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Monitoring vaccine protein glycosylation: analytics and recent developments

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In recent years, it has become increasingly clear that glycosylation of key pathogen glycoprotein antigens can significantly affect antigenic properties. For example, pathogens, such as human immunodeficiency virus and influenza, can develop a "glycoshield" over key antigens as they passage through host populations. In addition to shielding of antigenic sites key changes in glycosylation have been shown to modify host innate immune responses and both of these phenomena can potentially impact vaccine performance. A better understanding of glycosylation properties of vaccine antigens may better guide development of these products and management of their production processes. Due to the complexity of oligosaccharides, the analysis of these glycosylation states has been difficult and time consuming. With the advent of cutting edge mass spectrometry based techniques many of the barriers to glycan and glycoprotein analysis have been lowered. Combined with traditional techniques such as high field NMR, GC/MS, CE-LIF, and HPLC analyses, these new technologies now make it feasible to monitor glycosylation through vaccine development and during the manufacturing process. This situation can allow for better defined and controlled vaccine products that contain these chemical entities. In this review the major analytics that can be used in these analytical platforms are discussed in relation to rational for their use in vaccine products. A brief review of protein glycosylation is provided as is a discussion of the basic biology behind and examples of how glycosylation processes influence host immunity. Also discussed are special considerations when choosing cell substrates and fermentation processes for production of these vaccines. A range of analytical techniques are discussed along with the information that can be derived from each and their advantages and drawbacks. Information is provided in order to guide the reader towards the choice of a series of methods that combine to provide the structural information desired ranging from basic glycan compositional information to detailed fine structure of glycans and on protein site specific glycosylation patterns.

Introduction

Currently, there is a range of licensed and investigational viral vaccines that contain key glycoprotein antigens. Examples are influenza hemagglutinin (HA) and neuraminidase [1–4], hepatitis C virus E1 and E2 proteins [5] and HIVGP120 V1/V2 [6]. Influenza virus undergoes antigenic change which can result in new variant viruses; as a result of immune pressure these new antigenic variants can become dominant. This antigenic drift results in selection of amino acids that facilitate escape of the virus from neutralizing antibodies. In some instances, this may include changes in glycosylation sites that mask or alter antigenic regions [1,4]. HIV has dense glycosylation over major antigenic regions of GP120 referred to as the 'glycan shield'. HIV may be an example of a viral pathogen that has utilized glycosylation as a mask over key conserved peptide sequences for which mutation is not tolerated. Current efforts to develop HIV vaccines attempt to avoid this 'glycan shield' by targeting small nonglycosylated regions interspersed within this and surrounding

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Key term

Glycan shield: The presence of one or more carbohydrate moieties that are covalently linked within or in the vicinity of and antigenic site that may interfere with the host's immune response.

regions containing conserved amino acid residues [7–9]. Hepatitis C E1 and E2 envelope proteins, currently targets for vaccine development, may also undergo changes in glycosylation patterning as part of immune evasion [10]. This review addresses the major structural aspects of these modifications, common glycan entities that may be useful to monitor and how they might be characterized during development and monitored during vaccine manufacture.

For the purposes of this review some major concepts, such as antigenic masking and glycosylation drift during viral adaptation, are discussed using influenza vaccine antigen HA as an example. This review in no way specifically recommends product characterization, production monitoring or regulatory policies regarding these vaccines. Rather, key aspects of this antigen are well understood and discussions in the context of HA may facilitate a better understanding of viral pathogen strategies using glycosylation to avoid host immune responses and thus introduce concepts that may allow the reader to better understand how monitoring changes in glycosylation can be an advantage in vaccine development and production. Antigenic drift sometimes results in changes to HA glycosylation. Changes in the immune response to HA due to differences in HA glycosylation have been documented [1,2,11–13]. Because new variants circulate, this may necessitate review of the viruses to be included in the seasonal vaccine. Influenza vaccines can be produced in a number of cell substrates with different glycosylation properties. Most influenza vaccines are produced in embryonated chicken eggs, but a split, inactivated vaccine produced using virus grown in Madin–Darby canine kidney cells and a vaccine composed of purified HA expressed from recombinant baculovirus in insect cells were recently licensed. Glycosylation patterns of antigens originating from avian, mammalian and insect cells differ and it is not clear how changes in glycosylation might affect immunogenicity. The choice of cell fermentation mode can also influence glycosylation status and thus further indicates that monitoring of these modifications occurring in key vaccine antigens is warranted.

In order to understand glycosylation of vaccine antigens in detail at the structural level the composition, size, charge, general structure and sites of glycan occupancy can be assessed. However, glycosylation is complex and analytically challenging to characterize. As glycans are composed of cyclic polyols the monomeric components can be linked together in many configurations leading to levels of structural complexity that are potentially orders of magnitude higher in number than those of their protein or nucleic acid counterparts. The high structural diversity of glycans is largely due to their mode of synthesis, as unlike proteins and nucleic acids, their synthesis is not template driven. To understand the diversity encountered by glycosylation and how these structural attributes are analyzed a basic understanding of glycosylation processes is required.

Protein glycosylation

Glycan synthesis is driven primarily by the spatial and temporal distributions of the glycosylation active enzymes present along the secretory pathways and the process gives rise to a wide range of structural diversity. Glycans are composed of a variety of monosaccharides. The most common in higher eukaryotes are Man, Gal, Glc, Fuc, GlcNAc, GalNAc and sialic acids. They can be joined together in a wide range of configurations. As cyclic polyols the monosaccharides can be linked together at any ring hydroxyl. Fortunately, the actual structural diversity encountered is limited by enzyme specificity and the properties of the secretory system. Structural variation is essentially driven by five major structural attributes: mass, composition, linkage, branch configuration and anomeric configuration and these will be important in discussion of analytics later. However, first key aspects of the major forms of protein glycosylation will be discussed.

Protein glycosylation occurs in two major forms and these are *N*- and *O*-linked glycosylation. The *N*-glycans, or asparagine-linked glycans, are transferred to protein en-bloc from a dolichol lipid carrier as $Glc_3Man_9GlcNAc_2$. The nascent saccharide is then trimmed in the ER and early Golgi to a trimannosyl core in most eukaryotes and extended in the Golgi by the addition of a series of monosaccharaides. Overall structure and composition of the final forms vary with genus, species and cell type including cell lines used in biologics manufacture. *N*-glycans are added on the peptide backbone at specific peptide sequences, known as the *N*-glycosylation sequon, NX(S/T), where X is any amino acid except P. Cell lines may have differing fidelity for glycosylation at these sequons.

O-linked glycans are added at Ser and Thr residues along the polypeptide backbone. While they are typically smaller than *N*-glycans they can be more structurally diverse as a class because many core types are possible. *O*-glycan assembly is initiated in the early Golgi by addition of GalNAc to Ser/Thr followed by extension in the Golgi stacks with the addition of a series of

monosaccharaides that are similar or identical to those of *N*-glycans. The number of cores and overall composition, structure and complexity of *O*-glycans depend on the genus, species and cell type they are derived from as indicated for *N*-glycans. Unlike *N*-glycosylation there is no defined sequon for *O*-glycosylation, although the modification tends to occur where S and T form clusters along the polypeptide chain. The tendency is that *O*-glycans cluster in groups along these ST tracts, which can make site specific analysis difficult. Glycoproteins can be modified with only a few or many dozens of chemically distinct glycan entities. The individual glycan abundances can range over two orders of magnitude. For a review of glycosylation processes in eukaryotes see [14]. Specific concerns about some cell substrate specific glycosylation entities will be covered briefly later in the section 'Cell substrates special considerations'.

Glycosylation & the vaccine antigen

As pathogens such as HIV, hepatitis C virus and influenza propagate in the human host over time the glycosylation status on key antigenic glycoproteins changes. A prime example is influenza H3N2 virus. Based on the A/Hong Kong/1968 virus, between 1968 and 2002 up to seven additional glycosylation sites were added to the globular head region of HA, and this is where the majority of known antigenic sites reside [15]. Animal studies performed to compare antigenicity, and ability of various glycosylation states of HA representative of low glycosylation forms and higher glycosylation forms, to protect against subsequent infection, have been conducted. It has been shown that the highly glycosylated forms are less antigenic and afford much lower protection than low glycosylation forms [16,17]. Experimental influenza vaccines designed to be denuded of HA glycosylation, save that required for proper folding, have been shown to be highly protective and protective over a broader range of influenza strains [18,19]. Clearly, from these studies and others, glycosylation of key vaccine antigens can have measurable effects on vaccine properties as shown in animal models. Components of the innate immune system, such as collectins and dendritic cell receptors, can target glycosylation patterns on pathogen proteins leading to immune processing [20,21]. Therefore, glycosylation patterns of key antigens can affect both humoral and innate arms of the immune system.

As we gain insight into the effects of glycosylation changes through observation of such pathogen strategies it becomes clear that control or even engineering of key antigen glycosylation could be used to optimize vaccines for more productive immune response. Changes in glycosylation could be used to tailor vaccine response and the ability to detect these changes empowers vaccine manufacturers to make such changes during development or detect them when they may be less desirable during manufacture. As glycosylation can be affected by the choice of cell substrate, the fermentation method used, and other aspects of production, monitoring of glycosylation of key vaccine antigens is warranted both in manufacturing process development and manufacturing process monitoring.

During the last two decades analytical methods have become available to produce fine structural information that allows monitoring of glycosylation in more detail and in a more routine fashion. The range of glycans present at individual glycosylation sites can now be determined and this is possible in an ever increasingly routine fashion. This rise in capability is largely due to the advances in MS. The use of these relatively new technologies allows the analysis and monitoring of changes in glycosylation that can affect vaccine properties. Their use together with the constellation of other technologies discussed in this review can allow control over the effects of glycosylation in vaccine products that until now has remained largely unexplored.

Analytical methods

Detailed glycosylation characterization can be technically demanding relatively time consuming and generally not required for vaccine regulatory submissions. However, as these technologies have advanced these constraints continue to diminish. During drug development the manufacturer may wish to characterize the glycosylation patterns on major vaccine antigens. Typically product characterization produces greater structural detail than release tests the latter of which assure that product remains essentially the same throughout its life. In terms of glycosylation characterization, typically fine structural characterization of a glycoprotein involves determination of monosaccharide composition, linkages, branch structure and anomeric configurations. At the glycoprotein level this can also include glycosylation site heterogeneity and occupancy. This review presents a guide as to what analytics are available. In many instances structural aspects can be inferred through previous product characterizations or based on the knowledge of the cell system used in production. However, it should be noted that, in some cases, such inference can be misleading and must be approached with caution.

Full characterization of free oligosaccharides assesses the major areas that provide structural diversity: molecular weight, composition, branch structure, linkage and anomeric configuration. No single method can provide all of the required information to determine these structural attributes. A combination of techniques is required. Table 1 shows the most common methods currently

Table 1. Analytical methods for oligosaccharide analysis.

Method	Chemical modification required	Sample requirement	Dynamic range (order of magnitude)	Information yielded	Destructive	Advantages	Limitations	Ref.
GC/MS monosaccharide	O-trimethyl silyl ethers	5–20 mcg	2	Composition	Yes	Two types of information supplied: elusion position and fragmentation pattern	Requires high sample amount; no commercially available standards	[22]
HPAEC-PAD	None	5–20 mcg	2	Composition	Yes	Limited chemical manipulation	Assignment based only on elution position	[23]
UHPLC-MS	Arylthiocarbamate	1–2 mcg	3–5	Composition	Yes	Low sample amount required	Relatively newly applied to oligosaccharides	[24]
CE-LIF	Amination	1–2 mcg	3–5	Composition	Yes	Low sample amount required; wide dynamic range	Assignment based only on elution position	[25]
GC/MS linkage analysis	Permethylated alditol acetates	5–20 mcg	2	Composition and linkage	Yes	Two types of information supplied: elusion position and fragmentation pattern	Multistep chemistries and high sample amount requirement: no commercial standards not available	[26]
High-field NMR	None	100–300 nmol	1.5	Composition, linkage, sequence, anomericity	No	The most informative analytical platform	Does not provide mass information; high sample amounts required	[27]
MALDI-TOF MS	Permethylation or amination or can be unmodified	5 mcg	3–5	Composition, mass	Yes	Rapid; tolerant to salts and other contaminant	Ion suppression may mask low abundance compounds	[28,29]
MALDI-TOF/TOF with CID	Permethylation or amination or can be unmodified	2 mcg	3–5	Composition, mass, sequence, branch, linkage	Yes	Rapid; tolerant to salts and other contaminants; high information content in fragmentation spectra	Ion suppression may mask low abundance compounds	[30,31]
LC/MS ion trap with CID	Permethylation or amination or can be unmodified	2 mcg	3–5	Composition, mass, sequence, branch, linkage	Yes	Multistage decompositional analysis allows deep structural interrogation	Limited MS resolution	[32]

CE-LIF: Capillary electrophoresis with laser-induced fluorescence; CID: Collision-induced dissociation; HPAEC-PAD: High-pH anion exchange chromatography with amperometric detection.

Table 1. Analytical methods for oligosaccharide analysis (cont.).

Method	Chemical modification required	Sample requirement	Dynamic range (order of magnitude)	Information yielded	Destructive	Advantages	Limitations	Ref.
LC/MS Q-TOF or Triple Quad with CID	Permethylation or amination or can be unmodified	2 mcg	3–5	Composition, mass, sequence, branch, linkage	Yes	High MS resolution; rapid duty cycle for high sample coverage	Limited to MS^2 and pseudo MS^3 decompositional analysis	[33,34]
Exoglycosidase digestion	None	1–5 mcg	N/A	Linkage and anomeric configuraton of target	Yes	Determination of linkage and anomeric configuration with reduced sample amounts	Applications limited to available enzyme specificities	[35–37]

CE-LIF: Capillary electrophoresis with laser-induced fluorescence; CID: Collision-induced dissociation; HPAEC-PAD: High-pH anion exchange chromatography with amperometric detection.

used and the information yielded. In some instances it may be desired to perform glycosylation site specific characterization. In this case glycopeptide analysis may be used. Here the site of occupancy, heterogeneity of occupancy at each site, and the mole percent each site is occupied are the major structural attributes analyzed. Common methods used are listed in Table 2. Major methods that can be used for product development and monitoring will be discussed below.

In addition to product glycosylation characterization the manufacturer may wish to implement manufacturing specifications to monitor glycosylation. Such release assays can be implemented to ensure that the drug product remains essentially the same and these data can be linked to characterization data and clinical experience.

Glycan release

N-glycans can be efficiently released enzymatically or chemically. There are no enzymes that can efficiently release all *O*-glycans and therefore they must be released chemically. Chemical releasing methods can be found here [42]. *N*-glycan enzymatic releasing methods can be found here [43]. *N*-glycans are most commonly released using the peptide asparagine amidase, PNGase F. However, the enzyme can be sterically blocked by some core substitutions such as core linked Fucα1,3- substitution of the GlcNAc closest to the protein. Many plant and insect derived cell systems can produce this modification. In the case of insect or plant glycoproteins PNGase A can be used for release, which has broader specificity and can release glycans with this substitution. Alternatively *N*-glycans can be released chemically by hydrazinolysis [42]. However, this process can degrade the protein. So, if a goal is to analyze the protein an enzymatic releasing method may be more appropriate. Hydrazinolysis is more laborious and difficult to optimize but is less expensive and releases a broader range of *N*-glycans compared with PNGase F or A.

The most common release method for *O*-glycans is reductive β-elimination which yields the corresponding sugar alditols [44]. Some peeling can occur, especially at β1,3-linkages, but this is usually not significant. A drawback of the β-elimination methods is that the alditols are essentially nonreactive at the reduced end and cannot be modified. For example, reductive animation is not possible as the hemiacetal, a reactive group, is no longer present so downstream analysis that requires a strong chromaphore is not possible. However

Key term

Glycopeptide analysis: The analysis of a peptide or peptides that contain a covalently linked carbohydrate moiety.

Table 2. Glycopeptide analysis.

Method	Chemical modification	Sample requirement	Dynamic range (order of magnitude)	Information yielded†	Destructive	Ref.
MALDI-TOF MS	Proteolysis alkylation	50 ng	3–5	Composition, mass	Yes	[38]
MALDI-TOF/TOF with CID	Proteolysis alkylation	50 ng	3–5	Composition, mass, sequence	Yes	[39]
LC/MS orbitrap with HCD, ETD, CID and or ion mobility	Proteolysis alkylation	150 ng	3–5	Composition, mass, sequence		[40]
LC/MS Q-TOF with MS^E, ETD, CID and/or ion mobility	Proteolysis alkylation	150 ng	3–5	Composition, mass, sequence		[41]

Some branch information is possible for tandem MS analysis.
CID: Collision-induced dissociation; ETD: electron transfer dissociation; HCD: higher energy collision dissociation.

an advantage is that there is no interconversion between α- and β-anomers of the reducing end monosaccharide which can improve some HPLC separations. For detection purposes alditols containing HexNAc can be monitored with UV detection at 206 nm as electron resonance between the amide nitrogen and carbonyl absorb at this wavelength [45].

Monosaccharide analysis

Monosaccharide analysis is most often performed by GC/MS using trimethylsilylated monosaccharide derivatives of the hydrolyzed oligosaccharides [22]. An advantage to this method is that NIST and other libraries are available for spectral matching of most trimethylsilylated monosaccharide forms. In this method, the oligosaccharides are hydrolyzed and the resulting monosaccharides are rendered volatilizable after trimethylsilylation. The analytes are applied to the gas chromatograph where they are separated. The analytes then elute from the capillary, are ionized and fragmented by electron impact (EI) and detected, yielding ion trace and EI spectra that can be database matched or interpreted manually.

Another popular method used is high-pH anion exchange chromatography with amperometric detection (HPAEC-PAD). Here, the oligosaccharides are hydrolyzed to yield their monosaccharide components and subjected to HPLC separation using an anion exchange system that is stable under basic conditions. The hydroxyls of the monosaccharides are ionized and interact stereo specifically with the anion exchange system leading to monosaccharide specific elution profiles detected by amperometric detection [23].

Other methods are also available for monosaccharide analysis including ultrahigh performance liquid chromatography (UHPLC) and CE. UHPLC operates under the same principles as HPLC but takes advantage of a smaller solid phase particle size, increased flow rate and increased pressure on the order of 20,000 psi. These modifications increase resolving power and speed of analysis. A recently reported UHPLC method was shown to resolve 16 monosaccharide derivatives. The approach employed a reverse phase C18 solid phase coupled with an MS 'friendly' solvent system, methanol/isopropanol/acetonitrile 0.05% formic acid. The derivatization used, previously reported by Tanaka *et al.*, [46] produces arylthiocarbamate derivatives, which contain an excellent chromophore for UV detection and are amenable to MS analysis. D and L forms of the monosaccharides are produced. The method was coupled to a photodiode array detector, and a single quadrupole detector. Unique chromatographic elution position and select ion chromatogram detected fragmentation patterns were used to identify the D and L forms of monosaccharides allowing their identification [24].

CE is a powerful high-resolution separation technique and has been used for analysis of monosaccharides derives for more than two decades [25,47,48]. Separation is based on selective mobility of ions in solution. Monosaccharides from glycoproteins are derivatized with a charged fluorophore such as 8-aminopyrene-1,3,6-trisulfonate or 9-aminoacridone. Borate is often used as a separation enhancer as carbohydrates form anionic complexes with borate. Detection is usually performed by Laser-induced fluorescence (LIF) detection. Mass spectrometry has been coupled with CE. A major advantage of CE and UHPLC is high sensitivity. While sample requirements for GC/MS and HPAEC are in the 5–20 mcg

range, both UHPLC/UV and CE/LIF can be an order of magnitude more sensitive.

Linkage analysis

Linkage analysis can be achieved by GC/MS-based techniques, enzymatic digestion using linkage specific exoglycosidases or high-field NMR spectroscopy. GC/MS analysis is often performed on partially methylated alditol acetates (PMAA) of the processed sugars [26]. This requires a number of chemical steps thus requiring some chemical expertise. Glycans are first permethylated followed by hydrolysis. After hydrolysis the sample is reduced followed by peracetylation. The result is a series of structurally distinct derivatives representing the linkage arrangement of the original compound. The derivatives are rendered structurally distinct through the arrangement of acetyl and methyl substituents on the reduced carbohydrate backbone. The GC/MS analysis provides retention information as well as EI spectra as in GC/MS monosaccharide analysis. An example of EI spectra is shown in Figure 1 for two GlcNAc derivatives (see legend for details). Typically the method requires tens of micrograms of material. As with monosaccharide analysis NIST and other commercial compound libraries are available and contain many of the most common PMAA compounds produced from *N*- and *O*-glycans. A drawback of PMAA analysis is that no anomeric configuration information is rendered. Linkage specific exoglycosidases can also be used in conjunction with some of the other techniques in Table 1, such as MALDI-TOF MS, to analyze linkage and anomeric configurations. Sample strategies using exoglycosidases can be found here [35–37].

NMR analysis can be used to provide compositional, linkage, branch, sequence and anomeric configuration information. It can also be used to derive 3D structures of carbohydrates. However, this goes beyond the scope of most product characterization studies. Each atomic nucleus exists in a characteristic or, anisotropic, environment. The nuclei are excited using a series of radio frequency pulses and, as they relax back to equilibrium, the nuclei interact. This interaction is reflected in the magnetization energy that is detected. Essentially, by the transfer of magnetization energy 'through bond' and 'through space' key structural information about the analyte can be revealed. One and 2D experiments are commonly used. Useful 'through bond' transfer experiments can include ^{1}H COSY and ^{1}H TOCSY experiments, which are used to derive composition and linkage relationships in glycosidic pairs based upon the chemical shift and scalar coupling signals of hydrogen protons within each monosaccharide unit. Each carbon-linked hydrogen nucleus around the scalar coupled system in each monosaccharide unit has a defined chemical shift under controlled conditions. The splitting of these signals is primarily governed by their through bond scalar coupling to neighboring hydrogen nuclei. These signals can often reveal monosaccharide and linkage identities. Hydrogen nucleus specific information can be used to reveal 'through space' relationships as well, for instance, ^{1}H NOESY- or ^{1}H ROESY-based experiments can be used, which allow the proximity of structural components to one another to be determined. These experiments can be useful for determination of linkages. Heteronuclear

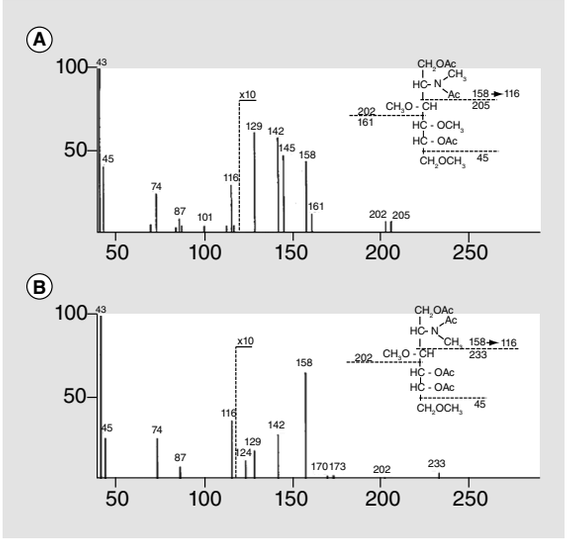

Figure 1. GC/MS analysis of oligosaccharides. In this example, EI spectra of representing the partially methylated alditol acetates derivatives of unsubstituted and C4-substituted GlcNAc are shown reproduced from [49]. Partially methylated alditol acetates derivatization occurs thorough the following sequential steps: permethylation, hydrolysis, reduction and peracetylation. The example illustrates the differentiation between a terminally located GlcNAc and a C-4 substituted GlcNAc in an oligosaccharide. In **(A)** the EI spectra of 3,4,6-tri-*O*-methyl-l,5-di-*O*-acetyl-2-deoxy-2-*N*-methylacetamidoglucitol and **(B)** 3,6-di-*O*-methyl-1,4,5-tri-*O*-acetyl-2-deoxy-2-*N*-methylacetamidoglucitol are shown. These derivatives result after sequential modification of the oligosaccharide by permethylation, hydrolysis, reduction and acetylation. In **(A)** the GlcNAc in the original oligosaccharide had a free hydroxyl at C-4 as it was not involved in a glycosidic bond and was available for methylation during the permethylation step as were C-3, C-6 and amide nitrogen at C2. In **(B)**, as C-4 was linked to a neighboring saccharide unit in the original oligosaccharide it was not available for methyl substitution during permethylation. However, after permethylation, hydrolysis and reduction the C4 is available for acetylation. The resulting substitutions reflect the original configuration of the particular monosaccharides in the original glycan. Note characteristic ion patterns such as m/z 161 and 205, which highlight the difference in substitution at C4.

Key terms

Permethylation profiling: The analysis of per-O-methylated oligosaccharides using a mass spectrometry-based methodology often performed under conditions that allows for quantitative comparisons of related samples.

experiments are often used, allowing more informed and complete structural assignment. An example is the ^{1}H - ^{13}C HMBC, which can be used to acquire multibond through space correlations between anomeric carbons and the proton of the adjacent sugar residues. A review of these and other useful NMR methods can be found here [27].

Although NMR analysis can be extremely informative, a drawback is its inherent insensitivity. Typically tens to hundreds of nmoles of material are required to produce the desired result depending on instrument and its configuration. However, the increasing magnetic field of NMR instruments over time and the use of cryoprobes are improving sensitivity. The dynamic range is generally less than two orders of magnitude. Although the instrumentation has low sensitivity, the methods are nondestructive allowing a single sample to be used for multiple analyses across different analytical platforms. Some NMR databases exist containing a range of *N*- and *O*-glycans but most are outdated though still useful [50].

Glycan MS structural analysis

Released glycans can be profiled using a range of mass spectrometric platforms. Common among these are MALDI-TOF MS, ion-trap MS, triple-quad MS and Q-TOF MS. The glycans can be analyzed in native form or in the form of a number of derivatives. As native glycans vary in ionization potential acquisition of quantitative data can be a challenge. Labile groups such as sialic acids and sulfate can also be lost in this form. Therefore, to render the glycans more quantifiable and stabile it is often best to analyze them in derivative form. The most common derivatives are arrived at through permethylation and reductive animation. Permethylation increases ionization potential by replacing all exchangeable hydroxyl and amine hydrogens with a methyl group and adds a protecting group to sialic acids by substitution of the carboxylic acid hydoxyl hydrogen also with a methyl group [28]. The method, permethylation profiling, can be modified to also detect sulfated glycans quantitatively [29]. Reductive animation also increases ionization potential and can be used to substitute the reducing end with a UV/fluorescence active amine.

Both permethylated and aminated glycans can be analyzed by MALDI-TOF MS to determine the range of glycan compositions present and their relative abundances. Both of these forms can also be analyzed in LC/MS format as well. Historically, a drawback of permethylated glycan analysis was the absence of an HPLC separation method due to the lack of a natural chromophore for detection. Two groups have independently developed HPLC methods allowing LC/MS analysis of these compounds [33,51] and therefore a natural chromophore is not needed for detection as the mass spectrometer serves this purpose by detection of ion current. Since the permethylated glycans in a chemically related series have very similar ionization efficiencies the data can be quantitative and representative of true abundances. Tandem LC/MS analysis of permethylated glycans provides mass, linkage, branch structure and compositional data potentially providing everything except diastereomer specific monosaccharide information and anomeric configurations.

Alternatively, the aminated derivatives can be analyzed by LC/MS. Positive mode analysis of aminated glycans generally yields abundant $[M+nH]^{n}$ ions, which produce mostly glycosidic fragments. These provide sequence and monosaccharide information but only limited linkage and branch information. Negative polarity mode analysis of the aminated glycans can produce abundant cross ring fragments giving linkage and some branch information in addition to compositional and sequence information. Dual polarity mode analysis has been reported [32], which can provide complementary data sets for better structural assignment than either mode used alone. However, a drawback of aminated glycans is that, upon fragmentation analysis, they produce no molecular mass marker to indicate branch structure like that of permethylated glycans.

Several HPLC platforms are commonly used with the amine derivatives [52–54] and often in conjunction with MS although quantitation requires UV or fluorescence detection. 2-aminobenzamide and 2-amino benzoic acid are most commonly used amines. Amination generally increases ionization efficiency two or more fold [55]. As previously mentioned, the chemical modification is limited to the reducing end so there is no protection of labile groups such as sialic acids when analysis is performed in positive mode. However, negative polarity mode MS analysis can be used which limits loss of sialic acids [32,56]. In positive mode migration of the mobile carboxyl hydrogen leads to glycosidic bond cleavage. Negative mode operation favors removal of this proton during ionization making it unavailable to facilitate glycosidic bond loss. An example profile of both permethylated and aminated glycan LC/MS performed in positive mode is shown in Figure 2. For details see [32,33]. Both of these methods can be optimized for glycan isomer separation as seen in Figure 2A (top) & B (inset), respectively.

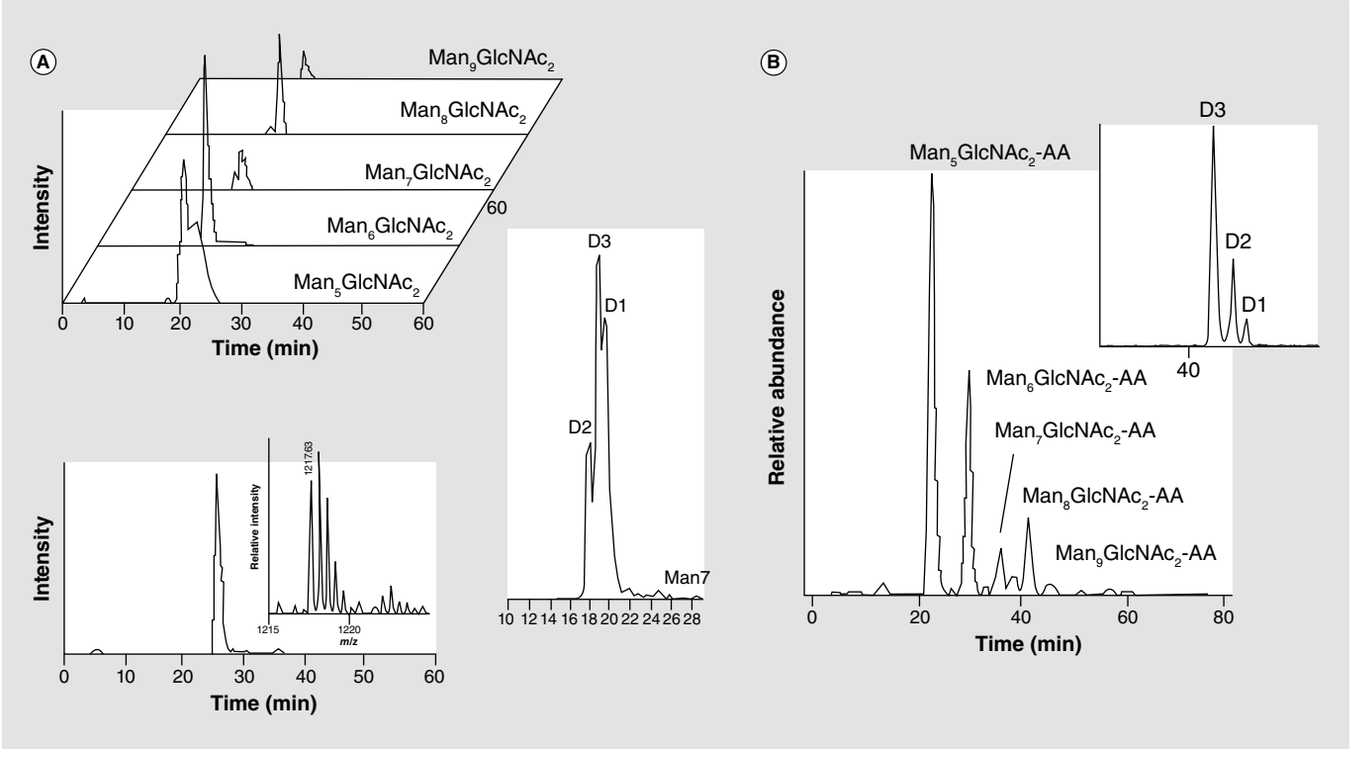

Figure 2. LC/MS glycan mass profiling analysis of permethylated alditol derivatives (A) and 2AB derivatives (B) of high mannose glycans released from bovine ribonuclease B. These are modified from [33] and [57], respectively. In **(A)** the stacked extracted ion chromatograms of $Man_5GlcNac_2$, $Man_6GlcNac_2$, $Man_7GlcNac_2$, $Man_8GlcNac_2$ and $Man_9GlcNac_2$ are shown. At the lower right is the partial separation of the isomer of $Man_7GlcNac_2$, D1-D3. At the lower left of (A) is the extracted ion chromatogram and mass spectrum of the permethylated alditol of $Man_9GlcNac_2$ detected at 160 fmol. In **(B)** the ribonuclease B released $Man_{5-9}GlcNac_2$ are analyzed. Inset: shows complete resolution of $Man_7GlcNac_2$ isomers D1-D3. In both cases these experiments can be performed in tandem MS format and the isomers can be positively identified through fragmentation patterns. See references for additional detail.

Glycopeptide enrichment

Glycopeptides produce much lower MS detector responses than their unglycosylated counterparts. To avoid low glycopeptide signal in a protein digest analysis a glycopeptide enrichment procedure is often used. A number of methods can be used for this purpose including size exclusion chromatography, lectin affinity, hydrophilic interaction chromatography (HILIC) and porous graphite chromatography. These have been reviewed elsewhere [58,59]. HILIC enrichment is one of the most successful forms of glycopeptide enrichment and has been shown to enrich glycopeptide signal up to 240-fold with little glycopeptide loss or bias [60] as shown in Figure 3.

LC/MS glycopeptide analysis

Glycopeptides are larger than their unmodified counterparts. If a maximum mass of 10,000 amu must be detected, which typically is present at a charge state of 3, then a resolving power of 10,000 or greater must be used to resolve isotopic forms. This exceeds the capacity of most conventional ion-trap or triple-quad instruments and thus these are not usually used for glycopeptide analysis. A review of current methods used in glycopeptide analysis can be found here [61]. State of the art LC/MS glycopeptide analysis approaches used currently employ Q-TOF or orbitrap MS. Examples can be found here [41] and here [40]. The former can have resolving power up to 60,000 and the latter in excess of 100,000, which is more than adequate. A complicating characteristic of glycopeptides is the difference in energy required to decompose their glycosidic versus peptidyl components. During collision-induced dissociation fragmentation analysis there is a tendency for the saccharidyl component to dissociate at lower energies than the peptidiyl component. Therefore scans performed using conditions that fragment the peptide backbone lead to excessive decomposition of the glycosyl moiety and information pertaining to glycan structure is lost. Alternatively, if the scan is acquired with only enough energy to disrupt the bonds of the glycan moiety the peptidyl component is often left intact leading to little peptide sequence information for use in identification. For this reason multiple scans at different energy levels may be required to obtain diagnostic fragment ions for both. This requirement can exceed the duty cycle capacity of some instruments, which must

be considered.

A recent approach, MS^E, allows for energy ramping during the dissociation scan, which allows accumulation of diagnostic fragments from both peptidic and glycosidic moieties as data are collected in the proper energy domains for each moiety [41]. During glycopeptide analysis the most useful diagnostic fragment ions include peptide+HexNAc, oxonium, glycosidic (peptide with partial loss of glycan) and peptide b- and y-ions [62]. Some informatics software incor-

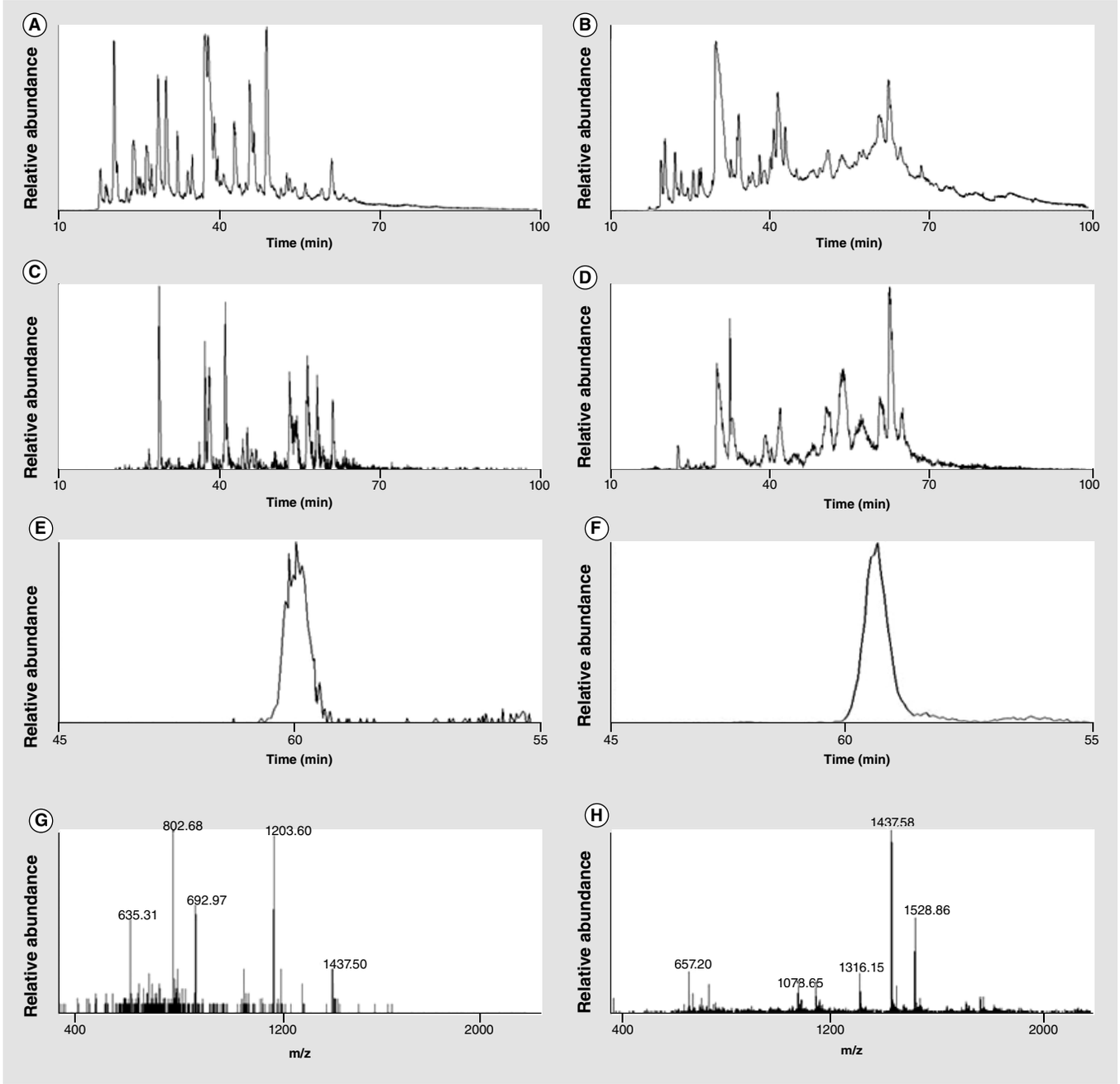

Figure 3. Q-time of flight LC/MS analysis of fetuin peptides before and after hydrophilic interaction chromatography solid-phase extraction. (A) TIC of fetuin tryptic peptides before HILIC enrichment. **(B)** TIC of fetuin tryptic peptides after HILIC enrichment (glycopeptides). **(C)** XIC of oxonium ion m/z 657 (HexHexNAcNeuAc) before HILIC enrichment. **(D)** XIC of ion m/z 657 after HILIC enrichment. **(E)** XIC of glycopeptide m/z 1437.58 before HILIC enrichment. **(F)** XIC of glycopeptide m/z 1437.58 after HILIC enrichment. **(G)** MS scan at 50 minutes before HILIC enrichment. **(H)** MS scan at 50 minutes after HILIC enrichment. Total integrated cps for the glycopeptide m/z 1437.58 was increased 240 fold on comparison of before and after sample enrichment. See [60] for details.
HILIC: Hydrophilic interaction chromatography; TIC: Total ion chromatogram; XIC: Extracted ion chromatogram.

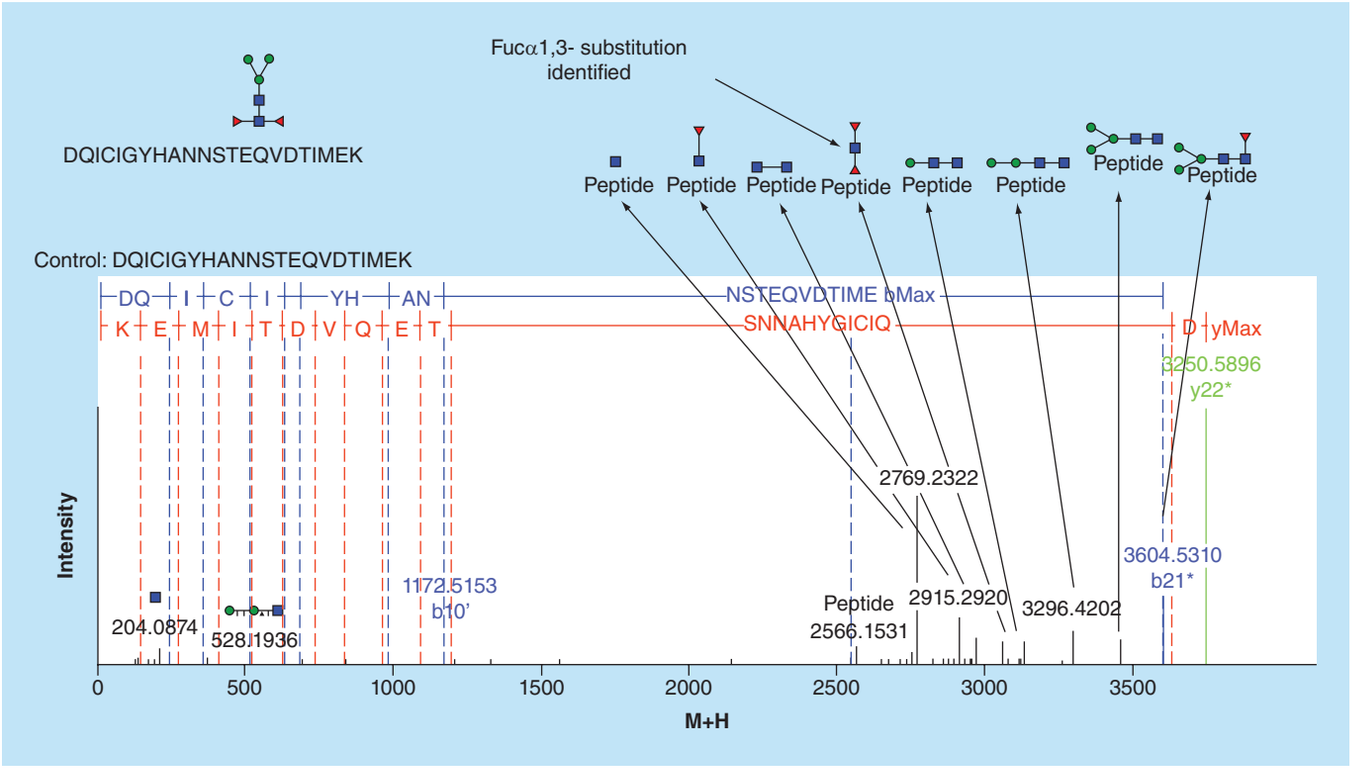

Figure 4. MS^E spectrum of hemagglutinin tryptic glycopeptide ($Hex_3dHex_2HexNAc_2$)DQICIGYHANNSTEQVDTIMEK derived from A/Bar-headed goose/Qinghai/14/2008 produced in high five cells containing site one. CCD Fuc-α1,3- core glycan substitution is identified. The colors in the spectrum represent: red, y ions; blue, b ions; green, y/b ions after neutral loss; gray-unassigned ions by biopharmalynx (some have been manually assigned as indicated). Monosaccharide symbols: blue square, GlcNAc; green circle, Man; red triangle, Fuc. Oxonium ions at m/z 204.09 and m/z 528.19 are indicated.

porates these components into their identification schemes [63]. A sample spectrum is shown in Figure 4. The figure shows the MS^E spectrum of the first glycosylation site of A/Bar-headed goose/Qinghai/14/2008 HA. The peptide+HexNAc, oxonium, glycosidic and peptide b- and *y*-ions are detected allowing high confidence identification as well as detection of the cross-reactive carbohydrate determinant (CCD) core Fucα1,3 substitution, which is associated with high IgE titers in response to natural Fucα1,3 containing allergens, although the clinical significance is not clear [64,65].

An alternative glycopeptide MS analysis method incorporates electron activated modes of dissociation. In this approach molecular disassembly proceeds through propagation of a free radical cascade preferentially targeting the peptide backbone leaving the majority of glycans intact at the resident Asn [66]. The method is more useful than MS^E for determining glycosylation sites when there is more than one present on a glycopeptide. Typically, the glycan is left intact and the peptide backbone dissociates yielding the precise position and mass of the glycan(s) [67]. Subsequent scans or additional experiments using higher energy collision dissociation, collision-induced dissociation or another fragmentation mode, such as infrared multiphoton dissociation, can be used to produce glycan sequence data. For examples see [40,68].

Glycosylation site occupancy

Glycosylation sites may be occupied fully, partially or not at all and this status may have implications for the vaccine properties especially where antigenic sites may be masked or altered. For this reason it may be necessary to determine the percentage of occupancy. Of the two types of protein glycosylation, *N*-linked and *O*-linked, the former is more tractable and will be discussed first. The most common *N*-glycosylation site occupancy analyses take advantage of the difference in mass between Asn and Asp. When *N*-glycosidase PNGase F or PNGase A is used to release *N*-glycans the Asn of an occupied sequon is converted to an Asp [69]. Any unoccupied sequon Asn will not be converted by the enzyme. The fractional percent of each can be used to calculate the approximate percent occupancy. The monoisotopic molecular weight difference between Asn and Asp

is 0.98401 Da. If the reaction is performed in the presence of ^{18}O water the difference is 1.98404 Da. Using high-resolution MS these differences can be easily resolved. The spectra are deconvoluted/deisotoped and the peak ratios of the Asn (unglycosylated fraction) and Asp (deglycosylated fraction) containing peptide areas are used to calculate the percent occupancy. Example data are shown in Figure 5. A caveat of this method is that Asn can spontaneously deaminate to Asp. If ^{18}O water is used then these Asp residues can be identified. However, if ^{18}O water is not used then any Asp resulting from spontaneous deamination can be incorporated into the site occupancy data incorrectly as occupied leading to a higher occupancy estimate.

O-linked glycosylation sites are more difficult to assess for percent occupancy. Unlike *N*-glycosylation, there is no enzyme or other method available that leaves behind a mass tag for *O*-glycosylation so a similar method to that used for *N*-glycosylation is not possible. There is no standard method for determining site occupancy for *O*-glycans. However, there have been some approaches proposed. One accounts for the fractional abundance of each glycoform plus the unoccupied form for each glycopeptide. A simple calculation then yields the apparent abundances of all forms thus providing percentage occupancy. Ion efficiency decreases with the size of the glycan so the approach has been used most successfully on glycopeptides with short *O*-glycans [70]. For larger glycans this approximation becomes less accurate.

Intact mass analysis can also be used to determine site occupancy. This is most often accomplished using high-resolution MS such as that available with Q-TOF or orbitrap mass spectrometers. Spectra are collected and deconvoluted. The number of glycosylation sites can be determined by the observation of masses above that of the protein backbone (not including other potential post translational modifications [PTMs]) in multiples of expected oligosaccharide masses. For instance if two sites are expected and can contain $Man_5GlcNAc_2$ the spec-

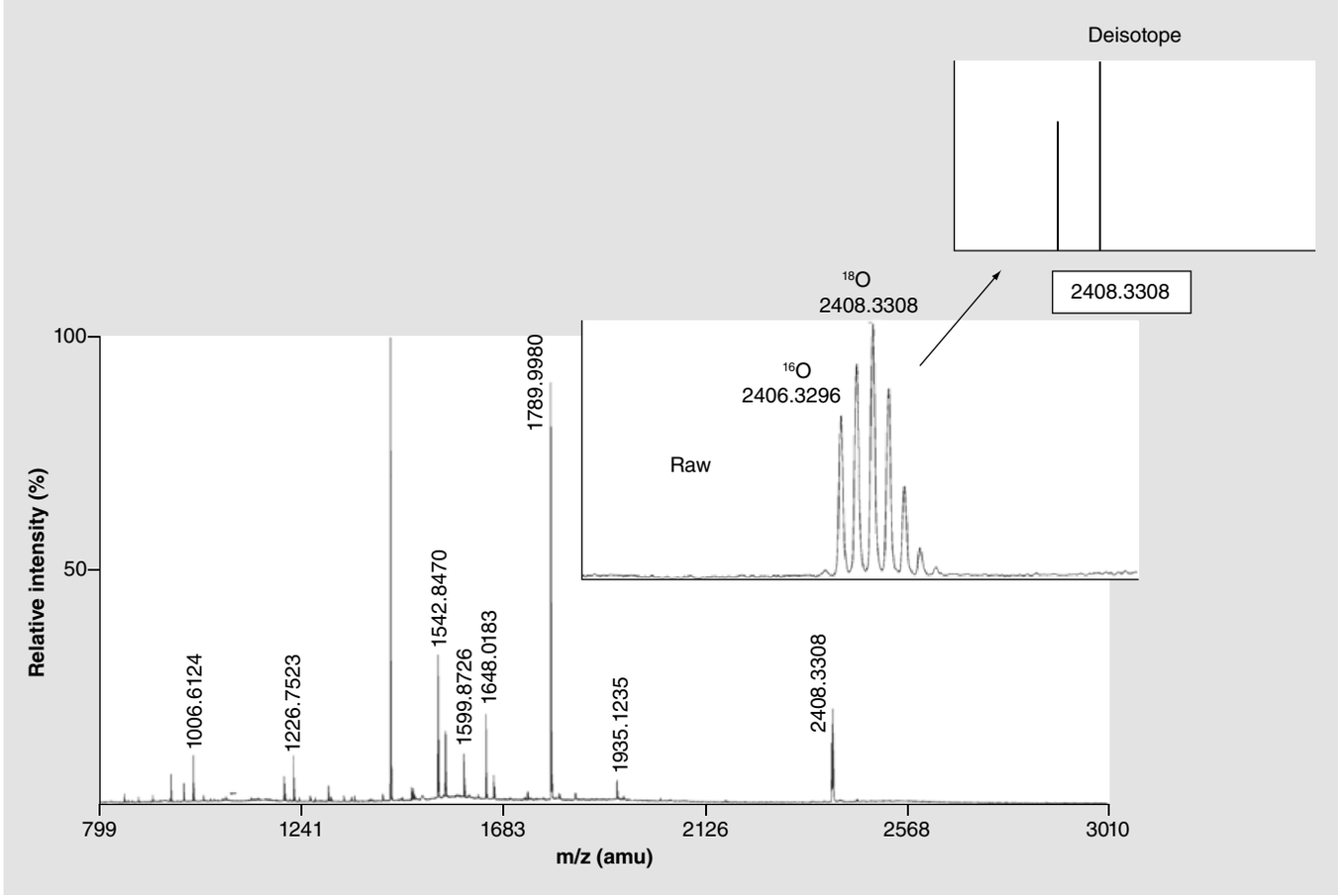

Figure 5. Matrix-assisted laser desorption/ionization-time of flight spectra of an nano-LC fraction ^{18}O labeled glycopeptide. The first inset shows the isotopic distribution of the glycopeptide with an unoccupied glycosylation site with an unmodified Asn ^{16}O, and one with an occupied site, which is converted to Asp and labeled with ^{18}O upon release of the glycan using PNGase F. Second inset represents deisotoped raw data. The deisotoped data can then be integrated and used to estimate site occupancy percentage. See [5] for details.

trum should contain the protein expected mass plus one and or two equivalents of the mass of that oligosaccharide (1217.4 amu). The complexity of such spectra increases as the diversity of oligosaccharide substitutions and number of potential glycosylation sites increases. The specific location of each oligosaccharide chain cannot be determined in this way. However, peak intensities are correlated to glycoform abundances. Therefore, the technique can be useful, for instance, in lot to lot consistency testing. Along with intact MS analysis, using similar rational, reducing gel PAGE and CE have also been used to determine glycosylation site occupancy in relatively simple glycoproteins such as monoclonal antibodies [71].

Manufacturing considerations

Glycosylation monitoring

There are a number of methods that may be appropriate for glycan monitoring during the manufacturing process. An assay for quantitation of released glycans can be employed. Methods such as HPLC analysis of aminated glycans, analysis of released glycans by HPAEC-PAD, and LC/MS analysis of permethylated or aminated glycans could be used as previously discussed. A simple method for quantitation involves release and permethylation of glycans followed by direct analysis by MALDI-TOF MS. The data can be collected in a quantitative fashion when an internal reference is used or semiquantitatively without an internal standard. The relative abundances of glycan compositions can be determined accurately and repeatably [60,72] making this method atractive for the purposes of batch analysis. While this does not directly detect site specific glycosylation or isomeric forms, in general, a change in overall abundance at any site or shift in isomeric forms will be reflected in the more general glycosylation pattern and thus in the permethylated free glycan pool profile. The assay is rapid and can be performed with a few micrograms of released saccharide. The procedure is robust and requires little sample manipulation. An example is shown in Figure 6. Here, the permethylated glycosylation profiles of HA-released glycans produced in two different insect cell substrates are shown. These are derived from Sf9 parent and SWT7 genetically modified cells. The SWT7 cells have been modified to produce human like glycans. As shown in the histogram below the SWT7 cells produced less pauci-mannose structures such as $Hex_3HexNAc_2$ and $dHex_1Hex_3HexNAc_2$ and more human-like hybrid and complex glycans such as $Hex_4HexNAc_3$ and $Hex_3HexNAc_4$, respectively.

Another example assay is a peptide mapping assay as it is closely interactive with glycosylation sites since the glycopeptides would be present in the chromatographic trace. Peptide mapping serves as an assay for more general protein concerns as well adding to its attraction.

Key terms

Glycan monitoring: The periodic analysis of free glycans samples.

Batch analysis: An analysis in which all of the samples collected for a specific, nonemergent assay undergo the same testing process at the same time or sequentially.

It is often preferable to have two or more assays rather than a single one, especially those that are interactive. As released glycan and peptide map-based assays would be interactive the use of both may be superior to either one used alone under some circumstances.

Cell substrates special considerations

A number of cell systems are used to make investigational and licensed vaccines containing glycoprotein antigens such as influenza HA. These include mammalian cell lines: MDCK (Canine), HEK293 (human embryonic kidney), Chinese hamster ovary (CHO), Vero (spider monkey, *Cercopithecus aethiops*); insect High Five™ (*Trichoplusia ni*), Boyce Thompson Institute for Plant Research, Ithaca, NY), Sf9 (*Spodoptera frugiperda*, ovarian), Sf21 (*Spodoptera frugiperda* Sf9 subclone); Avian, embryonated hen egg (*Gallus gallus*) and plant, such as *Nicotiana benthamiana*. The *N*- and *O*-glycans produced can be similar to human or quite different; essentially, depending on how related the expressing cells are phylogenetically to humans. While there is no data that is negatively associated with vaccine glycoprotein glycosylation currently, key glycosylation differences seen in some of these systems may be important.

In mammalian cell systems and embryonated hen eggs the *N*-glycans are quite similar to those found in human. There are three subtypes including high mannose, complex and hybrid. Some cell types, such as CHO and NS0 [73] (neither currently used in vaccine production) can produce a Galα1,3Gal substitution which has been associated with organ rejection after transplant [74] and has recently been identified as an emerging allergen in response to biting arthropods [75]. It can be present on either *N*- or *O*-glycans. CHO and MDCK cell lines may also substitute glycans with NeuGc, which is not found in humans. In total, 85% of human populations contain high titers of NeuGc antibody [76]. Insect cells can produce *N*-glycans containing core-linked Fucα1,3-, which is a known cross-reactive carbohydrate determinant (CCD) as previously stated [65]. The insect *N*-glycans are mostly of the pauci mannosidic type having less than five Man residues. The *O*-glycans of insect cells are less well characterized. In *Drosophila melanogaster* the *O*-glycans contain a mammalian core-1-like disaccharide but are short, and contain terminally linked GlcA, which is unlike any *O*-glycans of humans. *Drosophila melanogaster* also produces *O*-glycans with core Fuc in nonmamma-

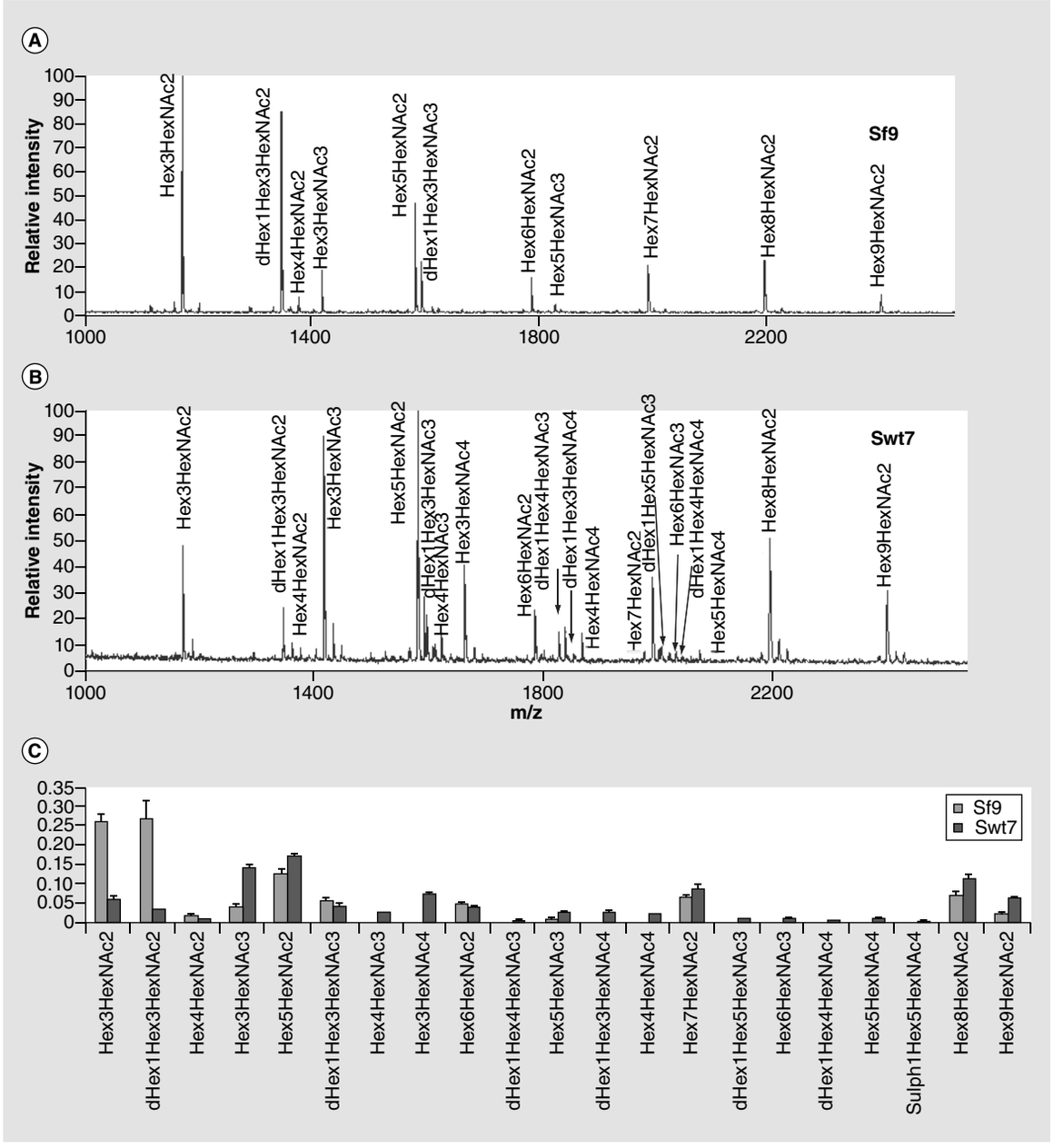

Figure 6. Permethylation profile comparison of glycans released from A/Vietnam/1203/2004 hemagglutinins derived from two insect cell lines, Sf9 insect cells and SWT7. The former is the parent of the later. The SWT7 cells are genetically modified to produce mammalian-like glycans [41]. The released glycans were permethylated, which replaces exchangeable hydrogens of hydroxyls and amines with *O*-methyl groups allowing nearly identical ionization potentials across related oligosaccharides such as those shown. The resulting MS spectra are highly reproducible and semiquantitative. Error bars in the bottom panel represent 1SD produced using triplicate analyses. It can be seen that pauci mannose glycans (insect type), such as those indicated by Hex3HexNAc2 and dHex1Hex3HexNAc2, are higher in sf9 cell-produced HA glycans, while mammalian-like complex ones such as Hex3HexNAc4 are more abundant in SWT7 cell produced HA glycans.
HA: Hemagglutinin.

lian configurations. *Drosophila melanogaster* cells are not used in vaccine production but may offer a glimpse into the glycan diversity seen in other insect cell lines. *Spodoptera frugiperda* produces *O*-glycans containing phosphocholine [77]. Helminth glycans containing phosphcholine have been linked to host immunosuppression in mammalian hosts [78,79]. Plant systems produce high mannose, complex and hybrid *N*-glycans like their mammalian counterparts. However they also produce core Xylβ1,2- and core Fucα1,3- moieties both of which

Xylβ1,2- and core Fucα1,3- moieties both of which are CCDs as previously mentioned. As described, some of these nonhuman carbohydrates have been associated with significant biological effects. For others the biological effects, if any, are not known. None of these entities have been negatively associated with currently licensed vaccines. However, as biological activities have been associated with some of these entities, it may be useful to consider the potential effects of glycosylation when these cellular substrates are used.

Fermentation modes

Many cell culture process variables can affect glycosylation. Manufacturing scale, fermentation type and fermentation conditions all must be considered. Bioreactor pH, manganese concentration, dissolved oxygen, ammonia concentration and temperature have all been shown to affect glycosylation patterns [32]. The three major modes of production, batch, fed-batch and perfusion, all have different waste product accumulation and nutrient depletion profiles and these can have profound effects on the glycosylation profiles. Glycosylation processes are sensitive to fermentation conditions [80,81]. If a manufacturer determines that glycosylation changes may affect vaccine qualities it may be useful to consider untoward effects on glycosylation if a change in manufacturing is considered such as changing fermentation type, scale or location.

Conclusion

Glycosylation has important roles in protein function and stability. In the vaccine setting glycosylation of key glycoprotein antigens or host interaction regions may theoretically influence vaccine characteristics. State of the art MS-based analytics are becoming available to aid in characterization of glycoprotein-based vaccines. These techniques allow deep structural analysis revealing fine structural detail of the carbohydrate chains as well as location and percent occupancy of specific glycosylation sites. Combined with more traditional analytical methods such as those based on NMR, HPLC and GC/MS, state of the art mass spectrometric methods can provide a wealth of structural information for these vaccines. Manufacturing choices such as cell substrate, culture media, fermentation type and production scale can significantly affect carbohydrate composition and content. Production of vaccines that are better characterized or designed with regard to carbohydrate content may be warranted for vaccines in which glycosylation impacts the vaccine product. In these cases, it may be useful to monitor glycosylation status during the manufacturing process the specification of which could be linked to clinical data and product characterization better ensuring consistency of the product characteristics throughout the shelf-life.

Key term

Glycomics: The study of the entire complement of sugars, free or as part of more complex molecules, in an organism or biological entity such as a virus.

Future perspective

Glycosylation is emerging as an important post-translational modification of key glycoprotein antigens in the vaccine setting. It follows that monitoring of these chemical entities as quality attributes will become more common. As the vaccine field progresses toward better defined vaccines analyses that define glycosylation, free released and protein linked, will become more desirable as more instances surface showing their impact on antigenic structure and immune processing. Analytics such as high-field NMR, gas chromatography and MS traditionally have proven to be extremely useful in the determination of glycan structure. New highly sensitive MS-based techniques can facilitate more rapid analyses that reveal great detail about oligosaccharide composition, configuration and heterogeneity as well as their site of occupancy. Some challenges remain in MS-based structural evaluation of protein glycosylation. Major areas that require improvement include methods for more complete coverage and quantitation of structural isomers, accurate determination of site occupancy in *N*- and especially *O*-glycosylation, complete determination of all glycosidic linkages in complex oligosaccharide mixtures and development of better informatics for rapid glycomics data processing. While some areas need improvement, the use of this constellation of MS-based analytics for glycosylation characterization in combination with other techniques as outlined in this review, will allow better, more well-defined, vaccines in the future.

Disclaimer

The contributions are an informal communication and represent author's own best judgment. These comments do not bind or obligate the US FDA.

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No writing assistance was utilized in the production of this manuscript.

Executive summary

- Immune system pressure and development of the pathogen glycan shield.
- Influenza hemagglutinin as a model glycoprotein antigen.
- Cell substrates and their differences in protein glycosylation.
- Glycosylation complexity and analytical challenges.

Protein glycosylation

- Principles of *N*- and *O*-glycosylation are reviewed.

Glycosylation & the vaccine antigen

- Pathogens use glycosylation strategies to avoid host immune response.
- Adaptation of influenza H3N2 to the human host demonstrates a pathogen's use of glycosylation in immune avoidance strategies.
- Understanding of pathogen glycosylation strategies aids in better design and monitoring of vaccines.
- Recent advances in MS have significantly increased the ability to monitor vaccine antigen glycosylation in a more routine fashion.
- The use of analytics to detect changes in glycosylation status of major vaccine antigens can aid in vaccine design, development and monitoring of the production process.

Analytical methods

- Current and state of the art methods for free glycan analysis are discussed including: glycan release, monosaccharide analysis, linkage analysis, glycan mass spectrometry structural analysis.
- Current and state-of-the-art methods for glycopeptide analysis are discussed including: glycopeptide enrichment, LC/MS glycopeptide analysis and glycosylation site occupancy are covered.

Manufacturing considerations

- Key topics of manufacturers to consider in process development and monitoring are discussed including: glycosylation monitoring, cell substrates special considerations and fermentation modes.

Future perspective

- The emergence of glycosylation as a tractable attribute is discussed.
- Remaining challenges faced by glycosylation analytics are discussed.

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