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Comparative Glycomics Analysis of Influenza Hemagglutinin (H5N1) Produced in Vaccine Relevant Cell Platforms

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Abstract

Hemagglutinin (HA) is the major antigen in influenza vaccines and glycosylation is known to influence its antigenicity. Embryonated hen eggs are traditionally used for influenza vaccine production, but vaccines produced in mammalian and insect cells were recently licensed. This raises the concern that vaccines produced with different cell systems might not be equivalent due to differences in their glycosylation patterns. Thus, we developed an analytical method to monitor vaccine glycosylation through a combination of nanoLC/MS^E and quantitative MALDI-TOF MS permethylation profiling. We then used this method to examine glycosylation of HA's from two different influenza H5N1 strains produced in five different platforms, including hen eggs, three different insect cell lines (High FiveTM, *expresSF*+[®] and glycoengineered *expresSF*+), and a human cell line (HEK293). Our results demonstrated that (1) sequon utilization is not necessarily equivalent in different cell types, (2) there are quantitative and qualitative differences in the overall *N*-glycosylation patterns and structures produced by different cell types, (3) ~20% of the *N*-glycans on the HAs produced by High FiveTM cells are core 1,3-fucosylated structures, which may be allergenic in humans, and (4) our method can be used to monitor differences in glycosylation during the cellular glycoengineering stages of vaccine development.

Keywords

Hemagglutinin; Influenza; glycosylation; mass spectrometry; MS^E; permethylation; cell substrate; NetNGlyc

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ASSOCIATED CONTENT

Supporting Information. The MS^E spectra of 4 additional prolinyl sequon glycopeptides from insect cell derived HAs and referred to in the text are shown in Figure S1–4; The MALDI-TOF MS profile of the glycans of HAs are shown in Figure S5–9. *N*-glycan abundances within subgroups in intermediate, pauci mannose, high mannose, hybrid and complex subgroups are shown for HAs derived from each cell platform. This material is available free of charge via the Internet at <http://pubs.acs.org>.

INTRODUCTION

Influenza virus causes three to five million cases of human illness and 250,000 to 500,000 deaths worldwide per year ¹. The influenza subtype A/H3N2, A/H1N1 and type B viruses cause the majority of these cases. The annual influenza vaccine is typically produced in embryonated eggs and contains one strain of each of these viruses, which are recommended by the World Health Organization (WHO) and regional health authorities ². H5N1 caused a total of 553 cases and 323 deaths (58.4%) in 15 countries between December 2003 and May 2011. WHO has identified several strains of H5N1 suitable for vaccine production should a pandemic be eminent ³.

Several alternative platforms for influenza vaccine production are currently being pursued. These include mammalian cell lines such as MDCK, CHO and Vero ^{4, 5}, plant systems such as *Nicotiana benthamiana* ⁶, and the baculovirus-insect cell system with cell lines derived from *Trichoplusia ni*, such as High FiveTM ⁷ or *Spodoptera frugiperda*, such as *expresSF*⁺ ^{8, 9}. Trivalent vaccines produced in MDCK cells (Flucelvax, Novartis) and SF⁺ insect cells (Flublok, Protein Sciences) were recently licensed. The former is produced by propagating influenza viruses and the later is a recombinant subunit vaccine produced using the baculovirus-insect cell system.

It is important to recognize that the protein glycosylation patterns provided by different cell lines are not necessarily equivalent. Different cell lines can have differences in sequon (three amino acid local sequence requirement for *N*-glycosylation) usage and produce different glycan structures, which could complicate antigen presentation and affect the properties of the influenza vaccine. Genetic shift of sequon position has been shown to alter influenza HA glycosylation in ways that can mask antigenic sites ^{10–14} and, therefore, differences in influenza vaccine glycosylation patterns may have an impact on antibody-based potency testing results that could translate to clinical efficacy.

The single radial immuno-diffusion assay (SRID) is the accepted way to measure the potency of influenza vaccines ^{15, 16}. Traditionally, antisera used in the assay are raised against HA derived from influenza viruses grown in embryonated hen eggs. However, as noted above, the glycosylation pattern obtained with eggs might differ markedly from the patterns obtained with various cell-based platforms, which also might differ markedly from each other. It has recently been shown that significant reagent-based differences occurred in SRID assay results (2-fold) with licensed egg-derived vaccine and rHA from the 2009 pandemic H1N1 strain A/California/07/2009 ¹⁷. Given the known impact of glycosylation differences on HA antigenicity, the use of the SRID assay with reference material prepared in a cell system different from that of the vaccine may not be appropriate. Clearly, there is a need to develop better analytical methods to monitor glycosylation differences to better understand the impact of this modification on influenza vaccine properties and better inform the choice of potency testing methods and reagents.

In addition plant and insects are known to produce glycoproteins that are allergenic in humans and these often contain Fuc 1,3-in the core region of *N*-glycans ^{18, 19}. The Fuc 1,3-substitution is known as a cross-reactive carbohydrate determinant (CCD) and reacts strongly with IgE of patients with plant or insect allergy. While there is still some controversy concerning the Fuc 1,3-core region *N*-glycan substitution being a clinically significant allergen, from a safety perspective, it is important to monitor such modifications. Currently there is no accepted method to monitor this component in influenza vaccine preparations.

To investigate these issues and study the glycosylation pattern in HAs derived from various cell substrates, we combined LC/MS, using a new data independent acquisition MS^E

methodology, and MALDI-TOF MS permethylation profiling of released glycans to analyze glycosylation of the HA's from two closely related influenza A/H5N1 viruses (see Table 1) with identical tryptic glycopeptides produced in five different platforms, including hen eggs, and HEK293, High Five™, *expressSF*⁺, or *SfSWT*-7 cells. *SfSWT*-7 cells are a subclone of *expressSF*⁺ cells that were glycoengineered to synthesize human-like biantennary, terminally galactosylated and sulfated *N*-glycans (see Experimental Section). Egg-derived HA represents the “gold standard” in this study because nearly all licensed influenza vaccines are currently produced in hen eggs. HEK293 cells are not currently used for vaccine manufacturing, but are used to produce HAs for research purposes²⁰. HA subunit vaccines produced in the baculovirus-insect cell system^{8, 21, 22} have been licensed as previously stated. The use of High Five™ cells also have been suggested as a host for baculovirus-mediated virus propagation⁷. Finally, *SfSWT*-7 may represent a potential next generation insect cell line designed to manufacture glycoproteins with *N*-glycosylation patterns that will more closely mimic those produced by human hosts.

In our two pronged approach first we analyzed seven glycosylation sites in tryptic peptides from HAs derived from each of the biosynthetic platforms. To accomplish this task we used nanoLC/MS^E, a new mass spectrometry technology that allows for efficient fragmentation of both the peptide and glycosidic moieties of glycopeptides allowing for sequencing of both parts²³. Secondly, we used permethylation analysis of enzymatically released *N*-glycans to determine the abundances of glycan compositions present on HA from each biosynthetic system. Permethylation imparts hydrophobic character leading to normalized ionization efficiency in a related glycan series allowing for quantitative analysis²⁴. Here we report differences revealed in *N*-glycosylation sequon usage and glycan compositions, including potential allergens, among the different HA proteins.

EXPERIMENTAL SECTION

Chemicals and reagents

Sep-Pak C18 Cartridges and RapiGest surfactant were purchased from Waters Corporation (Milford, MA). TSKgel Amide-80 particles were purchased from Tosoh Bioscience LLC (Montgomeryville, PA). Porous graphitic carbon (PGC) cartridges were purchased from Thermo Fisher Scientific Inc. (Waltham, MA). Sequencing grade modified trypsin was purchased from Promega Corp. (Madison, WI). *N*-glycosidase A was purchased from Roche Diagnostics Corporation (Indianapolis, IN). Iodomethane, dimethylsulfoxide (DMSO), sodium hydroxide beads and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). All solvents were HPLC grade and all other reagents were ACS grade or higher.

Influenza HAs

Two recombinant influenza A/Vietnam/1203/2004 (H5N1) HA preparations were produced by infecting either *expressSF*⁺ (Protein Sciences Corporation, Meriden, CT) or *SfSWT*-7 (this study) cells with recombinant baculovirus vectors, as described below. Two other recombinant influenza A/bar-headed goose/Qinghai/14/2008 (H5N1) HA preparations were produced in HEK293 or High Five™ cells and purchased from Sino Biological Inc. (Beijing, China). Finally, a native HA preparation was isolated from influenza A/Vietnam/1203/2004 (H5N1) virus cultivated in hen eggs, as described below.

Isolation of glycoengineered insect cells

SfSWT-7, which is a glycoengineered subclone of *expressSF*⁺ cells, was isolated by co-transfecting *expressSF*⁺ cells with dual *piggyBac* vectors²⁵ encoding mammalian *N*-acetylglucosaminyltransferase II (MGAT2), s4-galactosyltransferase I (B4GALT1),

carbohydrate sulfotransferase 2 (CHST2), and galactose-3-O-sulfotransferase 2 (GAL3ST2) under the transcriptional control of baculovirus immediate early 1 (*ie1*) promoter and homologous region 5 (*hr5*) enhancer elements, together with a hygromycin resistance marker. The transfected cells were then selected in growth medium containing hygromycin, cloned by limiting dilution, and screened for transgene expression by RT-PCR, as described previously^{26, 27}. *expresSF+* and *SfSWT-7* cells were routinely maintained at 28°C as suspension cultures in PSFM medium (Protein Sciences Corporation). The methods used to propagate and titer the recombinant baculovirus used in this study have been described previously²⁸.

Egg derived HA was produced as follows. Viruses were grown in 10-day-old embryonated hens' eggs by inoculation with 0.2 mL of diluted virus stock containing ~ 10⁴ pfu at 33°C. Allantoic fluid was harvested at 72 h post infection and clarified by centrifugation at 4000 rpm for 10 min at 4°C. Virus was pelleted by centrifugation in the Beckman 45 Ti rotor at 24,000 rpm for 90 min at 4°C. Viruses were purified by ultracentrifugation on 30% and 60% sucrose at 24,000 rpm for 90 min at 4°C in a Beckman SW32 Ti rotor. The virus band at the 30%–60% sucrose interface was collected and the virus was pelleted, and then resuspended in PBS, pH7.2, with aliquots stored at –80 °C.

Purified egg-derived virus was diluted to a concentration of 10 mg/mL in Tris–EDTA (TE) pH 8.0 and 1 mL of virus suspension was incubated with 50 U/mL bromelain (Sigma–Aldrich, Inc., St. Louis, MO) in the presence of 50 mM beta-mercaptoethanol for 4 h at 37°C with gentle shaking. The reactions were ultracentrifuged at 30,000 rpm for 2 h at 4°C in a Beckman Ti55 rotor (Beckman Optima TLX, Beckman Coulter Inc., Brea, CA) to separate the bromelain-cleaved HA from the viral cores. The bromelain cleaved HA in the supernatant was then purified on 5–20% continuous sucrose gradients, generated using a Gradient Master Model 107ip (BioComp, Fredericton New Brunswick, Canada) and ultracentrifuged in a Beckman SW40 Ti rotor for 35,000 rpm for 16 h at 10°C. The gradients were fractionated from top to bottom using an Auto Densi-Flow Density Gradient Fractionator (Labconco, Kansas City, MO), and each 0.5 mL fraction was analyzed by SDS-PAGE to identify fractions containing the HA trimer.

Glycopeptide production

Each HA protein preparation was dissolved in 50 mM ammonium bicarbonate containing 0.1% RapiGest and 5 mM dithiothreitol (DTT). The samples were incubated for 30 min at 60°C, then chilled to room temperature and treated in the dark with 15 mM iodoacetamide for 30 min at room temperature. Trypsin was added at an enzyme:protein ratio of 1:50 (w/w) and the samples were incubated at 37°C for 18h. After digestion, 99.9% pure trifluoroacetic acid (TFA) was added to the samples at a final concentration of 0.5%, the samples were incubated at 37°C for 45 min to degrade the RapiGest, centrifuged at 13,000 rpm for 10 min to remove insoluble by-products, and then the supernatant was vacuum dried for downstream analysis.

Enrichment of glycopeptides with hydrophilic interaction chromatography (HILIC)

Intact glycopeptides were enriched by solid phase extraction with TSKgel Amide 80 HILIC resin, as described previously²⁹. Briefly about 200 mg (400 SL of wet resin) of Amide-80 resin was placed into Supelco fritted 1 mL column, washed with 1 mL of 0.1% TFA/water, and conditioned with 1 mL of 0.1% TFA/80% acetonitrile (ACN). The tryptic peptides, produced from 100 to 200 µg of protein, were suspended in 0.1% TFA/80% ACN and applied onto the column. The hydrophobic species were washed through with 3 mL of 0.1% TFA/80% ACN, and then the glycopeptides were eluted with 1 mL of 0.1%TFA/60% ACN

followed by 1 mL of 0.1% TFA/40% ACN. The eluents were combined, vacuum dried, and analyzed by reverse phase LC-MS.

Reverse phase nanoLC/MS^E analysis of glycopeptides

The glycopeptides were reconstituted in 0.1% formic acid in water and approximately 5 – 10% of the sample was injected onto a C18 column (BEH nanocolumn 100Sm i.d.×100 mm, 1.7 Sm particle, Waters Corporation) for nanoLC/MS^E analysis. Approximating that 10% of peptides were present as glycopeptides based on tryptic peptide and capture efficiency. We estimate that 500 to 2000 ng of sample were analyzed per LC/MS experiment. A Waters nanoAcquity UPLC system was used for automatic sample loading and flow control. Solvent A was 100% water/0.1% FA, solvent B was 100% acetonitrile/0.1% FA, and the elution gradient was 1–50% solvent B for 110 min, 85% solvent B for 15 min, and 1% solvent B for 25 min. The effluent was introduced into a Waters SYNAPTTM G2 HDMS system (Waters Corp. Milford, MA) with an uncoated 15-Sm i.d. PicoTip Emitter (New Objective Inc., Woburn, MA) and a spray voltage of 3000 V. The mass spectrometer was operated in positive polarity mode and was set to perform MS^E experiments, a data independent acquisition, which uses a low collision energy (4V) for precursor ion scanning followed by an elevated collision energy (ramping from 15 to 45 V) for fragment ion scanning. The scan time was 0.9 second. An auxiliary pump was used to spray a solution of 200 fmol/SL Glu-fibrinopeptide B in 50/50 methanol/water with 1% acetic acid for mass calibration (lockmass channel), at a flow rate of 300 nL/min with sampling every 30 seconds. The system was tuned for a minimum resolution of 20,000 FWHM and calibrated using a 5 mM sodium formate infusion.

N-Glycan release and permethylation

Glycopeptides were suspended in 50 mM ammonium acetate (pH 5.0), and glycans were released by treatment with 2 SL of peptide *N*-glycosidase A (PNGase A, 5 mU/100 SL) for 16 h at 37°C. The reaction mixture was applied to a C18 cartridge preconditioned with 2 mL of ethanol and 2 mL of water and the *N*-glycans were eluted with 5 mL of water and applied to a porous graphitic carbon cartridge (PGC, Thermo Fisher Scientific Inc.) wetted with 1 mL of 0.1% TFA/ACN and sequentially equilibrated with 1 mL of 0.1% TFA/60% ACN, 1 mL of 0.1% TFA/30% ACN and 1 mL of 0.1% TFA/water. The cartridge was washed with 3 mL of 0.1% TFA/water, and then the *N*-glycans were eluted with 1 mL of 0.1% TFA/30% ACN and 1 mL of 0.1% TFA/60% ACN, and the two eluent fractions were pooled and vacuum dried. Solid-phase permethylation was conducted according to the published protocol of Mechref and colleagues³⁰ with modifications as previously described²⁹.

MALDI-TOF analysis of N-glycans

Permethyated *N*-glycans were suspended in 50% methanol/water, spotted onto a MALDI plate, mixed 1:1 with 2,5-DHB matrix in the same solution plus 1 mM sodium acetate, and analyzed using a Perseptive Biosystems Voyager DE RF MALDI-TOF mass spectrometer. Samples were analyzed in positive ion reflectron mode in the 800–5500 *m/z* range. The MS data were processed with the DataExplorer (Perseptive Biosystems).

Data analysis for peptide glycosylation identification

The nanoLC/MS^E data were processed using the ProteinLynx Global Server (PLGS) for peptide identification and BiopharmaLynx 1.3 to identify specific HA glycosylation sites. A database was created in BiopharmaLynx using *N*-glycan structures from GlycosuiteDB (<http://glycosuitedb.expasy.org/glycosuite/glycodb>), a curated *N*-glycan database. The search used trypsin digestion with one missed cleavage on the known sequences of HAs. Cysteine carbamidomethylation was set as a fixed modification, while oxidation on

methionine was set as a variable modification. All potential *N*-glycan compositions expressed in each cell system were documented in the glycan database and added as variable modifications in the search parameters. Identified glycopeptides were manually confirmed by MS^E spectra. The oxonium ions (such as *m/z* 204.09, 366.14, 528.19) present in MS^E spectra were used to help locate and determine the presence of glycopeptides. Assignment criteria included (1) Manual observation of oxonium ions, peptide ions with neutral loss of glycan fragment, and GlcNAc+peptide fragment ions, (2) BioPharmalynx identification, (3) GlycoMod verification of BiopharmaLynx identified glycopeptides, (4) PLGS identification of peptide moiety, (5) mass accuracy of less than 20 ppm and 30 ppm in parent and collision ion scans respectively.

As mentioned previously ExPASy's GlycoMod was also used to predict the possible *N*-glycan compositions from the masses of the glycopeptides in case our glycan database was not complete. The parameters set for the GlycoMod search allowed hexose, *N*-acetylhexosamine, deoxyhexose, *N*-acetylneuraminic acid and/or sulfate to be considered as potential components in the *N*-glycans according to reported observations in these cell systems. ExPASy's GlycoMod also was used to predict the possible glycan composition from the masses of the free glycans MALDI analysis using similar parameters.

CN3D 4.1 (<http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml>) was used to visualize the 3D structure of HA (A/Vietnam/1203/2004). The sequences of HA (A/Bar-headed goose/Qinghai/14/2008) and HA (A/Vietnam/1203/2004) were aligned. Localization of glycosylation sites are shown in Figure 6. The viruses studied here shared 94% identity at the protein level.

RESULTS

Glycomics strategy

An overview of the workflow used in this study is shown in Figure 1. Glycopeptides are relatively large and hydrophilic compared to their unmodified counterparts. Each glycopeptide will nearly always have more than one glycoform, which disperses signal. The glycan moiety is more labile than the peptide moiety and special approaches must be used if sequence data are desired from both. Additionally, due to the differences in ionization potential among different glycopeptides, quantitation is difficult. To address these challenges, we applied an analytical approach that includes (1) glycopeptide enrichment by hydrophilic interaction chromatography (HILIC) for improved detection, (2) an LC/MS method using high resolution C18 chromatography and MS^E mass spectrometry to facilitate separation, sample coverage and fragmentation, and (3) permethylation profiling of released and permethylated glycans for semi-quantitative analysis of glycoforms by MALDI-TOF MS.

HILIC enrichment removes most of the more ionizable hydrophobic peptides, which are nonglycosylated^{31, 32}, and simplifies the sample mixtures rendering the less ionizable hydrophilic glycopeptides more readily detectable. This strategy provides higher signal intensities and greater glycopeptide coverage, as we and others have reported previously^{29, 33–35}.

NanoLC/MS^E is a recently developed peptide mapping approach, which collects MS data in an alternating scan mode between low and elevated collision energies^{36, 37} and has been used previously to study HA glycosylation²³. No precursor ions are selected in the method and, therefore, scanning is data-independent. All precursor ions are subjected to collision conditions and MS/MS data are reconstructed according to the physiochemical properties of the parent ions and their fragments in association with their chromatographic positions

during the LC/MS experiment. The precursor ion data are collected during the low energy MS scan and then the collision energy is ramped increasing over a range of voltages in the dissociation stage to fragment the precursor ions. The data produced during the experiment are used to reconstruct MS/MS spectra without the bias associated with data-dependent ion scanning. One of the advantages of MS^E is that it ramps the collision energy from low to high in the elevated energy scan. This process can be optimized not only to fragment the glycan portion but, also to generate abundant b- and y-ions from the peptide backbone. In this way, information can be obtained from both moieties. As stated previously, the algorithms that drive the method are reliant upon LC resolution to accurately reconstruct the MS/MS spectra and, to facilitate this process herein, we used 1.7 μ m bead column ultrahigh pressure C18 chromatography.

ProteinLynx Global Server (PLGS) and Biopharmalynx were used to process the MS^E data and aid in assignments, as described in the Experimental section. To facilitate glycan identification we built a glycan library based on GlycoSuiteDB predictions for each cell type^{38–40}. Additionally, we examined the MALDI-TOF MS spectra of permethylated glycans from the five HAs and identified compositions using GlycoMod to bolster the library content. Glycopeptides also were verified using GlycoMod.

NetNGlyc 1.0 (<http://www.cbs.dtu.dk/services/NetNGlyc>) was used to predict glycosylation site occupancy. This publically accessible program uses nine neural net programs in its prediction algorithm. Each neural net is assigned as a juror and the prediction are tallied to produce qualitative likelihood of site occupancy and glycosylation probability scores⁴¹. A NetNGlyc potential score of 0.5 or higher indicates a high potential for occupancy. In cross-validated performance testing, this software identified 86% of the glycosylated and 61% of the non-glycosylated sequons with an overall accuracy of 76%⁴¹. We chose to pre-scan each of the seven predicted *N*-glycosylation sites in this way to evaluate predictive modeling versus the cell type-specific glycosylation patterns actually observed for each HA examined in this study.

N-glycans were released from HAs with peptide *N*-glycosidase A and permethylated. The permethylation process replaces sugar hydroxyl groups with *O*-methyl groups, rendering them more hydrophobic and ionizable than their native counterparts. In addition, the permethylated glycans in a chemically related series are similar in ionization potential, enabling semi-quantitative analysis. Using this process, ion abundances have been shown to be well correlated with glycan abundances determined using other quantitative methods^{24, 42, 43}. Herein, we used the method to compare relative abundances of glycans present on each of the HAs produced across the different cell systems. The purity of HAs analyzed was greater than 95% as estimated by SDS-PAGE.

Various *N*-glycan structures were divided into five groups, including high mannose, complex, hybrid, paucimannose and intermediate. Insect cells produce much higher levels of paucimannose and intermediate structures, whereas higher eukaryotes produce mostly high mannose, complex and hybrid type structures, which have been more thoroughly described^{44, 45}. Briefly, high mannose glycans have the composition, Man_{5–9}GlcNAc₂. Complex *N*-glycans consist of the trimannosyl core with both the alpha 1,3- and alpha 1,6-linked mannose residues substituted by a sugar other than mannose and typically extended by the addition of GlcNAc, GalNAc, Gal, Fuc, and/or Sialic acid. In addition, some of the terminal sugars can be sulfated. Hybrid *N*-glycans also consist of the trimannosyl core, but the alpha 1,6-linked core mannose residue retains some or all of its mannose substituents. Only the alpha 1,3-linked mannose arm is elongated, typically with GlcNAc, GalNAc, Gal, Fuc, and/or Sialic acid, in the same way that are complex *N*-glycans. Intermediate *N*-glycans, produced by insect cells, are similar to hybrid glycans but the alpha 1,3-linked arm

elongation is limited to the addition of GlcNAc. Herein, we define “intermediate” *N*-glycans as Man₃₋₅GlcNAc₃ structures with or without core fucose residues. The distinction between intermediate and hybrid glycans were important for comparisons made between cell platforms. The paucimannosidic *N*-glycans produced by insect cells consist of the trimannosyl core with no substituents on either terminal mannose residue. The core GlcNAc residue can be substituted with alpha 1,6- or alpha 1,3-linked Fuc residues.

Glycopeptide Analysis

The nanoLC/MS^E platform provides spectra rich in fragment ion abundance from both glycoside and peptide moieties of the glycopeptides analyzed. Figure 2 shows representative spectra from glycopeptides containing glycosylation site 4 for HAs produced on each cellular platform. Oxonium ions, peptide+GlcNAc, glycan denuded peptide and fragments resulting from neutral loss of carbohydrate provide glycan sequencing information. At higher energy ranges, that occur during collisional energy ramping, peptide fragmentation occurs yielding primarily b- and y-ion abundances providing peptide sequencing information.

Glycopeptide 1, DQICIGYHANNSTEQVDTIMEK, has two sequons, NNS and NST, with NetNGlyc potential scores of 0.379 and 0.783, respectively, indicating relatively lower and higher probabilities of occupancy respectfully (see Table 2). Indeed, the HAs produced in each platform examined in this study, including egg, HEK293, High FiveTM, *expres.SF⁺* and SfSWT-7, were exclusively *N*-glycosylated at the second sequon. An example of a spectrum obtained with (dHex₂Hex₃HexNAc₂)DQICIGYHANNSTEQVDTIMEK from High FiveTM cell-derived HA is shown in Figure 3. Glycosylation of the Asn in NST was confirmed by the b-10 peptide fragment ion at *m/z* 1172.52, which included Asn 26 and N-terminal sequence. The nanoLC/MS^E approach provided essential data for assignment of the glycan location due to collision energy ramping during the experiment, which allowed fragmentation of both peptide and glycan portions of the ion. It also should be noted that the human CCD Fuc 1,3-core substitution was detected in this glycopeptide as clearly evidenced by neutral losses of carbohydrate, which revealed glycan sequence (see Figure 3). The Fuc 1,3-core substitution was only detected in the High FiveTM cell-derived HA.

Glycopeptides 2–4 and 7 were found in the HAs produced in each platform, indicating that all platforms utilized sequons 2–4 and 7 as predicted by NetNGlyc. In contrast, although NetNGlyc potential scores were over 0.5, glycopeptides containing sequon 5 and 6 were not found in the HAs from all platforms and are further discussed below. As seen for glycopeptides 2–4 and 7 in Table 3, a range of glycoforms were detected across glycosylation sites in this study. Compositional and fragmentation data indicated the Egg- and HEK293-derived HAs contained *N*-glycans with up to 5 branches. HEK293 glycopeptides were highly sialylated. Glycan assignments are consistent with those reported previously for the egg-^{46, 47} and HEK293-derived proteins ^{48, 49}. HEK293 derived HA glycans were the largest (see Table 3). Antennae compositions were the most diverse of all cell systems studied. An example MS^E spectrum of an HEK293-derived HA biantennary glycopeptide containing sequon 4 is shown in Figure 4. Neutral losses of glycosidic fragments show that the antennae have two different compositions, NeuAc₁Hex₁HexNAc₁- and NeuAc₁HexNAc₂-, as shown in the figure. Oxonium ions at *m/z* 274.09 and 495.18 confirmed the NeuAc and NeuAc-HexNAc substitutions. Fragments arising from neutral losses of part of the glycan confirmed the sequences of both antennae. The peptide +FucHexNAc fragment at *m/z* 3456.62 confirmed the fucosyl substitution of the core. Peptide fragment data produced during CID energy ramping in the nanoLC/MS^E experiment confirmed the peptide identity with a series of b- and y-ions as shown in the figure.

Many of the High Five™ HA glycopeptides contained two fucosyl residues on the aglycone most core GlcNAc in the *N*-glycan core. Two fucoses present in this context are indicative of the human CCD and potentially allergenic core Fuc 1,3-substitution. There was no evidence of Fuc 1,3-substitutions in the HAs from *expresSF*⁺ or SfSWT-7 cells, supporting the idea that cell lines derived from *Spodoptera frugiperda* do not produce appreciable amounts of this undesirable substitution. The composition of the SfSWT-7 HA glycopeptides provided direct evidence that expression of the MGAT2 and B4GALT1 transgenes in these cells produces both enzyme activities. This evidence included the detection of *N*-glycans containing more than three HexNAc and more than three Hex residues (see Table 3) as well as fragmentation patterns observed in the MS^E spectra. Conversely, the glycopeptide data provided very little evidence of CHST2 or GAL3ST2 activity, despite RT-PCR data indicating that SfSWT-7 cells expressed both transgenes (data not shown). Sulfate substitutions are known to be labile and could have been lost through prompt decay in the mass spectrometer, but sulfation was detected in glycopeptides from a control protein, bovine thyroid stimulating hormone, under the conditions used in our study. Some sulfated glycopeptides were also detected in egg-derived HA glycopeptides (see Table 3). In contrast to the results obtained with glycopeptides, we did observe low abundance sulfation by analyzing released and permethylated *N*-glycans from the SfSWT-7-derived HA, and discussed later.

Glycosylation site 5 has a Pro in the X position of the NXS/T sequon. NetNGlyc predicted a glycosylation probability of 0.6843, but also gave a warning. Pro is known to sterically restrict the access of oligosaccharyltransferase^{50, 51} to *N*-glycosylation sites and reduce the probability the site will be filled. The nanoLC/MS^E analysis revealed no glycopeptides containing this sequon in HEK293- or egg-derived HA. However, the corresponding glycopeptides were detected in all insect cell-derived HAs. The *expresSF*⁺ and High Five™ cells both produced paucimannosidic glycoforms. In contrast *SfSWT*-7 cells produced three glycoforms, including both high mannose, intermediate and complex structures. Figure 5 shows an MS^E spectrum of the glycopeptide (dHex₁Hex₃HexNAc₃)LYQN*PTTYISVGTSTLNQR from the SfSWT-7-derived HA seen at *m/z* [M+H]⁺, 3559.61. Glycan sequence information was revealed by neutral losses resulting in fragment ions at *m/z* 2544.17, 2724.24, 2886.30 and 3091.38. Peptide sequence data are seen at *m/z* 701.35, 773.35, 1062.50, 1180.06 and 2156.03. Identities are indicated in the figure. The MS^E spectra from the remaining 4 prolinyl sequon glycopeptides are shown in Figures S1–4.

NetNGlyc predicted glycosylation site 6 to have an occupation probability of 0.6024. The corresponding glycopeptide, (glycan)CQTPMGAIN*SSMPFHNIHPLTIGECPK, was only detected in the insect cell-derived HAs, not in the egg- or HEK293 cell-derived HAs (see Table 3). This result provided another example of a difference in site occupancy, in which a specific sequon was utilized by insect cells, but not appreciably by higher eukaryotic cells glycosylated a specific sequon. The modeled placement of sequons in the 3D structure of the HAs can be seen in Figure 6. The significance of cell specific sequon occupancy is further discussed later.

MALDI-TOF MS Permethylation Analysis of Released Glycans

The HAs' detected glycan abundances from each individual subtype, high mannose, complex, hybrid, paucimannose and intermediate, were separated into groups to show subclass compositional complexity in Figures S5A–S9A. The compositions within each of the five subclasses were also summed and displayed as histograms to show relative abundances of each subclass as seen in Figure S5B–S9B. The summed subclasses from each cell platform are presented together in Figure S10 for comparison. These plots were

presented to show overall complexity, distribution among classes, and comparison of distribution of classes among the HAs derived from different cell sources.

The permethylated glycan MALDI-TOF MS profile histogram of the egg derived HA is shown in Figure S5. Approximately 35% of the *N*-glycans were high mannose, 21% were hybrid and 45% were complex. The complex *N*-glycans were highly branched, consistent with the glycopeptide data, indicating they occupy much larger volumes than the paucimannose, hybrid and intermediate structures.

The permethylated *N*-glycan MALDI-TOF MS profile histogram of the HEK293 cell-derived HA is shown in Figure S6. The relative abundances of complex, paucimannose and high mannose glycans were ~87%, 3% and 9%, respectively. Only trace intermediate and hybrid glycans were detected. Complex *N*-glycans dominated the spectra and the compositions predicted between 2 and 5 antennae, where nearly all were substituted with sialic acid. Some sulfate substitution also was observed. The small amount of paucimannose *N*-glycans was probably representative of processing intermediates. The abundances indicate that the vast majority of the *N*-glycans on HEK 293-derived HA are negatively charged in solution and large in volume, which could affect HA characteristics.

The MALDI-TOF MS permethylation profile histogram of the High Five™ cell-derived HA *N*-glycans is shown in Figure S7. Most (~88%) were paucimannosidic structures with and without core linked Fuc, but we also observed 8% high mannose, 2% intermediate, and trace complex *N*-glycans. Roughly 20% of the *N*-glycans contained 2 core Fuc residues, indicating high levels of the allergenic Fuc 1,3-substitution. The overall size of these glycans was smaller than those on the egg- and HEK293-derived HAs and they had primarily terminal Man residues.

The MALDI-TOF MS permethylation profile histogram for the *expresSF+* cell-derived HA glycans is shown in Figure S8. Like the High Five™ cell-derived HA *N*-glycans, the *expresSF+* HA *N*-glycans were also primarily paucimannosidic, but no evidence of the CCD Fuc 1,3-substitution was observed. The glycan abundances were ~56% paucimannose, 34% high mannose, and 10% intermediate. Like the High Five™-derived HA *N*-glycans, those produced by *expresSF+* cells also were smaller than those produced by eggs and HEK293 cells.

The MALDI-TOF MS permethylation profile histogram of SfSWT-7 cell derived HA glycans is shown in Figure S9. SfSWT-7 cells were engineered to be able to produce biantennary complex *N*-glycans with sulfation as previously stated. The permethylated *N*-glycan abundances observed were ~10% paucimannose, 48% high mannose, 19% intermediate 4% hybrid, and 18% complex. Evidence was seen for functional expression of the MGAT2, B4GALT1, and sulfotransferase transgenes. As an example, Hex₅HexNAc₄ is the predicted composition of an *N*-glycan produced by trimming to Man₃GlcNAc₂, the addition of one GlcNAc by the endogenous insect cell MGAT1, the addition of a second GlcNAc by the transgenic MGAT2, and the addition of two 2 Gal residues by the transgenic B4GALT1. Similarly, the Sulf₁Hex₅HexNAc₄ shown in the histogram (Figure S9) is the predicted composition of an *N*-glycan produced by the action of MGAT1, MGAT2 and B4GALT1, and GAL3ST2. A three dimensional plot of all five *N*-glycan sub-types from each cell platform is presented in Figure S10 for comparison.

A comparison of the permethylated *N*-glycan profiles obtained with the egg-, *expresSF+*- and SfSWT-7-derived HAs is shown in Figure 7. Compared to its parent cell line, *expresSF+*, the *N*-glycans produced by SfSWT-7 cells include lower proportions of paucimannose and higher proportions of high mannose, intermediate, hybrid, and complex *N*-glycans. These glycoengineered insect cells produced 18% complex *N*-glycans, as

compared to ~45% produced by eggs, the reference standard for influenza vaccines, which also produced more highly-branched complex *N*-glycans. Thus, *SfSWT-7* cells produced HA with an *N*-glycosylation profile that was more similar, but not identical to the profile obtained with egg-grown virus.

The glycan compositions detected for the HAs produced by different cell types using either MALDI-TOF permethylation analysis or nanoLC/MS^E glycopeptide analysis were in good agreement. Complete agreement cannot be expected due to differences in technique and physiochemical properties of the analytes. All *N*-glycans detected with the HAs produced by each platform were consistent with the known *N*-glycan processing pathways available in each cell type.

DISCUSSION

A method consisting of a nanoLC/MS^E glycan MALDI-TOF MS permethylation profiling of released *N*-glycans was developed and used to analyze the HAs produced in different platforms. The MS^E method uses a data-independent acquisition approach that allows fragmentation of parent ions without prior isolation. Ion chromatogram dependent reconstitution of MS/MS spectra then allows for very good tandem mass spectral analysis coverage of samples. Ramping of collision voltages during the fragmentation stage of the experiment facilitates generation of both glycosidic and peptide fragmentation data for assignment of both sequences. Using a combination of informatics programs PLGS (WatersTM), BiopharmaLynx (WatersTM), GlycoMod,⁵² and our custom glycan library, we assigned over 240 glycopeptides and over 80 glycan compositions in this study.

It should also be noted that the HAs studied here shared 94% identity at the protein level. The *N*-glycosylation site occupancy program NetNGlyc predicted that the HA sequences used in this study had the same 7 *N*-glycosylation sites with identical glycosylation potential scores. However, NetNGlyc provided only a first approximation of site occupancy, as shown by our glycopeptide data, which further revealed that site occupancy was platform-specific. One example of this specificity was observed with glycopeptide 5, which contained a Pro in the X position of the NXS/T sequon. While Pro in this context is known to restrict *N*-glycosylation site utilization^{50, 53, 54}, NetNGlyc predicted occupancy with a Pro warning, which indicated the surrounding peptide sequence was conducive to glycosylation. Interestingly, this prediction held up in all three insect cell lines, High FiveTM, *expressSF+* and *SfSWT-7*, but not in the higher eukaryotic systems, in which this site was not appreciably utilized. To our knowledge this difference in prolinyl sequon usage between insect and higher eukaryotes has not been previously reported. This aspect of insect cells should be considered when glycosylation changes may affect protein function. This prolinyl peptide contains Asn 209 (see Figure 6), which is in a region dense in antigenic sites⁵⁵. In A/Vietnam/1203/2004 HA, used in this study, two antibodies have been directly mapped to this region in the globular domain of the protein⁵⁶. As it is at least partially occupied in insect cells and not in egg or mammalian cell derived HAs, this case demonstrates a difference in glycosylation between cell substrates that could promote antigen uptake or mask antigenic sites and affect antigenicity. A mapping of the serum antibody repertoire⁵⁷ in these regions may shed further evidence of any epitope masking amongst HA antigens produced in the different substrates.

Another example of platform-specific *N*-glycosylation of HA was observed with glycopeptide 6, which contained Ser in the X and third positions of the NXS/T sequon. Sequons with Ser in the third position are less likely to be filled than those with Thr in this position^{51, 54, 58}. NetNGlyc gave a glycosylation potential score of 0.6024 for this site, which predicted a high probability of occupancy. Once again, however, this site was utilized

only in the insect cells, not in the higher eukaryotic platforms (egg and HEK293). Our data suggest that NetNGlyc may not be capable of predicting differences in sequon usage attributable to species or cell type origin. As seen in Figure 6, the Asn 302 in peptide 6 is in the stem region of HA, which is another area known to be antigenic^{11, 59, 60}. As for glycopeptides containing sequon 5, differences in glycosylation of sequon/peptide 6 are likely to impact vaccine performance by altering antigenicity. All licensed influenza vaccines are standardized for HA content using the SRID potency method, as it is the primary antigen to which humans, ferrets, swine, and avian species mount immune responses^{61, 62}. The globular and stalk domains of HA contains clusters of identified antigenic sites^{56, 63} and glycosylation near the receptor binding site has been shown to change receptor specificity^{64–66}. Glycosylation near antigenic sites can mask or alter host response^{14, 67–69}, or in the research setting, insidiously affect interpretation of results if the detail of glycosylation is not known. This work demonstrates differences in site occupancy, in addition to dramatic differences in resident glycan compositions, among the different cell platforms used to produce HA as subunit vaccine or as a component of inactivated virus. Such differences, could pose difficulties in vaccine potency testing and in accurate vaccine formulation that could subsequently impact the level of immune responses in vaccinated subjects. The potency assay used in influenza vaccine batch release measures the titer and corresponding amount of HA in vaccine preparations relative to a standard^{15, 16}. The serological reagent standards used in these tests are usually produced using egg-derived viral antigens. The presence, composition and structure of HA glycans are cell type-specific and can affect antigenic regions of the protein. Therefore, the same potency test reagents may not be appropriate for assaying influenza vaccines manufactured in different platforms. With the development of the methods we describe, knowledge of these platform-specific differences can be used in evaluating the suitability of serological reagents for batch validations and/or to develop more appropriate potency testing reagents and methods that are best suited for the manufacturing platform.

Egg-derived HA *N*-glycans had an average of ~8–9 monosaccharide units and were mostly neutral and highly branched. The recombinant HEK293 cell-derived HA *N*-glycans averaged ~12 residues, were highly sialylated, and also were highly branched. Thus, the HAs produced in higher eukaryotic systems have *N*-glycans that probably occupy relatively large volumes. In contrast, the insect cell-derived HA *N*-glycans had an average of ~5–6 monosaccharide units, indicating they occupy a smaller volume. The differences in the volumes occupied by the *N*-glycans on HAs produced in different systems could alter HA surface properties around the *N*-glycosylation sites. In addition, the *N*-glycans on influenza virus HAs do not normally contain sialic acid substitutions, as the viral neuraminidase removes those residues^{70, 71}. The presence of sialic acids on influenza HA has been shown to compromise infectivity and replicative ability^{72, 73}. Therefore, the use of influenza virus or HA produced in mammalian cells lines in the research setting should be carefully considered because the HA may be sialylated.

Approximately 20% of High FiveTM cell expressed HA *N*-glycans contained the human CCD Fuc 1,3-Substitution. High FiveTM cells systems have been proposed for use in influenza vaccine production because they can produce virus-like particles in high yield⁷. While CCDs are linked to plant and insect allergy their importance in clinical disease is still controversial^{18, 74, 75}. However, until they can be ruled out as clinically significant it would be advantageous to assess for their presence in biologics products such as vaccines. As the homologues of FUT3, the enzyme responsible for core 1,3-fucosylation, are well known^{76, 77} it might be possible to glycoengineer High FiveTM cells to eliminate its function. Alternatively, use of insect cells lines derived from *Spodoptera frugiperda*, such as *expresSF+* cells, minimizes the potential of allergenic risk as they do not appear to make a detectable amount of this CCD based on our findings and others⁷⁸.

Egg, *expresSF*⁺- and *SfSWT*-7-derived HAs had 45%, 0% and 18% complex *N*-glycans, respectively. Complex glycans in *SfSWT*-7 HA were approximately one third as abundant as those in the egg derived HA although branching was less complex due to genetic engineering design. This is the first time anyone has compared the HA *N*-glycosylation by insect cells, glycoengineered insect cells, and eggs, the traditional influenza vaccine production platform. Interestingly, the *SfSWT*-7 HA contained more high mannose glycans, while retaining a significant amount of paucimannosidic forms, as compared to the parent *expresSF*⁺ strain or egg. Terminal mannose is the target of dendritic cell mannose receptors⁷⁹ and these cells mediate both the innate and adaptive immune responses⁸⁰. Differences in glycosylation have been shown to alter the balance between cytotoxic and non-cytotoxic response to influenza infection^{69, 81}. These aspects of glycosylation and the ability to manipulate glycan presence and chemical properties may be useful in the vaccine setting as well as to facilitate a better understanding of the interaction between the different arms of the immune system in response to influenza infection.

CONCLUSIONS

We have developed a method for analyzing and monitoring HA glycosylation in influenza vaccines. The use of nanoLC/MS^E and permethylation profiling allowed us to establish the site-specific glycosylation and relative *N*-glycan abundances, respectively. This method can be used to track both quantitative and qualitative changes in glycosylation in the research or industrial setting, for instance, in vaccine cell system development, or lot-to-lot comparisons in product development.

Our analysis has revealed inherent differences in HA glycosylation site utilization in different cell types. We found that insect cells were more likely to utilize certain sequons than egg or mammalian cell platforms. Furthermore, the sizes, branching patterns, compositions and electrostatic charges of the *N*-glycans linked to HAs produced in different platforms were strikingly different. These differences could have an effect on vaccine properties particularly with influenza, where a standardized set of reagents are prepared from a single (hen egg) source. Thus, there can be differences in vaccines and vaccine study reagents produced in different cell types and these differences might inadvertently affect vaccine potency test results, efficacy and safety and could impact results obtained in the research setting. Methods such as the one presented here will be important to improve understanding of how glycosylation changes influence vaccine research and production.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Work flow diagram of the glycoprotein analysis approach used in this study. Branches indicate intact glycopeptide analysis by nanoLC/MS^E and glycan permethylation profiling.

Figure 2. Stacked plot of glycosylation site 4. An example is shown for each cell platform. The colors in the spectrum represent: red, y ions; blue, b ions; green, y/b ions after neutral loss; grey-unassigned ions by Biopharmalynx (some have been manually assigned as indicated). Monosaccharide symbols: ■, GlcNAc; ●, Man; ▲, Fuc. Oxonium ions at 204.09 and 528.19 are indicated.

Figure 3.
MS^E spectrum of High Five™ derived HA glycopeptide (Hex₃dHex₂HexNAc₂)DQICIGYHANNSTEQVDTIMEK containing site one. The colors in the spectrum represent: red, y ions; blue, b ions; green, y/b ions after neutral loss; grey-unassigned ions by Biopharmalynx (some have been manually assigned as indicated). Monosaccharide symbols: ■, GlcNAc; ●, Man; ▲, Fuc. Oxonium ions at 204.09 and 528.19 are indicated.

Figure 4.

MS^E spectrum of HEK293 derived HA glycopeptide (NeuAc₂dHex₁Hex₄HexNAc₅)-SYNNTNQEDLLVLWGIHHPNDAAEQTK, m/z [M+H]⁺ 5499.33, containing site 4. The colors in the figure indicate the following: red, y ions; blue, b ions; green, y/b ions after neutral loss; pink, glycopeptides after neutral loss assigned by Biopharmalynx; grey-unassigned ions by Biopharmalynx (some have been manually assigned as indicated). Monosaccharide symbols: ■, GlcNAc; ●, Man; ▲, Fuc; ■, GalNAc; ●, Gal; ◆, NeuAc. Oxonium ions at 274.09 and 495.18 are indicated.

Figure 5. MS^E spectrum of SfSWT-7 derived HA glycopeptide, m/z 3559.61 [M+H]⁺ (Hex₄dHex₁HexNAc₃)LYQNPTTYISVGTSTLNQR, containing site 4. The colors in the figure indicate the following: red, y ions; blue, b ions; green, y/b ions after neutral loss; pink- glycopeptides after neutral loss assigned by Biopharmalynx; gray; unassigned ions by the Biopharmalynx (some have been manually assigned as indicated). Monosaccharide symbols: ■, GlcNAc; ●, Man; ▲, Fuc. Peptide fragments with glycosyl neutral loss are indicated.

Figure 6.

3-D structure of HA A/Viet Nam/1203/2004 monomer analyzed in this study (PDB: 2IBX). All glycosylation sites are highlighted in yellow. Partial glycan structures observed in the X-ray structure are shown. The glycosylation site number is noted in parentheses. The glycosylation site ASN number and position is indicated. Protein data from the NCBI structural database were processed with CN3D 1.0 software. In this study glycosylation sites at ASN 209 and 302 were only occupied in insect cell derived HA and are therefore cell type dependant.

Figure 7.

The SfSWT-7 cell derived HA has glycan compositions that are more mammalian like those of its parent *expresSF+* cells. Comparison of egg, *expresSF+*, and *SfSWT-7* derived HA released and permethylated glycans as analyzed by MALDI-TOF MS are shown. Measurements were performed in triplicate and subclass individual isobars were summed. Error bars represent one standard deviation. Example compositions of each class are shown above histogram bars. The *SfSWT-7* mammalian like complex glycan subclass was approximately one third that of egg whereas parent *expresSF+* produced HA had no mammalian like complex glycans.

Table 1

Influenza HAs examined in this study

	Strain	Cell substrate
1	A/Vietnam/1203/2004	Egg
2	A/Vietnam/1203/2004	<i>expresSF+</i>
3	A/Vietnam/1203/2004	SfSWT-7
4	A/Bar-headed goose/Qinghai/14/2008	High Five™
5	A/Bar-headed goose/Qinghai/14/2008	HEK293

Table 2

Influenza HA glycopeptides and sequon use potentials as predicted by NetNGlyc

site	peptide position	Theoretical sequence ^a	NetNGlyc potential
1	16–38	(S)DQICIGYHANNSTEQVDTIMEK	0.783
2	39–51	NVTVTTHAQDILEK	0.7218
3	170–177	NSTYPTIK	0.53
4	179–205	SYNNTNQEDLLVLWGIHH PNDAAEQTK	0.6203
5	206–224	LYQNPTTYISVGTSTLNQR	0.6843 Warning:PRO-X1
6	294–320	CQTPMGAINSSMPFHNIHPLTIGECPK	0.6024
7	500–513	NGTYDYPQYSEEAR	0.5832

^aSequence position is relative to A/Vietnam/1203/2004

Table 3

N-glycans detected at various sites in influenza HAs from various sources

site	express ^{SF+}	SISWT-7	High Five™	HEK293	Egg
1	Fuc1Man2GlcNAc2 Fuc1Man3GlcNAc2 Fuc1Man3GlcNAc3 Man2GlcNAc2 Man3GlcNAc2 Man3GlcNAc3 Man3GlcNAc4 Man3GlcNAc5 Man6GlcNAc2 Man7GlcNAc2	Fuc1Man3GlcNAc3 Fuc1Man3GlcNAc4 Fuc1Man4GlcNAc3 Fuc1Man6GlcNAc3 Man3GlcNAc3 Man3GlcNAc4 Man3GlcNAc5 Gall1Man3GlcNAc3 Man5GlcNAc2 Man7GlcNAc2 Man8GlcNAc2	Fuc1Man3GlcNAc2 Fuc1Man3GlcNAc3 Fuc2Man2GlcNAc2 Fuc2Man3GlcNAc3 Man3HGlcNAc2 Man4GlcNAc3 Man5GlcNAc2	NeuAc1Fuc1Gal2Man3GlcNAc4 NeuAc1Fuc1Gal2Man3GlcNAc5 NeuAc1Fuc1Gal3Man3GlcNAc5 NeuAc1Fuc1Gal3Man3GlcNAc6 NeuAc2Fuc1Gal3Man3GlcNAc6 NeuAc2Fuc1Gal3Man3GlcNAc7 NeuAc2Fuc1Gal4Man3GlcNAc6 NeuAc2Fuc1Gal4Man3GlcNAc7 NeuAc3Fuc1Gal3Man3GlcNAc7 NeuAc3Fuc1Gal4Man3GlcNAc6 NeuAc3Fuc1Gal4Man3GlcNAc7	Fuc1Gal3Man3GlcNAc5 Fuc1Gal3Man3GlcNAc6 Gal3Man3GlcNAc5 Sulph1Fuc1Gal3Man3GlcNAc5
2	Fuc1Man2GlcNAc2 Fuc1Man3GlcNAc2 Fuc1Man3GlcNAc3 Man3GlcNAc2 Man3GlcNAc3 Man7GlcNAc2 Man8GlcNAc2 Man9GlcNAc2	Fuc1Gal2GlcNAc2 Fuc1Man3GlcNAc2 Fuc1Man3GlcNAc3 Fuc1Man4GlcNAc3 Fuc1Gall1Man3GlcNAc4 Fuc1Man5GlcNAc4 Man1GlcNAc2 Man2GlcNAc2 Man3GlcNAc2 Man3GlcNAc4 Man4GlcNAc2 Gall1Man3GlcNAc3 Gall1Man3GlcNAc4 Man5GlcNAc2 Man7GlcNAc2 Man8GlcNAc2 GlcNAc2	Fuc1Man2GlcNAc2 Fuc1Man3GlcNAc2 Fuc1Man3GlcNAc3 Fuc2Man2GlcNAc2 Fuc2Man3GlcNAc2 Man2GlcNAc2 Man5GlcNAc2	Fuc1Gall1Man3GlcNAc4 Fuc1Gal3Man3GlcNAc5 Fuc1Gal4Man3GlcNAc6 NeuAc1Fuc1Gal3Man3GlcNAc5 NeuAc1Fuc1Gal3Man3GlcNAc6 NeuAc1Fuc1Gal4Man3GlcNAc6 NeuAc2Fuc1Gal3Man3GlcNAc5 NeuAc2Fuc1Gal3Man3GlcNAc6 NeuAc2Fuc1Gal3Man3GlcNAc7 NeuAc2Fuc1Gal4Man3GlcNAc6 NeuAc3Fuc1Gal3Man3GlcNAc5 NeuAc3Fuc1Gal3Man3GlcNAc6 NeuAc3Fuc1Gal4Man3GlcNAc7	Fuc1Gal3Man3GlcNAc5 Fuc1Gal4Man3GlcNAc7 Gall1Man3GlcNAc3 Gall1Man4GlcNAc4 Gal2Man3GlcNAc4 Gal3Man3GlcNAc5 Gal3Man3GlcNAc7 Gal5Man3GlcNAc7
3	Fuc1Man2GlcNAc2 Fuc1Man3GlcNAc2 Man2GlcNAc2 Man3GlcNAc2 Man5GlcNAc2 Man7GlcNAc2	Fuc1Man2GlcNAc2 Fuc1Man3GlcNAc2 Fuc1Man3GlcNAc3 Fuc1Man3GlcNAc4 Fuc1Gall1Man3NAc4 Man2GlcNAc2 Man3GlcNAc2 Man3GlcNAc3 Man5GlcNAc2 Man7GlcNAc2 Man8GlcNAc2 Man9GlcNAc2 GlcNAc2	Fuc1Man3GlcNAc2 Fuc2Man2GlcNAc2 Fuc2Man3GlcNAc2	Fuc1Man3GlcNAc6 Fuc1Gall1Man3GlcNAc5 Fuc2Man3GlcNAc6 NeuAc1Fuc1Man3GlcNAc6 NeuAc1Fuc1Gall1Man3HexNAc5 NeuAc1Fuc1Gal2Man3GlcNAc5 NeuAc1Fuc2Gall1Man3GlcNAc5 NeuAc2Fuc1Gall1Man3GlcNAc5 NeuAc2Fuc1Gal2Man3GlcNAc5	Fuc1Gal1Man3GlcNAc4 Fuc1Gal1Man3GlcNAc5 Fuc1Gal2Man3GlcNAc5

site	expressSF+	SISWT-7	High Five TM	HEK293	Egg
4	Fuc1Man3GlcNAc2 Fuc1Man3GlcNAc3 Man3GlcNAc2 Man3GlcNAc3 Man3GlcNAc4 Man4GlcNAc2 Man4GlcNAc3 Man5GlcNAc2 Man5GlcNAc3 Man6GlcNAc2 Man7GlcNAc2 Man8GlcNAc2 Man9GlcNAc2	Fuc1Man3GlcNAc2 Fuc1Man3GlcNAc3 Fuc1Man3GlcNAc4 Man3GlcNAc2 Man3GlcNAc3 Man3GlcNAc4 Gal1Man3GlcNAc3 Man5GlcNAc2 Man8GlcNAc2	Fuc1Man3GlcNAc2 Fuc1Man3GlcNAc3 Fuc2Man2GlcNAc2 Fuc2Man3GlcNAc2 Fuc2Man3GlcNAc3 Fuc2Man4GlcNAc3 Man3GlcNAc2 Man5GlcNAc2	Fuc2Man3GlcNAc6 NeuAc1Fuc1Man3GlcNAc6 NeuAc1dFuc1Gal1Man3GlcNAc5 NeuAc1Fuc1Gal2Man3GlcNAc4 NeuAc1Fuc1Gal2Man3GlcNAc5 NeuAc1Fuc2Gal1Man3GlcNAc5 NeuAc2Fuc1Gal1Man3GlcNAc5 NeuAc2Fuc1Gal2Man3GlcNAc4 NeuAc2Fuc1Gal2Man3GlcNAc5 NeuAc3Fuc1Gal2Man3GlcNAc6 NeuAc3Fuc1Gal3Man3GlcNAc5 NeuAc4Fuc1Gal3Man3GlcNAc7	Fuc1Gal1Man3GlcNAc4 Fuc1Gal1Man3GlcNAc5 Fuc1Gal2Man3GlcNAc4 Man5GlcNAc2 Gal2Man3GlcNAc4 Gal2Man3GlcNAc5
5	Man3GlcNAc2	Fuc1Gal1Man3GlcNAc3 Man3GlcNAc4 Man8GlcNAc2	Fuc1Man3GlcNAc2		
6	Fuc1Man3GlcNAc2 Man3GlcNAc2 Man3GlcNAc3 Man8GlcNAc2	Man2GlcNAc2 Man5GlcNAc2 Man9GlcNAc2 GlcNAc2	Fuc1Man2GlcNAc2 Fuc1Man3GlcNAc2 Man3GlcNAc2 Man3GlcNAc3		
7	Fuc1Man2GlcNAc2 Fuc1Man3GlcNAc2 Fuc1Man3GlcNAc3 Man2GlcNAc2 Man3GlcNAc2 Man3GlcNAc4 Man4GlcNAc2 Man5GlcNAc2 Man6GlcNAc2 Man7GlcNAc2 Man8GlcNAc2 Man9GlcNAc2	Fuc1Man3GlcNAc2 Fuc1Man3GlcNAc3 Fuc1Man3GlcNAc4 Man2GlcNAc2 Man3GlcNAc2 Man3GlcNAc4 Man5GlcNAc2 Man6GlcNAc2 Man7GlcNAc2 Man8GlcNAc2 Man9GlcNAc2	Fuc1Man3GlcNAc2 Fuc2Man2GlcNAc2 Fuc2Man3GlcNAc2 Fuc2Man3GlcNAc3 Man2GlcNAc2 Man3GlcNAc2 Man4GlcNAc2	Fuc1Gal1Man3GlcNAc4 NeuAc1Fuc1Gal1Man3GlcNAc5 NeuAc1Fuc1Gal2Man3GlcNAc4 NeuAc1Fuc1Gal2Man3GlcNAc5 NeuAc1Fuc2Gal1Man3GlcNAc5 NeuAc2Fuc1Gal2Man3GlcNAc5	Fuc1Gal2Man3GlcNAc4 Man3GlcNAc3 Gal1Man3GlcNAc3 Gal2Man3GlcNAc4 Gal3Man3GlcNAc5