC of carbohydrates. A review addressing the recent development of HILIC systems and their carbohydrate applications has been published.[213]

Townsend and co-workers[219] devised a method for a simultaneous analysis of sialylated and nonsialylated oligosaccharides derived from glycoproteins. The method was based on labeling the released glycans with 2-aminobenzamide according to the method of Bigge et al.[220] prior to their separation on a polymeric, secondary-amine column by HILIC. The basicity of the amine-bonded stationary phases results in ionic interactions with the charged species, such as anionic oligosaccharides, thus eliminating their elution.[221] However, the ionic interactions on such columns can be largely suppressed by adding formic acid or acetic acid into the mobile phase. This modification of the mobile phase allows oligosaccharides bearing the same number and type of anionic moieties to be eluted in the order of size. Moreover, separation of neutral oligosaccharides remains unchanged in the presence of formic or acetic acid. Accordingly, the method afforded unambiguous separation between neutral and anionic oligosaccharides. The method was utilized for the analysis of *N*-glycans released from ribonuclease B and α_1-acid glycoprotein. The *N*-glycans released from ribonuclease B were separated not only by size but also by a branch location of the terminal Man α(1−2)-linked residues. On the other hand, the *N*-glycans released from α_1-acid glycoprotein were separated according to charge, their fucose content, and the number of Galβ(1−4)-Galβ(1−3) repeats. The minor fucosylated and polylactosamine species were well-separated from the major sialylated tetraantennary oligosaccharides. Volatile mobile phases were used to allow MALDI/MS analysis of the peak fractions with minimal sample handling.

Guile et al.[222] devised a sensitive and reproducible HILIC method capable of simultaneously resolving subpicomolar quantities of the mixtures of fluorescently labeled neutral and acidic glycans. The method was also based on the labeling of glycans released from glycoproteins with 2-aminobenzamide according to Bigge et al.[220] prior to separation on a silica-based column with amide functional groups; an acetonitrile−water gradient, buffered with volatile salts, was used. The elution positions of the standard glycans were determined in glucose units with reference to the dextran ladder, and incremental values for the

addition of monosaccharides to oligosaccharide cores were calculated. This information was used to interpret the full oligosaccharide profiles of glycoproteins in a predictive manner based on the arm specificity, linkage, and monosaccharide composition. The method was successfully applied to characterize a family of glycans isolated from the human parotid gland and a serum IgG glycan pool. The reproducibility and predictability of the glucose unit values, together with a quantitative response for the fluorescently labeled glycans, permitted the automatic analysis of neutral sugars using the combinations of enzymatic arrays. Moreover, the simultaneous resolution of both sialylated and nonsialylated products from the enzymatic digestion allowed direct analysis of sialylated structures. The sequential exoglycosidase digestions of the pool of glycans released from normal human serum IgG are illustrated in Figure 5. The dextran ladder used to calculate the glucose values is shown in the upper panel, while the second panel depicts the HILIC separation of normal IgG glycans. The results of incubating the glycan pool successively with the enzymes designated in each panel is shown in panels a−f. This method can be applied to characterization of both *O*- and *N*-glycan pools. However, the *O*-glycan pool must be chemically released from the glycoprotein in a way that preserves the reducing end required for labeling with a fluorescent reagent. This methodology was successfully utilized for characterization of the *N*- and *O*-glycan pools derived from the complement regulatory protein, human erythrocyte CD59,[223] and for identification of highly fucosylated N-linked oligosaccharides from the human parotid gland.[224]

Recently, the aforementioned methodology was adopted by Dwek and co-workers[225] to analyze the *N*-glycan pools derived from the soluble forms of glycoproteins belonging to the Ly-6 scavenger receptor and immunoglobulin superfamilies expressed in Chinese hamster ovary cells. The focus of their study was to examine the extent to which N-linked oligosaccharide processing might vary in superfamily-, domain-, or protein-dependent manner in a given cell. While no evidence for a superfamily-specific modification of the glycans was found, marked differences were determined in the type of oligosaccharides attached to individual proteins within a given superfamily. The glycan data from this study, together with the 3D structures of proteins and oligosaccharide structural database, were utilized to construct molecular models of the studied glycoproteins and examine the relative importance of local protein surface properties against the overall tertiary structure of the molecule in directing this protein-specific variation. The results indicated that both the overall organization of the domains and the local protein structures can have a large influence on the site-specific glycan modification of cells in stasis.[225]

In another study, the same group utilized this methodology for characterizing the O- and N-glycosylation of both natural human neutrophil gelatinase B and neutrophil gelatinase B-associated lipocalin (NGAL).[226] Gelatinase B is a matrix metalloproteinase involved in tissue remodeling, development,

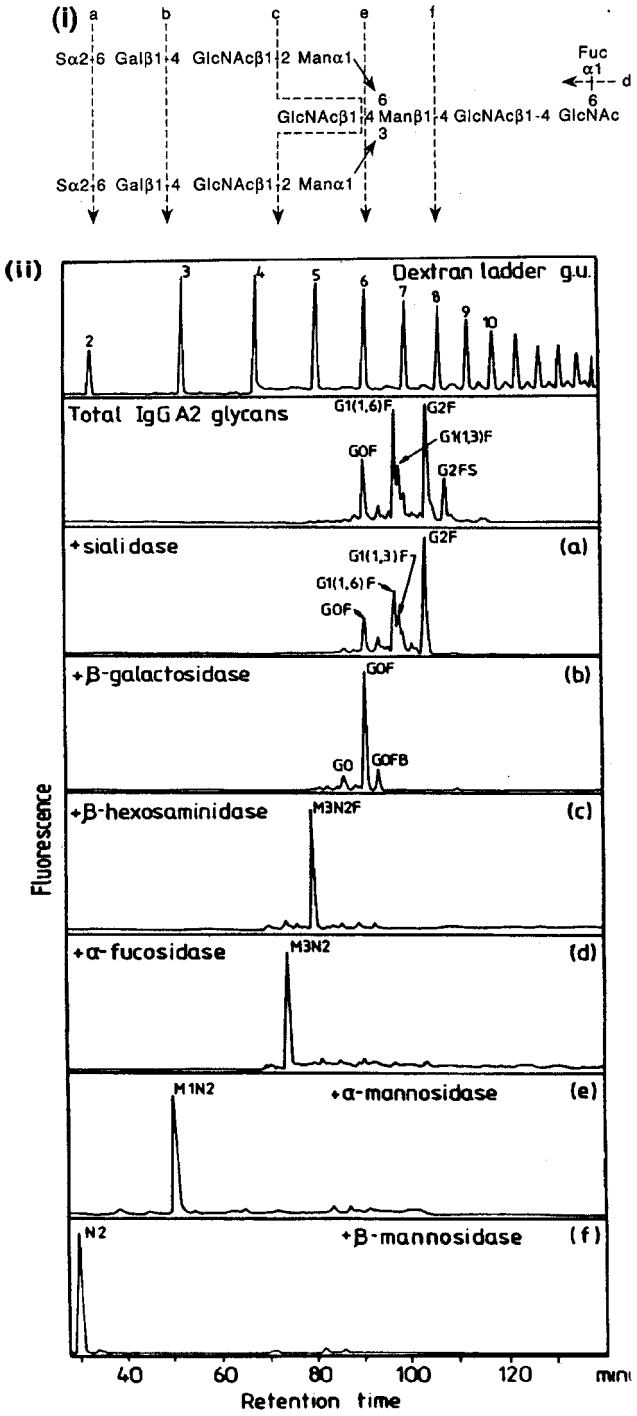


Figure 5. (i) The structure of a fully processed complex glycan associated with normal human serum IgG. The largest glycan associated with this IgG is indicated. All other IgG structures can be derived by removing residues from this fully sialylated, core-fucosylated glycan, which contains a bisecting GlcNAc residue. (ii) Sequential exoglycosidase digestions of the pool of glycans released from normal IgG, as monitored by HPLC. The first panel shows the dextran ladder used to calculate the GU values; a−f show the results of incubating the glycan pool successively with the enzyme designated in each panel. (Reprinted with permission from ref 222. Copyright 1996 Publisher.)

cancer, and inflammation. The methodology allowed a simultaneous determination of *O*- and *N*-glycan pools derived from the two glycoproteins. The structures of more than 95% of the N-linked glycans attached to both gelatinase B and NGAL were found

to be partially sialylated, core-fucosylated, biantennary structures with and without an outer-arm fucose. On the other hand, the O-linked glycans, which were estimated to constitute approximately 85% of the total sugars on gelatinase B, were mainly type-2 cores with Galβ1−4GlcNAc extensions, with or without a sialic acid or an outer-arm fucose. It was also determined that the *O*-glycans in NGAL were significantly smaller than those of gelatinase B.

The methodology was also utilized to compare N-glycosylation of a recombinant HIV evelope glycoprotein (gp160s-MN/LAI) purified by two different processes.[227] HILIC was performed on a secondary-amine stationary phase. The study revealed the presence of charged glycans, accounting for 77% and 80% of the total glycans from the glycoprotein purified by two different procedures. No differences were found in the glycan distributions from the two preparations in either their degree of sialylation or the relative proportion of each separated structures.

In a series of communications, Camilleri and coworkers reported on the utility of HILIC in the fractionation of 2-aminoacridone or 3-acetamido-6-aminoacridine derivatives of complex glycans released from glycoproteins.[83,186,228−231] The method devised in these communications was based on the labeling of released oligosaccharides with one of the fluorescent reagents, prior to fractionation on a polymeric amino column and a subsequent detection with a scanning fluorescence detector or MALDI/MS. This method allowed characterization of several glycoproteins, including ribonuclease B,[229] hen ovalbumin,[186,228] porcine thyroglobulin,[230] human IgG,[186,229,231] ovomucoid,[83] transferrin,[83] a recombinant glycoprotein,[83] and therapeutic Fc fusion protein.[186]

Birrell et al.[229] developed a protocol to characterize glycans that involves derivatization with 2-aminoacridone and coinjection with a dextran ladder derivatized with methyl 4-aminobenzoate (M-4AB). These two derivatizing agents have different UV absorbance and fluorescence characteristics. A HILIC separation followed by UV and fluorescence detection in series allowed a simultaneous analysis of the two mixtures of separately derivatized carbohydrates without interference. Using M-4AB dextran ladder derivatives as internal standards throughout the entire chromatogram allowed an accurate and detailed comparison of glycosylation profiles. A number of standard glycans were derivatized with 2-AMAC and coinjected with the M-4AB-labeled dextran ladder. The glucose unit (GU) values were subsequently calculated and used to determine the "monosaccharide rule",[222] i.e., the increment in GU value per additional sugar unit. The protocol was effective in characterizing *N*-glycans derived from ribonuclease B and human IgG.[229]

Under ionic suppression conditions, the ability of an amino-bonded column to separate sulfated oligosaccharides was recently tested by Thomson et al.[232] Two mixtures of sulfated oligosaccharide alditols from porcine stomach and the large intestine were analyzed by HILIC connected to a Q-TOF instrument. The sulfated oligosaccharides were characterized through tandem MS. Nine sulfated mucin-derived oligosaccharide alditols from porcine stomach were subsequently sequenced. The fragmentation generated primary, secondary, and tertiary fragment ions that are informative in the elucidation of saccharide sequence and localization of sulfate groups. The sequence of 28 different sulfated mucin oligosaccharide alditols, which had been purified from the porcine large intestine, was determined from a single chromatographic analysis.

Morelle and Strecker utilized HILIC on an amino-bonded column to fractionate *O*-glycans obtained by β-elimination from the oviducal mucins of a toad (*Bufo bufo*[233]) and *Rana utricularia*.[234,235] The *O*-glycans were characterized by one- and two-dimensional ^{1}H NMR spectroscopy and MALDI/MS; therefore, the different structures had to be resolved to permit such characterization. The use of this chromatographic method also allowed characterization of 12 oligosaccharide alditols derived from the jelly coat of *Rana utricularia*, which were either core-1 or core-2 type.[234] With a species, 10 more individuals were determined to have either core-1 or core-2 type glycans.[235] The method was further extended toward characterization of the oligosaccharide alditols released from *B. bufo*.[233] Thirty-four compounds, ranging in size from a trisaccharide to a dodecasaccharide, were fractionated using the same column.

2. Reversed-Phase LC

The hydrophobic interactions in LC, most commonly practiced with octadecyl-substituted siliceous packings, have been the most popular means of HPLC for a long time. However, appreciation for this separation mode in the analysis of oligosaccharides came relatively late.[233,236] Due to the polarity of underivatized oligosaccharides, only weak interactions are experienced with typical reversed-phase packings. Using water as the mobile phase, more polar oligosaccharides elute before the less polar ones.[237] Retention can be generally increased through addition of salts that modify the surface-penetrating characteristics of the mobile phase and enhance separations.

The retention of underivatized sugars in reversed-phase LC (RP-LC) roughly increases with molecular mass.[238] Moreover, some structural aspects modify appreciably retention, as is particularly evident in the case of branched oligosaccharides. Accordingly, it was observed that the retention times for branched oligosaccharides were shorter than those of the linear counterparts.[239] The presence of acetoamido sugars renders an oligosaccharide more hydrophobic, increasing retention on a reversed-phase packing using water as the mobile phase.[240] Fairly detailed reviews discussing the analytical utility of RP-LC for oligosaccharides were recently published.[214,241]

Although RP-LC with plain water as the mobile phase was found useful in the separation of O-linked oligosaccharide alditols derived from human meconium glycoproteins,[242] sample derivatization enhances the scope of analysis substantially. While the major reason for derivatization is to improve detection through the attachment of a chromophore or a

fluorophore, it can also render the solutes more hydrophobic and retainable in RP-LC.

Since its introduction in 1978 by Hase et al.,[243] reductive pyridylamination has been employed extensively for the RP-LC analysis of glycans cleaved from various glycoproteins. The method is based on the labeling of glycans with 2-aminopyridine (2-AP) by reductive amination prior to the analysis. Initially, this method was demonstrated for characterization of the glycans cleaved from as little as 10 μg of taka-amylase A.[244] However, a further optimization of the labeling conditions permitted the analysis of pyridylamino derivatives of glycans cleaved from as little as 0.15 nmol of taka-amylase A or bovine submaxillary mucin.[245] In a different study, the same group characterized *N*-glycans cleaved from a protease inhibitor of Barbados pride (*Caesalpinia pulcherrima*) seeds utilizing the same method.[246] The study employed stepwise exoglycosidase digestions and 500 MHz ^{1}H NMR spectroscopy to determine the structures of resulting 2-AP-glycans.

Tomiya et al.[247] devised a two-dimensional sugar mapping method as a simple, reproducible and sensitive means of *N*-glycan structural analysis. The method was based on the labeling of *N*-glycans with 2-AP and the separations by HILIC (on amide-bonded column) and RP-LC with octadecyl column. The database for the map was constructed through the use of 113 standard oligosaccharides; 58 structures were confirmed through ^{1}H NMR spectroscopy. The map was constructed by plotting the glucose units determined on the octadecyl column (*x*-axis) against those obtained on the amide-bonded column (*y*-axis). The GU values of the unknown samples were compared to those acquired with standards. The method also involved a sequential enzymatic digestion with exoglycosidases, together with HILIC and RP-LC analysis of the 2-AP-labeled digest. This allowed determinaton of the shifts in GU values caused by the enzymatic digestion, thus predicting the *N*-glycan structures. The method was successfully applied to *N*-glycans derived from hen ovalbumin.

Makino et al.[248] employed the aforementioned two-dimensional sugar map in conjunction with a partial acid hydrolysis for the analysis of oligosaccharide structures from the reducing-end terminal. A 2-AP-oligosaccharide was partially hydrolyzed, with the acid hydrolysis conditions being optimized so as to obtain various 2-AP-oligosaccharide fragments with high yields from different types of 2-AP-oligosaccharides. The acid hydrolysate was then fractionated through size-exclusion HPLC, and each fraction was further analyzed through RP-LC. The structure of each 2-PA-oligosaccharide fragment was identified on a two-dimensional sugar map prepared with standard PA-sugar chains, after which the original 2-AP-oligosaccharide was reconstructed from the reducing-end terminal based on the obtained structures of 2-PA-oligosaccharide fragments. The method was successfully used to determine the structures of *N*-glycans cleaved from human IgY.

Recently, Morelle et al.[249] used 2-AP for the labeling of *O*-glycans cleaved from glycoproteins prior to their fractionation by RP-LC. The method involved treatment of the oligosaccharide alditols with sodium *m*-periodate under conditions where core *N*-acetylgalactosaminitol was specifically degraded to generate a reactive aldehyde group, allowing the labeling of the resulting fragments with 2-AP. Next, the 2-AP-labeled *O*-glycans were fractioned by RP-LC and further characterized by ESI/MS and tandem MS. The use of tandem MS provided information on the sugar sequence and branching of oligosaccharides linked at C_3 and C_6 to the *N*-acetylgalactosaminitol. This method afforded characterization of *O*-glycans cleaved from mucin. However, the approach is not practical for the analysis of sialooligosaccharide alditols, since the required mild periodate oxidation step leads to degradation of up to 60% of the sialic acid residues.[250]

Varki and co-workers[251] synthesized a fluorescent reagent, 2-amino-6-(amidobiotinyl)pyridine. This reagent was demonstrated to derivatize oligosaccharides under nondegradative conditions with high efficiency. The labeled glycans were effectively fractionated by RP-LC with sensitive detection in the low picomole range. Combining this approach with sequential exoglycosidase digestion permitted a stepwise sequencing of the sugar chains. The biotinyl group can be used to recover the sugar chain from reaction mixtures. The high-affinity interaction of biotinyl group with the multivalent avidin or streptavidin can be utilized to create a functional equivalent of neoglycoproteins carrying multiple copies of the oligosaccharides with a defined structure. These complexes allow production of IgG antibodies directed against the oligosaccharide chain. They can also harness the power of (strept)avidin−biotin technology for the detection and isolation of oligosaccharide-specific receptors from native sources or recombinant libraries.

In 1994, another labeling procedure was introduced in which the reducing end of an *N*-glycan was modified with tyrosine.[252] The reducing ends of the oligosaccharides were converted to glycosylamine and reacted with the *N*-hydroxysuccinimide ester of Boc-tyrosine. The tyrosinated oligosaccharides were completely resolved into individual peaks by RP-LC and then characterized by ^{1}H NMR spectroscopy and FAB/MS. Each product contained a single Boc-Tyr residue attached to the reducing-end GlcNAc residue through a β-glycosylamide linkage. The procedure provided a new route for the purification of large quantities of *N*-glycans that contain a terminal tyrosine residue.

In a series of communications, Camilleri and co-workers[253−257] introduced the utility of 2-aminoacridone (AMAC) derivatizing agent for the characterization of *N*-glycans through RP-LC and MS. The used AMAC derivatizing agent is very hydrophobic and thus well-separated from the glycan derivatives, preventing interference from the excess reagent. RP-LC of AMAC-labeled *N*-glycans, in conjunction with MALDI and nano-ESI Q-TOF/MS, allowed characterization of the glycans cleaved from ribonuclease B,[253,255,256] human IgG,[255,257] α_1-acid glycoprotein,[257] and human transferrin.[254]

The mixtures of *N*-glycans released from α_1-acid glycoprotein and IgG were derivatized with AMAC.[257] The RP-LC profile obtained for the derivatized glycans in each case were compared to those obtained after digestion with sialidase and a two-enzyme array system made up of sialidase and α-fucosidase. These studies provided significant preliminary information about the degree of sialylation and core fucosylation in the corresponding parent glycans. Collection of glycans from a single injection was sufficient for molecular-weight determination by MALDI/MS. Additional insights into the structure of glycans fractionated by RP-LC were obtained through tandem MS using a nano-ESI/Q-TOF/MS. In another study by the same group, the AMAC-labeled glycans derived from ribonuclease B and IgG were fractionated with capillary RP-LC.[255] The fractions were robotically collected onto MALDI targets; MS information was acquired for the carbohydrates present at low picomole levels.

The same group synthesized a new fluorescent derivatizing agent, 3-(acetylamino)-6-aminoacridine (AA-Ac), to be employed for the analysis of picomole levels of *N*-glycans.[258] AA-Ac was determined to be an effective derivatizing reagent for the purpose, giving at least twice the fluorescence intensity of its predecessor, AMAC. AA-Ac-labeled glycans were successfully analyzed by both HILIC and RP-LC. They were also amenable to enzymatic sequencing and analysis by MALDI/MS and ESI/MS. The effectiveness of this derivatizing agent was illustrated for the analysis of *N*-glycans derived from ribonuclease B and IgG.

Recently, RP-HPLC was combined with the multiple-stage mass analysis capability of an ion-trap mass spectrometer to deal with permethylated oligosaccharide mixtures.[259] The method was employed to separate the components of a complex mixture of 2-aminobenzamide-labeled bi-, tri-, and tetraantennary standards and a mixture of *N*-glycans cleaved from the recombinant soluble CD4 glycoprotein. Permethylated mixture components including α- and *β*-anomers were separated by RP-LC on the basis of their structure. Fluorescent labeling with 2-aminobenzamide prior to permethylation was not required for the MS analysis, since the ionization and fragmentation characteristics of the molecules were improved by permethylation alone. The antennal composition of permethylated derivatives was determined in MS^2, where the fragmentation patterns of the Y- and B-ion series predominated. Additional information pertaining to branching was obtained from A and X cross-ring fragmentations observed in MS^3.

3. HPLC Using Graphitized Carbon Columns

Graphitized carbon columns (GCC) for HPLC are relatively new, having a unique ability to resolve isomeric and closely related compounds. Carbohydrate retention on GCC appears primarily due to adsorption. The unique selectivity of GCC and their unmatched ability to resolve isomeric and closely related compounds are brought about by the homogeneous adsorptive nature of this material.

The hydrophobicity of GCC is greater than what is observed with reversed-phase materials. Thus, a relatively large percentage of organic solvent in the mobile phase is required to assist the elution of carbohydrates. Another major advantage of GCC is their ruggedness, which apparently allows a repeated use without loss of performance or reproducibility. Moreover, these columns are unaffected by strongly acidic or alkaline conditions and can be used throughout the entire pH range with a wide range of solvents. Different aspects of this mode of chromatography have been addressed recently in two communications.[217,260]

Koizumi et al.[261] reported for the first time the chromatographic behavior of several mono- and disaccharides and cyclomaltoses on Hypercarb, which is the first commercially available graphitized carbon column manufactured by Shandon Scientific (Cheshire, UK). While monosaccharides were weakly retained on this column, it was feasible to accomplish anomeric separation of all solutes. The column was also effective in separating several disaccharides, cyclomaltohexaoses, cyclomaltoheptaose, and cyclomaltooctaose.

Next, Hounsell and co-workers[262] reported on the chromatographic behavior of oligosaccharide alditols and glycopeptides containing neutral and acetamido sugars and a sialic acid. The authors exploited the high resolving power of GCC for the analysis of oligosaccharide alditols derived from meconium glycoproteins. The alditols were substantially retained to be eluted with 0−25% acetonitrile/0.05% trifluoroacetic acid in 0.05% aqueous trifluoroacetic acid between 3 and 30 min. The separation was based on size, charge, and linkage, so that various isomeric compounds could be separated from each other. The same group also reported on the ability to use GCC for the analysis of *N*-glycans derived from glycoproteins.[263] The method allowed simultaneous separations of peptides, glycopeptides, reducing oligosaccharides, sialylated oligosaccharides, and oligosaccharide alditols under the same conditions. The salt-free mobile phase utilized with GCC allowed an off-line, sensitive liquid secondary-ion mass spectrometric (LSI/MS) analysis. Although LSI/MS revealed coelution of oligosaccharides of the standard glycoproteins (fetuin and bovine submaxillary mucin), GCC could still be considered as a useful complement to other HPLC profiling and separation techniques.

Recently, the chromatographic behavior of N-linked glycans derived from ribonuclease B and the glycopeptides generated through pronase digestion of ribonuclease B and soybean agglutinin was compared with GCC and C_{18} columns.[264] The glycopeptides derived from soybean agglutinin were not retained on a C_{18} column but could be separated on GCC with a gradient of 10−45% aqueous acetonitrile in 30 min. While the ribonuclease B glycopeptides obtained by pronase digestion were separated on GCC with a gradient of 10−30% acetonitrile, they were not retained on a C_{18} column, even with water used as the eluant. The N-linked glycans, enzymatically released from ribonuclease B, were separated from each other with a linear gradient of 10 mM am-

monium hydroxide to 10 mM ammonium hydroxide with 12.5% acetonitrile in 50 min at 70 °C. Most silica-based reversed-phase columns cannot withstand such an alkaline mobile phase.

The effectiveness of GCC in resolving the oligosaccharide branching isomers was demonstrated.[265] A mixture of two high-mannose isomers (Man_6-GlcNAc) from human IgM, as was determined through ^1H NMR spectroscopy, was completely resolved by GCC−HPLC. Also, Man_{10}-GlcNAc and Man_{11}-GlcNAc species from a recombinant invertase were resolved into three and five fractions, respectively, by GCC−HPLC, in agreement with the number of isomeric forms determined by one- and two-dimensional ^1H NMR spectroscopy. According to the authors, GCC−HPLC should prove invaluable in preparation of singular oligosaccharides to define exoglycosidase and glycosyl transferase branch specificity and for preparing the standards to develop more sensitive methods for structural elucidation of oligosaccharides.[265]

Recently, the interfacing of GCC to ESI/MS for the analysis of carbohydrate heterogeneity in a glycoprotein[266] and sulfated oligosaccharides from mucin glycoproteins[267] was discussed in two communications. As mentioned above, the salt-free mobile phase usually used with GCC is very advantageous for on-line MS analysis. Kawasaki et al.[266] investigated the utility of GCC−HPLC with on-line ESI/MS for the analysis of carbohydrate heterogeneity using ribonuclease B as a model glycoprotein. The oligosaccharides enzymatically released from the glycoprotein were reduced and separated on GCC. GCC−HPLC/MS in the positive-ion mode allowed a successful identification of one Man_5GlcNAc, three Man_6GlcNAc, three Man_7GlcNAc, three Man_8GlcNAc, one Man_9-GlcNAc, and an oligosaccharide having six hexose residues and two *N*-acetylhexosamine residues. Tandem MS permitted characterization of the branch structures of the three Man_7GlcNAc isomers. Moreover, LC−MS/MS analysis proved invaluable in detection and identification of the trace amount of $Hex_6HexNAc_2$ alditol as a hybrid oligosaccharide. Its structure was further confirmed by the use of LC−MS in conjunction with exoglycosidase digestions (using β-galactosidase and *N*-acetyl-β-glucosaminidase).

Thomsson et al.[267] compared the effectiveness of GCC−HPLC and HILIC in coupling to ESI/MS in the analysis of sulfated oligosaccharides derived from mucin glycoproteins from porcine stomach and colon. The two columns demonstrated different selectivities toward sulfated oligosaccharide alditols, and were both effective in resolving the mixtures. The use of both columns, coupled to ESI/MS in conjunction with the in-source collision-induced dissociation (CID), permitted a complete characterization.

4. High-Performance Anion-Exchange Chromatography

High-performance anion-exchange chromatography (HPAEC), coupled to pulsed amperometric detector (PAD), is now a time-honored method of carbohydrate analysis. HPAEC−PAD has several assets: (i) analysis speed; (ii) adequate separation of anomeric, structural, linkage, and branch isomers; and (iii) fairly high sensitivity without pre- or postcolumn solute derivatization.

The separation in HPAEC is based on ionic interactions between the negatively charged oxyanions of carbohydrates, which result from the use of strongly alkaline mobile phase (pH >12), and the anion-exchange resins. The strongly alkaline mobile phase is also needed to facilitate the oxidation reaction at the working gold electrode of PAD. However, the use of an alkaline mobile phase also bears disadvantages, as it induces side reactions. At pH 12, the equilibrium of epimerization of GlcNAc to ManNAc is virtually attained within an hour. Therefore, large oligosaccharides containing GlcNAc or GalNAc as their reducing terminus can suffer epimerization or degradation. This has prompted the reduction of alkali-sensitive oligosaccharides prior to HPAEC−PAD analyses to eliminate such possibilities.

Although quantification by PAD is very convenient, the molar response of analytes measured with PAD is dependent on the structures and varies from one compound to another. Therefore, accurate (absolute) quantification of unknown oligosaccharides can be quite difficult, if not impossible. Another disadvantage of HPAEC−PAD is the high-salt content commonly used with this methodology. Most certainly, this form of chromatography cannot be directly coupled to MS. However, several desalting approaches have reduced the general difficulties of off-line MS. When the salt content is not too high, an on-line membrane desalting device may also be satisfactory.[268−270] The commonly practiced methods for desalting the HPAEC fractions containing high levels of salts are dialysis, ion-exchange, and gel filtration. However, the risk of losing the sample as a result of this excessive sample handling is high, thus making it impossible to desalt small quantities of sample. Despite some of these difficulties, HPAEC−PAD has been used extensively for the analysis of N- and O-linked glycans released from sufficient amounts of glycoproteins. Different aspects of HPAEC−PAD have been addressed in a recently published review.[20]

Hardy and Townsend were the first to explore systematically the separation power of HPCE-PAD in the analysis of carbohydrates.[20] They succeeded in separating neutral oligosaccharides according to their molecular weight, sugar composition, and linkage of the monosaccharides residues. The method allowed resolution of 1 → 3, 1 → 4, and 1 → 6 positional isomers of neutral oligosaccharides that have the same number, sequence type, and anomeric configuration of monosaccharides but feature differences in linkage position of a single sugar. Moreover, the study also demonstrated high retention of reducing oligosaccharides relative to their reduced counterparts and a substantial decrease in retention time as a result of the presence of a Fuc(α1−3) linkage to GlcNAc. The authors also reported the effectiveness of this methodology in resolving N-linked glycans derived from glycoproteins and glycopeptides. However, the separation of only two triantennary oligosaccharides derived from fetuin was demonstrated.

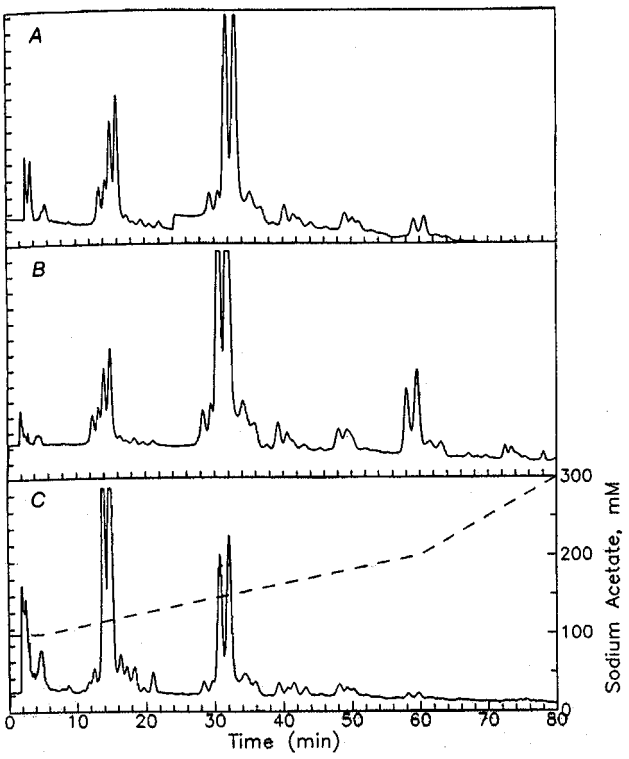


Figure 6. HPAEC−PAD of oligosaccharides released from bovine fetuin purified differently or obtained from different commercial sources. Oligosaccharides were released from Sigma Type III (A), Sigma Type IV (B), and Calbiochem (C) bovine fetuin. (Reprinted with permission from ref 271. Copyright 1989 Publisher.)

The same group communicated a more elaborate report demonstrating a high resolving power in the analysis of N-linked glycans derived from glycoproteins.[271] Ten sialylated oligosaccharides from bovine fetuin were analyzed. At near-neutral pH, oligosaccharides were separated according to their number of formal negative charges due to sialic acids; however, at alkaline pH, resolution was enhanced by the oxyanion formation, which induced a stronger interaction with the column packings. It was observed that triantennary oligosaccharides containing a Galβ-(1−3)GlcNAc sequence were more retained than those having Galβ(1−4)GlcNAc, with a consequent resolution from each other. Also, the oligosaccharides having their sialic acid residues as α(2−6)-linked were less retained than those having sialic acid residues in an α(2−3) linkage. HPAEC−PAD was also demonstrated to be a powerful tool in comparing the N-linked glycan profiles of different glycoproteins or the same glycoproteins from different sources. Figure 6 illustrates the oligosaccharide profiles obtained from fetuin that was purified using different methods and originated from different commercial sources. Figure 6A is the oligosaccharide profile obtained through the ammonium sulfate precipitation method, while Figure 6B shows oligosaccharides derived from the same type of fetuin that was subjected to a Sephadex G-75 purification to remove the high molecular weight contaminants. The oligosaccharide profile, shown in Figure 6C, was from fetuin purified by the ammonium sulfate precipitation method, but originating from a different source. The relative proportions of the major classes of sialylated oligosaccharides (bi, tri, tetra, and penta) varied significantly, depending on the source or purification technique.

HPAEC−PAD, when used in conjunction with other analytical techniques, such as specific endoglycosidase digestions, other HPLC techniques, FAB/MS, and ^{1}H NMR spectrometry, was utilized to determine the carbohydrate structures of recombinant human tissue plasminogen activator expressed in Chinese hamster ovary cells.[272] In this study, HPAEC−PAD permitted determination of the distribution of oligosaccharide structures at individual glycosylation sites. The oligosaccharides released from each glycosylation site were analyzed. This important methodology has been routinely used for the profiling of N- and O-linked glycans derived from glycoproteins such as mucin,[273−275] human IgG,[276] human α_1-antitrypsin,[276] human transferrin,[276,277] fetuin,[277] α_1-acid glycoprotein,[277,278] recombinant erythropoietin,[279] gastric H^+,K^+-ATPase,[280] recombinant plaminogen activator,[280] and bile-salt-stimulated lipase from human milk.[281,282]

Rice et al.[279] combined HPAEC−PAD with the two-dimensional quantitative mapping of 2-AP derivatized oligosaccharides (discussed in a previous section)[247] to analyze the N-linked glycans derived from recombinant erythropoietin (EPO). The overall approach involved fractionation of the enzymatically released N-linked glycans by HPAEC−PAD, followed by derivatization of the fractions with 2-AP prior to the two-dimensional HPLC mapping. Seven complex-type asialooligosaccharides, ranging from a biantennary structure to *N*-acetyllactosamine-extended tetraantennary structure, were identified. The method also allowed monitoring of the N-linked glycan constituents of EPO from different lots.

Weitzhandler et al.[280] demonstrated the applicability of HPAEC−PAD for the oligosaccharide mapping of glycoproteins separated by SDS−PAGE and transferred to polyvinylidene fluoride membranes. The method involved various endoglycosidases that release specific oligosaccharide types from SDS−PAGE-separated and membrane-transferred glycoproteins. The released N-linked glycans were then fractionated by HPAEC−PAD, with a further characterization through exoglycosidases and monitoring of their actions. This method was shown to be applicable to a variety of soluble glycoproteins, as well as to the membrane-bound protein gastric H^+,K^+-ATPase.

Barr et al.[201] developed a strategy for determining the structural types, branching types, and molecular microheterogeneity of N-linked glycans at specific attachment sites in glycoproteins. The method combined MS and HPAEC−PAD to take advantage of their high sensitivity and capability for the analysis of complex oligosaccharide mixtures. First, glycopeptides were identified and isolated by a comparative HPLC mapping of the proteolytic digests of proteins before and after an enzymatic release of N-linked glycans. The attachment sites on the isolated glycopeptides were determined by fragmentation using FAB/MS. Parts of the enzymatically released N-linked glycans were permethylated and analyzed by

FAB/MS to identify the composition and molecular heterogeneity. Fragmention of permethylated glycans in FAB/MS allowed linkage determination. The structural type of the carbohydrate at each attachment site was determined by FAB/MS. The remaining portions of released carbohydrates from a specific attachment site were preparatively fractionated by HPAEC−PAD, permethylated, and analyzed by FAB/MS. These analyses determined the charge state and composition of each fractionated peak. Permethylation of the HPAEC−PAD fractions was only possible after their exhaustive desalting. This is a major disadvantage of HPAEC−PAD, due to unavoidable sample losses.

Recently, a new desalting device for the on-line coupling of HPAEC−PAD with ESI/MS was described in two separate communications,[283,284] which detailed a design difference from the previous work.[268−270] The new desalting device is based on on-line microdialysis utilizing a cation-exchange membrane unit that permits the exchange of sodium ions with hydronium ions (for sodium ion concentrations up to 600 mM). An effective on-line desalting of the effluent before its introduction into the mass spectrometer was achieved through electrolysis of water with 500 mA current. The unit coupled to ion spray eliminated the need for a booster pump (usually used with the previous device), while the use of water instead of sulfuric acid improved sensitivity.

5. Lectin Affinity Column Chromatography

As described earlier, lectin chromatography is now commonly used for isolation and purification of glycoproteins from biological samples. During recent years, the carbohydrate specificities of various lectins were also exploited for analytical purposes. The approach is based on the use of a series of immobilized lectin columns with the known oligosaccharide-binding specificities and the subsequent fractionation of small amounts of radioactively labeled oligosaccharides or glycopeptides into structurally distinct groups. Accordingly, the serial lectin affinity chromatography, when used in conjunction with some other separation techniques, can purify oligosaccharides and yield substantial information about their nature. This methodology has been dealt with in several reviews.[88,92,216]

Since its introduction 2 decades ago by Cummings and Kornfeld,[285] over 40 studies have utilized serial lectin columns for the fractionation of complex N-linked glycan mixtures derived from various glycoproteins. Cummings and Kornfeld not only demonstrated the effectiveness of this methodology for the estimation of the entire spectrum of N-linked glycans synthesized by the lymphoma cell line but also suggested a general-purpose scheme employing various lectins. Next, the approach was utilized to compare the glycosylation of the two polypeptide chains of murine Ia antigens.[286] Fractionations and characterization were performed on the *N*-glycan structures derived from human immunoglobulin G,[287] human von Willebrand factor,[288] equine chromnicgonadotropin and lutropin,[289] natural and recombinant blood coagulation factor VIII,[290] rat liver β-glucuronidase,[291] human urinary kallikrein,[292,293] rat transferrin,[294] human serum transferrin,[199] human placental fibronectin,[295] human and rabbit testosterone-binding globulin,[296] natural and recombinant human interferon-β1,[297] recombinant human interleukin V,[298] recombinant human prorenin,[299] human leukocyte common antigen CD45,[300] human pancreatic bile-salt-dependent lipase,[301] zona pellucida 2 and zona pellucida 3 glycoproteins from mouse,[302] and human intercellular adhesion molecule-3 (CD50).[303]

Differences among rat alkaline phosphatases from various organs were established by the serial lectin affinity technique.[304] It was also used to determine glycosylation of the proteins attached to the cell surface of herpes virus-infected cells,[153] and in exploring whether thyrotropin-releasing hormone alters the carbohydrate structures of secreted mouse thyrotropin.[305] The sugar-chain heterogeneity of γ-glutamyl transferase from the human reproductive system (seminal plasma, prostate, and testis) and kidney was investigated by the same approach.[306] Several comparative studies of the N-linked glycans released from glycoproteins isolated from normal and carcinoma human cells were also performed.[217,218,307−309]

B. Capillary Electrophoresis

Several types of capillary electromigration techniques have become a significant part of carbohydrate analysis during the past decade: (a) capillary zone electrophoresis, using either free-buffer media or gels; (b) micellar electrokinetic chromatography (MEKC); and (c) capillary electrochromatography (CEC). The benefits of such techniques (resolution and speed of analysis) can only be derived when used in conjunction with sufficiently sensitive detectors. While still utilized in several applications, as listed below, the sensitivity of UV absorbance is marginal for carbohydrates, and fluorescence-tagging procedures are generally recommended.

The *N*-glycans enzymatically released from recombinant tissue plasminogen activator were separated through MEKC using sodium dodecyl sulfate (SDS) surfactant and detected by direct UV absorbance at 200 nm.[310] The best separation capacity was observed upon the addition of a divalent ion (Mg^{2+}) to the SDS electrolyte. The same group utilized this electrolyte system to study the difference between the oligosaccharide distribution of two rtPA variants.[311]

Hermentin et al.[312] developed a carbohydrate-mapping database that enables structural analysis through a simple comparison of migration times. Eighty native sialooligosaccharides derived from glycoproteins were separated and detected by CE at 194 nm to establish the database. The structures of *N*-glycans pooled from various glycoproteins such as recombinant human urinary erythropoietin, bovine fetuin, and α_1-acid glycoprotein were deduced from the database. However, this mapping database is only useful for structural determination of sialylated structures.

Native *N*-glycans derived from various immunoglobulin G antibodies and CTLA4Ig, a biologic fusion protein, were analyzed by CE using a phytic acid−

Table 3: Analysis of *N*-Glycans by HPCE

derivatizing agent	glycoprotein	HPCE mode	detection	ref
2-aminopyridine (2-AP)	ovalbumin	CZE	fluorescence	315, 321
	ribonuclease B	CZE	UV	316
6-aminoquinoline (6-AQ)	ribonuclease B	CZE	UV	316
3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA)	fetuin	CZE	LIF (argon ion laser)	317
8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS)	human immunoglobulin G	CZE	LIF (HeCd laser)	319
	ovalbumin	CZE	LIF (HeCd laser)	319, 321
	fetuin	CZE	LIF (HeCd laser)	320, 322
	ribonuclease B	CZE	LIF (HeCd laser)	318
	recombinant HIV gp-120	CZE	LIF (HeCd laser)	322
	α_1-acid glycoprotein	CZE	LIF (HeCd laser)	322
2-aminonaphthalene-1-sulfonic acid (ANS)	ribonuclease B	CZE	LIF (HeCd laser)	318
7-aminonaphthalene-1,3-disulfonic acid	ribonuclease B	CZE	LIF (HeCd laser)	318
1-aminopyrene-3,6,8-trisulfonic acid (APTS)	ribonuclease B	CGE	LIF (argon ion laser)	323, 326
	fetuin	CGE	LIF (argon ion laser)	324, 325
	ribonuclease B	CZE	LIF (argon ion laser)	327, 328
	fetuin	CZE	LIF (argon ion laser)	327, 328
	ribonuclease B	CZE	UV 254 nm off-line MALDI/MS	329
	recombinant human erythropoietin	CZE	LIF (argon ion laser)	328
	kallikrein	CZE	LIF (argon ion laser)	328
	chimeric recombinant monoclonal antibody	CZE	LIF (argon ion laser)	330
2-aminobenzamide (AB)	ribonuclease B	MEKC	UV 254 nm	334
	α_1-acid glycoprotein	MEKC	UV 254 nm	334
2-aminoacridone (AMAC)	ovalbumine	MEKC	LIF (argon ion laser)	331
	fetuin	MEKC	LIF (argon ion laser)	331, 332
	ribonuclease B	MEKC	LIF (argon ion laser)	331, 256
	IgG antibodies	MEKC	LIF (argon ion laser)	332
	ribonuclease B	ultrahigh voltage MEKC	LIF (442 nm HeCd laser)	333
	human IgG	ultrahigh voltage MEKC	LIF (442 nm HeCd laser)	333
7-nitro-2,1,3-benzoxadiazole (NBD)	fetuin	MEKC	LIF (argon ion laser)	335

borate buffer with UV detection at 200 nm.[313] Resolution between the *N*-glycans in complex mixtures was substantially improved by the presence of phytic acid, an ion-pairing agent.

Since *O*-glycans are released chemically through β-elimination (producing nonreducing sugars), only one communication reported the analysis of *O*-glycans by CE. Alditol derivatives of the *O*-glycans derived from bovine submaxillary mucin and swallow nest material were analyzed by CE with UV detection at 185 nm.[314] The study demonstrated only minor differences between the use of phosphate or borate buffers as background electrolytes, suggesting no significant contributions of borate complex formation. *N*-acetyl- and *N*-glycolylneuraminic acid-containing oligosaccharide pairs were only resolved from each other using MEKC with SDS micelles.

The analysis of *N*-glycans by CE has most often been done through solute labeling with a chromophore or fluorophore. Since not all *N*-glycans possess a charge, the derivatization is also expected to introduce ionic groups needed for migration. However, some neutral derivatizing agents have also been utilized, but only if used in the MEKC mode or with a borate buffer that permits complexation with oligosaccharides, imparting the charge to the neutral structures.

In 1990, Honda et al.[315] demonstrated for the first time the high resolving power of CE in the mapping of *N*-glycans chemically cleaved from ovalbumin. The analyte molecules were labeled with 2-aminopyridine (2-AP) via reductive amination, separated, and detected by fluorescence. Although the actual amount analyzed in the capillary tube was quite small (ca. 5 ng), a large amount (ca. 25 mg) was required to make the sample concentration sufficiently high to be detected. Nashabeh and El Rassi[316] compared the detection limits attained with 2-AP and 6-aminoquinoline derivatives of the ribonuclease B associated *N*-glycans using a UV detector. 6-Aminoquinoline provided a signal that was 8 times higher than with 2-AP.

Novotny and co-workers reported a substantial improvement in detectability, utilizing 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA) as a derivatizing agent in conjunction with argon ion laser-induced fluorescence.[317] This allowed separation and detection of *N*-glycans derived from bovine fetuin at attomole levels. The CBQCA-labeled *N*-glycans were resolved into four major (expected) peaks as well as some minor peaks.

Since then, additional efforts have been concentrated on exploring various ways of derivatizing sugars for a high-sensitivity characterization of *N*-glycans by CE. Table 3 summarizes different chemical approaches together with pertinent references.

The role of charged groups in the derivatizing molecules in the CE analysis of *N*-glycans was investigated using oligosaccharides derived from ribonuclease B.[318] The labeling agents included in

this study were 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS), 7-aminonaphthalene-1,3-disulfonic acid (ANDS), and 2-aminonaphthalene-1-sulfonic acid (ANS). As expected, a greater charge caused faster analyses and higher resolution, making ANTS the most effective derivatizing agent for the CE analysis of *N*-glycans derived from glycoproteins. Additional glycans that were derived from other glycoproteins were successfully labeled with ANTS and separated by CE, including human immunoglobulin G,[319] ovalbumin,[320,321] fetuin,[320,322] recombinant HIV gp-120,[322] and α_1-acid glycoprotein.[322] ANTS is commonly used for labeling *N*-glycans for polyacrylamide slab gel electrophoresis, in the methodology now commercially known as fluorophore-assisted carbohydrate electrophoresis (FACE). The analysis of *N*-glycans derived from glycoproteins by CE and FACE was recently reported.[322] CE has several advantages over FACE, including automated sample handling, short run times, high resolution, high sensitivity, and a low sample consumption.

Although ANTS has been effective in labeling *N*-glycans for CE with laser-induced fluorescence (LIF), it requires the use of a He−Cd laser, which is more operationally expensive and less stable than the argon ion laser, another convenient light source. Therefore, additional derivatization agents, such as 1-aminopyrene-3,6,8-trisulfonic acid (APTS), have been sought for LIF detection of glycans.[323] APTS was utilized for the labeling of *N*-glycans released from ribonuclease B and fetuin prior to their analysis by gel CE.[323−325] It was also utilized in the analysis of *N*-glycans by others, including glycoproteins such as ribonuclease B,[326−329] fetuin,[327,328] recombinant human erythropoietin,[328] kallikrein,[328] and a chimeric recombinant monoclonal antibody.[330]

Recently, Chen and Evangelista[328] devised a complete method for the analysis of *N*-glycans derived from glycoproteins. It is based on a combination of specific chemical and enzymatic conversions coupled with CE/LIF. *N*-Glycans are released enzymatically from glycoproteins and derivatized with APTS under mild reductive amination conditions to preserve sialic acid and fucose residues. The method successfully profiled the heavily sialylated *N*-glycans.

Guttman devised a method for multistructure sequencing of *N*-glycans by gel CE and exoglycosidase digestions.[324] It involves a carefully designed exoglycosidase matrix with a subsequent comparison of the positions of the separated exoglycosidase digest fragments to maltooligosaccharides of known size, in terms of relative migration shifts. Accordingly, the appropriate linkage information can be easily deduced from the positions of separated peaks and combined with the shifts resulting from treatment with a specific exoglycosidase.

Ma and Nashabeh[330] extended the use of CE and APTS to monitor the glycosylation variations during the manufacture of rituximba, a chimeric recombinant monoclonal antibody. The *N*-glycans derived from rituximba are neutral, complex biantennary oligosaccharides with zero, one, or two terminal galactose residues (G0, G1, or G2, respectively). The method was based on releasing *N*-glycans from the glycoprotein via PNGase F and then derivatizing them with APTS prior to a CE mapping. All observed glycans were fully resolved, including the positional isomers of G1. The two G1 positional isomers were identified by comparing CE profiles obtained from sequential enzymatic digestions. *N*-Glycans were first released enzymatically by treating the glycoprotein with PNGase F; a CE profile of APTS-derivatized *N*-glycans is illustrated in Figure 7a. The *N*-glycans were further incubated with β-*N*-acetylglucosaminidase to remove the terminal GlcNAc residues on the G0 and G1 isomers, as illustrated in Figure 7b. The migration time of G2 remained unchanged, since it does not possess terminal GlcNAc. On the other hand, G0 exhibited a larger change in migration time than G1 isomers, because of the removal of two GlcNAc residues from G0 relative to one from G1 (see Figure 7b). Finally, the use of α(1−2,3) mannosidase, which specifically cleaves the terminal α(1−3) linkage, in conjunction with a change in the CE profile, indicated the predominance of G1 with a galactose residue on the mannose α(1−6) arm. The method has several advantages over other schemes, including simplicity, accuracy, precision, high throughput, and robustness.

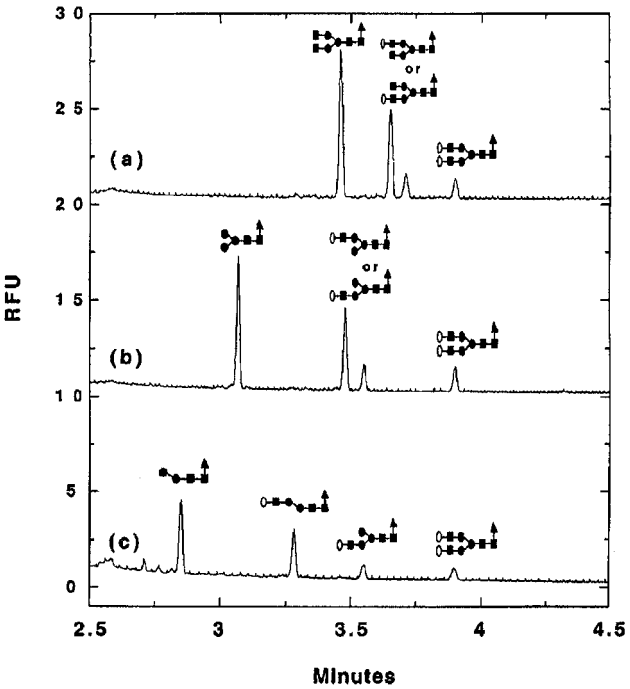


Figure 7. Electropherograms of the glycans obtained from rituximab after sequential enzymatic digestion steps: (a) PNGase F, (b) β-*N*-acetylhexosaminidase, and (c) α1−2,3-mannosidase. Samples were derivatized with APTS before CE analysis. Separation conditions: capillary, 50 μm i.d. and 27/20 cm coated; voltage, 740 V/cm. (Reprinted with permission from ref 330. Copyright 1999 American Chemical Society.)

2-Aminoacridone is yet another derivatizing agent that has been recently used for derivatization of *N*-glycans. This is a neutral fluorophore, so that a borate buffer or MEKC must be employed to allow the separation of neutral oligosaccharides. The *N*-glycans derived from glycoproteins profiled by CE using this procedure include ovalbumin,[331] fetuin,[331,332] ribonuclease B,[256,331,333] and IgG antibodies.[332,333]

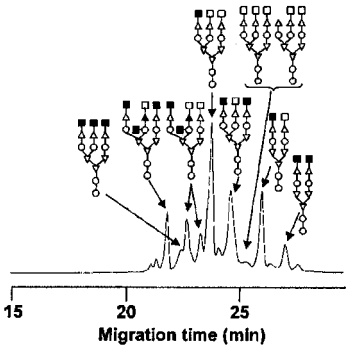


Figure 8. CE/LIFof *N*-glycans from bovine fetuin. The *N*-glycans were released from fetuin through digestion with PNGase F. The digest was derivatized, and excess substances were removed by extraction with ethyl acetate. Peaks are tentatively assigned to the structures given above. Separation conditions: capillary, 50 μm i.d. and 50 cm in length; buffer, 200 mM borate, containing 100 mM SDS, pH 10.8; voltage, 13 kV. (Reprinted with permission from ref 330. Copyright 1999 American Chemical Society.)

2-Aminobenzamide is another neutral fluorophore to be used in conjunction with MEKC or CZE (in the presence of anionic cyclodextrin as a pseudostationary phases) for profiling glycoprotein *N*-glycans.[334]

Honda et al.[335] introduced an ultramicroscale method for the analysis of carbohydrates using CE-LIF. The method is based on labeling carbohydrates through their initial conversion to *N*-methylglycamines, before tagging those products with 7-nitro-2,1,3-benzoxadiazole (NBD). The tagged carbohydrates are then analyzed by CE-LIF. Conversion of *N*-glycans to *N*-methylglycamines was achieved by incubating the sample with dimethylamine−borane complex at 40 °C. Next, *N*-methylglycamines were coupled to NBD, where the coupling was achieved in 5 min at 70 °C. Although the derivatization involves two steps, the procedure is carried out simultaneously in "one pot" without a loss in derivatization efficiency. This method allowed a high-resolution profiling of *N*-glycans derived from fetuin through the use of borate buffer (see Figure 8).

C. Capillary Electrochromatography

Although capillary electrochromatography (CEC) is gaining ground as a powerful separation technique that can combine some of the best features of HPLC and HPCE, it has not been fully exploited in the analysis of carbohydrates, in general, and glycans derived from glycoproteins, in particular. Palm and Novotny were first to illustrate the effectiveness of CEC in separating 2-aminobenzamide-derivatized maltooligosaccharides.[336] They utilized a monolithic capillary bed formed by copolymerization of polyacrylamide and poly(ethylene glycol), with added ratios of acrylic or vinyl sulfonic acid to generate the desired electroosmotic flow. Hydrophobic ligands, including C_4, C_6, and C_{12}, were introduced through various copolymerized acrylate esters. The CEC column allowed separation of labeled oligosaccharides with very high efficiency (ca. 200 000 theoretical plates/meter). The isocratic electrochromatogram of a maltose oligosaccharides mixture is illustrated in Figure 9.

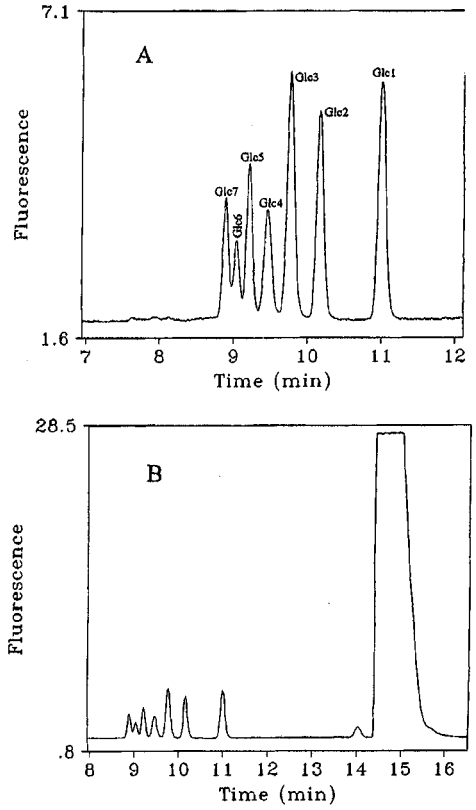


Figure 9. Isocratic electrochromatography of maltooligosaccharides (glucose (Glc1)-maltohexaose (Glc6)) in a capillary filled with a macroporous polyacrylamide/poly(ethylene glycol) matrix, derivatized with C4 ligand (15%) and containing vinylsulfonic acid (10%). 2-Aminobenzamide was used to "tag" the oligosaccharides for the laser-induced fluorescence detection. Panel B shows the same analysis as in panel A, including the peak of derivatization agent, which appears at 14−16 min. Conditions: capillary, 32 cm (25 cm effective length); field strength, 900 V/cm, 20 A. (Reprinted with permission from ref 336. Copyright 1997 American Chemical Society.)

More recently, the application of these polymer matrixes was further extended to the analysis of isomeric oligosaccharides and *N*- and *O*-glycans using amino and cyano ligands instead of the previously described hydrophobic entities. The effectiveness of interfacing CEC with MS for the analysis of oligosaccharides was also demonstrated.[32,33] Monolithic packing with a cyano or an aminoalkyl functional group allowed the separation of maltoheptaose α(1−4) and dextran DP7 α(1−6), which are linkage isomers.[32] The same packing also permitted separation of compositional isomers such as maltotetraose and stachyose. Structural isomers, anomers, and *N*-glycans derived from ribonuclease B were also successfully resolved using the same monolithic column. The electrochromatogram of *N*-glycans derived from ribonuclease B and analyzed on an aminoalkyl monolithic column is illustrated in Figure 10.

El Rassi and co-workers designed a special octadecyl-silica (ODS) stationary phase for CEC that had a limited amount of hydrocarbon coverage to leave 75% of the surface silanols unreacted, thus yielding a moderate flow without loss of the reversed-phase behavior.[337,338] This CEC packing was utilized for the analysis of *p*-nitrophenyl-labeled mono- and oligosac-

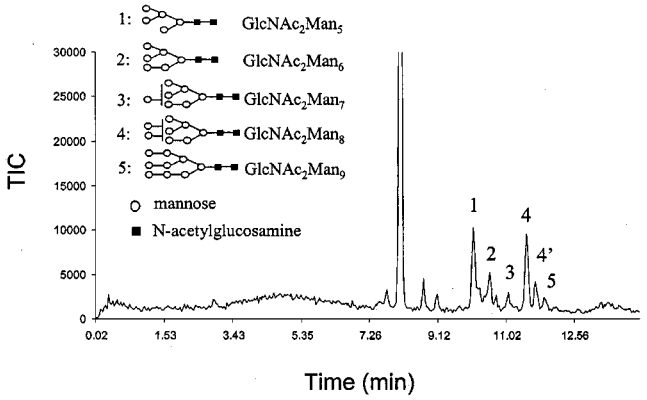


Figure 10. Mass electrochromatogram of *N*-glycans derived from ribonuclease B. Conditions: column length 28 cm; field strength 460 V/cm; mobile phase acetonitrile: water:240 mM ammonium acetate (pH 3) (5.5:4.4:0.1 v/v/v); sheath liquid 1% (v/v) acetic acid in 1 mM sodium acetate prepared in acetonitrile/water (1:1 v/v); injection, 1 kV, 5 s. (Reprinted with permission from ref 32. Copyright 2001 American Chemical Society.)

charides. The labeling was needed to increase the sensitivity of detection as well as to provide the hydrophobicity required for reversed-phase CEC. Several saccharides were analyzed using this packing, including α- and β-anomers of glycopyranoside derivatives.

Suzuki et al.[339] utilized a Hypersil ODS column with a mixture of 50 mM *N*-(2-hydroxyethyl)piperazine-2′-(2-ethanesulfonic acid) buffer, pH 6.0, and acetonitrile (2.2:1 v/v) as eluent for a CEC separation of 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatives of component monosaccharides in glycoproteins and epimeric aldopentoses. Elution of these compounds showed a relatively strong dependence on pH and concentration of the buffer salts contained in the eluent. The labeling with PMP was required to provide both sensitivity and hydrophobicity, thus enhancing detection and separation, respectively. CEC separation of PMP-derivatized monosaccharides was substantially better than that attained through HPLC using the same packing material.

D. Fluorophore-Assisted Carbohydrate Electrophoresis

Fluorophore-assisted carbohydrate electrophoresis (FACE) is a specialized technique that has been routinely employed to profile and elucidate the structures of *N*-glycans derived from glycoproteins. This technology was first introduced by Jackson[340] to separate both mono- and oligosaccharides, combining the simplicity of polyacrylamide gel electrophoresis (PAGE) with the sensitivity of fluorescence.

The method is based on the enzymatic release of *N*-glycans and their labeling with a fluorophore prior to separation by PAGE. The fluorescently derivatized *N*-glycans are detected and quantified by monitoring the fluorescence of the separated bands under UV illumination. Recently, several reviews discussed in detail the principles and procedures of FACE.[341−344]

The commonly used fluorophore is 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS), which facilitates good resolution, enabling in some cases the separation of anomers, epimers, and other isomers;[340,345,346] however, 2-aminoacridone (AMAC)[346] and more recently 1-aminopyrene-3,6,8-trisulfonic cid (APTS)[347] have also been used. ANTS and APTS confer fluorescence on *N*-glycans as well as the electrical charges that enable PAGE of the originally neutral oligosaccharides. The other fluorophore, AMAC, is uncharged under neutral and alkaline conditions. The AMAC derivatives of neutral sugars do not migrate in the Tris-glycin buffer system that is used with the other derivatizing reagents but can migrate in a borate buffer as the result of forming charged complexes between the borate and the *cis*-hydroxyl groups of the glycans. Accordingly, AMAC can be used to differentiate between sialylated and nonsialylated glycans by comparing electrophoresis in borate and borate-free buffers.

FACE gels can be viewed and recorded simply with a standard UV light box and the type of arrangements commonly used for photographing stained gels. A substantial enhancement in sensitivity can be achieved by recording a digitized image of the FACE gel with a cooled, charged-coupled device (CCD) imager. As little as 0.1 pmol/band can be detected with the Glycoscan system of Millipore (Bedford, MA) or the FACE imaging system of Glyko, Inc. (Novato, CA).

FACE profiling of *N*-glycans has been utilized for comparing the glycosylation patterns of glycoproteins. Multiple samples were analyzed side-by-side on the same gel, thus allowing the ability to determine differences in glycosylation of a glycoprotein isolated from different species or at different stages during development or cellular differentiation.[348−350] It has also been used to identify changes in glycosylation that accompany certain physiological[351] or pathological[352,353] conditions. Moreover, the glycosylation of recombinant glycoproteins from conditioned media was monitored by FACE for quality-control purposes.[354,355]

Oligosaccharide sequencing is yet another application of FACE technology. This is accomplished by digesting the oligosaccharides before or after labeling with the fluorophore and monitoring the effect of digestion on mobility. It allows the determination of monosaccharide sequences and linkages based on comparison of the migration of bands prior to and after treatment with a specific exoglycosidase. The position and anomeric character of a glycosidic linkage can be determined through the specificity of an exoglycosidase in use. A protocol involving the use of a series of mixtures of exoglycosidases used to degrade an ANTS-labeled *N*-glycan was described.[356] Numerous digestions in parallel were performed using as little as 5−10 pmol/digestion tube. Susceptibility of the ANTS-labeled *N*-glycan to each group of enzymes was demonstrated, and the presence of a particular monosaccharide residue at the nonreducing end was deduced. Mobilities of the degradation products could be compared (on the same gel) either with a known glycan standard or with the ANTS-labeled maltose ladder extending from two sugar units to 20.[356] This protocol allowed determination of *N*-glycan structures derived from different glyco-

proteins, including recombinant iduronidase,[357] plant glycoproteins,[358] soluble human interferon receptor 2,[359] recombinant human factor VIII,[360] *Candida* mannoprotein,[361] recombinant human erythropoietin,[362,363] and recombinant human prolactin.[364]

Hu and Vallee[365] devised a method using FACE to study interactions between proteins and carbohydrates. The method involved labeling a carbohydrate mixture with ANTS, incubation with a protein under investigation, and then separation, visualization, and quantification by the FACE system. The protein-bound carbohydrate remains at the top of the gel, while the free carbohydrate migrates into the gel and becomes thereby separated. Employing the mixtures of carbohydrates, a side-by-side comparison between protein-present and protein-absent samples allows an easy identification of a protein ligand. The method can also be employed to assess the association constants between proteins and carbohydrates.

Since identification of *N*-glycan structures through FACE is mainly based on their electrophoretic mobility after labeling with ANTS, the analysis of highly complex mixtures cannot be achieved in a single FACE analysis. Comigration of structurally related glycans on gels could easily occur. Therefore, protocols have been developed where FACE is employed as complementary to other techniques. Quintero et al.[366] described a simple and sensitive two-dimensional oligosaccharide-mapping technique at the picomole level. The method is based on derivatization of enzymatically released *N*-glycans with ANTS, prior to their simultaneous separations by FACE and NH_2-HPLC column under ion-suppression conditions, and plotting the relative migration indices against the relative retention times. Such a map was shown useful in characterizing *N*-glycans, at least in terms of the number of antennas, the presence or absence of inner-core fucosylation, and the number and type of sialic acids. However, the map is not able to define branch substitution and, hence, provide a complete structural characterization. This problem was surmounted by combining the two-dimensional mapping with the use of specific exoglycosidases.

Recently, the common limitations of FACE were overcome by utilizing MALDI/MS. First, *N*-glycans were separated by FACE. Then, the ANTS-labeled glycans were eluted from the FACE gel slices and analyzed by MALDI/MS. This allowed accurate determination of the molecular weights of the glycan bands visualized on FACE gels as well as a determination of the presence or absence of comigrating glycan structures.

E. Mass Spectrometry

1. MALDI/MS

a. Matrixes. Mock et al.[140] introduced 3-amino-4-hydroxybenzoic acid as the first matrix specifically designed for the MALDI analysis of dextran hydrolysate and N-linked glycans derived from glycoproteins. However, this matrix was soon replaced by 2,5-dihydroxybenzoic acid (DHB),[367] which remains today the most widely used matrix for the analysis of carbohydrates, in general, and N- and O-linked glycans, in particular. Like 3-amino-4-hydroxybenzoic acid, DHB produces $[M + Na]^+$ ions.

Since the nature of the matrix and the method of sample preparation are critical to obtain strong signals, crystallization of the DHB matrix and its influence on the signal of analyzed glycans were investigated.[368,369] DHB typically crystallizes as long needle-shaped crystals that originate at the periphery of the spot and project toward the center when a mixture of acetonitrile or methanol and water is used. An amorphous mixture of the analyte, contaminants, and salts are present in the central region of the spot. Stahl et al.[369] reported that in a mixture of glycans and glycoproteins, glycans were fractioned in the central region of the spot, while the glycoproteins were in the periphery, as concluded from the acquired spectra. Therefore, a more even film of crystals is produced by redissolving the spot in dry ethanol and allowing it to recrystalize.[368] In addition to producing a thin and even film of crystals, this technique also increased sensitivity by an order of magnitude as a result of more efficient mixing of matrix and analyte from a single solvent.

Strupat et al.[370] introduced a matrix that is commonly referred to as "super-DHB." This matrix consists of DHB and other substituted benzoic acids or related compounds, of which 2-hydroxy-5-methoxybenzoic acid seems the most effective. This additive causes disorder of the crystal lattice, thus allowing a"softer" desorption. The mixture allowed a 2−3-fold increase in sensitivity for a standard dextrin as well as a resolution increase attributed to reduction in metastable ion formation.

The use of carbohydrates as matrix additives was also demonstrated to be effective. The addition of α-L-fucose to DHB improved reproducibility and resolution, as was demonstrated by Gusev et al.[371] in improving crystal homogeneity. Better results were observed when super-DHB was doped with fucose. Also, rapid drying of the target spot improved performance.

Another DHB matrix additive that was found to be very effective at ionizing carbohydrates is 1-hydroxyisoquinoline (HIQ).[372] A stronger signal was attained by using fast vacuum-drying, which also produced fine crystals. HIQ alone was determined to be a poor matrix for carbohydrates; however, its inclusion in DHB caused formation of much finer crystals. Since HIQ is less soluble than DHB, the authors believed that HIQ crystallized first and seeded the crystallization of DHB. A major advantage of this matrix is its tolerance to the presence of a number of salts and additives such as SDS.

Chen et al.[373] synthesized and evaluated 15 sugar osazones for their use as matrixes. However, D- or L-arabinosazone (ara), prepared from D- or L-arabinose and phenylhydrazine, gave the best overall performance. The microcrystalline nature of ara facilitates formation of a highly uniform layer on the sample plate. This matrix also requires low laser irradiation for desorption, resulting in higher mass resolution and lower matrix background. As can be seen in Figure 11, the spectrum of laminarin, a linear D-glucan from brown algae, exhibited higher intensity

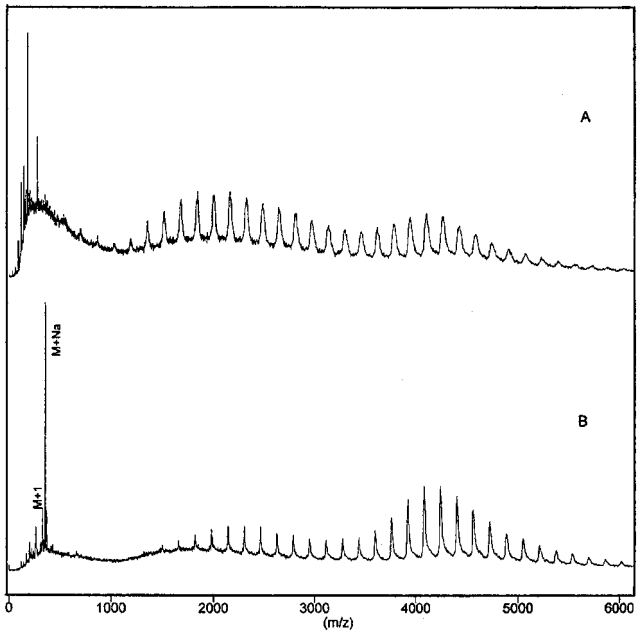


Figure 11. Mass spectra of laminarin in (A) DHB matrix, recrystallized on the plate with ethanol, and (B) sodium-rich D-arabinosazone. (Reprinted with permission from ref 373. Copyright 1997 Publisher.)

for the higher mass constituents when acquired with ara rather than DHB. The osazone matrix needed less laser energy.

Although other matrixes were developed for the analysis of carbohydrates, none have enjoyed the popularity of the aforementioned matrixes. Table 4 summarizes different matrixes that have been successfully used for the analysis of carbohydrates, including 3-aminoquinoline,[374] potassium hexacyanoferrate and glycerol,[375] hygroscopic tetrabutylammonium bromide, chloride, or acetate,[376] 5-chloro-2-mercaptobenzothiazole (CBMT),[377] β-carbolines,[378] ferulic acid,[379] and 2,5-dihydroxyacetophenone.[380]

Sialylated N-linked glycans generally give poor MALDI spectra when ionized with matrixes such as DHB. Most of the time, sialylated and some sulfated glycans undergo fragmentation by loss of either sialic acid or carbon dioxide from the sialic acid or a sulfate. It is now clear that this problem can be rectified by a suitable choice of matrix. Papac et al.[381] determined that 6-aza-2-thiothymine (ATT) gives a significant increase in sensitivity for acidic glycans over that produced by the more common matrixes. This matrix improved detection of N-linked glycans by approximately 10-fold over DHB. However, this matrix did not prevent fragmentation and a loss of sialic acid in the linear mode of detection. The loss of sialic acid in the reflectron mode was substantial.

In the same report, the authors also found a mixture of 2,4,6-trihydroxyacetophenone (THAP) and ammonium citrate to be effective in the analysis of sialylated glycans.[381] This mixture gave a single negative ion peak from sialylated N-linked glycans and allowed their detection at the 10 fmol level in the linear mode with no evidence of fragmentation. However, like with the other matrixes, a major loss of sialic acid in the reflectron mode was observed. Sensitivity was dependent on the conditions used for sample preparation. Vacuum drying of the sample spot was used to prevent large crystal formation, and then the sample was allowed to absorb water to promote formation of small crystals.

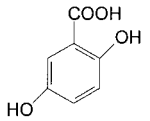
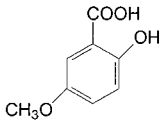
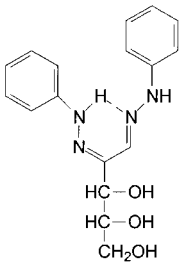
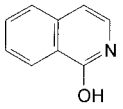
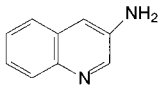
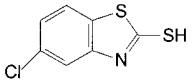
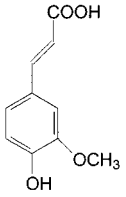
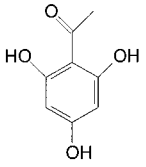
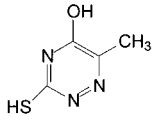
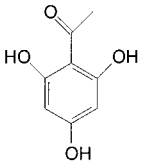
Mechref and Novotny[382] used the base spermine as a DHB co-matrix for the determination of sialylated glycans. Since the carboxylic acid groups of sialic acids have a tendency to form salts with alkali metals, distributing the signal intensity among multiple peaks in the spectra, the addition of spermine was found to reduce (if not eliminate) sodium salt formation. Moreover, spermine also provides a good crystalline surface. As a result, the detection limits down to ca. 50 fmol became attainable. The matrix was effective in analyzing standard sialylated N-linked glycans as well as the glycans derived from fetuin and α_1-acid glycoprotein.

Recently, Pfenninger et al.[383] demonstrated that the use of 5-chloro-2-mercaptobenzothiazol (CMBT) as a first layer for ATT-NaCl or DHB enhances the signal intensities dramatically when used as a substrate for a "two-layer" preparation. The procedure was thus recommended for sample mixtures containing components in low concentrations. It was also determined that CMBT is effective as a thin-layer preparation for samples with very low analyte concentrations. Analysis of a complex mixture of oligosaccharides with a total concentration of <0.005 g/L was easily possible with this matrix. To determine the number of sialic acids, a special preparation protocol was suggested. It involves investigating a sample in both the negative- and positive-ion modes using two different matrixes, namely, ATT-dihydroxyacetophenone and DHB−NaCl in cocrystallization with the sample. Three MALDI experiments are necessary: one in the negative-ion mode for the sample with ATT as the matrix and then in the negative- and positive-ion modes for the sample with DHB. The molecular pattern arising from a monosialylated oligosaccharide is different from that of a disialylated species, allowing determination of the extent of sialylation.

b. Removal of Contaminants. Since the ion yield and crystal formation in MALDI/MS analysis are adversely influenced by the presence of salts and buffers, their prior removal becomes desirable. Mock et al.[384] investigated the maximum tolerable amounts of contaminants. Carbohydrates were found to be generally less tolerant than proteins to salts and other compounds. This is despite the fact that small amounts of sodium or other alkali metals are required for efficient ionization. Recently, it was determined that metals cause clustering between the matrix and sample, adversely affecting resolution.[373] Therefore, many methods have been developed for the removal of salts and buffers.

Although salts and buffers can be removed from glycoproteins simply by washing the dried sample spot with cold water[385] or a dilute trifluoroacetic acid aqueous solution,[386] this procedure cannot be generally recommended for glycans because of their high water solubility. However, glycans are commonly desalted by drop dialysis on a 500 Da cutoff dialysis membrane.[387,388] This method involves floating of a

Table 4: Matrixes and Additives Utilized for the Analysis of N-Linked Glycans

matrix	abbreviation	structure
2,5-dihydroxybenzoic acid	2,5-DHB	
2-hydroxy-5-methoxybenzoic acid	—	
arabinosazone	ara	
1-hydroxyisoquinoline	HIQ	
3-aminoquinoline	3-AQ	
5-chloro-2-mercaptobenzothiazole	CBMT	
ferulic acid	—	
2,5-dihydroxyacetophenone	DHA	
6-aza-2-thiothymine	ATT	
2,4,6-trihydroxyacetophenone	THAP	

small piece of the dialysis membrane on the surface of water, on top of which the carbohydrate sample is loaded. Evaporation is prevented by covering the used water container, while the sample is removed after 10−60 min. This time is sufficient for a partial desalting of the sample that should be adequate for MALDI/MS.

Nafion-117 membranes have also been used for purification of glycans.[389] This membrane was first pretreated by heating at 80 °C in nitric acid or hydrochloric acid for 2 h to protonate extensively the sulfate groups, after which the membrane was washed with water. This membrane not only allows desalting of glycan samples, but it also adsorbs proteins and peptides. The membrane combined with ion-exchange chromatography was also used for cleaning the glycans released by hydrazinolysis.[390]

Worrall et al.[391] replaced the conventional stainless steel probe with activated synthetic membranes. Contaminated samples were directly spotted onto the activated synthetic membranes (such as polyethylene and polypropylene membranes), while various impurities, including salts, glycerol, and detergents, were washed from the sample.

Recently, Jacobs and Dahlman[392] were able to substantially enhance the quality of MALDI mass spectra of highly acidic oligosaccharides by coating the MALDI probe surface with a film consisting of a perfluorosulfonated ionomer (Nafion) prior to addition of the sample/matrix mixture. The effectiveness of this coating in improving the quality of MALDI spectra of highly acidic oligosaccharides was compared to that of uncoated and nitrocellulose-coated target plates. While the MALDI spectra of oligouronates (oligomers containing mannuronic/glucuronic and galacturonic acid residues) obtained through the use of uncoated or nitrocellulose-coated probes consisted of a series of broad and multiple peaks, the corresponding spectra obtained with Nafion-coated probes contained only a single series of sharp peaks of nondissociated oligomers exhibiting chain lengths of up to 15 uronic acid residues. This improvement was attributed to the ability of the Nafion coating to remove the sodium counterions remaining in the deposit of the sample/matrix mixture on the probe.

Another approach to purification of glycans has been the use of short columns of ion-exchange or hydrophobic resins packed into disposable pipet tips. Kussmann et al.[393] constructed disposable micro-LC columns with 1−2 μL volume using Gel Loader tips packed with porous reversed-phase materials. Such disposable tips were very effective in removal of sample contaminants. Currently, such disposable tips with various packing materials (such as cation- and anion-exchange, reversed-phase, and normal-phase resins) are commercially available from Millipore (Bedford, MA) and Harvard Bioscience (Holliston, MA).

Rouse and Vath[394] developed an on-the-probe sample cleanup technique in which small amounts of resins were codeposited with a matrix on the sample spot on the probe. The technique involved first drying the sample on the sample plate. Next, the matrix was added, followed by the addition of appropriate adsorbing media, depending on which contaminants are present. The sample was dried again and the adsorbent was loosened with a microspatula before being removed by an air stream. Detergents were removed with Extracti-gel-D resin, cations with AG-50W-X8, and anions with Mono-Q resin. However, the authors recommended removal of acetate ions by drop dialysis prior to their on-probe cleanup technique.

Novotny and co-workers[395] reported a simple microscale procedure that substantially removes detergents from the small quantities of oligosaccharides recovered after the common endoglycosidase or exoglycosidase enzymatic digestion of glycoproteins. Their technique involved the addition of a small quantity of a hydrophobic resin (Supelco SP20SS) directly into the dilute digest solutions. The resin adsorbs detergents, remaining deglycosylated proteins, and possible hydrophobic contaminants while leaving the glycans intact in the solution. This contaminant removal substantially enhances the sensitivity of oligosaccharide detection and sequencing by MALDI/MS. The effectiveness of this technique was demonstrated with submicrogram quantities of several model glycoproteins as well as with characterization of the N-linked glycans of the sperm-binding protein isolated from an extremely small biological sample from the egg vitelline envelope of a frog species. The effectiveness of this resin in removing contaminants was compared to that of C_8- and C_{18}-bonded silica materials. As can be seen in Figure 12, SP20SS is superior in removing contaminants.

Packer et al.[396] investigated extensively the use of graphitized carbon as a solid extraction cartridge. It was demonstrated that such a cartridge allows purification of glycans from solutions containing one or more of the following contaminants: salts, monosaccharides that do not interact with the graphitized carbon, detergents (sodium dodecyl sulfate and Triton X-100), proteins (including enzymes), and reagents used for the release of glycans (such as hydrazine and sodium borohydride). The authors demonstrated utilization of such cartridges for (i) purification of N-linked glycans after enzymatic or chemical removal from glycoproteins, (ii) desalting of O-linked glycans after alkaline removal from glycoproteins, (iii) on-line desalting of HPAEC-separated oligosaccharides, and (iv) purification of oligosaccharides from urine.

c. Fragmentation of Glycans in MALDI/MS. While mass determination through MALDI/MS can often lead to compositional data (in terms of isobaric monosaccharides), additional information must be secured through other methodologies. Monosaccharide sequences, branching, and, in some cases, linkages can be determined through fragmentation that a glycan may experience in either a postsource decay or a collision-induced dissociation. However, the amounts of material may not be sufficient for a full characterization, and conclusions can often be vague. Fortunately, the combination of MALDI/MS and enzymatic sequencing using exoglycosidases provides the necessary information related to sequence, branching, and linkage of a glycan. The principle of oligosaccharide sequencing is based on the ability of a

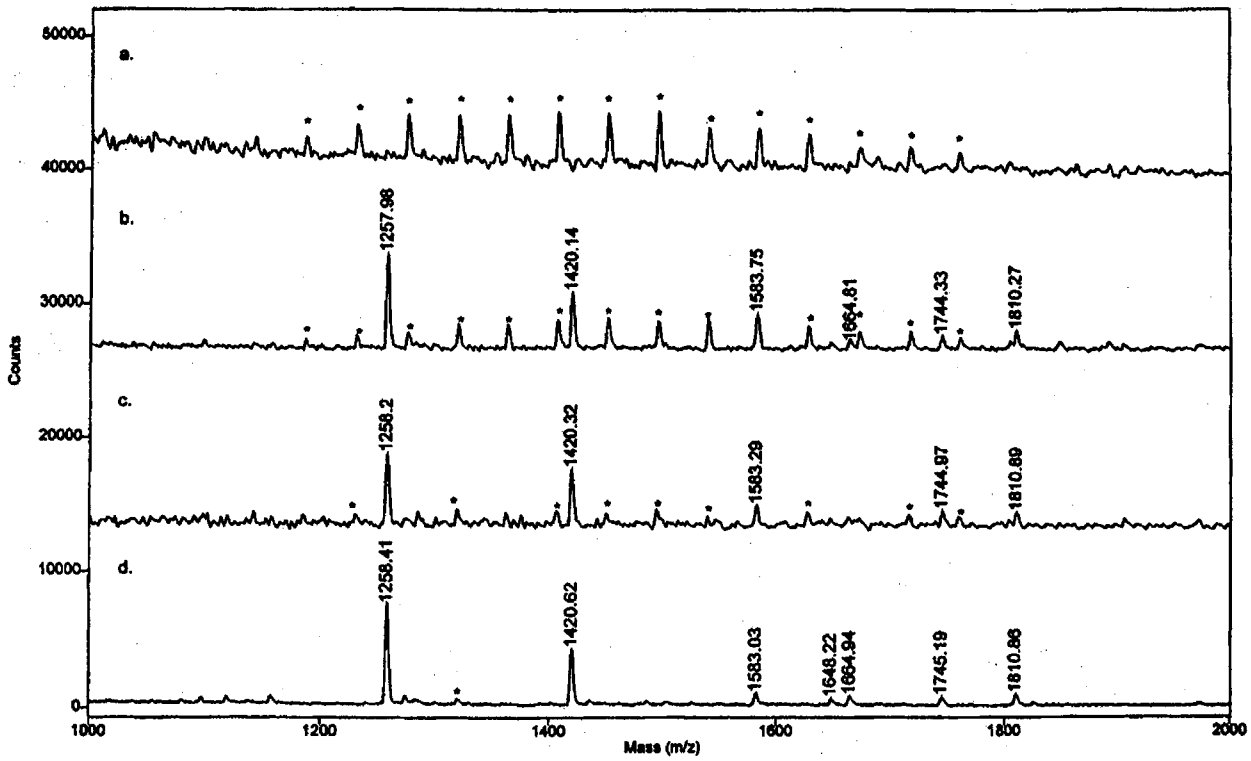


Figure 12. Positive-ion MALDI mass spectra of the N-linked glycans derived from 1 μg of ribonuclease B: (a) before PNGase F digestion, (b) enzymatic digest without purification, (c) enzymatic digest treated with C_{18} packing, and (d) enzymatic digest treated with SP20SS. (Reprinted with permission from ref 395. Copyright 2000 Publisher.)

specific exoglycosidase to remove terminal monosaccharides from the nonreducing end of glycans. Obviously, this is made possible in part by the availability of several exoglycosidases that are highly specific to anomericity, monosaccharide type, and linkage. Thus, a glycan structure can be deduced through the information obtained from a series of digestion experiments involving different arrays of exoglycosidases.

Fragmentation of glycans observed in MALDI/MS is similar to that observed in FAB/MS and ESI/MS and is dependent on factors such as ion formation, its charge state, the energy deposited into an ion, and the time available for fragmentation. In general, glycans undergo two types of cleavages: glycosidic cleavages that result from breaking the bond linking two sugar residues and cross-ring cleavages that involve rupturing two bonds on the same sugar residue. The former provides information pertaining mainly to the sequence and branching, while the latter may reveal some details on a linkage.

Domon and Costello[397] introduced the nomenclature pertaining to fragmentation of carbohydrates. According to this nomenclature, the ions retaining the charge at the reducing terminus are designated as X for cross-ring cleavages and as Y and Z for glycosidic cleavages. Those retaining a charge at the nonreducing terminus are designated as A for cross-ring cleavages and as B and C for glycosidic cleavages. Ions are designated by a subscript number that follows the letter showing the fragment type. Sugar rings are numbered from the nonreducing end for A, B, and C ions and from the reducing end for the others. Greek letters are used to distinguish fragments from branched-chain glycans, with the letter α representing the largest branch. In the case of ring cleavages, superscript numbers are given to show the ruptured bonds.

Ngoka et al.[398] and Cancilla et al.[399] determined that protonated species decompose much more readily than metal-cationized species and that the order in which cationized species decompose is $Li^+ > Na^+ > K^+ > Cs^+$. This is prompted by the exothermicity of cation binding, which follows the same order, so that protonated and lithiated species are formed with the excess energy that can be utilized to initiate fragmentations. Moreover, it was also observed that linear carbohydrates produce more fragmentation than branched ones.[399] This has also been attributed to coordination of the metal ions.

Fragmentations in MALDI/MS can result from (a) the postsource decay (PSD), which designates the fragments formed after ion extraction from the ion source; (b) in-source decay (ISD), which designates the fragments formed within the ion source; and (c) collision-induced dissociation (CID), which designates the fragments formed in a collision cell filled with a gas. PSD spectra of sodiated ions from neutral carbohydrates tend to be dominated by the glycosidic and internal cleavages with very weak cross-ring ions.[400] This abundance of internal and glycosidic cleavages makes spectral interpretation somewhat difficult. Major ions are usually the result of B and Y cleavages, and the information is related to sequence and branching. A lack of abundant cross-ring cleavages limits the linkage information to be deduced. The lack of cross-ring fragmentation in PSD is attributed to their high energy requirements.

The most useful cross-ring cleavage ions in the spectra of N-linked glycans are the $^{3,5}A$ and $^{0,4}A$ ions

produced by cleavages of the core-branching mannose residues. These ions contain only the antenna attached to the 6-position, providing a wealth of information on the composition of each antenna. Spengler et al.[400] reported ^{0,4}A ions in the PSD spectra of bi- and triantennary N-linked glycans. These two cross-ring cleavage ions were used to determine the antenna configuration in high-mannose N-linked glycans derived from hemoglobin extracted from tube worms.[401]

As discussed above, the incorporation of delayed extraction (DE) into MALDI/MS caused a dramatic improvement in sensitivity, mass resolution, and mass accuracy of the precursor ions up to ~10 kDa. However, under DE the loss of total PSD fragment ion yield can be as large as 1 order of magnitude.[402] A part of this loss was balanced by a better signal-to-noise ratio, which resulted from a significantly improved mass resolution of the PSD fragment ions. While this compensating effect was true for the middle to high mass range of the PSD fragment ions, it gradually diminished toward the low mass scale. As shown in the case of linear peptides, some important information can be lost.[402] Although DE adversely affects PSD fragmentation, it improves the overall quality of PSD spectra, while any loss of analytical information can be compensated for by CID.

Lemoine et al.[403] demonstrated that derivatization can significantly enhance the quality of PSD mass spectra and thus the information they facilitate. Peracetylated and, to some extent, permethylated oligosaccharides yielded useful preliminary information. However, deuterioacetylation allowed a clear distinction between the hexose and *N*-acetylhexosamine residues. The best results were obtained with benzylamino derivatives, which offered a basic site allowing the formation of protonated ions (which, as discussed above, easily fragment), thus providing sequence and branching information.[403]

Rouse et al.[404] devised a strategy to characterize unknown isomeric N-linked glycan structures that was referred to as "knowledge-based." The strategy was based on comparing specific fragment ion types and their distributions in the unknown PSD spectrum to those in the PSD spectra of standards possessing similar structural features. A precursor ion selection device was employed to isolate the component of interest from the mass profile without chromatographic isolation steps. This PSD knowledge-based isomeric differentiation strategy allowed the respective isomers for certain high-mannose and asialo complex *N*-glycan standards that were characterized previously by NMR spectroscopy to be distinguished. This strategy provided the means to identify oligosaccharide isomer types, to pinpoint the locations of structural variations, to recognize isomerically pure oligosaccharides from isomeric mixtures, and to estimate the major and minor oligosaccharide isomer species in a mixture.

Recently, Yamagaki and Nakanishi exploited the potential of a MALDI mass spectrometer with the curved-field reflectron for PSD fragmentation analyses of the linkage isomers of oligosaccharides.[405,406] A reflectron of the curved voltage gradient type, which was first introduced by Cornish and Cotter,[407,408] allows a total fragment spectrum to be aquired in a single experiment. This type of reflectron employs a modified single-stage reflector; the gradient differential increases of its axial voltage produce an alignment of energy focal points for product ions. Therefore, the parent ion and all the fragment ions are detected under the same measurement conditions. A major advantage of this reflectron is an accurate comparison of the intensity of the differenially observed fragments, since they are all detected under the same conditions. As in the case of a conventional reflectron, glycosidic cleavage ions are predominant, while cross-ring cleavage ions are weak or absent.

Generally, CID fragmentation spectra contain more abundant cross-ring fragments than are normally produced under ISD and PSD conditions.[409] The very abundant ^{1,5}X ions were of particular significance. Their masses could be used to determine the branching patterns of glycans such as the high-mannose sugars.

The effect of the reducing-terminal substituents on the high-energy CID MALDI spectra of oligosacharides has been investigated.[410] The study compared the CID spectra of oligosaccharides bearing different commonly encountered, reducing-terminal modifications including hydroxyl, 2-aminobenzamide-labeled, and asparagine- and a tetrapeptide-linked substances. All compounds formed abundant sodiated molecular and fragment ions, the latter corresponding to glycosidic and cross-ring clevages as well as to internal fragment ions. However, the nature of the modification at the reducing end considerably influenced CID behavior. The strongest and most complete series of glycosidic cleavage ions were observed for the underivatized oligossaccharides, while most cross-ring fragment ions (diagnostic of a linkage) were observed for the glycopeptides. A-type cross-ring clevage ions were abundant in the spectrum of asparagine derivatives. Reductive amination of the reducing end with 2-aminobenzamide resulted in suppression of the cross-ring fragment ions, prompted by the presence of an opened reducing terminal sugar as a result of labeling.

Penn et al.[411] recently reported MALDI/CID spectra of milk sugars and N-linked glycans using a Fourier transform mass spectrometer (FT/MS) fitted with an external ion source. A fucose loss was observed as the favorable glycosidic cleavage with the lowest relative dissociation threshold. This threshold increased when cesium was used as the cationization species, being attributed to complexation between cesium and fucose. Dissociation thresholds for the appearance of cross-ring cleavage ions were determined to be higher than those due to glycosidic cleavages, correlating well with the appearance of these ions in the CID spectra. The ability to measure dissociation threshold values with the FT-MS instrument can be employed to distinguish between different monosaccharide residues, thus allowing the determination of glycan composition. This type of instrument also permits a storage of ions for consid-

erable periods of time, making it possible to carry MS^n.

In a different communication, the same group proposed a method for obtaining the relative dissociation thresholds using a multiple-collision CID.[412] The multiple-collision dissociation threshold (MCDT) values were used to examine the relative affinities of the alkali metal ions for oligosaccharides and the effect of alkali metal ions on oligosaccharide fragmentation (including glycosidic bond and cross-ring cleavages). According to these MCDT values, the activation barriers for glycosidic bond cleavages were found to be dependent on the size of alkali metal ions, as was determined previously for MALDI/TOF/MS (see above). Moreover, cross-ring cleavages were found to be independent of the alkali metal ion, but dependent on a linkage type. The results also suggested that glycosidic bond cleavages are charge-induced, while cross-ring cleavages were a charge-remote process.

Recently, the application of MALDI/FT/MS was extended to characterization of permethylated oligosaccharides.[413] The study evaluated several aspects of MALDI/FT/MS for carbohydrate structural analyses, including sustained off-resonance irradiation (SORI), collision-induced dissociation (CID), quadrupolar axialization, multiple stages of isolation and dissociation (MS^n), and ion remeasurement. It was determined that SORI/CID internal energies were adequate for the linkage analysis of permethylated glucose oligomers. In FT/MS, ions continually drift out of the trap as a result of the exponential increase in the off-axis displacement of the center of an ion cyclotron orbit (i.e., the magnetron radius) with time. This magnetron radial expansion is also observed during sequential dissociation. Nevertheless, magnetron radial expansion is rectified by ion axialization through cyclotron-resonant quadrupolar excitation in the presence of collision damping. Therefore, ion axialization greatly improves measurement efficiency, being a crucial component of the ion control required for MS^n. Accordingly, ion remeasurement and axialization techniques enhance the sensitivity of ion fragmentation analysis.

Harvey et al.[414] reported for the first time the utility of quadrupole/time-of-flight mass spectrometer fitted with the MALDI ionization source for ionization and fragmentation of complex glycans, particularly the N-linked glycans derived from glycoproteins. Positive-ion spectra of sialylated glycans obtained on this instrument were simpler than those obtained with a conventional MALDI instrument, because of the absence of ions resulting from metastable fragmentations occurring in the flight tube. MS/MS spectra of the sodiated ions from all compounds were far superior to MALDI−PSD spectra recorded with a conventional MALDI instrument. Fragmentation of the parent ion was dominated by B- and Y-type glycosidic cleavages, leading to both primary and internal fragment ions. Less abundant cross-ring cleavage ions providing linkage information were also detected. The utility of this instrument was demonstrated for the native and derivatized N-linked glycans cleaved from ribonuclease B and human secretory IgA and Apo-B 100 from human low-density lipoproteins.

d. Use of Exoglycosidases for the Analysis of N-Linked Glycans. Although MALDI−PSD and −CID are useful in determining the structures of glycans, the use of enzymatic sequencing is superior for a conclusive, complete, and accurate determination of glycan structures.

Küster et al.[415] were the first to devise a protocol combining exoglycosidase digestion with MALDI/MS in the structures of underivatized oligosaccharides at low picomole amounts of a starting material. The key feature of this approach is that an oligosaccharide sample can be recovered after a MALDI experiment and a series of sequential exoglycosidase digestions can be carried out on that sample within a single day. The method involves the following steps: (i) acquiring the molecular mass profile of N-linked glycans derived from a glycoprotein by MALDI/MS, (ii) sample recovery from the target and removal of the matrix by drop dialysis using 500 Da cutoff dialysis membrane, (iii) exoglycosidase digestion in a volume of 1 μL, (iv) removal of the incubation buffer by drop dialysis, (v) removal of the enzyme by adsorption on a Nafion membrane; (vi) MS acquisition; and (vii) repeating the same steps with another exoglycosidase. This methodology offers several advantages over the conventional oligosacharide sequencing techniques, including faster analysis, no need for analyte derivatization, faster exoglycosidase digestions in small volumes, and no need for sample splitting, allowing characterization of small amounts of sample.

Mechref and Novotny[36] demonstrated for the first time the enzymatic release of N-linked glycans from glycoproteins directly on the MALDI target plate using submicrogram quantities. The on-plate digestion with PNGase F released effectively the corresponding oligosaccharides in less than 2 h, irrespective of the molecular weight of a glycoprotein under investigation. It was demonstrated that the on-plate release of oligosaccharides from glycoproteins was as effective as the in-solution release with overnight incubation (see Figure 13). Moreover, the authors demonstrated the possibility of simultaneous enzymatic release and sequencing of the released oligosaccharides, using the same buffer. A buffer of pH 6.5 with a very low salt concentration (10 mM) was sufficient to release N-linked glycans without adversely affecting the activity of exoglycosidases used for enzymatic sequencing.

This methodology allowed complete characterizations of the N-linked glycans released and sequenced simultaneously by using four sample spots with submicrogram quantities of a glycoprotein. All spots contained PNGase F, while a different enzymatic array was included in each spot. The four spots were sufficient for a complete characterization of N-linked glycans in a few hours. The effectiveness of this method was demonstrated with ribonuclease B, bovine fetuin, ovalbumin, human α_1-acid glycoprotein, and diamino oxidase from porcine kidney (see Figure 14). The ability to characterize N-linked glycans released from more than one glycoprotein is

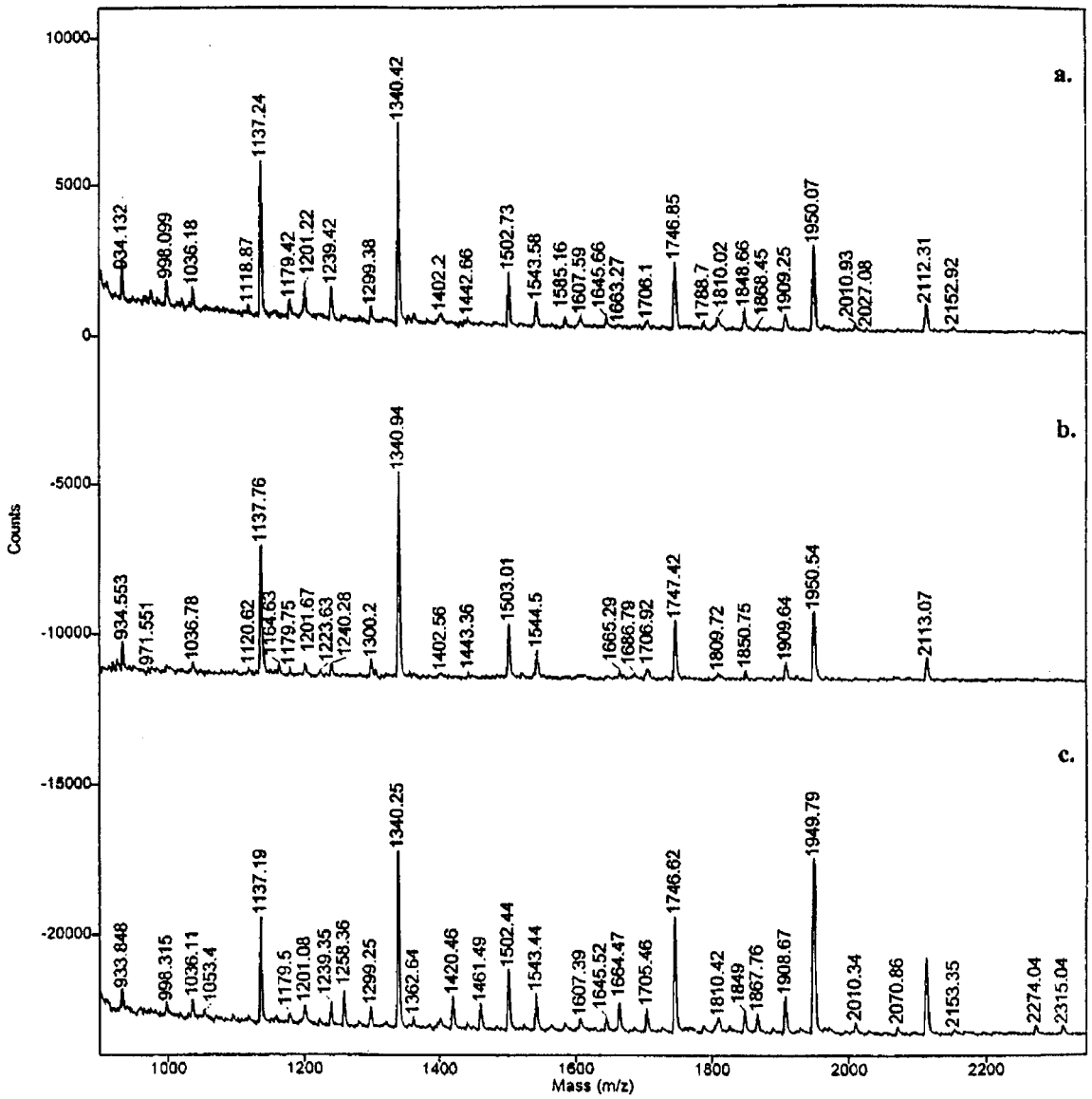


Figure 13. MALDI mass spectra representing the N-linked oligosaccharide profiles derived from ovalbumin by digestion overnight (a), digestion of 5 μg for 3 h on the plate (b), and digestion of 5 μg on the plate, after thermal denaturation of the glycoprotein (c). (Reprinted with permission from ref 36. Copyright 1996 American Chemical Society.)

possible with a typical MALDI target plate having 100 spots. Theoretically, N-linked glycans released from up to 25 glycoproteins could be characterized in 1 day using one plate. The method appears ideal for high-throughput glycomics. The method was also utilized by Colangelo and Orlando[416] and Geyer et al.[417] for characterization of primary structures.

Recently, a 96-well MultiScreen assay system containing a poly(vinylidene difluoride) (PVDF) membrane was employed to immobilize glycoproteins for a subsequent enzymatic deglycosylation.[417] This method enabled deglycosylation of recombinant tissue plasminogen activator (rtPA) from samples as small as 0.1 μg (see Figure 15). The method was based on immobilizing glycoprotein on a high protein-binding membrane, PVDF, followed by reduction with dithiothreitol and alkylation with iodoacetic acid prior to treatment with PNGase F. Reduction and alkylation of the glycoprotein were performed to avoid the use of detergents, which are often required for a complete deglycosylation. PNGase F or any other endoglycosidases were prevented from adsorption to the membrane by treating it with poly(vinylpyrrolidone) (PVP) 360. The membrane was extensively washed with water following the blocking step to remove excess PVP 360, which could interfere with MALDI/MS. Immobilization of rtPA on the PVDF membrane did not sterically hinder PNGase F release of its oligosaccharides, while a complete deglycosylation was achieved after incubation for 3 h, as determined from tryptic mapping experiments. Moreover, no difference was noted between the HPAEC−PAD map of the oligosaccharides released by this method and those released from the same sample in solution. This method allows 60 glycoprotein samples to be deglycosylated in 1 day with MALDI/MS or HPCE-PAD analysis being performed on the following day.

Since proteins and glycoproteins are commonly analyzed by SDS−PAGE, Küster et al.[418] described a method for the analysis of N-linked glycans released enzymatically within the gel, following SDS−

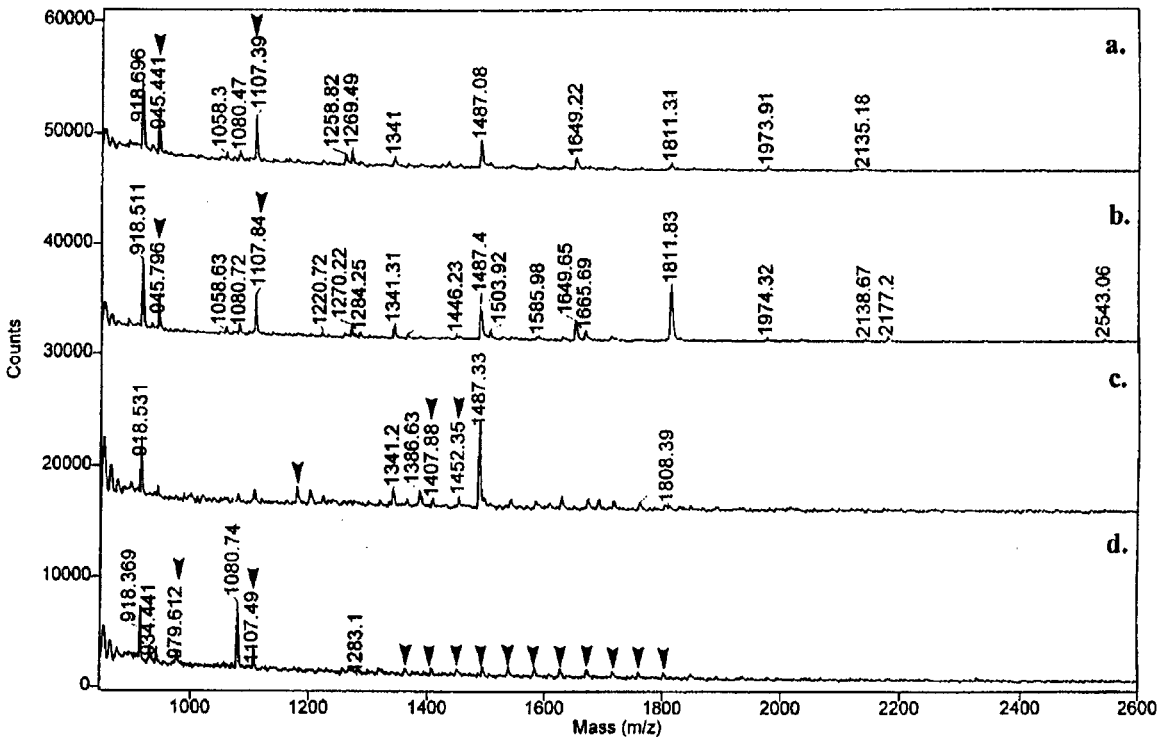


Figure 14. MALDI mass spectra of the N-linked oligosaccharides derived from 5 μg of DAO after treatment with PNGase F (a); PNGase F and neuraminidase (b); PNGase F, neuraminidase, and galactosidase (c); and PNGase F, neuraminidase, galactosidase, and *N*-acetylglucosaminidase (d). The peaks marked with an arrow are contaminants from the enzyme preparations and the sample itself (presumably, poly(ethylene glycol) used as a stabilizer). (Reprinted with permission from ref 36. Copyright 1996 American Chemical Society.)

PAGE separation of glycoproteins. The method involves the following steps: (i) cutting, destaining, reducing, and alkylation of the separated bands containing glycoproteins; (ii) in-gel incubation with PNGase F to release N-linked glycans; (iii) extracting N-linked glycans with water and acetonitrile; (iv) desalting of N-linked glycans by employing a short mixed-bed ion-exchange column; and (iv) MALDI/MS profiling of the released N-linked glycans. Structural determination of the released glycans was achieved using exoglycosidase digestion. The method allowed determination of N-linked glycan structures derived from α_1-acid glycoprotein from man, cow, sheep, and dog.

Recently, Hoja-Lukowicz and co-workers[419] characterized the N-linked glycans derived from human placental arylsulfatase A using a slightly modified protocol. The glycoproteins were separated through SDS−PAGE and Western-blotted on Immobilon P. Next, the glycoproteins were deglycosylated on-blot, using PNGase F. The profiles of N-linked glycans were subsequently obtained by MALDI/MS, while the oligosaccharides were sequenced using specific exoglycosidases and digestion products were monitored by MALDI/MS. The N-linked glycans of human placental arylsulfatase were determined by this method to be of the high-mannose type, of which almost one-half were core-fucosylated.

e. N-Linked Glycan Analysis Using Alkaline Degradation. Cancilla et al.[420] used the base-induced "peeling reaction" as an alternative to exoglycosidase sequencing. The method is based on the fact that a strong base causes successive loss of the residues from the reducing end by a glycosidic cleavage and production of a new reducing end. The reaction products were sampled directly with a minimum cleanup and monitored by MALDI/FT/MS to elucidate the oligosaccharide sequence. This allowed a direct determination of sequence and branching. The linkage information was acquired by cross-ring fragmentation of the new reducing end, created by either a MALDI in-source fragmentation or SORI/CID. This method allowed sequencing and linkage determination of neutral, branched, fucosylated, and sialylated oligosaccharides. An FT/MS instrument furnishes high resolution, an exact mass, and facile tandem MS experiments of the MALDI-produced ions.

2. ESI/MS

Due to the sensitivity problems with large glycans, excessive sample consumption, and mass discrimination, ESI/MS of the native glycans did not originally match the popularity of MALDI/MS. However, this is now changing because of the development of nanospray.

In 1992, Duffin et al.[146] were the first to exploit the potential of ESI/MS in the characterization of native N-linked glycans derived from glycoproteins. Sialylated oligosaccharides were detected with the best sensitivity in the negative-ion mode, while the asialylated species performed best in the positive-ion detection mode. The primary structural features of the studied oligosaccharides were determined by tandem MS. The MS/MS characterization of the native complex oligosaccharides yielded fragment ions that resulted from the cleavages of glycosidic bonds, while no linkage determination was feasible.

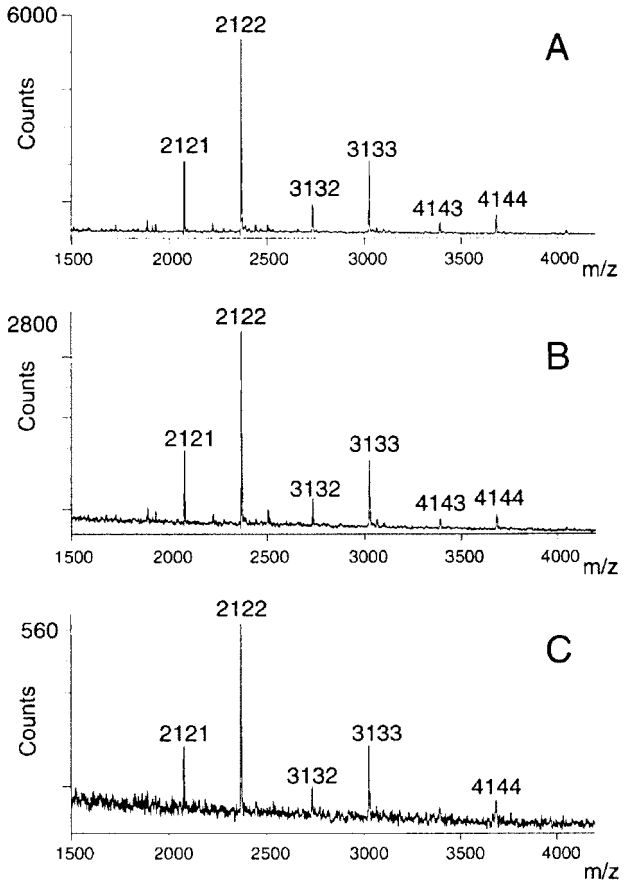


Figure 15. Negative-ion MALDI mass spectrum of 2.5% of the N-linked oligosaccharides released from (A) 1 mg, (B) 0.5 mg, and (C) 0.1 mg of recombinant tissue-type plasminogen activator. The spectra was acquired using THAP as the matrix and smoothed with a 19-point Savitzky−Golay function. (Reprinted with permission from ref 417. Copyright 1999 American Chemical Society.)

The composition and relative abundances of the ovalbumin-derived oligosaccharides, as analyzed by ESI/MS, matched the results obtained by HPAEC−PAD and gel permeation chromatography.

Permethylation or other derivatization of glycan structures has been demonstrated to improve substantially the sensitivity of ESI/MS. This was demonstrated through permethylation by Linsley et al.[421] in the characterization of recombinant erythropoietin N- and O-linked glycans. The glycans were permethylated and profiled with ESI/MS without desialylation. The O-linked glycans were also analyzed directly by ESI/MS. On the other hand, Okamoto et al.[422] illustrated an improvement in sensitivity of ESI/MS through derivatization of glycans with trimethyl(*p*-aminophenyl)ammonium chloride (TMAPA). The TMAPA derivatives exhibited extremely high sensitivity in the positive-ion ESI/MS and gave Y and Z fragment ions by ESI/MS/MS. Since then, the utility of ESI/MS for the analysis of glycoprotein glycans has been growing continuously.

Naven and Harvey[423] devised a cationic derivatization of oligosaccharides with Girard's T reagent for improved performance in ESI/MS. The oligosaccharides were derivatized to form hydrazones in order to introduce a cationic site needed for detection by ESI/MS. The derivative exhibited a high yield and did not require extensive cleanup prior to MS examination, which was an advantage over reductive amination. The derivatives offered a 10-fold increase in detection sensitivity over the underivatized oligosaccharides and provided intense spectra in the positive-ion ESI without the need to add cations to the solvent.

ESI/MS was used in conjunction with RP-LC to characterize permethylated oligosaccharides.[424] *N*-Acetylhexosamine-containing carbohydrates yielded protonated molecular ions that underwent extensive fragmentation, even under low-energy CID. MS/MS recordings of the protonated molecular ions were characterized by simple fragmentation patterns that result from the cleavage of glycosidic bonds, allowing a straightforward interpretation. The nature of some internal fragments was established on the basis of MS/MS of the derivatives labeled with ^{18}O at the reducing end.

Weiskopf et al.,[425,426] Viseux et al.,[427] and Sheeley and Reinhold[428] described the use of ESI/quadrupole ion trap (QIT)/MS, with its capacity to perform multiple stages of fragmentation (MS^n), for the characterization of permethylated N-linked glycans derived from glycoproteins. Collisional activation of permethylated oligosaccharide molecular ions (MS^2) produced abundant fragments from the glycosidic bond cleavages that depicted composition and sequence. However, these fragment ions do not furnish any information related to linkages. Therefore, the fragments were trapped and further dissociated (during MS^n), now permitting interpretation and confirmation of cross-ring cleavage products. The mixtures of isobaric oligosaccharides, which were ionized and introduced into the trap simultaneously, were resolved and further examined in isolation by selection of MS^2 or MS^n specific to only one glycomer. This potential of ion trap was demonstrated for the $GlcNac_2Man_5$ oligosaccharide from ribonuclease B and two isobaric HexNAc5 oligosaccharides from ovalbumin.[425] Higher order experiments further illustrated the potential of ESI/QIT/MS for oligosaccharide analysis, as MS^8 was used to produce significant branching information for an oligosaccharide from ovalbumin (see Figure 16).[426] ESI/QIT/MS was also effective in characterization of monosialylated N-linked glycans.[428] The applicability of this technique was further extended to characterize a series of subunits generated from fucosylated and sialylated oligosaccharides.[427]

Recently, Perreault and co-workers[429,430] discussed the advantages of labeling free N-linked glycans with 1-phenyl-3-methyl-5-pyrrazolone (PMP), for HPLC and ESI/MS. The studies focused on some asialo and sialylated glycans, comparing HPLC and ESI/MS behaviors of the PMP-labeled glycans against the native counterparts. PMP-asialo glycans did not yield a significant increase in sensitivity relative to the native structures; however, fragmentation produced by in-source CID of the PMP-labeled glycans was less complex and more informative than that for the native glycans. This feature is particularly useful when structural determination of an unknown glycan structure is desired. The PMP-labeled monosialogly-

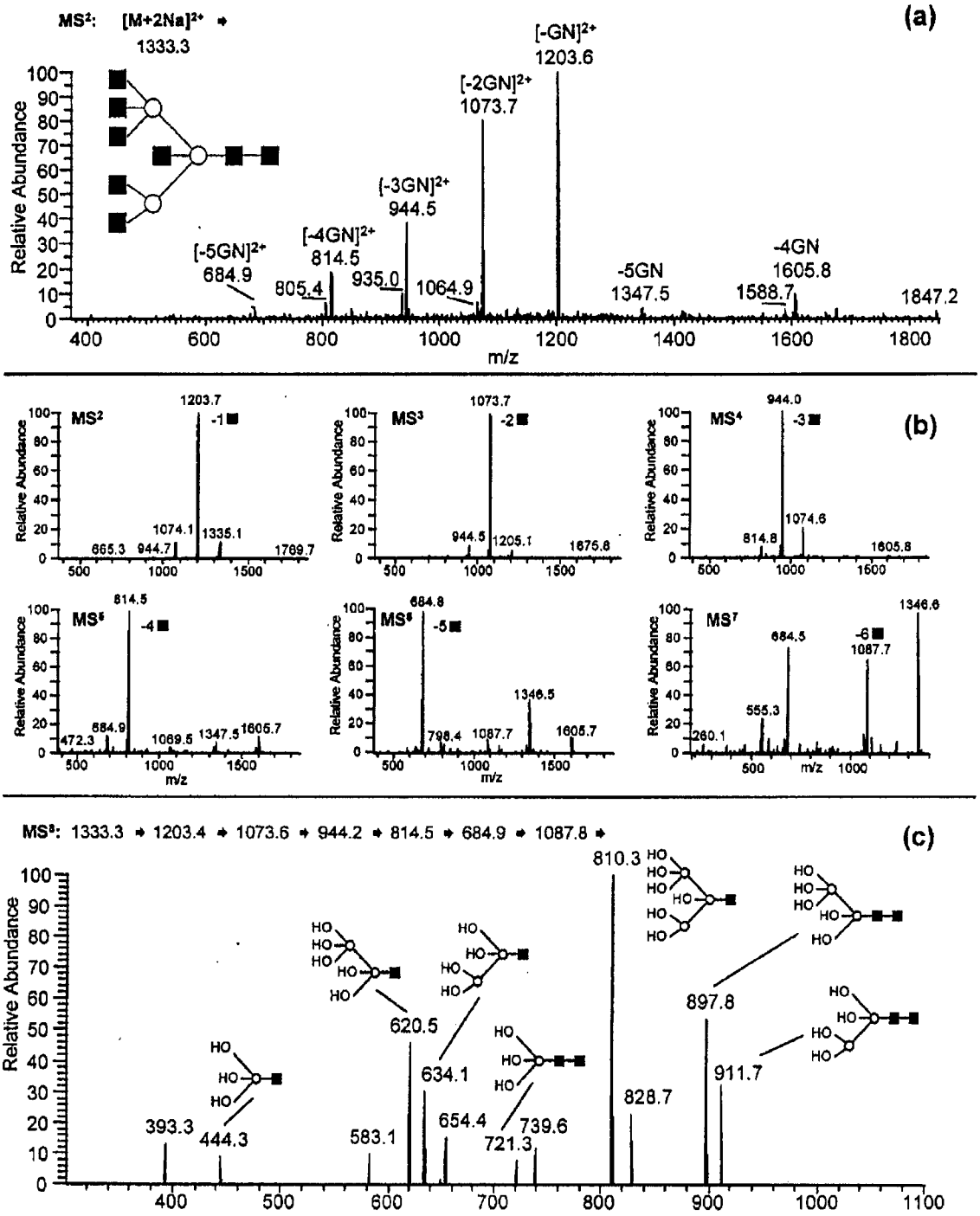


Figure 16. High-order MS^n analysis of the $GlcNAc_8Man_3$ from ovalbumin: (a) MS^2 of the $[M + 2Na]^{2+}$ parent ion, *m/z* 1333.3; (b) MS^2–MS^7 stepwise removal of *N*-acetylglucosamine residues; (c) MS^8 analysis of the exposed saccharide core for determination of branching patterns. Symbol key: (■) GlcNAc and (○) Man. (Reprinted with permission from ref 426. Copyright 1998 American Chemical Society.)

cans yielded a 100-fold sensitivity improvement, while a 100% sensitivity improvement was realized for a disialylated compound.[429]

Suzuki et al.[431] compared the sensitivity of various derivatives of oligosaccharides in ESI/MS. Their study compared the sensitivities for oligosaccharides labeled with α-aminopyridine, 4-aminobenzoic acid ethyl ester, 1-phenyl-3-methyl-5-pyrazolone (PMP), 2-aminoethanethiol, and 2-aminobenzenethiol. PMP derivatives gave the highest sensitivities in ESI/MS under the optimized conditions. Although the comparison study was based on linear oligosaccharides, PMP labeling allowed the microanalysis of N-linked glycans derived from ovalbumin and porcine thyroglobulin.

A hybride quadrupole orthogonal acceleration time-of-flight mass spectrometer (Q-TOF) in the precursor ion scanning and the MS/MS modes was utilized for the analysis of 2-aminoacridone-labeled N-linked glycan mixtures.[432] The use of a precursor ion scanning strategy on this instrument provides a rapid and sensitive method for screening glycan mixtures without a prior separation by chromatographic methods. Although this will not furnish information related to

linkages between the various sugar residues, it allows a facile and preliminary characterization of glycans into different classes such as high-mannose or complex types. Using the MS/MS mode of this instrumentation permits, with selected glycans, the determination of sequences and branching information. This methodology allowed characterization of the N-linked glycans derived from ovomucoid, ovalbumin, and porcine thyroglobulin. The same instrument was also utilized for the analysis of 2-aminobenzamide-labeled N-linked glycans from ovalbumin and related glycoproteins from egg white;[421] *N*-(2-diethylamino)ethyl-4-aminobenzamide-labeled standard complex, hybrid, and high-mannose N-linked glycans;[433] and 2-aminoacridone-labeled standard high-mannose, hybrid, and complex N-linked glycnas;[434] and 4-aminobenzoic acid 2-(diethylamino)ethyl ester-labeled N-linked glycans from IgY and thyroglobulin.[435]

Recently, Harvey[436] investigated the influence of alkali metals on the ionization and fragmentation of N-linked glycans examined with ESI/Q-TOF mass spectrometer. The glycans were ionized most effectively as adducts of alkaline metals, with lithium providing the most abundant signal and cesium the least. The energy required for CID fragmentation increased linearly as a function of mass with the sodiated molecular ions requiring about 4 times as much energy as the protonated molecular ions for a complete fragmentation of the molecular ions. Fragmentation of the protonated molecular ions was predominated by B- and Y-type glycosidic fragments, while the sodiated and lithiated molecular ions produced a number of additional fragments, including those derived from cross-ring cleavages. The sodiated and lithiated molecular ions from all N-linked glycans gave abundant fragments resulting from a loss of the terminal GlcNAc moiety and prominent, yet weaker, ^{0,2}A and ^{2,4}A cross-ring cleavage ions of this residue. Most other ions were the result of successive losses of residues from the nonreducing terminus. MS/MS data also allowed the high-mannose and complex N-linked glycans to be distinguished.

Electrospray fragmentation spectra of 12 sialylated carbohydrates were recorded on a Q-TOF instrument.[437] The fragmentation mechanism was investigated with the aid of several synthesized analogues of the sugars labeled with ^{13}C and ^{2}H. A substitution of the sialic acid dramatically effected the overall fragmentation pattern for these compounds. The appearance of an ion at *m*/*z* 306 appeared to be diagnostic of the presence of an α(2−6) linked sialic acid. Selection and further fragmentation of the in-source fragment ion corresponding to the trisaccharide Neu5Acα(2−3)(or 6)Galβ1−4GlcNAc from larger, N-linked glycans, ionized by electrospray, gave fragmentation patterns identical to those of the reference trisaccharides, thus furnishing a method for confirming a sialic acid linkage.

Recently, Cancilla et al.[438] described a general oligosaccharide acid hydrolysis method that is amenable to ESI/MS. The method allows for hydrolysis of the glycosidic bonds for both hexose- and *N*-acetylhexosamine-containing oligosaccharides. A partial acid hydrolysis of oligosaccharides was achieved by using a cation-exchange resin as the acid catalyst. A ladder sequence of the glycan is produced in solution that is directly analyzed by ESI employing both IT and FT-ICR mass spectrometers, providing sequence and linkage information. This acid hydrolysis did not promote excessive degradation of the monosaccharide residues or deacetylation of *N*-acetylhexosamines. The stereochemistry of the released monosaccharides and the anomeric configuration of disaccharides was determined by a direct derivatization of the hydrolysate with Zn(dien)-Cl_2 prior to ESI/MS/MS.

F. Nuclear Magnetic Resonance Spectrometry

NMR spectrometry is a valuable analytical tool for the characterization of protein-bound glycans, as it furnishes an unmatched wealth of information related to the number of sugar residues, constituent monosaccharides, anomeric configuration, linkage sequence, and position of the appended groups of these glycans. Moreover, an NMR spectrometer is a nondestructive detector, allowing recovery of the intact sample after analysis. However, one major drawback from the use of NMR for the analysis of glycans is the relatively large amounts of material needed (commonly, at the microgram level). Naturally the amount of needed sample is dependent on the type of NMR data required. Over the past few years, several approaches have been introduced to increase NMR sensitivity, including an increase in magnetic field strength,[4] developments in hardware,[439−441] probe designs, and RF coils,[442,443] and new sample tubes.[444] Since this review is concerned with highly sensitive methods, we shall review only the applications in the analysis of glycans derived from glycoproteins. The other aspects of NMR spectroscopy have been reviewed several times over the past several years.[445−449] A full description of the advances and aspects in the design of small volume probes has been communicated recently by Lacey et al.[444]

Although a substantial improvement in sensitivity of NMR and its coupling with separation methods such as HPLC,[450,451] CE,[452−459] and CEC[455,457,459] has been reported for a number of years, currently only one publication describes the utility of nano-NMR spectroscopy for the analysis of glycan mixtures.[460] The report appeared to analyze entire mixtures of N- and O-linked glycans, thus complementing HPLC profiling methods in elucidating structural details.

G. Miscellaneous Methods

A methodology permitting a medium-throughput analysis of N-linked glycans derived from low-picomole amounts of glycoproteins was recently described.[461] It uses the standard DNA-sequencing equipment available in most life science laboratories. The method involves several steps, including (i) deglycosylation of glycoproteins bound to Immobilon-P membrane in a Multiscreen plate through a

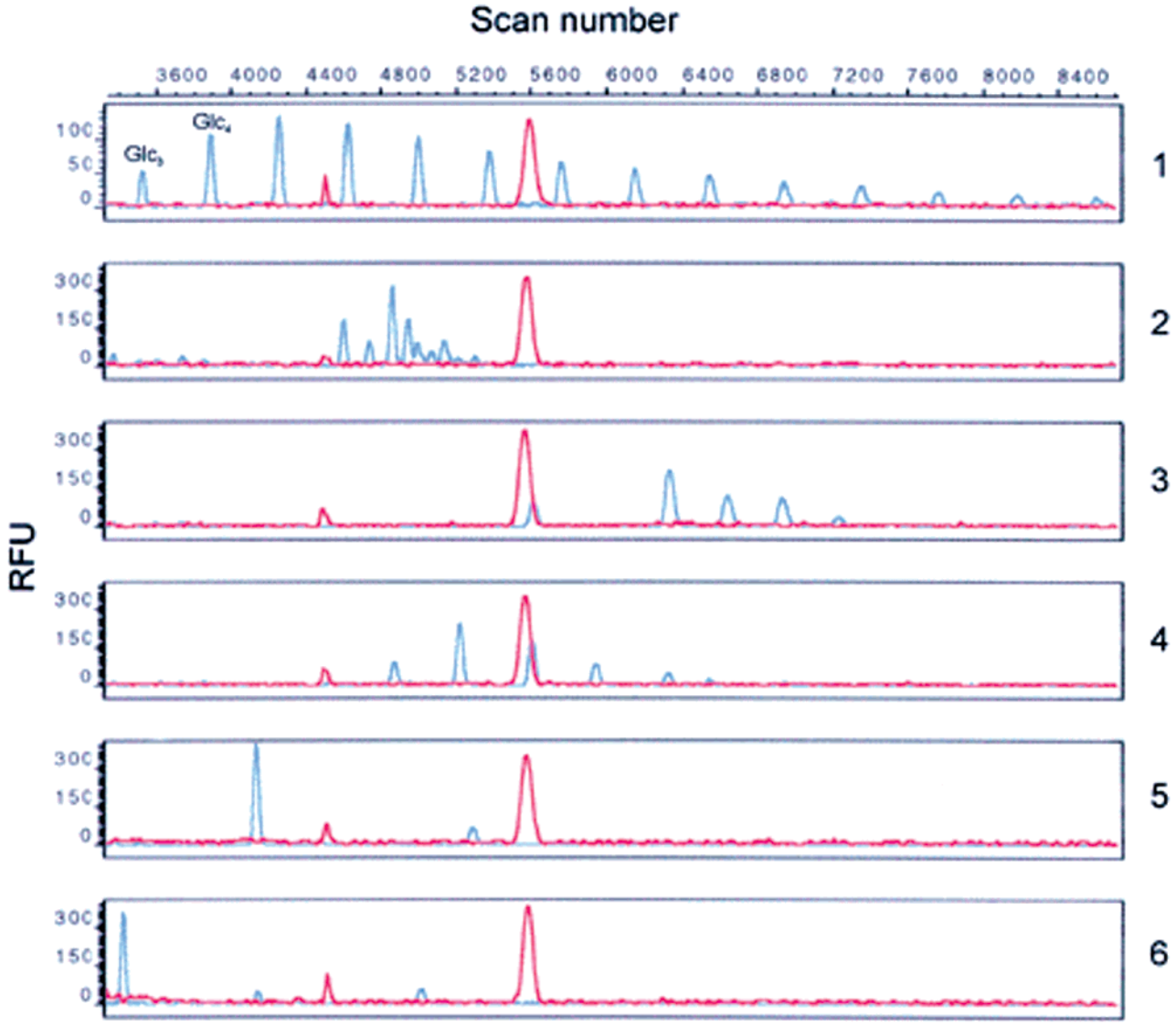


Figure 17. Profiling and sequencing of N-linked glycans derived from 500 ng of human α_1-acid glycoprotein. Deglycosylation after immobilization of the glycoprotein on Immobilon P, derivatization, sample clean up, and exoglycosidase digestions were as described. Blue peaks represent APTS-derivatized N-linked glycans, red peaks are ROX-labeled oligonucleotides serving as internal standards. The scan numbers are lower solely due to a slower laser scanning speed set in the instrument software. Panel 1: maltooligosaccharide sizing reference standard. Panel 2: nondigested α_1-acid glycoprotein derived N-linked glycans. Panel 3: *Arthrobacter ureafaciens* sialidase digest. Panel 4: sialidase + *Diplococcus pneumoniae* β-1,4-galactosidase digest. Panel 5: sialidase + β-1,4-galactosidase + jack bean β-*N*-acetylhexosaminidase digest. Panel 6: sialidase + β-1,4-galactosidase + β-*N*-acetylhexosaminidase + Jack bean α-mannosidase digest. (Reprinted with permission from ref 461. Copyright 2001 Publisher.)

treatment with PNGase F, (ii) dividing the resulting mixture into two parts, (iii) treating the part destined for MALDI/MS with acetic acid to obtain full conversion to reducing glycans, (iv) desalting this mixture using a Multiscreen Durapore plate filled with AG-50-WX8 cation-exchange resin to remove cations and PNGase F prior to the MALDI/MS analysis, (v) derivatization of the part destined for an electrophoretic analysis with APTS in a tapered-well plate, and (vi) removal of the excess derivatizing agent by passing the mixture through a partially dry Sephadex G10 bed packed in a Multiscreen Durapore plate. Centrifugal force was used in both AG-50-WX8 and Sephadex G10 chromatography procedures. A combination of the described approaches with exoglycosidase digestions provided structural information at the low femtomole level. Profiling and sequencing of N-linked glycans derived from 500 ng of human α_1-acid glycoprotein is illustrated in Figure 17.

VIII. Strategies for Analysis

A. Analysis of Glycoproteins

A strategy for the analysis of glycoproteins is summarized in Figure 18. This involves first the isolation of glycoproteins by immobilized-lectin affinity chromatography. Due to the reasons outlined earlier, further separations of the glycoprotein pools isolated by lectin chromatography are achieved through additional chromatographic and electrophoretic techniques, including anion-exchange and/or reversed-phase LC and SDS−PAGE and/or 2-dimensional (2-D) electrophoresis. These separation

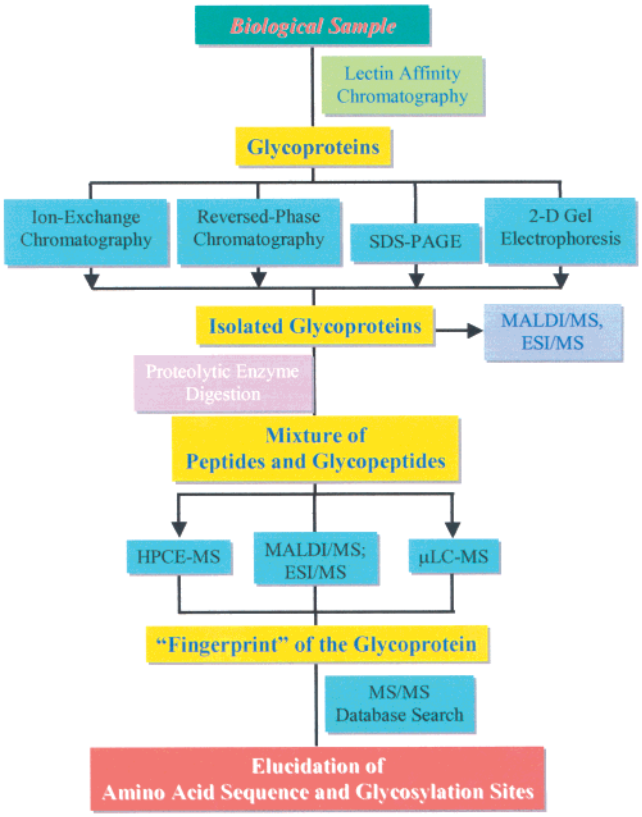


Figure 18. Strategy for the analysis of glycoproteins.

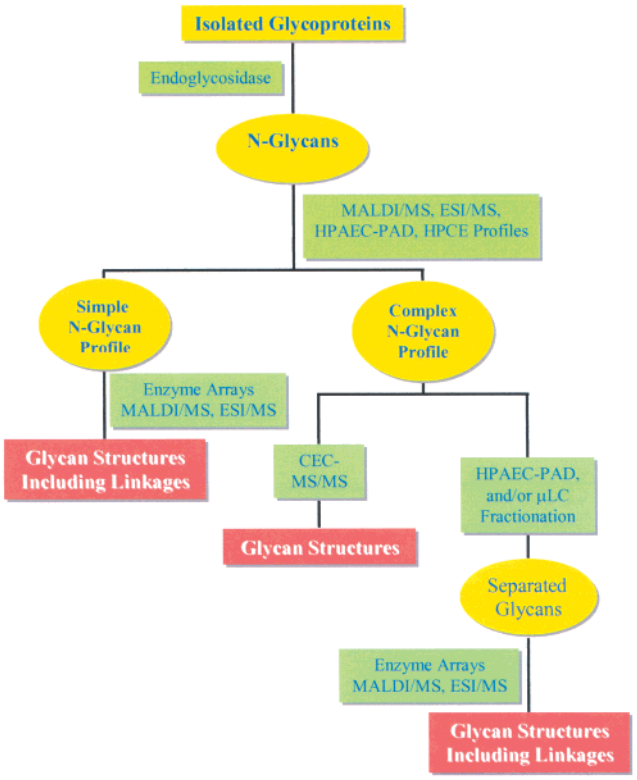


Figure 19. Strategy for the analysis of N-linked glycans derived from glycoproteins.

methods probe very different properties of the analyte because of their different separation mechanisms.

The next step in a glycoprotein analysis may involve determination of its molecular weight by MALDI/MS or ESI/MS. However, if information pertaining to the amino acid sequence and glycosylation sites is needed, an enzymatic digestion of an isolated glycoprotein, followed by either MALDI/MS analysis of the peptide map or a separation of the resulting peptides, logically followed by microcolumn LC or HPCE and then by MS detection, is best employed for the purpose. A glycoprotein proteolytic digest can further be digested with endoglycosidases prior to separation, thus permitting a confirmation of the glycosylation sites. Such steps provide a "fingerprint" of the glycoprotein, which is often adequate for comparison of samples from different cells, tissues, or species. Moreover, a complete amino acid map and glycan structures of this glycoprotein can be achieved through an on-line MS sequencing of the separated peptides and glycopeptides. Map information related to other types of posttranslational modification such as phosphorylation could be also determined either by MALDI[462−470] or ESI/MS[463,465−467,471−475] analysis of the digest. Finally, the MS data so produced can be searched for a match against the exponentially expanding databases of protein sequences, using search algorithms[476,477] to unmistakably identify peptides and, consequently, the protein from which they were derived.

B. Analysis of N-Linked Glycans

A strategy for the analysis of N-linked glycans is outlined in Figure 19. This strategy first involves an enzymatic release of N-linked glycans from the isolated glycoprotein, as highly efficient and specific enzymes are available (see above). The released N-linked glycans can then be profiled by many analytical approaches, including HPAEC−PAD, CE, and MS. Although HPAEC−PAD and CE possess high resolving power, MS is the ultimate approach, since it provides molecular weight profiles from which tentative structures can be deduced.

The next step in the analysis of N-linked glycans is dictated by a complexity of the acquired MS profile. A simple *N*-glycan mixture could be adequately characterized by tandem MS in conjunction with the enzyme-array sequencing. This allows determination of monosaccharide sequences, branching, and linkages for all *N*-glycan structures.

A complex *N*-glycan profile necessitates an incorporation of a separation technique prior to MS and enzymatic sequencing. Complex profiles can be substantially simplified through fractionation with multidimensional LC (or μLC), or CEC that are coupled to MS (on- or off-line). The on-line coupling will minimize sample handling and will provide information pertaining to monosaccharide sequences, branching, and some linkages, if tandem MS is performed. On the other hand, the off-line coupling will allow the enzymatic sequencing with exoglycosidase enzyme arrays and the acquisition of spectra from a digested mixture. These procedures can provide a wealth of information related especially to linkages.

Recently, determination of glycan structures from MS data has been made simpler through the introduction of several types of software and databases. Glycomode (http://www.expasy.ch/tools/glycomod/) is a software tool designed to find all possible composi-

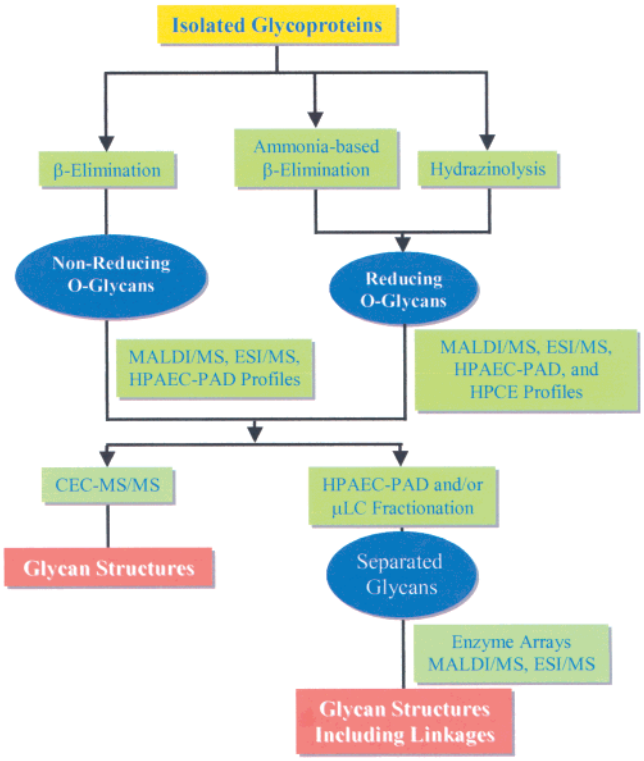


Figure 20. Strategy for the analysis of O-linked glycans derived from glycoproteins.

tions of a glycan structure from its determined mass.[460] The program can be used to predict the composition of any glycan derived from a glycoprotein comprised of either underivatized, methylated, or acetylated monosaccharides or with a derivatized reducing terminus. A biological O-linked database (BOLD), which was introduced recently, is a relational database that contains information on O-linked glycan structures, their biological sources, the references in which a glycan has been described, and the method used to determine its structure.[478]

C. Analysis of O-Linked Glycans

A strategy for the analysis of O-linked glycans derived from glycoproteins is outlined in Figure 20. As discussed previously, there are very limited options for the use of endoglycosidases specific to O-linked glycans; therefore, *O*-glycans are commonly released by chemical means. O-Linked glycans released by hydrazinolysis or ammonia-based β-elimination can be derivatized with a fluorophore and profiled by CE, CEC, or LC with LIF. HPAEC−PAD and MS can also profile the glycans released by these two approaches. On the other hand, HPAEC−PAD and MS analyses can mainly be utilized when a classical β-elimination is employed. A profile complexity will dictate the use of fractionation prior to the enzymatic sequencing. A multidimensional chromatographic approach is applicable to fractionations prior to enzymatic sequencing of each fraction and MS analysis. CEC-MS/MS can be also used to characterize reducing and nonreducing *O*-glycans. However, the lack of a reducing end limits the cross-ring fragmentations required for linkage determination; therefore, linkage information in the case of the reduced *O*-glycans is available only through enzymatic sequencing.

Acknowledgments

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