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Glycosylation of viral surface proteins probed by mass spectrometry

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Glycosylation is a common and biologically significant post-translational modification that is found on numerous virus surface proteins (VSPs). Many of these glycans affect virulence through modulating virus receptor binding, masking antigenic sites, or by stimulating the host immune response. Mass spectrometry (MS) has arisen as a pivotal technique for the characterization of VSP glycosylation. This review will cover how MS-based analyses, such as released glycan profiles, glycan site localization, site-occupancy, and site-specific heterogeneity, are being utilized to map VSP glycosylation. Furthermore, this review will provide information on how MS glycoprofilng data are being used in conjunction with molecular and structural experiments to provide a better understanding of the role of specific glycans in VSP function.

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Introduction

Glycosylation produces an abundant, diverse, and highly regulated repertoire of cellular glycans that are frequently attached to proteins and lipids. Viral surface proteins (VSPs) are no exception, often hijacking host cell's molecular machinery in order to obtain O-linked, N-linked, or C-linked glycans [1]. Although the cell cannot distinguish between host and viral proteins, one difference that is observed is an increase in glycosylation compared to host glycoproteins. The high levels of glycosylation observed in the envelope protein of human immunodeficiency virus type 1 (HIV-1), Ebola virus (EBOV), and Lassa virus (LASV), as well as others serve as a protective shield from the host's immune system [2–4,5*,6*]. Some specific glycans are important for protein structural stability and

the loss or gain of glycans can influence protein function. For example, the loss of either the N2 glycan or N4 glycan from hepatitis C virus (HCV) envelope 2 results in total loss of HCV infectivity [7]. In contrast, hemagglutinin (HA), a major surface protein on the influenza (Flu) virus, can lose activity when glycans are positioned close to the HA cleavage site, preventing protease access and virus entry [8,9]. During viral evolution, glycosylation sites are often added and deleted. With this diversity of modification, the complexity of viral glycoproteins that exists in an individual host or host population is increased [10]. Alteration of glycosylation site(s) can have dramatic impacts on virus survival and transmissibility [11,12]. The location of these highly mutable regions is often in the globular head of viral fusion proteins with each virus having a varying number of glycosylation sites (Figure 1).

Glycosylation of VSPs occurs as they pass through the cellular secretory pathway, which is also where cellular protein glycosylation occurs [13]. The diversity of glycosidase and glycosyltransferase expression in different cells [14], and the non-template driven process by which all types of glycosylation occurs results in each VSP being a heterogeneous population differentiated by their glycans [13].

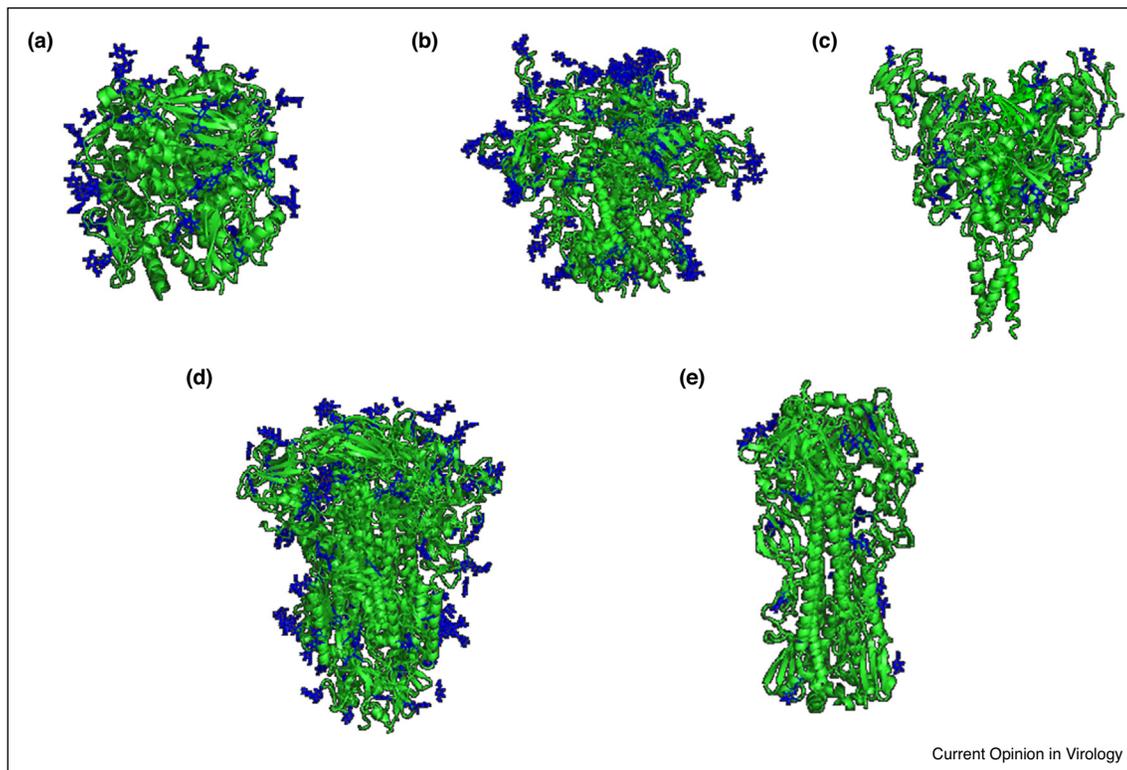
This heterogeneity is an integral part in how viruses escape the host immune system [15] but has always been considered a complex task. To tackle this analytical challenge, mass spectrometry (MS) has become the standard tool to map protein glycosylation. This review will briefly cover developments in MS and how the resulting analytical glycomic information can be coupled with other biological data to better understand the role of glycosylation in the structure and function of VSPs.

Viral surface protein glycosylation

There are three main types of glycosylation, O-linked, N-linked, and C-linked. C-linked or C-Mannosylation is the rarest form, occurring at the carbon of the first Tryptophan residue in the consensus sequence W-X-X-W, W-X-X-C, or W-X-X-F (where X represents any amino acid) [16]. C-Mannosylation has only been reported at one site on the soluble glycoprotein (GP) of the EBOV even though the consensus site can be observed on other VSPs [17].

In N-linked glycosylation, the initial glycan moiety, comprises three glucose (Glc), nine mannose (Man), and two *N*-acetylglucosamine (GlcNAc), is transferred to asparagine residues within N-X-S/T sequon (X ≠ P)

Figure 1



Viral Fusion Protein Structures and *N*-glycosylation Sites (NGS). Viral fusion proteins are typically trimers of heterodimers with a globular head domain and a stalk/transmembrane domain. A GlucNAc₁ or GlucNAc₂ are modeled at each NGS (Blue). **(a)** Lassa virus (LASV) glycoprotein (GP) complex contains 11 (NGS) with 7 in GP1 and 4 in GP2 (PDB: 5VK2) [27]. **(b)** Human immunodeficiency virus (HIV-1) Envelope (Env) GP contains around 29 NGS with 25 in gp120 the outer Env domain and 4 in gp41 the transmembrane domain (PDB: 5FYK) [49**]. **(c)** Ebola virus (EBOV) GP has 17 NGS with 15 on GP1 and 2 on GP2 (PDB: 6G9B) [101]. **(d)** Coronavirus (CoV) spike GP has 26 NGS with 15 in subunit 1 and 11 in subunit 2 (PDB: 6BFU) [89]. **(e)** Influenza virus hemagglutinin is a homotrimer with 11 NGS (PDB: 4FNK) [92].

co-translationally as the protein folds in the endoplasmic reticulum (ER) [18–20]. The initial glycan is processed first in the ER by trimming three Glc sugars resulting in a high-mannose *N*-glycan. As the protein passes through the Golgi apparatus, differentially expressed glycosidases and glycosyltransferases further trim, elongate, and branch the glycan moiety resulting in hybrid or complex-type *N*-glycans [21,22]. Most VSPs contain *N*-glycosylation with HIV-1, coronavirus (CoV), and EBOV surface proteins containing more than 20 *N*-glycosylation sites [5*,23*,24**] and Flu and LASV containing fewer than 11 *N*-glycosylation sites [25*,26,27].

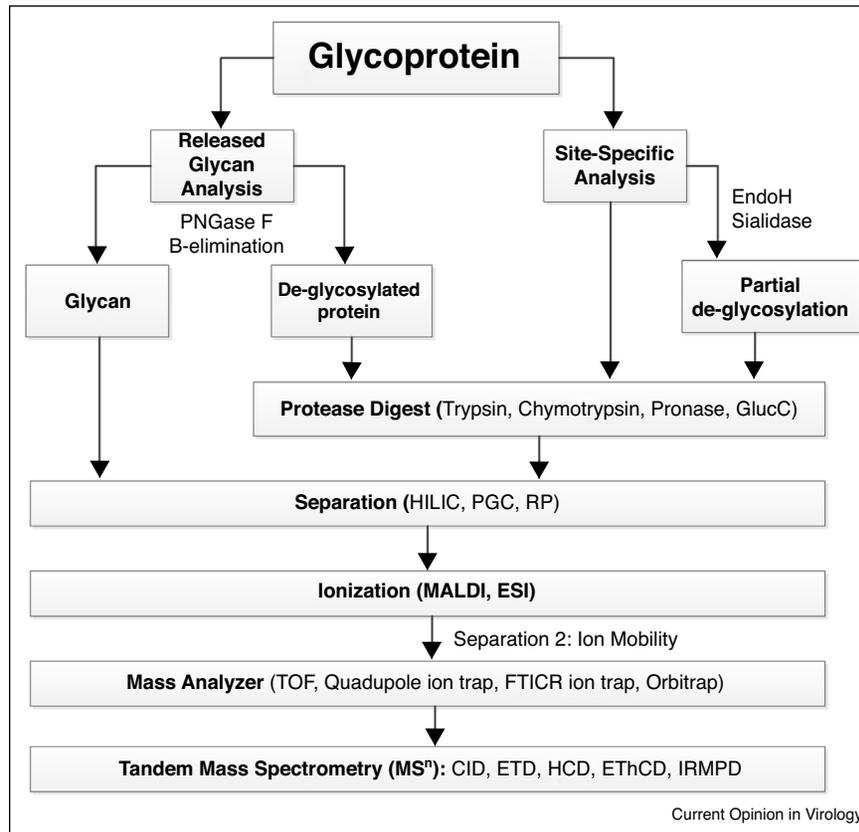
While there are many types of O-linked glycosylation [13], the GalNAc-type or mucin-type *O*-glycosylation is the most common on VSPs [28]. Mucin-type *O*-glycan modifications are initiated by a family of 20 GalNAc-transferases in the Golgi apparatus that add the initial GalNAc monosaccharide to S, T, and sometimes Y residues [29]. The O-linked glycans can be further elongated by competing glycosyltransferases to form up to eight core structures [30,31]. Human cytomegalovirus

(HCMV), Epstein Barr Virus (EBV), and HCV contain VSPs that are extensively *O*-glycosylated [32**,33].

Mass spectrometry in the analysis of viral surface protein glycosylation

MS, the production and detection of ions separated according to their mass-to-charge (m/z) ratios, can provide structural identification of glycans, site-specific glycan characterization and be utilized to track changes in glycan composition, pattern, and site-occupancy [34–36,37*]. Analysis of protein glycosylation by MS is typically achieved using two main approaches: glycans are released from the peptide backbone either enzymatically or chemically, or the glycoprotein can be subjected to protease digestion, producing a mixture of peptides and glycopeptides. These glycans/glycopeptides are then chromatographically separated before ionization into the mass spectrometer where MS and tandem MS data are obtained (Figure 2, for review of MS approaches to glycomic and glycoproteomic analyses, see Ruhaak *et al.* [38**]).

Figure 2



Workflow diagram of glycoprotein analysis commonly used to characterize viral surface protein glycosylation.

The analytical benefits of MS have established it as the standard tool for the analysis of glycans and glycoproteins [35,37,39]. Two specific areas have led to significant advances in MS analysis of glycoproteins. 1) The advent of chromatography separation platforms that were based on the hydrophilic properties of glycans [40–42]. 2) The development of novel ion fragmentation techniques, especially electron radical methods that fragment peptide ions while leaving labile amino acid modifications (i.e. glycosylation) intact [43,44]. These advances were quickly adopted by virologists to provide comprehensive glycan profiles for VSPs.

Glycan profiling viral surface proteins

Applying modern MS-based methods for the robust characterization of VSP glycosylation involves using a variety of techniques that provide different levels of glycoprotein detail [32]. For example, MS of released glycans gives an overall profile of the type of oligosaccharides that exist for a given VSP. Analyzing the peptides obtained after glycan release by MS contributes information about glycan site-occupancy. Further analysis of glycopeptides by MS provides site-specific heterogeneity profiles for each *N*-glycosylation site (NGS). The combination of these

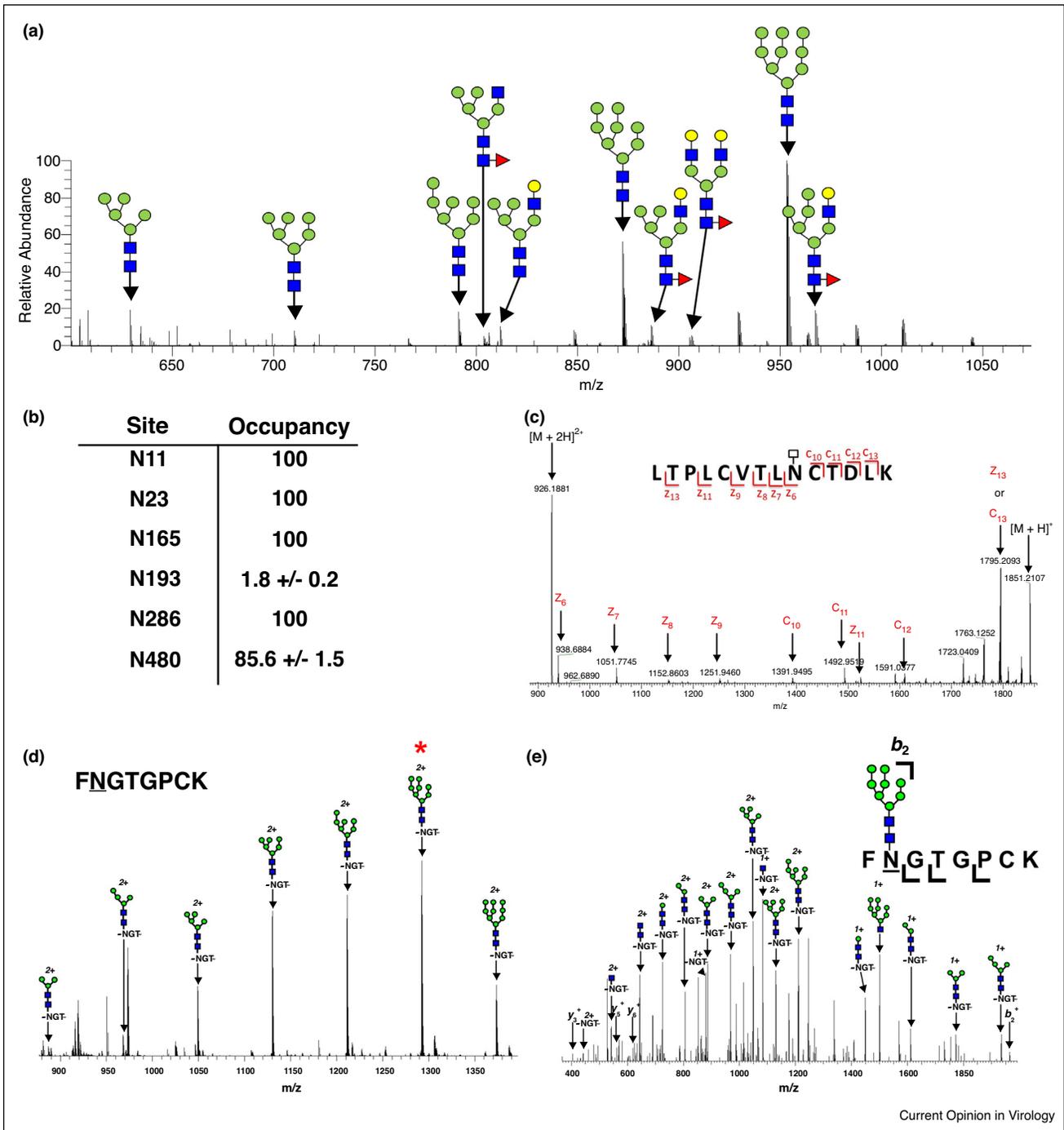
techniques and others provide glycan profiles for VSPs that can be utilized by researchers to better understand the role of oligosaccharides in the structure and function of VSPs [15]. Figure 3 illustrates the typical information obtained from MS glycoprofiling data.

Released glycan profiles

A common starting point for initial characterization of VSPs is MS analysis of released glycans (Figure 3a). This type of VSP glycan analysis confirmed VSP glycosylation was dependent on the cells expressing the virus [24,45,46,47]. These profiles also helped explain differences in antibody (Ab) neutralization capacity of virus produced in different cell types [2,48,49].

Both *O*-glycans and *N*-glycans can be released either chemically or enzymatically [50]. *O*-glycans are typically released chemically by hydrazinolysis or β -elimination due to a lack of glycosidases with broad specificity [51–53]. These techniques were used to identify 8–10 *O*-glycans on EBOV GP_{1,2S} [54]. When the *O*-glycans from five pathogenic EBOV GP_{1,2S} were compared, differences in *O*-glycan patterns were observed [23].

Figure 3

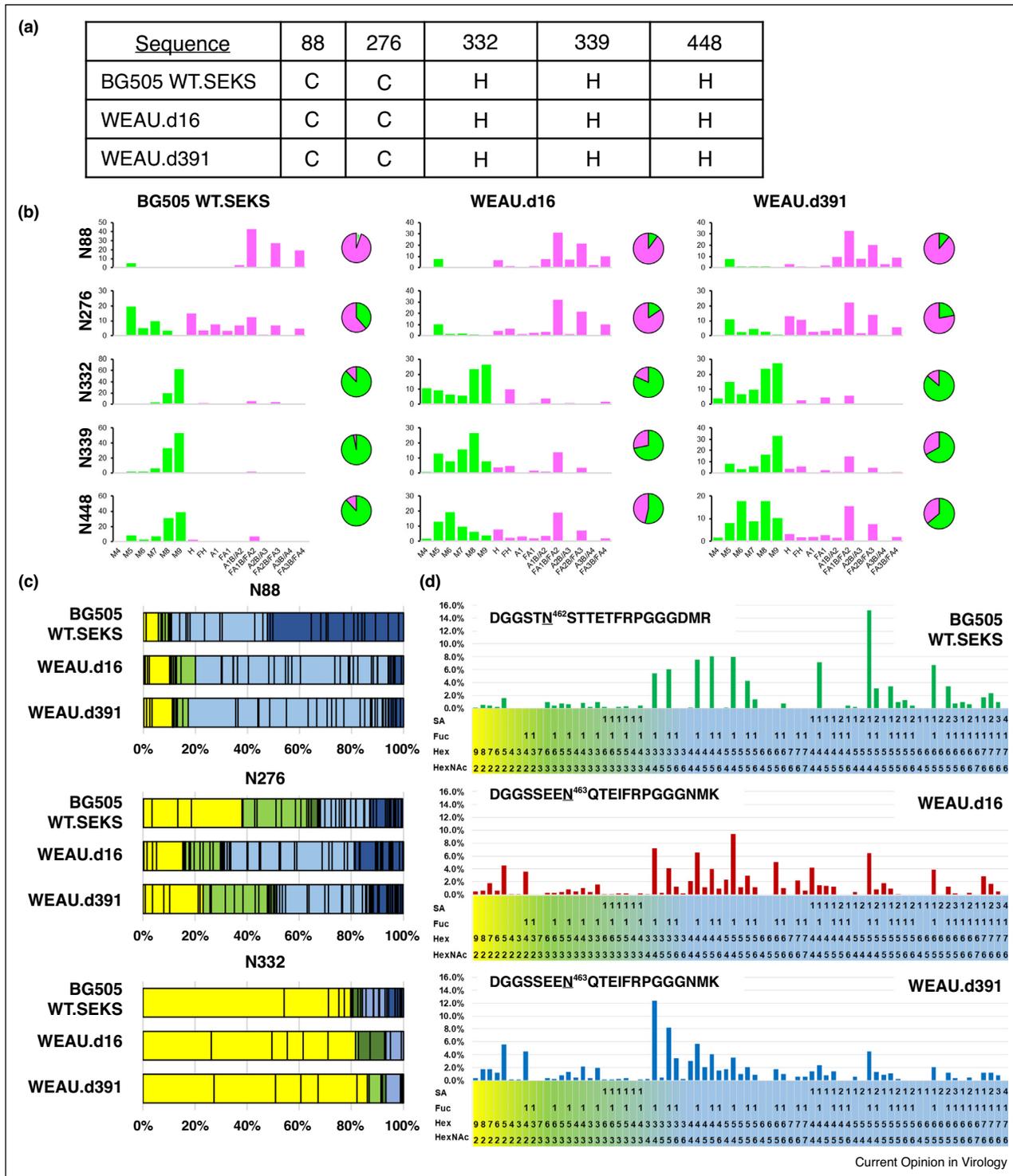


Types of *N*-glycan profiling data. **(a)** Released glycan profile for HIV-1 gp120 produced in 293T cells and treated with neuraminidase. **(b)** Site Occupancy data for Flu HA *N*-glycosylation sites [25]. **(c)** Tandem MS spectra confirming site localization for an HIV-1 gp120 glycopeptide after EndoH digestion. **(d)** Site-specific heterogeneity profile for a HIV-1 gp120 *N*-glycosylation site. **(e)** Tandem Mass spectra confirming peptide sequence in the MS1 spectra shown in panel d.

N-Glycans are usually released via a peptide-*N*-glycosidase F (PNGase F) enzymatic digestion. PNGase F cleaves all types of *N*-glycans except those with $\alpha(1-3)$ -linked core fucose. In this case, PNGase A is

utilized; however, it is less efficient [55,56]. Machupo GP₁ [57], HIV-1 gp120 [45,58], Dengue virus GP E [59], chikungunya virus E1 and E2 proteins [60], and the LASV GP complex (GPC) [6] are a few examples of

Figure 4



Examples of site-specific glycan profile outputs. All data obtained from publicly available HIV-1 recombinant gp120 data sets [24**,94**]. Each panel demonstrates how single-site glycan heterogeneity for 3–5 individual HIV-1 Env *N*-glycosylation sites are commonly represented in the literature. (a) Table of 5 NGS from three gp120 trimers indicating their predominant *N*-glycan type observed from site-specific MS data similar to reports from Go *et al.* [46]. (b) Quantitative site-specific *N*-glycosylation bar graph of the same NGSs categorized as oligomannose series (M5–M9), hybrids (H), and fucosylated hybrids (FH), and also by the number of branching antennae (a) of complex type glycans. A summary pie chart that compares the amount of high mannose (green) to processed glycans (pink) is provided next to each bar graph similar to data reported by

VSPs where the *N*-glycans have been released to provide a profile of the overall oligosaccharides present. Recently, a study compared the *N*-glycosylation profiles of Flu HA produced in hen egg, Madin–Darby canine kidney (MDCK), or insect (Sf9) expression systems where the observed differences could have implications for immune processing and immune surveillance of vaccines that use HA produced in these cell lines [47].

Glycan site localization

Another level of glycoprofiling is to localize the amino acid site of attachment of the oligosaccharide on the protein. For researchers, the site of attachment provides information that can be applied to mutational studies to identify glycan sites that are critical to the proteins' functional role and/or identify sites that play a role in immune evasion [15,24**,47,61,62*].

N-Glycan site localization can be accomplished by partial or complete enzymatic release of *N*-glycans. Complete release of glycans by PNGase F results in the deamidation of the asparagine to aspartic acid which is confirmed by tandem MS to identify the site of glycosylation, such as for the SARS spike protein [63]. Alternatively, to mitigate error caused by spontaneous deamidation, an endoglycosidase (Endo) is used to identify the site of glycosylation by consolidating the glycan heterogeneity into one oligosaccharide through a partial glycan cleavage [64]. Each Endo has specificity for a different type of *N*-glycan, but all cleave between the two GlcNAc residues in the diacetylchitobiose core of the oligosaccharide leaving a single GlcNAc or a disaccharide of FucGlcNAc linked to the asparagine [65–67]. These glycopeptides are identified by tandem MS using electron transfer dissociation (ETD) fragmentation, which preferentially cleaves along the peptide backbone (N–C α bond) leaving the sugar moiety intact [68] (Figure 3c). These methods are especially important for VSPs that have a high number of potential NGS such as HIV-1 Env, Flu HA, and LASV GP among others.

For localizing sites of *O*-glycosylation, samples that contain both *N*-glycans and *O*-glycans are pretreated with PNGase F to enrich for sites of *O*-glycosylation. Bagdonite *et al.* reported on the profiling of *O*-glycans from the herpes viruses, HSV-1, varicella zoster virus (VZV), HCMV, and EBV to identify the sites of attachment [32**]. Other glycosidases, such as sialidase, have been used for the HBV surface protein to liberate the sialic acids from the glycan moiety. This consolidates the sugar

moiety into the de-sialyated forms increasing the likelihood of observing the site of attachment by MS [69].

Glycan site occupancy

N-Glycan site occupancy is an additional MS assessment that can be utilized in the VSP glycoprofile (Figure 3b). In order to obtain site occupancy, *N*-glycans are released using PNGase F (as mentioned above converting N to D) [56]. The resultant peptides containing aspartic acid are used to determine the extent of glycan occupancy at a given NGS. Go *et al.* produced a heatmap generated from the clustering analysis of the glycosylation site occupancy analysis of 40 NGS observed among early and late HIV-1 Env immunogens [70]. The ratio of the intensities of peptides with aspartic acid (occupied sites) to peptides with asparagine residues (unoccupied sites) was calculated to estimate site occupancy. When this was done for flu HA (HAA/Hong Kong/1/1968) >90% occupancy of 9 of the 11 sites was reported [25*]. A similar method was utilized in the analysis of ninety-four HIV-1 Env variants and reported that 83% of possible sequons were present with 92% of those sequons exhibiting full or partial occupancy [62*]. For VSPs such as HIV-1 Env and Flu HA with many sites of *N*-glycosylation, the possibility of unoccupied NGS exists and their presence or absence could alter key interactions that influence protein function.

Site-specific glycan profiles

Because many VSPs are heavily glycosylated, profiling released glycans and confirming the sites of attachment cannot provide all the needed glycan information, especially, when specific glycans types have a functional role for viral fitness (i.e. immune evasion or viral entry) [71,72]. Specific high-mannose glycans on LASV GPC mediate viral entry into host cells through an interaction with DC-SIGN [73]. Another example are the glycan-specific Abs that target HIV-1 Env which require specific sites and glycans to neutralize the virus [49**].

Accomplishing site-specific glycoproteomic analysis for heavily glycosylated VSPs requires multiple protease and glycosidase digestions. Multiple proteases (*e.g.*, trypsin, chymotrypsin, pronase, pepsin) are employed to produce peptides that contain only one or two potential glycosylation site(s). The Desaire and Crispin groups have developed combinations of MS methodologies (including those described above) for the characterization of VSP glycosylation, namely for HIV-1 Env [2,6*,46*,49**,57,58,67,70,74]. A recent protocol for the global site-specific analysis of *N*-glycan processing made use of two Endo treatments to

(Figure 4 Legend Continued) Behrens *et al.* [94**] **(c)** Three of the same NGS presented as a weighted distribution graph of every oligosaccharide observed organized based on how *N*-glycans are processed (high-mannose glycans (yellow), hybrid glycans (green), complex glycans (blue)). A darker shading is used to indicate oligosaccharides that contain a sialic acid. **(d)** Relative abundance bar graph of every oligosaccharide observed at the N463 site of the WEAU.d16 and WEAU.d391 from a recombinant gp120 trimer organized in the same order as the weighted distribution graph similar to Hargett *et al.* [24**].

introduce unique mass signatures for (potential) sites of glycosylation that carry no glycan, high-mannose/hybrid type glycans, and complex glycans [75]. These strategies have arisen from the need for a HIV-1 vaccine that utilizes HIV-1 Env, which contains 75–90 *N*-glycans [76].

For VSPs with numerous sites of glycosylation, large spreadsheets catalogue the observed *N*-glycans and *O*-glycans [32,70]. In some cases of *N*-glycosylation (e.g. N276 of HIV-1 Env), up to 68 oligosaccharides can be observed at one NGS [24]. More recently, MS analysis of heavily glycosylated VSPs has progressed toward expressing glycan heterogeneity as quantitative profiles of different oligosaccharides at individual sites [2,24,46,77]. While the methodology utilized is the same, how the data are presented (i.e. table, pie charts, bar graphs) and the amount of information reported (type of glycan, glycan branches or all oligosaccharides observed) has varied (Figure 4). The high degree of reproducibility between technical and biological replicates along with reports demonstrating glycopeptide ionization is driven by the peptide (not the glycan) has resulted in quantitative profiles becoming a standard mode of reporting single-site heterogeneity [78–80]. After its initial use with HIV-1 gp120, these quantitative profiles have been reported for HCV E2, Flu HA, and LASV GPC [6,25,47,81].

These groups have transitioned the field of VSP glycan analysis from a discovery glycomics field into an analytical glycoprotein biosimilar assessment where individual preparations, constructs, and viral strains are compared to each other [24,32,46,47,82]. This has allowed MS to bridge the gap between analytically characterizing VSP glycosylation and making that information accessible and usable. Thus, allowing researchers to do comparative VSP glycan profiling analysis in the context of their own molecular experiments.

Applying viral surface protein glycoprofiles

It is now becoming routine practice to combine glycan profiling data with other techniques to determine the role of specific glycans and glycosylation sites on the structure and function of VSPs [2,5,6,24,49,71]. Crystallography and electron microscopy (EM), both require homogeneity in order to get higher resolution glycan structures for VSPs. To overcome the heterogeneity problem, groups have used Ab binding enrichment or glycosidase deficient cell lines to select for specific glycan types [5,49,83]. Modeling glycan profile data onto the solved VSP structures or reporting glycan profile data in the context of the overall structure provides an additional layer of information. Viral mutagenesis studies, infectivity, viral neutralization experiments all benefit from VSP glycoprofile to corroborate their findings. By combining glycan profiling, structural experiments, and molecular experiment of VSPs, virologists can design better vaccines and therapeutics to combat viral invasion.

Structural studies

The structure of CoV spike protein, HIV-1 Env, Flu HA, and other VSPs have been solved [5,49,84–92]; however, these structures lack an accurate representation of their glycan profiles. This is because the glycoproteins are produced in a manner that limits their glycosylation. A study of the CoV spike glycoprotein combined EM data and MS data and confirmed 26 of 27 potential NGS that were subsequently mapped onto the structure or resulting schematic. From this analysis, glycans masking the receptor binding loops of conserved regions were observed [5]. Similar studies have been done for HIV-1 Env, LASV GP, and EBOV GPs which all show glycan structures masking conserved receptor binding regions [3,6,49,93].

Alternatively, modeling/mapping MS glycan profiling data to their location on solved structures corroborates the overall type of site-specific heterogeneity profiles. For *N*-glycosylation, heterogeneity profiles can be subdivided into three categories: high-mannose, mixed, and complex or predominantly processed sites [2,6,24,94,95]. A 2016 study of HIV-1 Env used this method to determine that complex sites were located in dispersed regions of Env, and high-mannose sites were located in buried or NGS dense regions of the structure [2]. A second study in 2019 utilized a panel of HIV-1 mutants to confirm *N*-glycan microdomains based off changes in site-specific *N*-glycan heterogeneity profiles [24].

Molecular experiments

Glycan mutagenesis studies of West Nile virus (WNV), Hendra virus, Nipah virus, and HCV to name a few have all been shown to have glycosylation sites with vital roles in infectivity, protein folding, tropism, proteolytic processing, and immune evasion [7,11,96–99]. However, when these results vary based off viral strain or the glycosylation site is not in an obvious inhibitory location on the structure, it can be difficult to understand the mechanism by which the glycan alters the VSP function. MS glycan profiling data, such as site-occupancy and site-specific heterogeneity profiles provide a means to better understand why specific glycosylation sites are important [62]. The N301 glycan on HIV-1 Env, for example, has been shown to be essential for PGT128 neutralization in some viral variants, but not all [100]. When this information is coupled with the known shift in site-specific *N*-glycan heterogeneity, based off presence or absence of neighboring glycans [24], researchers can begin to better understand the mechanism by which certain glycan mutations (but not others) help the virus to evade the immune system.

Conclusion

MS has become the standard tool for the analysis of VSP glycosylation. The efforts of multiple groups have led to a set of essential MS analyses that provide specific details about VSP glycosylation, such as released glycan profiles,

glycan site localization, site-occupancy, and site-specific glycan heterogeneity profiles. When the glycoprofiling data are used in conjunction with molecular and structural experiments, virologist can begin to establish how specific glycans and glycan types may affect virulence through modulating virus receptor binding, masking antigenic sites, or by stimulating the host immune response. Understanding how this heterogeneous post-translational modification can fine-tune VSP function will provide unique insights into virus biology, which can only be addressed by considering the VSPs and the attached glycan as one complete biomolecule.

Conflict of interest statement

Nothing declared.

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