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Recent Trends in Analytical and Structural Glycobiology

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Abstract

The great complexity of glycosylated biomolecules necessitates a set of powerful analytical methodologies to reveal functionally important structural features. Mass spectrometry (MS), with its different ionization techniques, mass analyzers, and detection strategies, has become the most important analytical method in glycomic and glycoproteomic investigations. In combination with MS, microscale separations (based on capillary chromatography and electrophoresis) and carbohydrate microchemistry, we feature here conceptually important applications of the recent years. This review focuses on methodological advances pertaining to disease biomarker research, immunology, developmental biology, and measurements of importance to biopharmaceuticals. High-sensitivity determinations and sample enrichment/preconcentration are particularly emphasized in glycomic and glycoproteomic profiling.

Introduction

The enormous structural diversity of glycoconjugates reflects their multilateral importance in biochemical recognition. Extensive glycosylation of proteins is featured inside different cells, on their surfaces, and the extracellular spaces of diverse organisms. While glycosylated structures were traditionally considered within the domain of multicellular eukaryotic systems, studies of the last decade have documented the presence of oligosaccharides (often with unusual monosaccharides) in the microbial world as well [1,2]. Although the methodologies for glycoanalysis have advanced substantially during the last several years [3,4], identifying and quantifying the glycome and glycoproteome still represents a daunting task for the current and future generations of glycoscientists. Many modern glycoconjugate analytical techniques rely on mass spectrometry (MS), which has gradually become the most prominent tool in the structural characterization of glycoproteins. Additionally, capillary-based separation methods coupled with MS enhance the positive identification of glycan isomers, describe the sites of glycosylation, and decipher their microheterogeneity. The structural complexity of the resulting glycomic and glycoproteomic data needs extensive use of bioinformatic tools [5] for structural interpretation. Yet different approaches to understanding glycan-protein interactions have been pursued through the technologies of glycan and lectin arrays pioneered a decade ago (see the review by L. Mahal, this issue), which appear complementary to MS-based systems.

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Scope of Investigations

From microbes to the most sophisticated multicellular organisms, glycoconjugates are increasingly recognized as the key determinants in both extracellular and intracellular functions. Biological investigators with interests ranging from embryology and developmental biology, to evolutionary development and physiology, increasingly subscribe to the "glycobiology approach." At different levels of experimental difficulties, there are now methodological options to tackle some of the most difficult problems of glycoprotein structural characterization. In the time-honored approach, some investigators isolate the glycoproteins of interest through affinity chromatography or gel electrophoresis. The isolated and purified glycoproteins can then be subjected to a controlled protease-based degradation, followed by a further chromatographic separation and measurement of glycopeptides (glycoproteomic approach), and additionally or alternatively, a sample aliquot can be deglycosylated, either enzymatically or chemically, to yield a series of oligosaccharides for further (glycomic) measurements. The glycoprotein amounts available through such isolations often determine the success of structural characterization. Fortunately, the sensitivity, mass resolution, and mass accuracy of today's MS-related techniques enable extensive characterization of both the polypeptide and glycosylated parts of fairly complex biomolecules. This is seen in examples of identifying the microbial virulence factors [6,7]. In less frequent situations, sufficient quantities of isolated glycoconjugates permit the use of protein crystallography and NMR techniques to appreciate the most intimate details of the glycan interactions with their biologically relevant binding proteins [8].

While research activities in mammalian (and human) glycobiology continue, there has been increasing interest in microbial and parasitic systems during the recent years. Apparently, a number of surface-layer glycoproteins have now been described in a number of species, featuring both N- and O-linked glycans and some unusual monosaccharides in their glycan structures [6,7]. While there is less information available at this time [9] on other parasites, genomic studies point to the occurrence of glycosylation throughout the entire range of eukaryotic systems (worms, insects, plants, fish, etc.). Here, glycomic and glycoproteomic comparative measurements are likely to yield information of value to developmental biologists studying appropriate "model systems" (as an example, see *Drosophila* [10]). In particular, isotopic labeling, MS-techniques, and appropriate uses of knock-out technologies for glycosyltransferase genes can all provide valuable information. From the human health viewpoint, protozoans [9], parasitic helminthes [11] and ticks [12] have already been targets of glycomic studies.

The biotechnology industry has been producing a number of glycoprotein-based drugs, most notably monoclonal antibodies, as therapeutics against cancer and inflammatory diseases. In turn, the use of recombinant antibodies and the more recently introduced "biosimilars" necessitate very stringent analytical control of their chemical compositions and physicochemical properties. The glycans incorporated into the biopharmaceuticals are known to influence important properties, such as *in vivo* circulatory half-life and possible antibody-dependent cytotoxicity [13,14]. Additionally, antigenic epitopes could possibly be introduced during the manufacturing process involving non-human cell lines [15]. Thus, the immunogenicity of unusual glycans must be closely examined in the selection of different eukaryotic expression systems. Tracking "correct glycosylation" in recombinant products has resulted in the design of different analytical platforms and methodologies, some based on MS and the others on fluorescence labeling together with HPLC and CE. Several analytical strategies concerning IgG glycosylation have been reviewed [16]. The current needs for better standards for biological functions and structural attributes of antibody-based pharmaceuticals is likely to drive further methodological developments.

Among the most challenging areas of contemporary glycobiology are high-sensitivity, multicomponent analyses of complex biological mixtures, such as physiological fluids and tissue extracts (as an example, see Figure 1). This area has largely been driven by the search for biomarkers of human diseases and the many connections that glycobiology has to health-related issues. Many known human diseases and metabolic disorders have been tentatively linked to aberrantly glycosylated proteins for a number of years [17], but only recently has it become possible, through methodological improvements in analytical glycobiology, to appreciate quantitatively the extent in which a “pathological glycome” could be distinguished from normal conditions and how such quantitative measurements could potentially be used in clinics. Common physiological fluids, such as blood serum or plasma, can nominally be analyzed, but other biological samples (cell lines, cysts, tumor biopsies, etc.) are also applicable.

In-depth investigations of mammalian glycomes and glycoproteomes in complex biological samples are complicated by the very extensive concentration range in which various (glyco)proteins occur [18]. Whereas “total” (or “global”) glycomic profiling of small aliquots of blood serum from cancer patients can yield diagnostically or prognostically useful information [19], it will be necessary to cover a multitude of glycoproteins spanning the range of nearly 10 orders of magnitude. This task is difficult to accomplish without developing effective preconcentration strategies.

A more complete understanding of the roles of glycosylation in the immune system has also been important. While it is established that both the innate and adaptive immunity involve glycoproteins, the use of highly sensitive glycomic and glycoproteomic techniques will be needed to address the intricacies of the immune system dealing with inflammation and cancer [20]. Glycomic methodologies can be utilized in the structural characterization of neutrophils [21], eosinophils, basophils, and mast cells [22]. With reference to bodily fluids, the abundant IgGs and their different chains have been the obvious target of current investigations ; as the microisolation techniques further evolve [3,23], less abundant, albeit biologically important, minor immunoglobulins can also be measured. Glycan isomerism in sialylation and fucosylation could potentially be involved in the intricate functions in immunity. Recent reviews specifically endorse glycomic and glycoproteomic techniques [3,24].

Preconcentration of Glycoproteins from Complex Mixtures

Structural characterization of trace glycoproteins in complex biological materials continues to rely on the methodologies that can selectively isolate the target molecules or groups of glycosylated species from non-glycosylated molecules which otherwise mask their presence during analytical measurements. A typical example of such interferences is encountered during a coincidental chromatographic elution of glycopeptides with non-glycosylated peptides, in which competitive ionization results in a signal suppression during MS interrogations. During analyses of serum or plasma, it is common to deplete the major proteins by one of the commercially available immunoaffinity chromatographic columns prior to glycomic or glycoproteomic measurements. Immunoaffinity steps can also facilitate identification and quantification of *a priori* known minor glycoprotein target molecules through a design of specialized capture materials [25]. A recent example includes isolation of haptoglobin [26], followed by MS measurements demonstrating increased glycan fucosylation in pancreatic cancer, as well as similar measurements in hepatocellular cancer [27]. However, the immunoaffinity approach is somewhat limited by the availability of specific antibodies and their price.

For high-sensitivity measurements, the availability of miniaturized reactors constructed from optimal solid supports with minimum irreversible and nonspecific interactions is essential to sample recovery. A miniaturized serial trapping of the major and minor immunoglobulins has been demonstrated [23] prior to their glycomic profiling from small (a few μL) volumes of blood serum. While glycosylation of major proteins encountered in biological fluids could be informative in different health-related situations, measurements of tissue-derived glycoproteins (putative disease biomarkers) will undoubtedly require a new set of detection approaches [28].

Whereas different lectins have long been utilized in biochemical and histological practice, their analytical (quantitative) explorations are relatively recent. The degree of selectivity toward different glycan structures (differently linked sialic acids, high-mannose structures, fucosylation, etc.) can be utilized in complex fractionation and glycoprotein preconcentration schemes, as well as in the fabrication of lectin arrays (L. Mahal, this issue). Microscale affinity techniques have been essential in numerous investigations to characterize the subglycoproteomes from a wide array of biological matrices, including human urine, saliva, ovarian tissue [29], pancreatic cysts [30] and blood serum [31]. In the quantitative uses of lectin preconcentration, the immobilized lectins should ideally be bound to pressure-resistant support materials, such as silica particles [32] or monolithic polymers [33], which can then be used in conjunction with LC/MS-MS as a part of an integrated on-line system capable of comparing quantitatively hundreds of different glycoproteins [31,34] through bottom-up proteomic analysis. A recent review [35] estimates that there are 160 easily obtainable lectins, some of which could be selectively chosen for different enrichment schemes. The currently available lectins feature both overlapping and fairly unique interactions with glycoproteins [33].

Glycopeptide Enrichment

Specific enrichments or fractionations of peptidase digests are generally advisable before high-sensitivity MS or LC/MS-MS measurements of glycopeptides in order to reduce interferences from other peptides. Enrichments can be broad in their specificity, as is exemplified by the use of hydrophilic interaction chromatography (HILIC) [36,37], demonstrated in Figure 2, which depicts the fractionation and enrichment of both O- and N-glycopeptides in a single chromatographic experiment. Glycopeptides exhibiting certain saccharide motifs may also be enriched through lectin chromatography [38], although these procedures appear more effective for intact glycoprotein mixtures. Sialylated glycopeptides are quite selectively enriched with TiO_2 particles using very acidic buffer conditions [39]. An innovative approach to enrich cell-surface glycoproteins is to incorporate unnatural azide-modified carbohydrates into the glycan structures through metabolic labeling [40]. The azide (or alkyne) moiety is subsequently reacted via a “click” mechanism to a phosphine-FLAG peptide, and the so-labeled glycoconjugates are, in turn, isolated using an anti-FLAG antibody [41].

Non-specific enrichment procedures, in which the carbohydrate becomes covalently attached to appropriately functionalized solid supports, have become increasingly common in the practice of proteomic measurements. Since the initial report on the “hydrazide capture” [42], a modified procedure was reported [43] where samples are tryptically digested prior to periodate oxidation of carbohydrates’ vicinal diols to aldehydes and formation of hydrazones with the surface of functionalized beads. Subsequent variations in the protocols for the oxidation/hydrazide capture [44] aim at further optimization for quantification purposes. Boronic acid-functionalized materials provide another option for glycopeptide enrichment through utilization of the vicinal diol chemistry [45], although they seem less popular, presumably due to the weak binding constants.

Mass Spectrometry Advances

MS capabilities, particularly the tandem MS modes, have further evolved for the benefits of both glycomics and glycoproteomics. While collision-induced dissociation (CID) is still extensively used, the tandem MS innovations emphasizing electron-transfer dissociation (ETD) hold significant potential for the characterization of glycopeptides. This radical-initiated process is generally advantageous for post-translationally modified peptides, as the modification is largely unaffected by the fragmentation process, allowing ETD to assess the site of attachment and yield information on the multiple glycans associated with each site [46,47]. Another recent development in tandem MS is the higher-energy collisional dissociation (HCD) method that is performed in the octopole of Orbitrap instruments [48]. One of the most intense product ions is the so-called Y_1-fragment (peptide + GlcNAc ion), which can be re-isolated, and in a subsequent HCD experiment, allowed to dissociate, yielding the amino-acid sequence [49]. Moreover, this approach can be used to selectively "trigger" ETD procedures if the oxonium ions (carbohydrate-specific fragments) are detected [50].

Tandem MS of glycans also continues to mature toward the negative-ion mode techniques, permitting extensive cross-ring fragmentations and thus yielding linkage-specific and branching information. While first reported some time ago [51], the general approach continues to be refined and combined with nano-scale LC [52] using porous graphitic carbon (PGC), a medium known for its ability to resolve isomeric glycans, as the stationary phase, with fluoride ions as a mobile-phase additive.

Ion-mobility spectrometry (IMS) coupled to MS is becoming more widely adapted within the glycoscience community due to the recent availability of commercial instrumentation. IMS holds potential as a gas-phase separative technique to resolve some isomeric structures, based on their unique collisional cross-sections. Moreover, in a recent report [53], the potential of IMS/MS has eloquently been demonstrated by the application to crude enzymatic digests (containing both peptides and glycans without desalting) to minimize sample preparation and handling steps. Peptides and carbohydrates could be separately detected, as they occupied different regions of the IMS drift space, with good sensitivity and reproducibility.

Glycan Profiling

Scientists who emphasize the integrated roles of the glycomes in different biological phenomena are beginning to appreciate new methodologies that enable entire profiles of glycolipids, oligosaccharides released from glycoprotein mixtures, or other glycoconjugates to be displayed quantitatively. These "glycan mapping" efforts can be increasingly correlated with screening the genes coding for corresponding glycosyltranferases [54], or even *in vivo* imaging of glycosylation through metabolic labeling [55] in model biological systems. How complex can the individual glycomes be for different species in their entire extent? From the nearly endless combinations of monosaccharide units and isomerism to form hypothetical glycans [56], functional arguments seem to restrict the number to less than 10,000 structures [57], This is still a respectable task for analytical profiling measurements, but knowing precisely the structural details of even trace carbohydrates will likely advance the knowledge of the binding protein interactions and the means to control them pharmaceutically. Consequently, the effective ways of glycan profiling should be: (a) as inclusive as possible; (b) structurally informative; (c) highly sensitive and quantitative; and (d) facilitating detection and measurements in a wide dynamic concentration range.

MS in its different ionization modes and mass-analyzing capabilities, provides a nearly ideal and the most sensitive means to cover the expected range of structures for N- and O-linked

oligosaccharides [58]. However, MS is not always informative concerning the frequent isomerism of glycans and its biological significance, unless particular isomeric species are selectively derivatized [59]. Fortunately, capillary liquid-chromatographic and electrophoretic methods can often resolve structural isomers, and when used in combination with MS, they lead to effective analytical platforms for glycan profiling.

To begin with, glycans must be liberated quantitatively from the glycoproteins of interest. A quantitative and reproducible release of oligosaccharides has acquired great importance with today's emphasis on high-sensitivity measurements. The traditionally-used chemical release procedures relying on hydrazinolysis or Carlson-type β-elimination have now been replaced by enzymatic N-deglycosylations (through the use of N-glycanases, largely the commercially available peptide N-glycosidases F and A), and microscale β-elimination procedures for threonine/serine-linked oligosaccharides. Largely due to the availability of N-glycanases, N-glycans are generally easier to analyze than O-glycans. Reducing digestion times without sacrificing digestion efficiency has been pursued in the recent studies involving ultra-high pressure cycling [60] or microwave radiation [61], and the use of immobilized reactors [62]. In the area of O-glycans, alternative chemical cleavage procedures were actively sought, including the use of dimethylamine coupled with microwave radiation [63] and the O-glycan recovery utilizing Pronase for a complete protein digestion, followed by a solid-phase permethylation and its coincident β-elimination from serine and threonine sites [64]. Profiles of released glycans can be quantitatively displayed through MALDI-MS, as exemplified by Figure 3 for blood-serum originated N-glycans, or ESI-MS, with the latter typically combined with liquid chromatography (LC). Both approaches are finding their utilization across different biochemical areas. The distinct advantage of MALDI-based techniques is their sensitivity and relative tolerance to sample impurities. ESI has the disadvantage of producing a complex set of differently-charged ions for the same parent mass. With regards to MS-fragile glycans (due to sialylation, or fucosylation, and the occasional presence of sulfate or phosphate groups), it is often recommended to derivatize glycans prior to their MS analysis. The time-honored approach of permethylation has now been widely utilized [3] to (a) stabilize the analytes; (b) include both the neutral and acidic glycans in a single profile; and (c) enhance sensitivity and create more discernible tandem MS fragmentation patterns. Permethylation is also desirable in making the glycans of interest sufficiently hydrophobic for reversed-phase LC separations in combination with MS [65]. Isotopic labeling through permethylation [66,67] further enhances the use of this derivatization for comparative profiling measurements.

Glycomic profiling can also be performed, and often significantly enhanced, through the use of separation techniques such as HPLC, capillary LC, or capillary or chip-based electrophoresis (CE). Fluorescence derivatization at the microscale is usually important for the detection of profile constituents. This is perhaps best demonstrated by successful applications of HPLC/fluorescence detection in search for disease biomarkers [68,69], and the increasing use of hydrophilic interaction chromatography (HILIC) featuring smaller particles [36,37], as well as the demonstrations of HILIC in resolution of glycan isomers [37].

The rising popularity of a unique chromatographic material, porous graphitized carbon (PGC), can largely be attributed its effectiveness in separating isomeric glycans [70]. PGC small columns, combined with MS, have been utilized in the highly sensitive analyses of very small biological samples [71].

Microfabricated devices (microchips) have recently become popular in various glycan separations. Not only can the separatory channels within some microchips be tightly packed with chromatographic particles, but it is also possible to include various trapping columns,

microreactors, and switching valves into an integrated analytical unit; this reduces the number of manual operations and sample transfers and, moreover, allows the microchip to be connected as a specialized “MS inlet”. The LC-based microchips, employing different packing materials have been demonstrated with the ESI-MS analysis of permethylated glycans extracted from blood serum [65]. Using graphite chips, both serum [72] and breast milk samples [73] were extensively characterized by the Lebrilla group. Another separation technique, CE, has also been applicable to very efficient separations of glycan mixtures (including isomeric structures) which had been labeled with a suitable fluorophore for detection. Here, too, the originally-used capillary formats quite easily translate to the microchip designs [74,75], achieving greater separation efficiencies and shorter run times. Unfortunately, CE is not easily combined with MS for the needed positive identification of separated glycans. The general lack of authentic glycan standards has also been a serious hindrance to positive identification of CE-separated glycan mixtures. Advances in coupling CE with MS have recently been reviewed [76,77].

Conclusions

In structural terms, the current and future explorations of glycomes and glycoproteomes are strongly dependent on the further evolution of MS instrumentation. The emphasis on high measurement sensitivity and information content is justified by the needs to characterize trace-level constituents of complex biological mixtures, as exemplified by the search for disease biomarkers. Selective sample preconcentration, glycan derivatization at the microscale, stable-isotopic labeling, and small-scale separations, such as capillary HILIC and CE, will continue to be used to resolve isomeric structures and simplify the task of MS measurements. There is also a significant trend to integrate the entire glycomic and glycoproteomic analytical platforms into a microchip format. The lack of authentic glycans still hinders the field of analytical glycobiology; it will hopefully be overcome by the current efforts in carbohydrate synthesis.

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Highlights

- In-depth structural details of biologically diverse glycomes and glycoproteomes remain important to study.
- Mass spectrometry has become the most important tool in structural glycobiology.
- Quantitative glycomic profiling has been greatly assisted through the use of capillary and microfabricated separation devices.

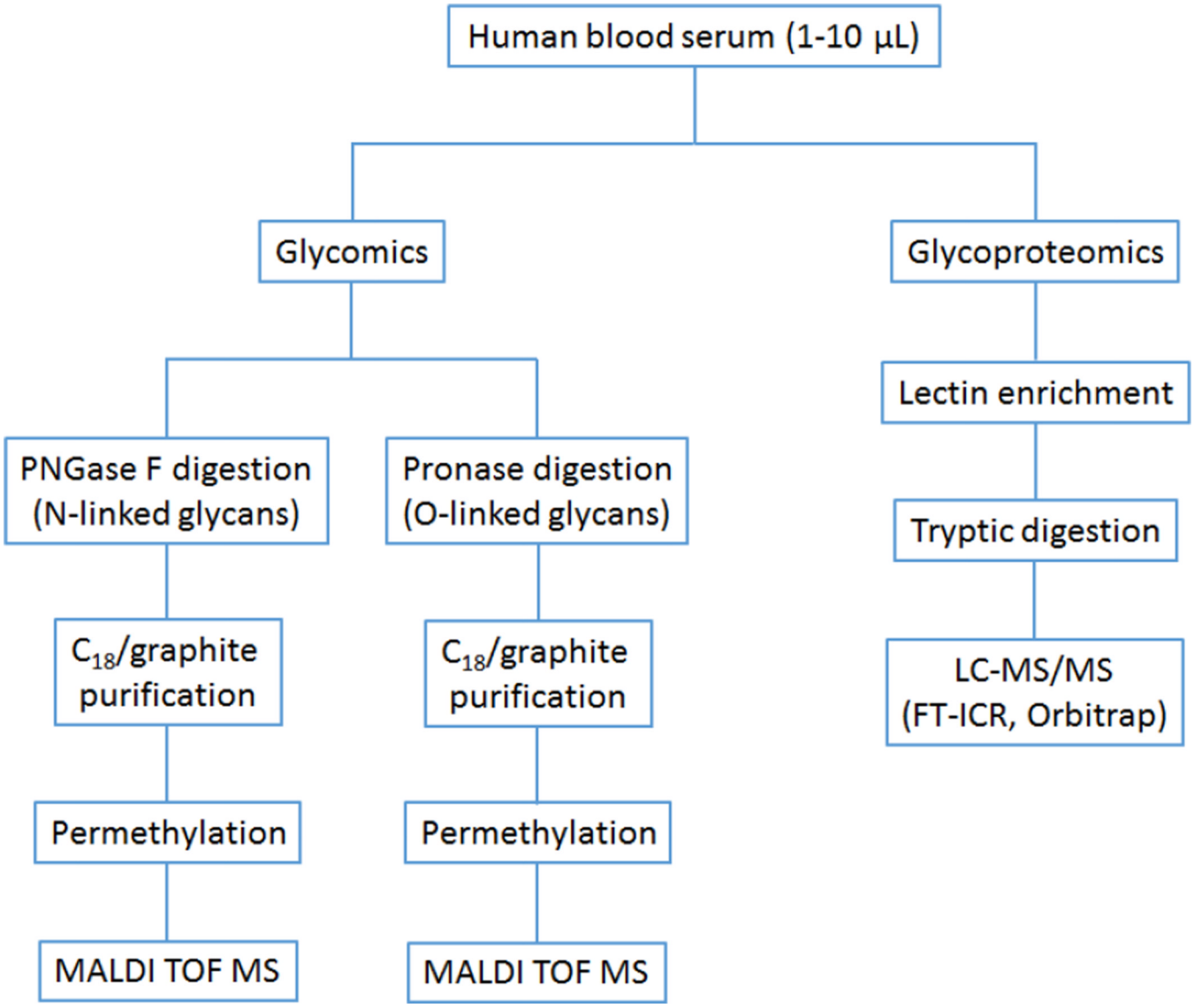

Figure 1.
Workflow for high-sensitivity glycoprotein analysis used in our laboratory for complex biological samples.

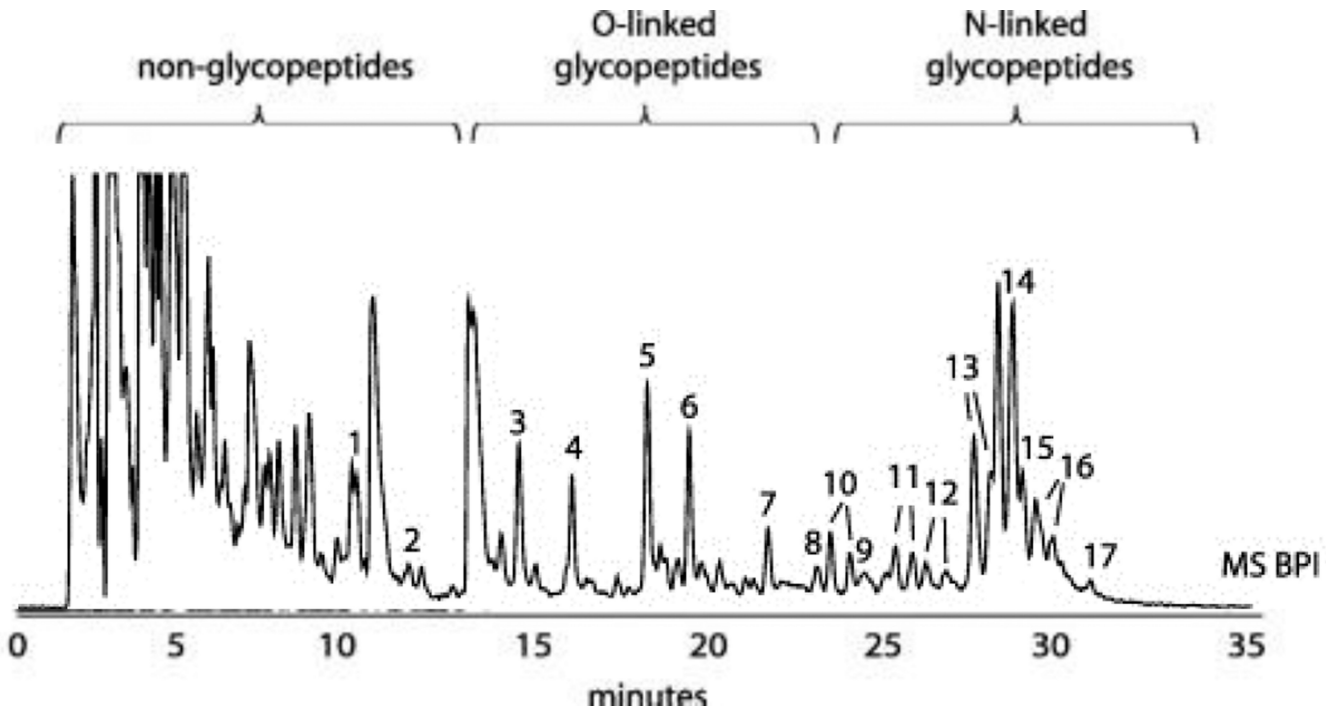

Figure 2.
An example of the power of HILIC enrichment for tryptic glycopeptides (derived from bovine fetuin). Adapted from Ref. 37.

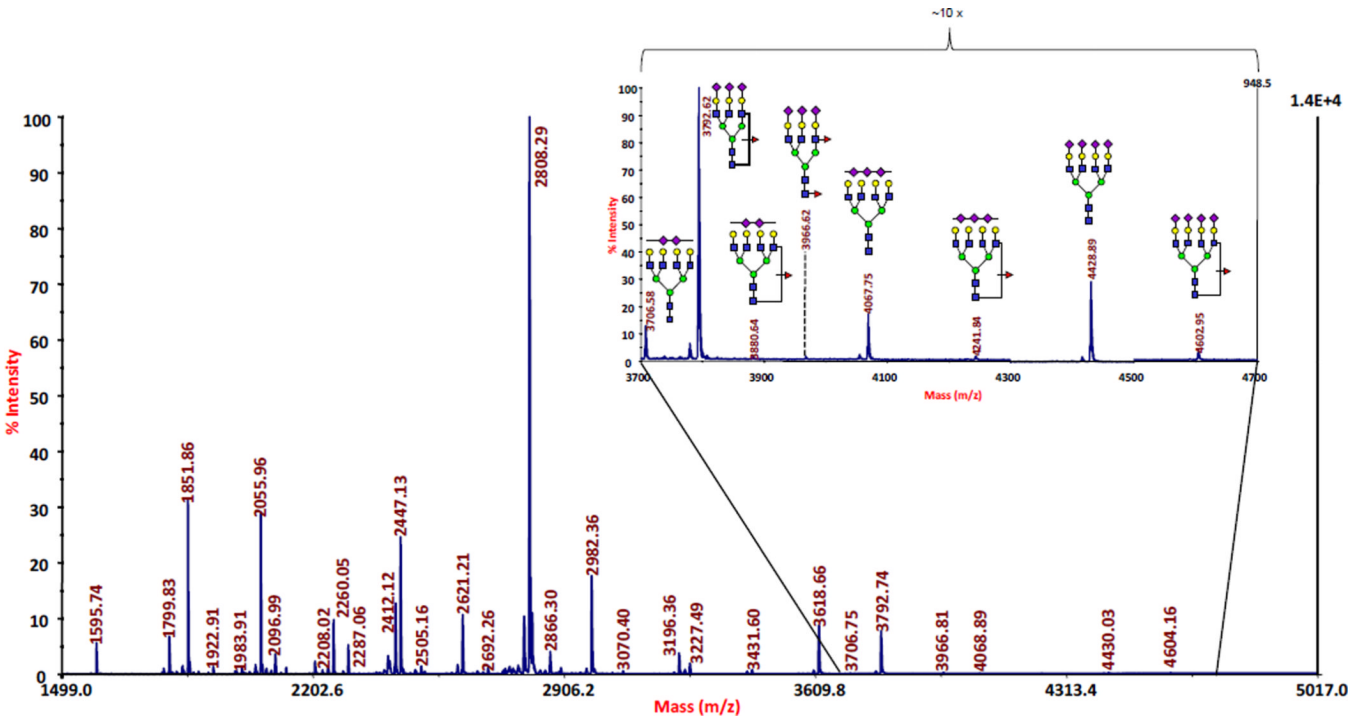

Figure 3.

A MALDI-MS profile of permethylated glycans derived from a blood serum sample originated from an ovarian cancer patient. The series of glycans marked in the high-mass region are diagnostically important. Adapted from Ref. 19.