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Review

# Mass Spectrometry and the Emerging Field of Glycomics

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The biological significance of protein and lipid glycosylation is well established. For example, cells respond to environmental stimuli by altering glycan structures on their surfaces, and cancer cells evade normal growth regulation in part by remodeling their surface glycans. In general, glycan chemical properties differ significantly from those of proteins, lipids, nucleic acids, and small molecule metabolites. Thus, advances in glycomics, a comprehensive study to identify all glycans in an organism, rely on the development of specialized analytical methods. Mass spectrometry (MS) is emerging as an enabling technology in the field of glycomics. This review summarizes recent developments in mass spectrometric analysis methods for protein-based glycomics and glycoproteomics workflows.

## Introduction

Glycosylation is the most common posttranslational modification, occurring in up to half of all gene products (Apweiler et al., 1999). As illustrated in Figure 1A, all cells are coated with a dense layer of glycans, a fact that implicates the importance of glycobiology in multicellular life. Glycan expression figures prominently in cell-cell and cell-matrix interactions. Glycans are expressed in a cell-type specific and a temporally regulated manner to allow cell phenotypes to change dynamically in response to environmental stimuli (Varki, 2006). Their biosynthesis occurs in the lumen of the Golgi apparatus in a nontemplate-controlled manner, and mature structures arise through the activities of a series of enzymatic reactions that are specific to each glycoconjugate class. The mature structures are cell-type specific and depend on multiple factors, including enzyme and nucleotide sugar availability and kinetics of glycoconjugate transport. As a result, glyconjugate glycans are mixtures of variants (glycoforms) on a core structure. This serves to diversify the biological functions of the limited number of genes in higher animal genomes to create tissue structures with defined boundaries and controlled functions.

Glycosylation has the potential to affect all stages of protein lifetimes, from folding and delivery to the cell surface to their interactions with binding partners, their degradation, and their turnover (Varki, 2007). As illustrated in Figure 1B, glycans-modifying proteins in animal cells include *N*-linked (bound to an asparagine side chain in an NXT or NXS amino acid consensus sequence with X ≠ proline), *O*-linked (with glycans attached via serine or threonine side chains), and glycosaminoglycans (bound to a serine side chain).

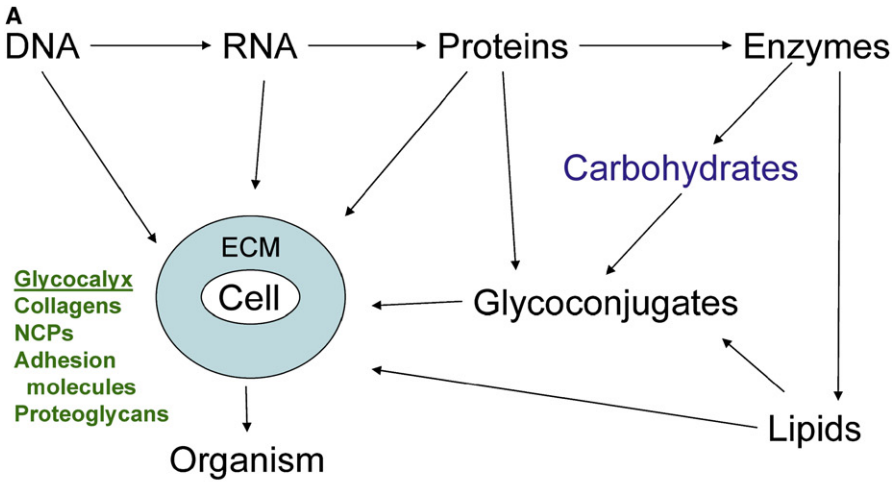
All *N*-glycans contain a conserved $GlcNAc_2Man_3$ core structure, termed the chitobiose core. High mannose *N*-glycans contain only mannose attached to the core. Complex *N*-glycans have had all mannose residues removed by enzymatic processing and replaced to form antenna structures. Hybrid *N*-glycans have been incompletely processed and have at least one antenna containing only mannose residues. Many cytoplasmic and nuclear proteins are modified by β-O-GlcNAc at Ser/Thr side chains. Secreted and cell-surface proteins may be modified by *O*-linked glycans that are generated by the elaboration of GalNAc residues at serine or threonine side chains. Several *O*-glycan core structures exist, and there is considerable structural diversity in this glycan class. Glycosaminoglycans are linear-sulfated glycans that are bound to serine residues through a characteristic tetrasaccharide linker. In some cases, *N*-glycans may be elaborated with glycosaminoglycan chains.

Glycomics is the study of the significance of glycoconjugate expression in biological systems. Because glycosylation involves a series of metabolic events, some of the concepts in lipidomics (German et al., 2007) and metabolomics (Dettmer et al., 2007) are common to glycomics. All three fields lend themselves to database-searching algorithms that reference compound libraries rather than the genomic databases used in proteomics. In all three, the mass spectrometry (MS) dimension defines the chemical composition of observed biomolecules in a given sample, and tandem MS is useful for determining structural details and defining isomeric mixture compositions. Complex glycans have masses that exceed the range typically used for metabolomics datasets (<1000 Da). Their chemistry differs significantly from those of proteins, lipids, and small molecule metabolites, and different sample preparation, chromatography, and ionization methods must be used.

The purpose of this review is to summarize recent developments in mass spectrometric glycomics related to protein-based glycoconjugates. To illustrate the methods used in emerging glycomics workflows, mass spectrometric ionization and chromatographic interfaces and their application in quantitative studies are summarized. Particular attention is paid to the uses of tandem MS for analysis of released glycan mixtures. Lastly, the review summarizes emerging glycoproteomics methods. Due to space limitation and the vastness of the filed, it is not feasible to discuss all the publications and contributions, and the understanding of authors whose work may have been omitted is requested.

## Mass Spectrometry of Glycans

Sample preparation and ionization are critical components of MS glycomics workflow. Thus, this section will briefly discuss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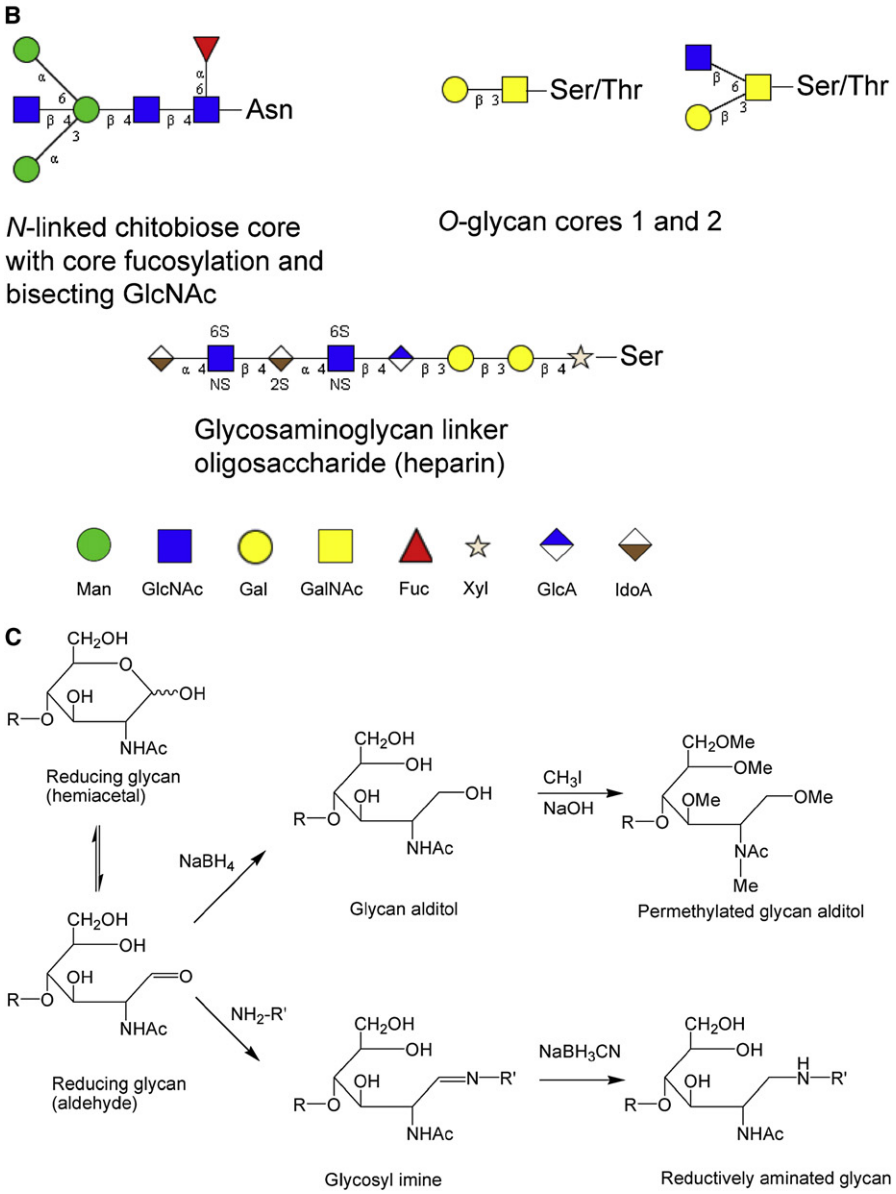
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Ser
Glycosaminoglycan linker oligosaccharide (heparin)
Man
GlcNAc
Gal
GalNAc
Fuc
Xyl
GlcA
IdoA
C
Reducing glycan (hemiacetal)
NaBH4
Glycan alditol
CH3I
NaOH
Permethylated glycan alditol
Reducing glycan (aldehyde)
NH2-R'
Glycosyl imine
NaBH3CN
Reductively aminated glycan

different ionization methods used, as well as sample preparation and separation strategies that lead to improved analytical results.

### Derivatization of Glycans for Mass Spectrometry

Glycans released by enzymes or nonreducing chemical methods have reducing ends that are an equilibrium between cyclic hemiacetals and open-ring aldehydes. Carbohydrates are derivatized to increase their volatility and stability for MS analysis. As shown in Figure 1C, glycans are often reduced to alditols to prevent the separation of anomeric forms of the reducing-end residue. Permethylation of glycan alditols results in the conversion of all hydrogen atoms that are bound to oxygen and nitrogen atoms to methyl groups and serves to render glycans hydrophobic. Permethylated carbohydrates are considerably more stable than native glycans and produce more informative tandem mass spectra. Permethylation is not compatible with some glycan modification groups (sulfate and phosphate). In addition, sample losses occur during the derivatization workup.

Reductive amination is widely used for the derivatization of reducing glycans (Anumula, 2006), as shown in Figure 1C. The advantage to this reaction is that it is reliable and may be used to attach a chromophore, fluorophore, or stable isotope tag to a single position of a glycan. Reductive amination works for *N*-linked glycans released enzymatically from proteins and to any glycan class that contains a reducing end. It is not applicable to *O*-glycans released under reductive conditions, under which the aldehyde group is reduced to an alditol. Glycans that are reductively aminated with hydrophobic groups produce stronger mass spectrometric signals than do native glycans (Harvey, 2000b).

Permethylation and reductive amination are very effective means of derivatization for mass spectral analysis of glycans. There remains substantial interest in analysis of native glycans, however, primarily due to the desire to avoid losses during the derivatization workup procedures.

### Ionization Methods

Mass spectrometric ionization of carbohydrates has been reviewed recently (Zaia, 2006). Therefore, this section will provide a brief description of two main ionization methods, matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI), and highlight some specific requirements for glycan sample preparation that improve overall efficiency of the MS ionization process.

The analysis of complex mixtures by mass spectrometry is limited by the phenomenon of ion suppression, in which the most abundant molecules suppress ionization of those in lower abundance. Ion suppression is minimized when analyzing mixtures with equivalent acidic and hydrophobic properties. Since native-released carbohydrates are hydrophilic and range from neutral to acidic, it is best to separate them according to their acidity prior to MS to minimize ion suppression.

MALDI entails mixing analyte molecules with an organic matrix, such as 2,5-dihydroxybenzoic acid. A small volume of the mixture (1–2 μl) is dried on a metal target, allowing crystals to form. The target is placed in the vacuum source of a mass spectrometer. See Harvey (1999, 2003) for more information on MALDI matrices for glyconjugates. Singly charged ions predominate using MALDI. It is typically used for analysis of chromatographic fractions that are collected offline. MALDI MS has the advantage of sample preparation and a relatively high tolerance to salts and other contaminants. However, acidic (sialylated, sulfated, or phosphorylated) native carbohydrates undergo fragmentation during MALDI ionization. As indicated above, permethylation protects glycans from fragmentation during the MALDI process.

ESI entails spraying a solution containing the analyte through a needle, to which electrical potential is applied. The electrical potential creates a condition in which very fine droplets are formed. Droplets are either repelled from or attracted toward the needle according to their charge. In this way, appropriately charged droplets move toward the mass spectrometer orifice while undergoing solvent evaporation. This process results in the formation of multiple charged gas phase ions that are analyzed in the mass spectrometer. ESI is used with many types of mass spectrometers and is particularly well suited to the analysis of liquid chromatographic effluents.

The extent of fragmentation of acidic glycans and other fragile ions is much lower than observed using MALDI. ESI produces inherently better resolved peaks for glycoconjugates due to the absence of matrix adduct peaks (Satterfield and Welch, 2005), but has the disadvantage that sample preparation is more labor intensive than in MALDI. Samples must be free of salts prior to introduction into the ion source to avoid the problem of multiple cation adduction. This entails the use of liquid chromatography (LC), either off- or online prior to ESI MS.

In conclusion, both MALDI and ESI can be used to ionize glycan samples for large scale glycomics analysis. The performance of either method depends on the sample composition and can be substantially enhanced by using chromatography to separate sample components prior to ionization.

### Chromatographic Interfaces for Mass Spectral Glycomics

The combination of LC with MS provides a powerful analytical tool for both qualitative and quantitative glycan analysis and dramatically improves the amount of obtainable information. Although the development of glycan online LC/MS has lagged behind that for proteins, a number of practical approaches are emerging, as discussed below.

In order to use reversed phase LC/MS, reductive amination (Anumula, 2000, 2006) is applied to increase the hydrophobicity

**Figure 1. Structures and Functions of Protein Glycoconjugates**

(A) Glycoconjugates form key structural elements that mediate cell-cell and cell-matrix interactions. The structures of glycoconjugate glycans reflect enzymatic biosynthetic reactions that are under complex control. Biosynthetic reactions do not go to completion, giving rise to distributions of glycoforms built on a core structure. Glycoconjugates mediate the antigenic character of cell surfaces and add a level of plasticity to the functions of the genetically coded protein gene products. NCP, collagenous glycoprotein. Modified after (Varki et al., 1999).

(B) Common core structures for protein glycoconjugate glycans. Symbolic glycan structures were drawn using GlycoWorkbench (Ceroni et al., 2008), which uses the symbolic nomenclature proposed by the Consortium for Functional Glycomics (Stevens et al., 2006).

(C) Common glycan derivatizations. Glycans released by enzymatic means and nonreducing chemical conditions exist as an equilibrium between close-ring hemiacetal and open-ring aldehyde forms (shown for example with *N*-acetylglucosamine). Aldehyde forms may be reduced to alditols. Permethylation converts all OH groups to O-methyl and NH groups to N-methyl. Reductive amination entails reaction of the aldehyde with a primary amine to form a glycosyl imine (also known as a Schiff base). The imine is reduced to a secondary amine to increase its stability. R, glycan moiety, R′,primary amine alkyl group.

of glycans. Aminopyridine (Kuraya and Hase, 1996), 2-aminobenzamide (Chen and Flynn, 2007), and 1-phenyl-3-methyl pyrazolone (Mason et al., 2006) are widely used for derivatization of *N*-glycans prior to online LC/MS. Under these conditions, the compound with the longest retention time is the unmodified reductive amination reagent. As the size and polarity of the glycan increases, the degree to which it binds the stationary phase decreases.

Acidic glycans can also be induced to interact with reversed stationary phases through the use of amines, such as dibutyl amine, tributyl amine, or triethyl amine as ion pairs in the mobile phase. This approach has been used with online LC/MS of glycosaminoglycans (Thanawiroon et al., 2004). Reductive amination with 8-aminonaphthalene-1,3,6-trisulfonate has been used to increase the acidity of *N*-glycans to obtain isomeric resolution using reversed phase ion-pairing LC/MS (Gennaro et al., 2003). Here, the advantages of high resolution using the reversed phase ion-pairing mechanism are balanced against the disadvantages of the need to infuse millimolar concentrations of amines continuously into the ion source.

Graphitized carbon chromatography has been used for online LC/MS analysis of *N*- and *O*-glycans, utilizing packed capillary (Kawasaki et al., 2003) and chip-based (Niñonuevo et al., 2005) chromatography systems. Graphitized carbon chromatography can be used to separate structural isomers. Therefore, to maximize its performance, it is best to reduce or reductively aminate glycans to prevent the splitting of peaks based on reducing-end anomers. Mobile phases containing a formic acid modifier are appropriate for analysis of native or reductively aminated neutral and sialylated glycans and permethylated glycans (Costello et al., 2007).

Hydrophilic interaction chromatography (HILIC), used to separate glycans based on their size and hydrophilicity, is an effective chromatography mode for online LC/MS. HILIC chromatographic retention time information may be correlated with the glycan structure using chromatographic standards (Guile et al., 1996). Its use with a mass spectral detector facilitates the development of retention time libraries, against which unknown glycan compositions may be referenced (Pabst et al., 2007). Capillary amide-HILC has been used for online LC/MS of *N*-glycans (Wuhrer et al., 2004), glycopeptides (Wuhrer et al., 2005), and glycosaminoglycans (Naimy et al., 2008; Hitchcock et al., 2008), as well as for offline MALDI MS of glycosphingolipids (Zarei et al., 2008). A disadvantage of HILIC is that the resolution of structural isomers is generally not observed.

In conclusion, there are several effective options for glycan chromatography that have been demonstrated with LC/MS in glycomics.

### *Ion Mobility MS Applied to Glycomics*

One of the challenges for LC is achieving the separation of glycan structural isomers. Gas phase ion mobility separations (IMS) have the potential to overcome this obstacle and resolve glycan structural isomers based on differences in the three-dimensional shape of such ions (Clowers et al., 2005; Clowers and Hill, 2005). In IMS, ions are moved through a chamber of relatively high gas pressure, so as to provide a degree of separation for structural isomers prior to MS or tandem to MS. Profiling of glycans released from biological sources often results in ions that have overlapping isotopic clusters. As shown in Figure 2, glycans isolated from the urine of a patient with a congenital disorder of glycosylation were analyzed by nanoscale ESI using an MS instrument in which a mobility separation chamber preceded the TOF-MS analyzer (Vakhrushev et al., 2008). This instrument permits ions exiting the mobility chamber to be detected directly using MS or fragmented in a collision cell prior to MS detection. Figure 2A shows that two glycan compositions are observed in the *m/z* 1007–1012 window. Charge states are segregated into separate windows defined by ion mobility and *m/z*. As a result, it is possible to extract the two glycans, as shown in Figures 2B and 2C. It is also possible to visualize the differing product ion profile resulting from dissociation of the two compositions, as shown in Figure 2D. The *m/z* values for the monoisotopic ion for the two glycans have distinct mobility chromatograms, as shown in Figure 2E.

In conclusion, mobility separations demonstrate clear potential as a means of analyzing complex released glycan mixtures in conjunction with MS detection. Although the mobility resolution is modest at present, the technology is advancing rapidly.

## Quantitative Glycomics

Glycan quantification is essential for determination for both fundamental studies of their biological activities and biomarker identification efforts. As in proteomics, quantitative methods for glycomics have been developed using label-free and stable isotope-labeled approaches. These trends are summarized below.

### *Label-free Approaches*

*N-Linked Glycomics*. Since *N*-glycosylation represents one of the most common posttranslational modifications in eukaryotes, developing methodologies to accurately and efficiently perform glycomics scale analysis of *N*-glycans is critical. A recent multi-institutional study of quantitative profiling of glycoprotein glycans highlights the strengths and weaknesses of methods in widespread use at the present time (Wada et al., 2007). The study revealed that there was a significant variance among laboratories using chromatographic quantitation due to differences in reductive amination methods used. On the other hand, laboratories using permethylation of the released *N*-glycans followed by MALDI-TOF MS produced consistently good results. Those using graphitized carbon LC/MS with negative ion ESI MS detection gave consistent results with underivatized oligosaccharide alditols. For analysis of underivatized sialylated *N*-glycans, the ESI LC/MS approach was found to be superior to MALDI since it did not lead to fragmentation during ionization. However, the ionization responses depended on the degree of sialylation, which somewhat complicated the task of obtaining quantitative results.

Further efforts are likely to refine the methods for recovery of submicrogram quantities of glycans from the permethylation reaction. LC/MS approaches will be useful for N-glycans modified by groups not compatible with the permethylation chemistry, including sulfation and phosphorylation.

*O-Linked Glycomics*. Mucin oligosaccharides represent the most common type of *O*-linked glycosylation and have high value as potential disease biomarkers. For example, mucin *O*-glycans have been profiled from gastric biopsies of Rhesus monkeys as a function of *Helicobacter pylori* infection (Cooke et al., 2007). Such glycans are typically released as alditols by

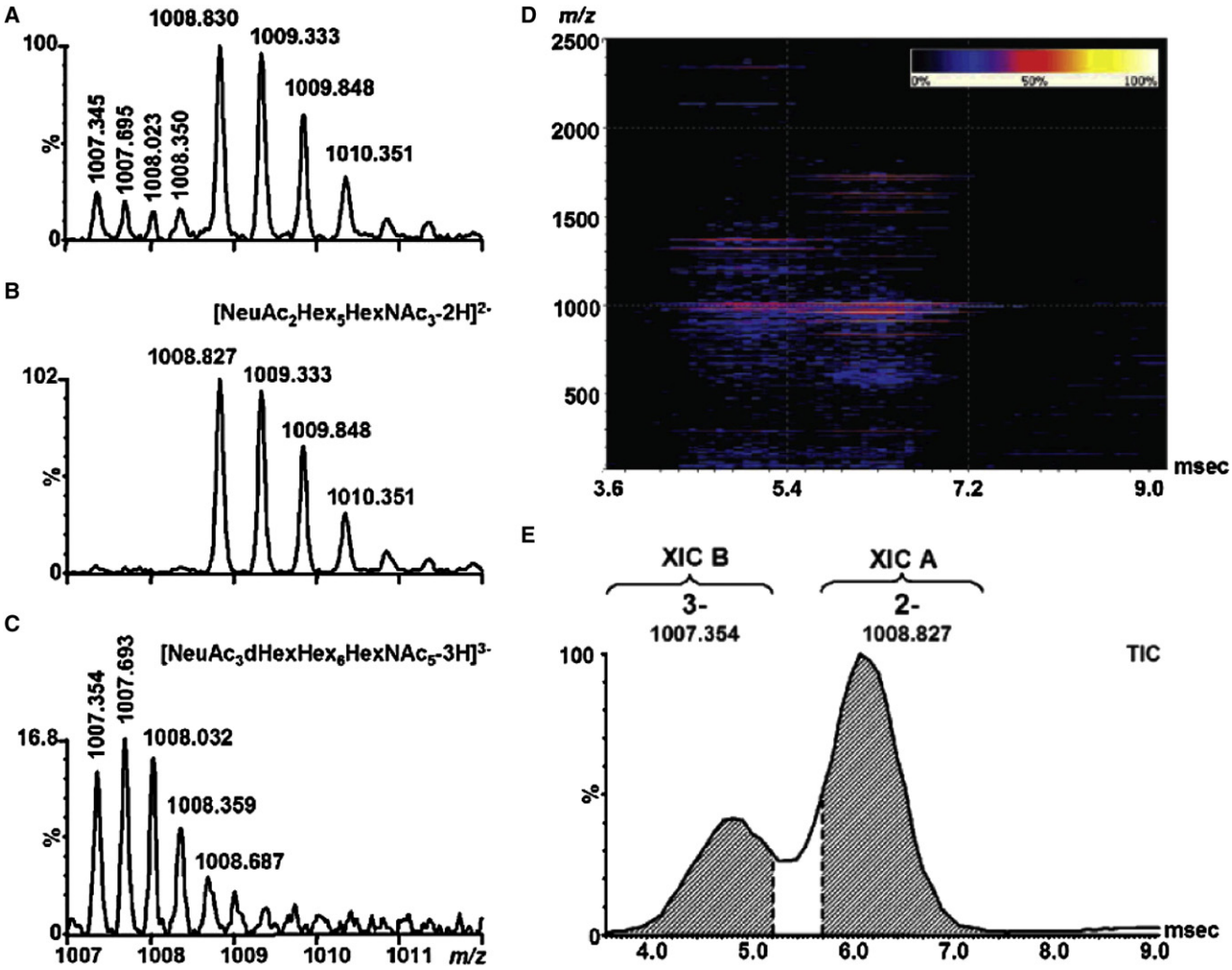



**Figure 2. Ion Mobility Spectrometry of Human Urine Glycans from a Congenital Disorder of Glycosylation Patient**

(A) Expansion of the mass range at *m/z* 1007-1012 of the negative ion mode nano-ESI Q-TOF MS of the urine fraction, acquired from the total ion chromatogram over all mobility drift times.

(B) Expansion of the mass range at *m/z* 1007-1012 of the spectrum acquired from extracted ion chromatogram of doubly charged distributed area after ion mobility separation.

(C) Expansion of the mass range at *m/z* 1007-1012 of the spectrum acquired from extracted ion chromatogram of triply charged distributed area after IM separation.

(D) Plot of fragment ions obtained by CID of overlapped precursor ions at *m/z* 1007.354 (left) and 1008.827 (right) versus *m/z* values.

(E) Total ion current chromatogram with retained drift time of overlapped precursor ions at *m/z* 1007.354 (left) and 1008.827 (right). Selected areas indicate extracted ion current chromatogram A for the precursor ions at *m/z* 1008.827 and chromatogram B for the precursor ions at *m/z* 1007.354. XIC, extracted ion chromatogram. © 2008, American Chemical Society (Vakhrushev et al., 2008).

alkaline β elimination in the presence of a reducing agent. The *O*-glycans released by β elimination were analyzed by ESI and MALDI Fourier transform (FT) MS. The data demonstrate that there is a significant difference in composition of core *O*-glycan structures between samples from infected and uninfected animals, suggesting that the overall hallmark of infection is the loss of mucin-type core structures. A related glycomics approach has been developed to identify breast cancer glycan biomarkers based on the analysis of released *O*-glycans from cancer cell lines and serum samples (Kirmiz et al., 2007). In addition, an online graphitized carbon LC/MS approach was used to analyze the composition of mucins in the sputum of cystic fibrosis patients (Schulz et al., 2007). The study revealed clear differences in glycan structures between cystic fibrosis patients and healthy controls.

In summary, MALDI and ESI-based methods for *O*-glycans are similar to those for *N*-glycans. Several groups are developing nonreductive release strategies for *O*-glycans that may prove effective.

*Glycosaminoglycan Glycomics*. The ability to measure the mass of glycosaminoglycan oligosaccharides forms the core of glycomics approaches aimed at the correlation of structure with biological function (Sasisekharan et al., 2006). Glycosaminoglycans can also be analyzed using MALDI MS when paired with basic peptides (Venkataraman et al., 1999). Glycosaminoglycan oligosaccharides can also be analyzed using LC/MS

setups, including size exclusion (Henriksen et al., 2004), reversed phase ion pairing (Kuberan et al., 2002), and hydrophilic interaction chromatography approaches (Naimy et al., 2008; Hitchcock et al., 2008). The online LC/MS approaches facilitate comparative profiling of glycosaminoglycan oligosaccharides as a function of tissue type, protein binding, or other biological variables.

The challenging chemistry of these and other sulfated glycan classes is no longer a barrier since effective analytical techniques have been developed.

### Stable Isotope Labels for Glycomics

Stable isotope labels are used commonly in glycomics as a means to improve determination of relative amounts of glycans in the sample. Depending on the sample and extent of the information needed, several different labels can be used, as described below.

*Permethylation Labeling*. Use of deuterated methyl iodide is a straightforward means of introducing stable isotope labels into permethylated *N*-glycans (Viseux et al., 1999). Labeled and unlabeled permethylated *N*-glycans would likely be resolved by graphitized carbon chromatography. Additionally, it is important to keep in mind that mass shift varies according to the glycan composition. An example of this labeling approach is demonstrated using a comparative glycoform mapping method (C-GlycoMAP), developed based on differential stable isotope labeling ($CH_3I/CD_3I$) and permethylation (Kang et al., 2007). The differentially isotope labeled samples are combined prior to MALDI MS analysis to minimize sample-to-sample variation in peak abundances and maximize the ability to perform the comparison of two samples. Stable isotope labels have also been introduced using $^{13}CH_3I$ (Aoki et al., 2007).

Recently, the concept of quantification by isobaric labeling was introduced (Atwood et al., 2008), under which glycans are permethylated with $^{13}CH_3I$ or $CH_2DI$. These groups have exact masses differing by 0.002922 Da and permethylated glycans modified with these reagents have the same nominal mass but their exact masses differ by multiples of this value. The resolution required to separate the exact masses of typical *N*- and *O*-glycans differentially labeled with these reagents is approximately 30,000, well within the operating range of FTMS and Orbitrap mass spectrometers. Thus, it is possible to analyze the mixtures directly in the MS mode, or to select the nominal masses for subsequent tandem mass spectrometric analysis of isobaric glycoform mixtures.

*Reductive Amination Labeling*. A number of reductive amination reagents are commercially available in deuterated form, allowing easy incorporation of stable isotope labels through reductive amination (Yuan et al., 2005; Hitchcock et al., 2006a). In addition, the reduction step may be conducted with a deuterated reducing agent to introduce a 1 Da mass shift (Hsu et al., 2006). For many applications, however, a larger mass shift is desired to separate the glycan isotopic clusters. Mono- and oligosaccharides differentially isotope-labeled with $d_0/d_4$ pyridyl amine (PA) can be analyzed using graphitized carbon LC/MS, and the isotopes are partially separated chromatographically (Yuan et al., 2005). Here, *N*-glycans labeled with the heavy form of PA are used as internal standards to facilitate quantitative comparison among glycan samples. Glycosaminoglycan oligosaccharides can be differentially labeled with $d_0/d_4$ 2-anthranilic acid and analyzed by size exclusion LC/MS/MS (Hitchcock et al., 2006a) or amide-HILIC LC/MS/MS (Hitchcock et al., 2008). A reductive amination tag has been synthesized in four stable isotope forms ($d_0$, $d_4$, $d_8$ *and* $d_{12}$) and applied to MS analysis of *N*-glycans and glycosaminoglycans (Bowman and Zaia, 2007). The advantage to the tetraplex tags is that four samples may be mixed and analyzed simultaneously.

In summary, isotopic labeling techniques have the advantage over label-free methods that a stable isotope-labeled internal standard can be used to compensate for changes in instrument performance over time. For extremely complex samples, such internal standards may not be appropriate since they increase spectral complexity.

## Tandem MS and the Analysis of Isomeric Glycan Mixtures

### Principles of Glycan Tandem MS

The use of tandem MS for glycomics is driven by the need to produce structural information on glycans expressed in serum/plasma or tissue relevant to the understanding of disease processes and biomarker discovery (Packer et al., 2008). The challenge of this task resides in the fact that glycoconjugates are expressed as a distribution of glycosylation variants relative to a core structure. Thus, a glycan composition, indicated by its mass, typically reflects a mixture of positional isomers. One approach is to purify the glycans to homogeneity before tandem MS analysis and interpret obtained mass spectra directly or in comparison to the spectra of synthetic standards. In many cases, such purification is not possible. Thus, another option for the tandem MS experiment is to analyze a mixture of positional isomers directly. In such an experiment, sequential stages of tandem MS are performed in series. Stages of MS are abbreviated $MS^n$, where n = the stage. For MS measurement, n = 1; for the first stage of tandem MS, n = 2. Subsequent dissociation of a product ion from $MS^2$ is termed $MS^3$, and so on. The masses of the product ions indicate compositions of glycan substructures, any of which may be selected for dissociation in further stages of tandem MS.

Most glycan tandem mass spectra are produced by collisional induced dissociation (CAD), a technique in which selected precursor ions are dissociated by collision with gas atoms in a collision cell. The collisions increase the vibrational energy of the ions to the point that bond rupture occurs. Typically, the weakest bonds rupture to produce the most abundant product ions. It is possible to dissociate permethylated glycans using high energy CAD that uses a MALDI TOF/TOF instrument, under which conditions bond rupture is kinetically controlled and cross-ring cleavage ions are more abundant. This approach is not generally applicable to native and reductively aminated glycans due to fragmentation during the MALDI process.

The nomenclature for glycan and glycoconjugate product ions (Domon and Costello, 1988) is shown in Figure 3A and will be used throughout this section. Product ions containing a nonreducing terminus are labeled as A, B, C, and those containing the nonreducing end or aglycon are labeled X, Y, Z. Cleavages across residue rings (A-and X-type ions) are particularly useful for determining linkages. In addition, as shown in Figure 3B, the D-ion (not part of the original nomenclature) consists of combined

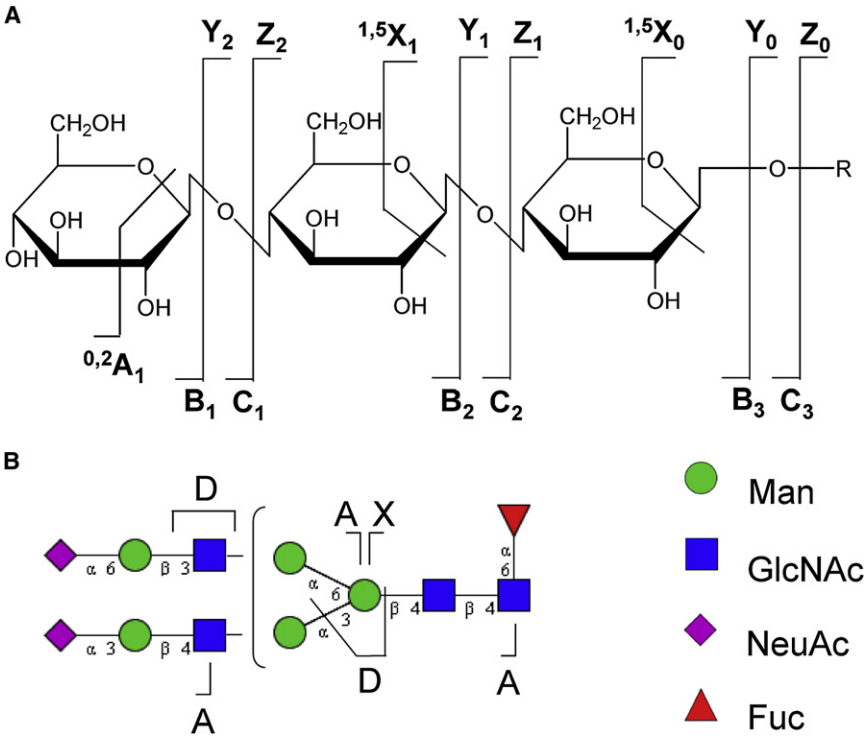



**Figure 3. Nomenclature for Tandem Mass Spectromic Product Ions of Glycans and Glycoconjugates**

(A) Illustration of the A, B, C and X, Y, Z nomenclature from Domon and Costello (1988).

(B) Illustration of the use of D-type ions to differentiate *N*-acetyllactosamine linkages, mannose branching structure, and core fucosylation.

C- and Z-type fragmentation about a Hex or HexNAc residue that is 3-linked and may be used to differentiate *N*-acetyllactosamine linkages and mannose-branching structure (Chai et al., 2001).

## Differentiation of Glycan Isomers Using Tandem MS

Tandem MS of glycan classes has been reviewed (Zaia, 2004). The following section summarizes how key glycan substructures may be differentiated using tandem MS, highlighting recent developments in the field. A detailed discussion is beyond the scope of this review, but relevant references are given in Figure 4.

### *Permethylated Glycans*

Strategies for acquisition and interpretation of multistage MS have been most fully developed for permethylated glycans (Ashline et al., 2005). The advantage to this approach is that tandem mass spectrometric dissociation of a glycosidic bond leaves a site that lacks a methyl group that is clearly indicated by mass. Thus, the linkage position is indicated by the mass of crossring cleavage ions (A- or X-types) (Figure 5). It is possible to differentiate some types of positional isomers based on the formation of specific product ion types. Tandem MS of permethylated glycans produces more structural detail than does that of native and reductively aminated glycans.

### *Native and Reductively Aminated Glycans*

Tandem MS of native and reductively aminated glycans may be acquired using either positive or negative ions. Generally speaking, positive ion tandem MS results in abundant glycosidic bond product ions, with those that occur adjacent to HexNAc, sialic acid, and Fuc particularly abundant. It must be noted that glycans must be cationized with sodium or other metals rather than analyzed as protonated ions. Protonated ions have been observed to undergo rearrangements during tandem MS. For *N*-glycans, A-type cross-ring cleavages to the core HexNAc residues are also abundant. The problem is that crossring cleavages to branching residues are typically low in abundances, as are D-type ions that similarly differentiate glycan branches. As a result, tandem MS of positive ions produces very limited detail on the branching structure of glycans and glycosidic linkages.

The structural detail produced from dissociation of negative ions is greater than in the positive mode. For neutral glycan classes, an abundant series of C-ions is often observed. Such ions readily form A-type ions for monosaccharide residues that have an unmodified hydroxyl group in the 3-position. In the event that this position is occupied (i.e., by a glycan branch), a D-type ion is formed. This pattern forms the basis of differentiation of lactosamine isomers and antenna branches, as detailed in Figure 4.

### *Activated Ion Dissociation of Glycans*

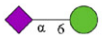
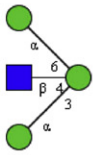
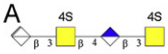
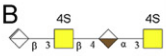
Electron capture dissociation (ECD) is an activated electron fragmentation method for positive ions using FTMS instruments that has been applied to glycans recently (Adamson and Hakansson, 2007a). Electron detachment dissociation (EDD) generates dissociation in the negative mode and has been shown to produce relatively abundant cross-ring cleavage ions for *N*-glycans and milk oligosaccharides (Adamson and Hakansson, 2007b) and glycosaminoglycans (Wolff et al., 2007a, 2007b). As shown in Figure 4, EDD generates product ions that differentiate glycan isomers. This approach therefore has great potential as a glycan analysis method.

In summary, tandem MS produces the greatest structural detail on permethylated glycans. Negative ion tandem MS is effective for producing useful structural information on native and reductively aminated glycans, including those classes not compatible with permethylation. Activated electron dissociation techniques, EDD in particular, show potential for incorporation into strategies for glycomics analysis.

## Glycoproteomics: Analysis of Glycopeptides

### *MS Analysis of the β-O-GlcNAc Glycopeptides*

Mass spectrometric analysis of glycopeptides is made challenging by the differing chemical properties of glycans and peptides. For example, although the β-*O*-GlcNAc modification occurs to Ser/Thr residues of many nuclear and cytoplasmic proteins, it was not detected until fairly recently because it is both uncharged and labile (Hart et al., 2007).

| Epitope | Symbol | Glycan[a] | Dissociation | Precursor ion[b] | Stage | Product ion | References[c] |
|---|---|---|---|---|---|---|---|
| *N*-acetyllactosamine isomers<br>Type 1 Chain (neo-lactosamine)  | | N, RA | CAD | $[M-nH]^{n-}$ | $MS^2$ | D, $^{0,2}A$ | (1-3) |
| Type 2 Chain (lactosamine)  | | P | CAD | $[M+nNa]^{n+}$ | $MS^n$ | $\rightarrow B_2\rightarrow$ | (4) |
| Neuraminic acid linkage isomers | | N, RA | CAD | $[M(X)+nH]^{n+}$ | $MS^2$ | X, Y, Z | (5) |
| (α2-3)  <br>(α2-6)  | | N, RA | CAD | $[M-nH]^{n-}$ | $MS^2$<br>$MS^n$ | $^{0,4}A_2$-$CO_2$,<br>$\rightarrow C_n\rightarrow$,<br>$\rightarrow D_n\rightarrow$ | (6-9) |
| | | P | CAD | $[M+nNa]^{n+}$ | $MS^2$<br>$MS^n$ | $A_2$<br>$\rightarrow 211\rightarrow$ | (10-12) |
| Differentiation of branching isomers 3-, 4-, 6- antennae  | | N, RA | CAD | $[M(Na)+nH]^{n+}$ | $MS^2$ | A- | (13, 14) |
| | | N, RA | CAD | $[M-nH]^{n-}$ | $MS^2$ | A- and D- | (15-19) |
| | | N, RA | EDD | $[M-nH]^{n-}$ | $MS^2$ | A-, B-,C-, X-, Y-, Z- | (20) |
| | | P | | $[M+nNa]^{n+}$ | $MS^2$ | $^{0,4}A$-, $^{3,5}A$- | (21, 22) |
| | | P | ECD | $[M+nNa]^{n+}$ | $MS^2$ | $^{3,5}A$ | (22) |
| Fucosylated positional isomers<br>Core *vs* antenna | | N, RA | CAD | $[M(Na)+nH]^{n+}$ | $MS^2$ | Core $^{0,2}A$, B, Y | (23, 24) |
| | | N, RA | | $[M-nH]^{n-}$ | $MS^2$ | C-, A-, D-, | (16, 19) |
| | | P | | $[M+nNa]^{n+}$ | $MS^2$ | B-, Y-, A- | (25) |
| Disaccharide linkage: (1,6) *vs* (1-4) vs (1-3) | | P | CAD | $[M+nNa]^{n+}$ | $MS^n$ | $\rightarrow B_2\rightarrow$ | (4, 26) |
| GAG disaccharide analysis | | N | CAD | $[M-nH]^{n-}$ | $MS^2$<br>$MS^3$ | B-, Y-, X- | (27-31) |
| GAG oligosaccharide analysis | | N | CAD | $[M-nH]^{n-}$ | $MS^2$ | B-, Y-, X- | (32-34) |
| CS type A  <br>CS type B  <br>CS type C  | | RA | CAD | $[M-nH]^{n-}$ | $MS^2$ | Y-, X- | (35-38) |
| GAG structural determination | | N | EDD | $[M-nH]^{n-}$ | $MS^2$ | A- and X- | (39-41) |
| Symbols  | | | | | | | |

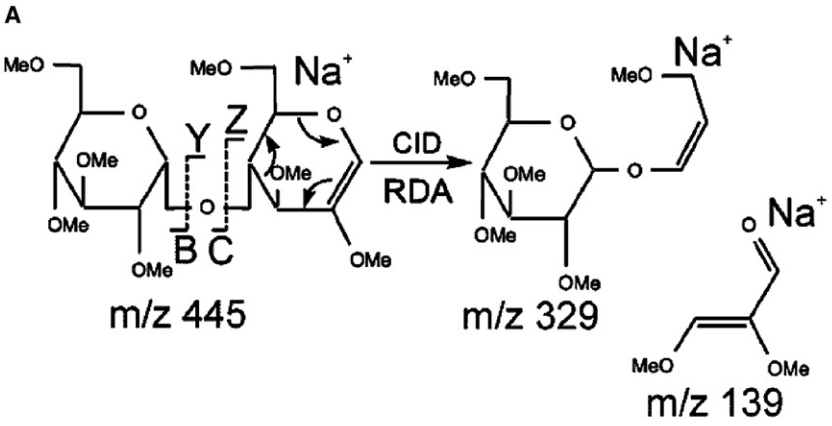
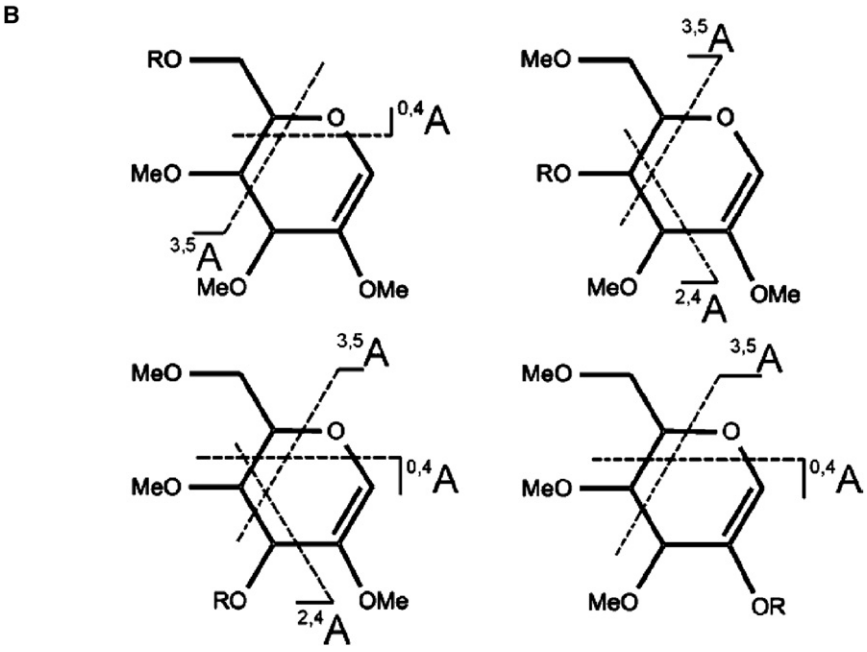



**Figure 5. Example of the Utility of Multistage Fragmentation of B-Ions Generated from Permethylated Glycans**

A retro-Diels-Alder reaction in a 1,4-linked $B_2$-type Hex-Hex disaccharide shows formation of an *m/z* 329 ion from an *m/z* 445 ion.

(A) The *m/z* 139 ion is generally of low abundance or absent, presumably due to the stronger affinity for the metal ion charge carrier by the larger fragments.

(B) The generic crossring cleavages that may be formed from B-type ions of various linkages. R indicates the location of mono- or oligosaccharide substituents. (Ashline et al., 2005). © 2005, American Chemical Society, used with permission.




### *MS Analysis of N- and O-Glycopeptides*

The ultimate goal of glycoproteomics is to quantify the site occupancy of glycosylation in the proteome and the structures of glycoforms at each site. Glycoproteomics schemes typically involve enrichment of glycoproteins or glycopeptides using affinity techniques. Enrichment approaches for glycoproteomics have been reviewed recently (Xin et al., 2008; Wuhrer et al., 2007a) and only a few approaches will be mentioned here. Several groups have published workflows for serum glycoproteomics based on use of lectins for affinity capture of glycoproteins or glycopeptides (Geng et al., 2001; Wang et al., 2006; Madera et al., 2006). Glycopeptides may also be isolated by hydrophilic interaction solid phase extraction (Wada et al., 2004) or chromatography (Wuhrer et al., 2005), and by graphitized carbon solid phase extraction (Larsen et al., 2005). They may also be enriched based on their high molecular weight using size exclusion chromatography (Alvarez-Manilla et al., 2006).

### *Tandem MS of Glycopeptides*

One of the challenges to glycopeptidomics is that the glycan moiety has different chemical properties than the peptide backbone and dissociates under different mass spectrometric conditions. Since tandem MS for glycoproteomics has been reviewed recently (Wuhrer et al., 2007a), only an outline of methodology will be given below, describing key aspects of fragmentation procedures routinely applied.

*Collision-Induced Dissociation and Infrared Multiphoton Dissociation*. IRMPD is a technique for dissociation that involves absorption of infrared light by the ions, causing vibrational excitation and subsequent bond fragmentation. Dissociation of glycopeptides using CID or IRMPD tends to produce abundant ions from cleavage of glycosidic bonds and low abundance ions from peptide backbone scission (Wuhrer et al., 2007a). The balance of glycosidic versus peptide backbone dissociation types depends on the glycopeptide structure. In particular, high mannose *N*-glycans do not undergo glycosidic bond cleavage as readily as complex *N*-glycans, and produce relatively abundant ions from peptide backbone cleavage using IRMPD (Adamson and Hakansson, 2006).

*Electron Capture Dissociation*. Whereas IRMPD and CID, by contrast, tend to produce abundant glycosidic bond cleavages for glycopeptides (Wuhrer et al., 2007a), ECD selectively fragments the peptide backbone of glycopeptides, allowing the site of glycosylation to be determined (Håkansson et al., 2001). Thus, a combination of ECD and dissociation based on increasing internal bond energy (CID or IRMPD) produces the greatest

**Figure 4. Summary of Tandem Mass Spectrometric Methods for Differentiating Isomeric Glycan Epitopes**

[a]N, native glycan; RA, reductively aminated glycan; P, permethylated glycan.

[b]X, metal cation.

[c]The numbered references are listed in Supplemental Data available online.

amount of information on the glycopeptide structure, and commercial mass spectrometers with these capabilities are now available. The ECD/IRMPD combination was used to analyze a xylosylated neutral *N*-linked glycopeptide (Håkansson et al., 2001), a high mannose *N*-linked glycopeptide (Adamson and Hakansson, 2006), and a monosialylated *N*-linked glycopeptide that also carried an *O*-glycosylation site (Kjeldsen et al., 2003). There do not appear to be any reports demonstrating the incorporation of ECD into an applied *N*-glycan glycoproteomics workflow.

*Electron Transfer Dissociation*. ETD is a dissociation method in which an electron is transferred between a reagent gas and an analyte ion inside an ion trap mass spectrometer, creating an activated electron species that undergoes dissociation similar to those observed in ECD (Coon et al., 2005). The capability of ETD in an ion trap instrument has been demonstrated using the same glycopeptide used with earlier ECD work (Hogan et al., 2005). In summary, abundant peptide backbone dissociation is observed for glycopeptides using ETD. CAD results in preferential fragmentation of the glycan moiety of glycopeptides. The combination of ETD and CAD that has been used with online LC/MS was used to analyze a high mannose *N*-glycosylated peptide from human epidermal growth factor receptor (Wu et al., 2007). Nano-LC/MS with a combination of CID and ETD dissociation was used to analyze complex immunoglobulin *N*-glycopeptides from human serum (Wuhrer et al., 2007b). Sialylated glycopeptides have not yet been analyzed by ETD.

In summary, incorporation of ETD into glycoproteomic workflows has the advantage of the wide availability of ion trap MS instrumentation. The incorporation of ETD into such workflows has advanced faster than those using ECD at the time of this writing.

## Conclusions

It has been the intention of this review to summarize the convergence of mass spectral methods into glycomics workflows to study glycan expression in areas of fundamental biochemistry and biomarker development. The structural information obtained from tandem mass spectrometric studies is greatest when the glycans are released from the peptide or protein backbone. For glycan classes compatible with the derivatization conditions, permethylation remains the clear methodological choice. Using permethylation, ionization responses are increased over those of underivatized glycans, and the chemical stability improved. Multistage tandem mass spectrometric dissociation of permethylated glycans produces the greatest level of detail possible using mass spectral techniques. Glycan classes modified with sulfate or other fragile substituents are not compatible with permethylation, but may be reductively aminated. Native and reductively aminated glycans produce the most informative tandem mass spectra for negatively charged ions. A number of LC/MS approaches have been incorporated into glycomics workflows for permethylated, native, and reductively aminated glycans. Glycopeptides may be analyzed using a combination of activated electron dissociation (ECD/ETD) and CID or IRMPD.

## SUPPLEMENTAL DATA

Supplemental Data include Supplemental References and can be found with this article online at http://www.chembiol.com/cgi/content/full/15/9/881/DC1/.

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