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Generation of a Natural Glycan Microarray Using 9-Fluorenylmethyl Chloroformate (FmocCl) as a Cleavable Fluorescent Tag

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Abstract

Glycan microarray technology has become a successful tool for studying protein-carbohydrate interactions, but a limitation has been the laborious synthesis of glycan structures by enzymatic and chemical methods. Here we describe a new method to generate quantifiable glycan libraries from natural sources by combining widely used protease digestion of glycoproteins and Fmoc chemistry. Glycoproteins including chicken ovalbumin, bovine fetuin, and horseradish peroxidase (HRP) were digested by pronase, protected by FmocCl, and efficiently separated by 2D-HPLC. We show that glycans from HRP glycopeptides separated by HPLC and fluorescence monitoring retained their natural reducing end structures, mostly core α 1,3-fucose and core α 1,2-xylose. After simple Fmoc-deprotection, the glycans were printed on NHS-activated glass slides. The glycans were interrogated using plant lectins and antibodies in sera from mice infected with *Schistosoma mansoni*, which revealed the presence of both IgM and IgG antibody responses to HRP-glycopeptides. This simple approach to glycopeptide purification and conjugation allows for the development of natural glycopeptide microarrays without the need to remove and derivatize glycans and potentially compromise their reducing end determinants.

Keywords

Glycan array; fluorescent labeling; immobilization; functional glycomics

Introductory Statement

The field of functional glycomics [1,2] has recently seen enormous progress by the development of high throughput methods such as glycan microarrays [3–8]. Glycan structures are immobilized onto activated glass surfaces and interrogated with glycoproteins, antibodies, or whole microorganisms. Many methodologies enabling solid phase immobilization of glycan derivatives onto various activated surfaces have been developed for the purpose of microarray. Non-covalent attachment mechanisms include TLC plate-protein overlay [9,10], neoglycolipids-nitrocellulose membrane [11,12] and biotinylated glycoconjugates-streptavidin coated substrates [3]. Covalent attachment generally utilizes inter-reactive pairs,

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such as activated ester-primary amine [5,13], epoxy-amine [14–16], sulfhydryl-maleimide [17–19], azide-alkyne [20] etc. While each methodology has its own advantages and disadvantages, their preference depends on the availability of the solid phase substrate and the convenience of the chemistry. The glycan microarray of the Consortium of Functional Glycomics, utilizing commercially available NHS-activated glass slides and including more than 400 glycans, has been one of the most successful glycan microarrays.

Despite the enormous success, the progress of glycan microarray methodology has been limited by the relatively slow expansion of glycan libraries relative to the large size of the glycome. An alternative approach is to develop natural glycan libraries, where glycans from natural sources are released, tagged, and separated. These tagged glycan libraries (TGLs) can be prepared directly from any biological system such as cells, tissues, and microorganisms, and can be interrogated with relevant carbohydrate binding proteins (GBPs), antibodies, and microorganisms. The most crucial component in TGL methodology is the glycan derivatization chemistry, which needs not only to facilitate the separation by adding a sensitive detectable tag, but also to functionalize the glycan for further conjugation such as solid phase immobilization. Widely used fluorescent labels such as 2-aminobenzamide (2-AB) and 2-aminobenzoic acid (2-AA) [21], are good tags for HPLC separation, but are not suitable for further reaction. The bifunctional alkoxyamine linker [22] enables further solid phase immobilization, but it is not fluorescent or UV active, which limits its application to structurally-defined glycans at relatively large scale. To address these questions, we have developed new methods utilizing fluorescent homo- or hetero-bifunctional linkers, i.e., 2,6-diaminopyridine (DAP) [15,23] and N-aminoethyl 2-aminobenzamide (AEAB) [24] for the generation of TGLs and successfully applied them for the identification of galectin ligands. In this paper, we report a new strategy utilizing well known 9-fluorenylmethyl chloroformate (FmocCl) chemistry to generate TGLs for functional study. Arsequell et. al. reported the derivatization of glycosylamines derivatives of free oligosaccharides with Fmoc-glycine for oligosaccharide fractionation [25]. Here we show that Fmoc serves as a fluorescent tag for glycoamino acids and glycopeptides and facilitates their separation, characterization and quantification. Most importantly, Fmoc is conveniently removed to expose amino groups on the peptide moiety for further reaction such as solid phase immobilization. This new strategy avoids the release of glycans from glycoproteins by hazardous chemicals and expensive enzymes, can be applied to both N- and O-linked glycans as glycopeptides, and serves as a powerful tool for functional glycomics studies.

Materials and Methods

Materials

HPLC solvents were purchased from Fisher Scientific, Pittsburgh, PA. An Ultraflex-II TOF/TOF system from Bruker Daltonics was used for MALDI-TOF mass spectrometry analysis of glycan conjugates. Pronase was purchased from EMD Chemicals Inc., Darmstadt, Germany. All chemicals were purchased from Sigma-Aldrich, St. Louis, MO. Chicken egg glycopeptide was prepared as described previously [26].

Pronase digestion of glycoproteins

The chicken egg glycopeptide and glycoproteins (200 mg) were dissolved in 0.1 M Tris buffer, pH 8.0 so that the final concentration was 20 mg/mL. Pronase was added to a final concentration of 0.1 mg/mL. At 24 hours and 48 hours of incubation at 37°C, an equivalent amount of Pronase was added, respectively. The final digestion was passed through a 2 g C18 Sep-pak, from which the flow-through was passed through a 1g Carbograph and washed by 6 column volumes (c.v.) water. The glycoamino acids and glycopeptides were eluted from the Carbograph by 3 c.v. 50% acetonitrile with 0.1% trifluoroacetic acid (TFA). The elution was dried by Speed-vac for

Fmoc derivatization. Chicken ovalbumin, bovine fetuin, and horseradish peroxidase were digested in a similar fashion.

Fmoc derivatization of glycopeptides

The lyophilized glycopeptides were reconstituted into cold water so that the concentration was 100 mg/mL. An equal volume of 50 mg/mL sodium bicarbonate (NaHCO₃) solution, an equal volume of water, and an equal volume of 20 mg/mL FmocCl in tetrahydrofuran (THF) was added. The mixture was then agitated vigorously by vortexing for 30 minutes, after which 3 × 1 volume of ethyl acetate was used to extract the mixture. The aqueous phase was applied on C18 Sep-pak and washed by 6 c.v. water. The Fmoc glycopeptides were eluted by 50% acetonitrile with 0.1% TFA.

High performance liquid chromatography (HPLC)

A Shimadzu HPLC CBM-20A system was used for HPLC analysis and separation of Fmoc-glycopeptides, which was coupled with a UV detector SPD-20A and a fluorescence detector RF-10Ax1. UV absorption at 254 nm or fluorescence at 254 nm excitation (Ex) and 340 nm emission (Em) was used to detect and quantify Fmoc-tagged glycopeptides.

For normal phase HPLC separation, a Zorbax NH₂ column (250mm × 4.6mm) was used; the mobile phases were acetonitrile, water, and 250 mM ammonium acetate (pH 4.5). The concentration of water increased from 16% to 40% and the concentration of ammonium acetate buffer increased from 4% to 50% over 60 minutes.

For reverse phase HPLC with a C18 column, a Vydec C18 column was used. The mobile phase was acetonitrile and water with 0.1% TFA. The concentration of acetonitrile increased from 15% to 45% in 30 minutes.

Generation of normal and 20-week *Schistosoma mansoni* infected mice sera

Ten 8 week-old C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA) and infected with 50–60 *Schistosoma mansoni* cercariae (Puerto Rican strain) subcutaneously. Ten age-matched uninfected C57BL/6 mice served as controls. Both groups of mice were housed at the Emory University laboratory animal facility. After 20 weeks, the mice were bled via retro-orbital route and blood collected in microtainer serum separator tubes (BD and Company, Franklin Lakes, NJ). The microtainer tubes with blood were centrifuged at 6,000 rpm in an Eppendorf bench-top microcentrifuge (Westbury, NY) for 10 minutes at room temperature. The sera were aspirated, pooled separately as either infected or uninfected normal mouse sera, and stored at –20°C in small aliquots.

Printing, binding assay, and scanning

NHS-activated slides were purchased from Schott, Louisville, KY. Epoxy slides were purchased from Corning, Lowell, MA. Non-contact printing was performed using a Piezoarray printer from Perkin Elmer. The average spot volume was within 10% variation of 1/3 nL. All the samples were printed in phosphate buffer (300 mM sodium phosphates, pH 8.5). After printing, the slides were boxed loosely and put in a high moisture chamber at 50°C and incubated for 1 h. The slides were then washed and blocked with 50 mM ethanolamine in 0.1 M Tris buffer (pH 9.0) for 1 h. The slides were dried by centrifugation and stored desiccated at –20°C for future use. Before assay, the slides were rehydrated for 5 minutes in TSM buffer (20 mM Tris- HCL, 150 mM sodium chloride (NaCl), 0.2 mM calcium chloride (CaCl₂) and 0.2 mM magnesium chloride (MgCl₂). Biotinylated lectins were used in the binding assay and the bound lectins were detected by a secondary incubation with cyanine 5-streptavidin. For multi-panel experiment on a single slide, the array layout was designed using Piezoarray

software according to the dimension of a standard 16-chamber adaptor. The adaptor was applied on the slide to separate a single slide into 16 chambers sealed from each other during the assay.

The slides were scanned with a Perkin Elmer ProScanarray microarray scanner equipped with 4 lasers covering an excitation range from 488 nm to 637 nm. The scanned images were analyzed with the ScanArray Express software. Detection of bound biotinylated lectins was carried out by incubation with cyanine5-streptavidin. Detection of bound mouse sera antibodies was carried out by incubation with Alexa568 labeled goat anti-mouse IgG and Alexa488 labeled goat anti-mouse IgM. For cyanine5 fluorescence, 649 nm (Ex) and 670 nm (Em) were used. For Alexa488, 495 nm (Ex) and 519 nm (Em) were used. For Alexa568, 579 nm (Ex) and 604 nm (Em) were used. All images obtained from the scanner were in grayscale and colored for easy discrimination.

Results

Fmoc derivatization of glycoamino acids and glycopeptides

The general strategy is schematically represented in Fig. 1a. Glycoproteins can be digested with Pronase to glycopeptides bearing O- or N-linked glycan, and in most cases either a single Asn residue, or one or two additional residues [27–29]. The amino groups of the glycopeptides are reactive with FmocCl under mild conditions. The resulting fluorescent glycopeptide mixture can easily be separated chromatographically and monitored by fluorescence to generate a tagged-glycan library or TGL. This Fmoc-protected TGL can be used as building blocks in microscale glycopeptide synthesis by prevalent solid phase peptide synthesis (SPPS). After Fmoc deprotection, this TGL can be immobilized onto various solid surfaces such as microarray slides for further functional studies.

A glycopeptide prepared from chicken egg yolk termed chicken egg glycopeptide (Fig. 1b) [26], a disialo-biantennary N-glycan attached to peptide KVANKT, was utilized in the preliminary study because of its availability and relative homogeneity. After Pronase digestion and mild acid hydrolysis to remove all sialic acids, it was treated with FmocCl. MALDI-TOF profiles of the mixture before and after FmocCl treatment are shown in Fig. 2a. The major component (Hex)₅(HexNAc)₄-Asn (NA2-N) and two minor components (Hex)₄(HexNAc)₄-Asn, Ala-((Hex)₅(HexNAc)₄-)Asn were successfully derivatized with Fmoc-group, as evidenced by a mass shift of 222 Daltons. In contrast, one minor free oligosaccharide component, (Hex)₅(HexNAc)₃-OH, was not derivatized with Fmoc-group and shows no corresponding mass shift after FmocCl treatment. Thus, the Fmoc-derivatization is specific for amino groups and suitable for labeling glycoamino acids and glycopeptides.

This strategy was further tested on a more complex system. Chicken ovalbumin is an abundant egg white glycoprotein with a single N-glycosylation site [30]. After extensive Pronase digestion, solid phase extraction columns, including C18 Sep-pak and carbograph, were used to purify the glycoamino acids and glycopeptides. The mixture was treated with FmocCl and profiled by MALDI-TOF. The MALDI-TOF profiles of ovalbumin glycopeptides before and after Fmoc-derivatization are shown in Fig. 2b. Except for several free reducing glycans present as contaminants of the commercial glycoprotein, all of the glycopeptides generated from ovalbumin were successfully derivatized and showed a $\Delta 222$ Dalton mass shift (Fig. 2c). The very similar MALDI-TOF profiles suggested a non-selective and quantitative derivatization of Fmoc. Furthermore, most of the peaks can be assigned to a glycan attached to a single asparagine, suggesting high efficiency of Pronase digestion of ovalbumin.

Solid phase immobilization of Fmoc derivatized glycoconjugates

The practicability of Fmoc-protected glycoamino acids and glycopeptides for microarray application was evaluated by applying a simple deprotection procedure to the Fmoc-derivatives before the standard printing procedure. Fig. 3a shows the HPLC profiles of (Hex)₅(HexNAc)₄-Asn-Fmoc before and after standard Fmoc-deprotection by treatment with 20% piperidine in dimethylformamide (DMF) for 30 minutes. Full deprotection was confirmed by the complete disappearance of the starting material and generation of an Fmoc-piperidine adduct (Fig. 3a). The regenerated glycoamino acid (Hex)₅(HexNAc)₄-Asn is not fluorescent and is not detected. Thus, the loss of fluorescence can be used to monitor the completion of the reaction.

After evaporation of all the solvents, the deprotected glycoamino acid was printed on NHS-activated glass slides without any further purification, along with other compounds, including Fmoc-protected glycoamino acid, buffer control, and biotin-hydrazine. The microarray slide was then assayed with the biotinylated plant lectins RCA I and ConA [31,32] followed by incubation with cyanine5-streptavidin (Fig. 3b). For deprotected (Hex)₅(HexNAc)₄-sn, both RCA I and ConA (each at 10 μ) showed binding with increasing printing concentrations of glycopeptide up to 100 μ. The binding of the lectins corresponded to the terminal βlactosylated N-glycan structure recognized by RCA I [33,34] and the hybrid-type N-glycan structure recognized by ConA [31,32,35]. The lowest detectable printing concentration at ~1 μM, shows the suitability of this strategy for glycan microarray development. RCA I did not show any binding to Fmoc-protected (Hex)₅(HexNAc)₄-Asn, however, ConA did show significant binding signals to Fmoc-protected (Hex)₅(HexNAc)₄-Asn. This is presumably due to trace amounts of deprotected NA2-N during the printing process at pH 8.5, which is detected by ConA. Nevertheless, the lowest detectable concentration (~3 μM) for NA2-N-Fmoc suggests an extremely high sensitivity of NA2-N to ConA binding on the microarray platform.

Generation of an Fmoc-derivatized TGL from HRP

To evaluate whether Fmoc-derivatized glycopeptides from pronase digestion of glycoproteins can be separated by HPLC to near-homogeneous fractions, we tested a 2D-HPLC (normal phase followed by reverse phase) strategy on chicken ovalbumin and bovine fetuin. The profiles of 1st dimension normal phase HPLC are shown in Figs. 4a and b. Fractions collected from normal phase HPLC were subjected to 2nd dimension reverse phase (C18) HPLC (Figs. 4c and d). Although it is usually challenging to separate oligosaccharides or glycopeptides efficiently only by RP-HPLC [36], the reasonably well-separated peaks in Fig. 4 indicated that 2D-HPLC is sufficient to resolve most Fmoc-glycopeptides, which is essential for generation of a TGL. Horseradish peroxidase (HRP) has multiple N-glycosylation sites with predominantly pausi-mannose-like structures [37, 38] (Fig. 1b). These N-glycans are resistant to widely used PNGase F and can only be released by treatment with PNGase A or harsh chemicals, such as sodium hydroxide and hydrazine. As an economic alternative, HRP was treated with Pronase so that the linkages between glycans and peptide chain remain intact. These glycopeptides were then derivatized with FmocCl. The MALDI-TOF spectra of the glycopeptides before and after FmocCl treatment are shown in Fig. 5a. Similar to egg yolk glycopeptides and ovalbumin glycopeptides (Fig. 2), all the glycopeptides showed a characteristic mass shift of 222 after FmocCl treatment, without affecting the general mass profile. It is interesting to note that the dominant peaks shown in both spectra (1459.78[M+H]⁺ before FmocCl and 1681.88[M+H]⁺ after FmocCl) have the composition of (Hex)₃(HexNAc)₂(Xyl)₁(Fuc)₁-Asn-Arg. This peak, however, is not the most abundant species as shown below by the 2D-HPLC analysis. The Fmoc-glycopeptides were purified by 2D-HPLC using a semi-preparative normal phase column for the first dimension (Fig. 5b) and a C18 reverse phase analytical column for the second dimension (Fig. 5c). Peaks were collected, characterized by MALDI-TOF/TOF, and re-quantified based on fluorescence. The selected

fractions were saved as the TGL from HRP (Table 1a). Their glycan and peptide compositions are proposed based on MALDI/TOF-TOF experiments and known structures. Based on the fluorescence, it is possible to quantify the relative abundance of each glycopeptide. The HRP-glycans can be roughly categorized as fucosylated, xylosylated, both fucosylated and xylosylated, and high-mannose structures (Fig. 1b). Their relative abundance are listed in Table 1b, which shows that $(\text{Hex})_3(\text{HexNAc})_2(\text{Xyl})_1(\text{Fuc})_1$ is the dominant glycan structure. These results are largely consistent with previous reports [37, 38], although the identified structures and their abundance may vary due to the source of HRP and the analytical methods applied.

Glycan microarray of HRP TGL

The Fmoc-derivatized glycopeptides from HRP were printed after simple deprotection by piperidine and interrogated with biotinylated lectins followed by incubation with cyanine5-streptavidin. ConA showed strong binding to several high-mannose-type structures (#25, #27, #28) (Fig. 6a). The presence of the core α 3-linked fucose residue slightly decreased the binding (#6 and #8), whereas the presence of the β 2-linked xylose residue largely abolished the binding. The elimination of ConA binding to core-xylosylated N-glycans is consistent with previous findings [39]. AAL, an α -fucose binding protein [40], showed binding to most of the glycans (Fig. 6b), in agreement with the fact that most of the N-glycans of HRP possess a core α 3-linked fucose. AAL did not bind to glycans lacking fucosylation (#3, #5, #7, #25, #27, and #28). Several glycopeptide fractions, although confirmed by mass spectra to possess a fucose (#1, 2, 8, 21, 23), did not show significant binding by AAL. While the reason is not clear, it is possible that amino acids in proximity to the glycan as it is derivatized on the slide surface may interfere with lectin accessibility, since the core-fucose is very close to the peptide chain. This is a unique possibility that should be explored further using defined glycans within a large number of peptide sequences, since the compromised recognition by AAL could also be reflected in compromised recognition by anti-glycan antibodies that recognize the α 3-linked fucose residue as a partial determinant. Thus, this is not a limitation of the approach, but an advantage, since multiple peptide species are likely to arise from Pronase digestion, giving a range of glycopeptides with similar glycans to test.

We then screened the HRP microarrays with sera from mice infected with *Schistosoma mansoni*. This vascular parasitic worm synthesizes N-glycans that contain core α 3-linked fucose and core β 2-linked xylose residues [41–43], and antibodies to those glycans are produced during infection [44,45]. It is believed that glycoconjugates play important roles in host-parasite interactions [46–48]. To test recognition of glycopeptides on the microarray, sera from normal, uninfected mice and 20-week infected mice were tested, followed by fluorescently labeled anti-mouse IgG (Fig. 6c) and IgM (Fig. 6d). While there was no antibody binding observed with normal mouse serum, strong reactivity was observed with 20-week infected sera for both IgG and IgM. Both IgG and IgM showed significant binding to most core-fucosylated and xylosylated glycans to different degrees. When glycopeptides are immobilized onto glass slides through the N-terminus, the peptide segments adjacent to the glycans could significantly affect the presentation of the glycans to antibodies, and therefore change the binding pattern. The existence of antibodies recognizing the structures close to the glycan-peptide linkages is also possible. The 20-week infected mouse sera also showed antibody recognition of high-mannose structures, which has also been observed previously [49]. These results demonstrate the presence of antibodies from the sera of *S. mansoni*-infected mice to core-fucosylated and xylosylated glycans, and confirm the utility of the microarray presentation in order to identify these glycan antigens [41,43]. These results also show the value of preparing glycopeptides with multiple peptide moieties to enhance glycan presentation, since clearly the linking peptide and other potential linking strategies could influence the recognition of antigenic glycan determinants that are near the reducing end of the glycan.

Discussion

Glycan microarrays have become powerful tools for exploring and defining protein-carbohydrate interactions, which are important in numerous biological processes, including cell-cell adhesion, cell signaling, glycoprotein turnover and biosynthesis, and genetic disorders and infectious diseases. The success of a glycan microarray essentially depends on the size, diversity, and representative nature of the glycan library included in the microarray. By developing a novel heterobifunctional linker, 2-amino-N-(2-amino-ethyl)-benzamide (AEAB), we have demonstrated that natural tagged glycan libraries (TGLs) provide a feasible and efficient way to expand glycan microarrays for functional glycomics studies [24]. Although this strategy is generally useful for most studies, the reducing end structures are lost due to the reductive amination. As a supplementary study, we developed this new strategy for glycanase-resistant N-glycans, O-glycans, and other situations where reductive amination is not favored and glycan release from peptides would compromise their structural integrity and recognition.

The 9-fluorenylmethyl chloroformate (Fmoc)-based solid phase peptide synthesis (SPPS) is the most popular peptide synthesis method, since it involves the easy installation of Fmoc to amino groups and its removal by mild conditions [50,51]. Fmoc-protected glycoamino acids have also been widely used for assembly of glycopeptides [52–57]. In our study, we have explored the application of Fmoc as a fluorescent tag for generating TGLs. This approach has several advantages. The fluorescent property of the Fmoc is essential for sensitive detection and quantification for microscale glycopeptides. The hydrophobic nature of Fmoc can also act as an affinity tag during the separation, since it imparts a unique hydrophobic group to the highly hydrophilic glycopeptides. In addition, the active amino group on the peptide that is required for covalent linking in glycan microarrays can be easily regenerated without interference of other structures. Preliminary study using glycoamino acid and glycopeptide mixtures from chicken egg yolk and ovalbumin showed successful Fmoc-derivatization of both glycoamino acids and glycopeptides, but not free reducing glycans (Fig. 2). The hydrophobicity of Fmoc group provide an easy clean-up of the material away from salts, free reducing glycans, and trace amounts of underivatized glycopeptides.

The printing of deprotected Fmoc-glycoamino acids was validated by lectin binding (Fig. 3), which shows that this array is a good platform for screening glycan-protein interactions. The printed glycans retained their natural linkages between the glycans and the peptides. Although in most cases protein-carbohydrate interactions occur at the non-reducing termini, there are many examples of the importance of the reducing termini and the close-ring structure of the reducing end, along with the adjacent aglycon. It is apparent that the easiest way to retain all such information is not to break the glycosidic linkages. It is noteworthy that although the deprotection of Fmoc by 20% piperidine in DMF at room temperature is generally considered safe for most base-catalyzed reactions including β -elimination, this has not been validated for O-glycopeptides. Extra caution may be needed when these systems are being studied.

Digestion of glycoproteins with Pronase, which is a protease mixture capable of cleaving virtually all peptide linkages [29], represents an inexpensive and convenient way to release glycoamino acids and glycopeptides of both N- and O-glycan related structures from glycoproteins. While PNGase F has been established as an efficient way to release most N-glycans from glycoproteins, it is too expensive to be applied to relatively large scale reactions, and more importantly, some N-glycans are resistant to PNGase F cleavage. Such is the case for N-glycans with core α 3-linked fucose attached to the reducing GlcNAc residue, for which PNGase A has to be used [58]. The most established chemical method, hydrazinolysis, is known to be too harsh for labile groups, such as sialic acids and O- and N-acetylation, and requires chemical re-N-acetylation of glycans. There are no enzymatic methods to release large-sized O-glycans containing more than 2 sugar residues, and the only methods to release large O-

glycans from Ser/Thr residues is base-catalyzed release, which may compromise the integrity of the glycans [59–61]. In most cases release of O-glycans by chemical treatment is accompanied by inclusion of sodium borohydride to reduce the glycan or the production of glycosylamines that have to be converted to reducing free glycans, with loss of efficiency. Thus, there are many advantages in avoiding chemical release methods for O-glycans and using protease treatments. Pronase digestion is a commonly used method to extract glycoamino acids and glycopeptides from glycoproteins. Other non-glycan components could be easily separated out based on the highly hydrophilic property of glycoamino acids and glycopeptides. Solid phase extraction (SPE) including C18 Sep-pak and Carbograph has been employed in the enrichment/purification of glycopeptides. One critical issue is to avoid over-saturation of the capacities of SPE columns. This can be easily confirmed by the traditional phenol-sulfuric assay for hexose, in which >95% hexose should be observed in the final elution from the Carbograph column.

It is also interesting that Pronase digestion often does not result in complete digestion to a glycan linked to a single Asn residue. While the heterogeneity of the peptide portion of glycopeptides could increase the complexity of the product mixture, making the separation and characterization of individual glycans difficult, this heterogeneity in peptide regions could offer an advantage, since it allows the exploration of the glycan recognition in the context of several types of peptide presentations. Extensive Pronase digestion can reduce the heterogeneity of the glycopeptide mixture, as in the case of chicken ovalbumin (Fig. 2b, c), which has only a single N-glycosylation site. While digestion of all glycopeptides from all sources to single asparagine-glycans may not be practical, the HPLC profiles of Pronase-digested and Fmoc-derivatized glycopeptide mixture from HRP suggests that chromatographic separation of such mixtures is achievable (Fig. 5a, b), especially by 2D-HPLC. It is noteworthy that although the glycan attached to a single asparagine was the most dominant molecular species (Table 1a, fraction 11), it did not appear as the major peak in MALDI-TOF analysis of the glycopeptide mixture (Fig. 5a) either before or after Fmoc-treatment. This suggests that regular MALDI-TOF of a glycan mixture is not reliable for quantification where tagging is necessary.

Horseshoe peroxidase (HRP) is known to include pausi-mannose glycan structures, which are resistant to PNGase F digestion. These glycan structures are known to be allergens and showed cross-reactivity towards Schistosoma-specific antibodies [41–45]. We therefore applied the Pronase-Fmoc strategy to generate a TGL of HRP for microarray studies. The Fmoc-glycoamino acids and glycopeptides could be efficiently separated by 2D-HPLC separation (Fig. 5) and the fractions can be characterized by MALDI-TOF/TOF. The interrogation of this natural glycan microarray from HRP showed expected lectin interactions. It also showed significant response to *Schistosoma*-infected mouse sera, confirming the cross-reactivity between typical plant glycans and *Schistosoma* related antibodies. It is interesting that the results show that glycopeptides containing glycans with both core-fucose and xylose are best recognized by anti-sera from schistosoma-infected mice, whereas the individual epitopes as either core xylose or core fucose alone are weakly recognized. Future studies should be aimed at further defining these important immunodeterminants using immobilized glycopeptides of varying peptide moieties. These data support the general applicability of this strategy for analyzing various glycan binding proteins and antibodies, and should make this approach especially useful for exploring glycan determinants in association with their natural peptide linkages.

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Abbreviations

AAL	<i>Aleuria aurantia</i> Lectin
AEAB	2-amino-N-(2-aminoethyl)-benzamide
ConA	Concanavalin A
c.v	column volumes
DAP	2,6-diaminopyridine
DMF	dimethylformamide
FmocCL	9-fluorenylmethyl chloroformate
GBP	glycan binding protein
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
NHS	N-hydroxysuccinimide
RCA I	<i>Ricinus communis</i> Agglutinin I
RFU	relative fluorescence unit
SPPS	solid phase peptide synthesis
TFA	trifluoroacetic acid
TGL	Tagged Glycan Library
THF	tetrahydrofuran

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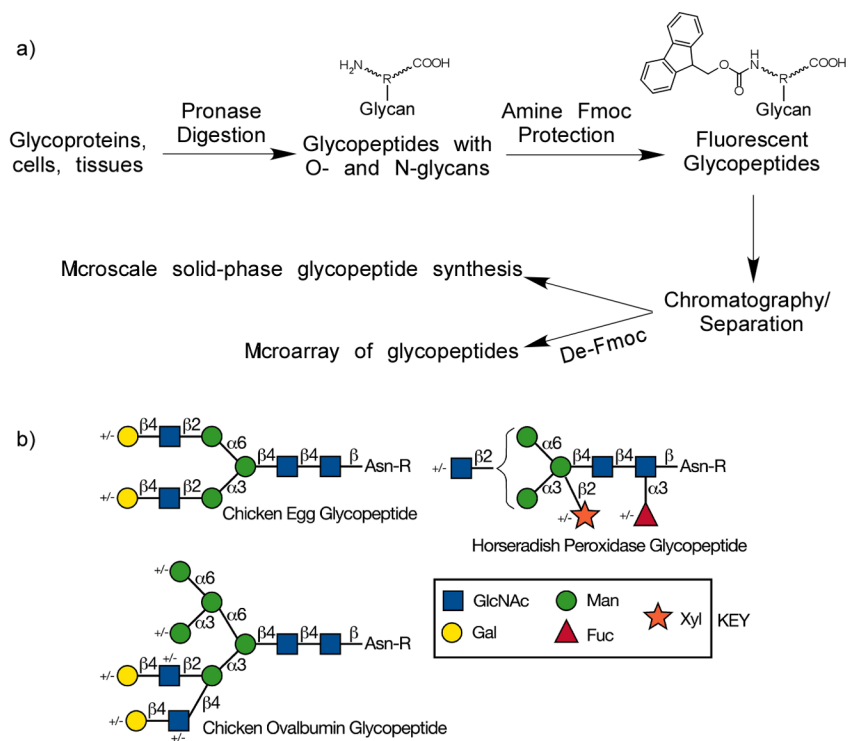


Figure 1. a) The general strategy of utilization of Fmoc-tag for fluorescent glycopeptide library construction and its possible applications. b) The types of glycopeptides with N-glycans used in this study, labeled chicken egg glycopeptide, ovalbumin glycopeptide, and horseradish peroxidase glycopeptide. The symbols used for monosaccharides are shown in the key.

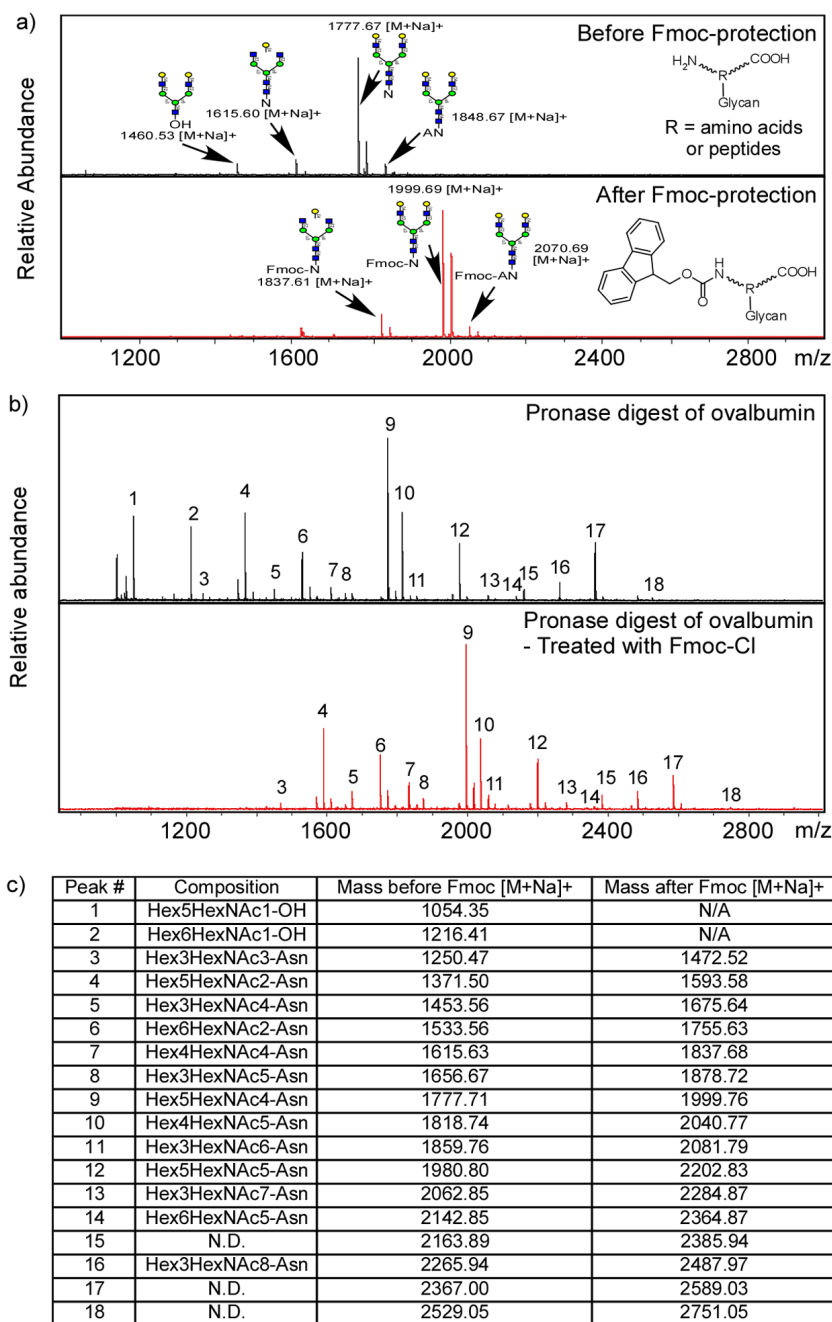


Figure 2. Fmoc-protected of glycoamino acids and glycopeptides: a) MALDI-TOF spectra of Pronase treated egg yolk glycopeptide before (top) and after (bottom) FmocCl treatment; b) MALDI-TOF spectra of Pronase treated chicken ovalbumin before (top) and after (bottom) FmocCl treatment; c) The MALDI-TOF spectra mass lists of Pronase treated chicken ovalbumin before and after FmocCl treatment. Quantitative Fmoc-protection of glycoamino acids and glycopeptides are shown by a mass increase of 222.

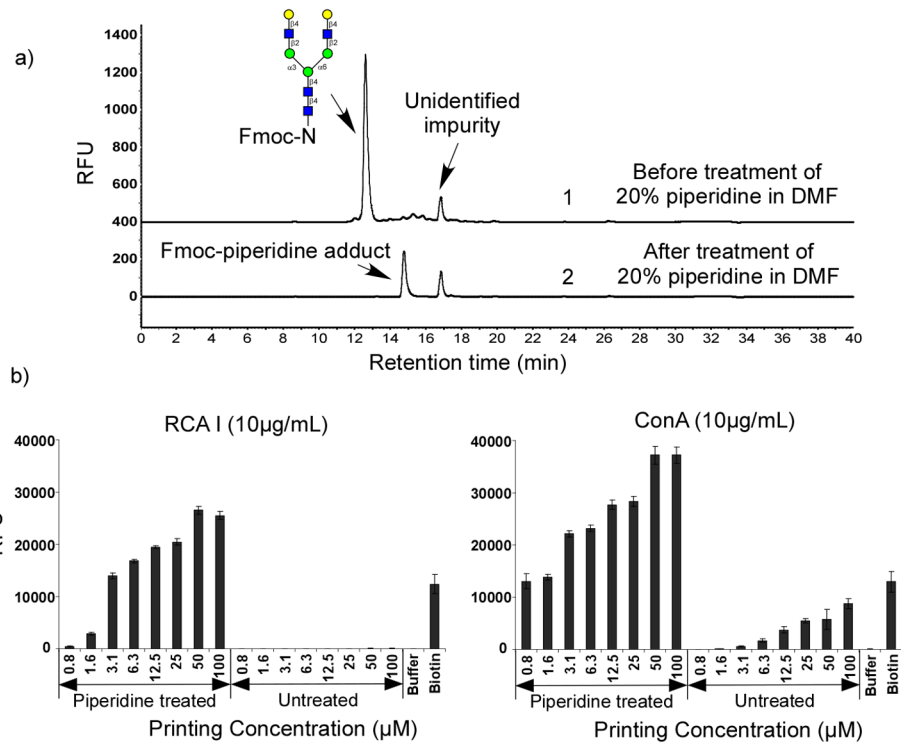


Figure 3. The printing of N-NA2-Fmoc: a) HPLC profiles of N-NA2-Fmoc (top) and after 20% piperidine treatment (bottom); b) The printed N-NA2-Fmoc before and after deprotection validated by RCA I and ConA binding.

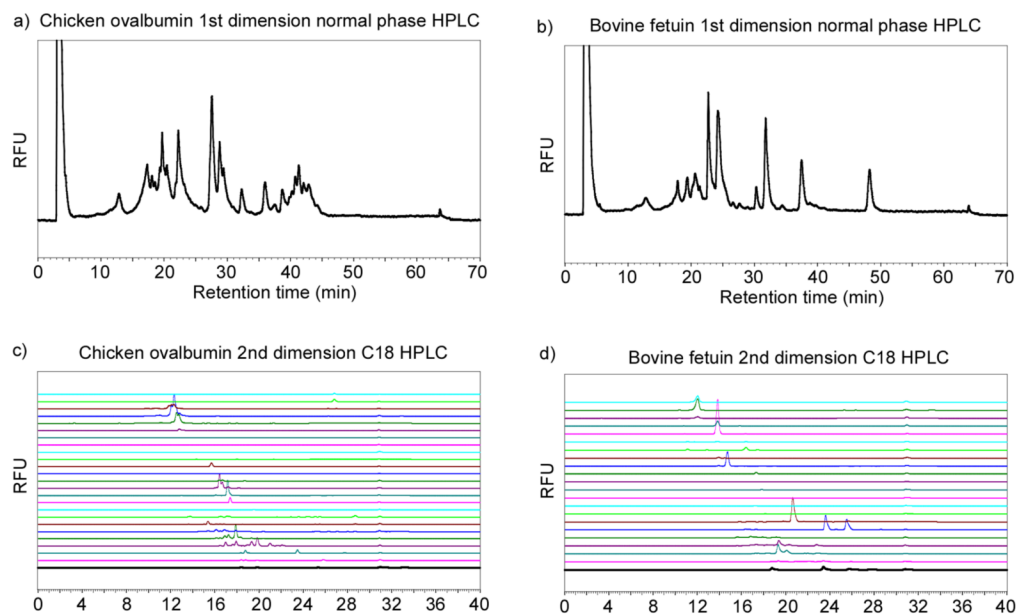


Figure 4. 2D HPLC separation of Fmoc-glycopeptide from pronase digested glycoproteins: a) 1st dimension normal phase HPLC profile of Fmoc-glycopeptides from pronase digested chicken ovalbumin; b) 1st dimension normal phase HPLC profile of Fmoc-glycopeptides from pronase digested bovine fetuin; c) 2nd dimension reverse phase (C18) HPLC profiles of fractions collected from normal phase HPLC of chicken ovalbumin Fmoc-glycopeptides; d) 2nd dimension reverse phase (C18) HPLC profiles of fractions collected from normal phase HPLC of bovine fetuin Fmoc-glycopeptides. Fluorescence detection at Ex 254 nm/Em 340 nm is shown.

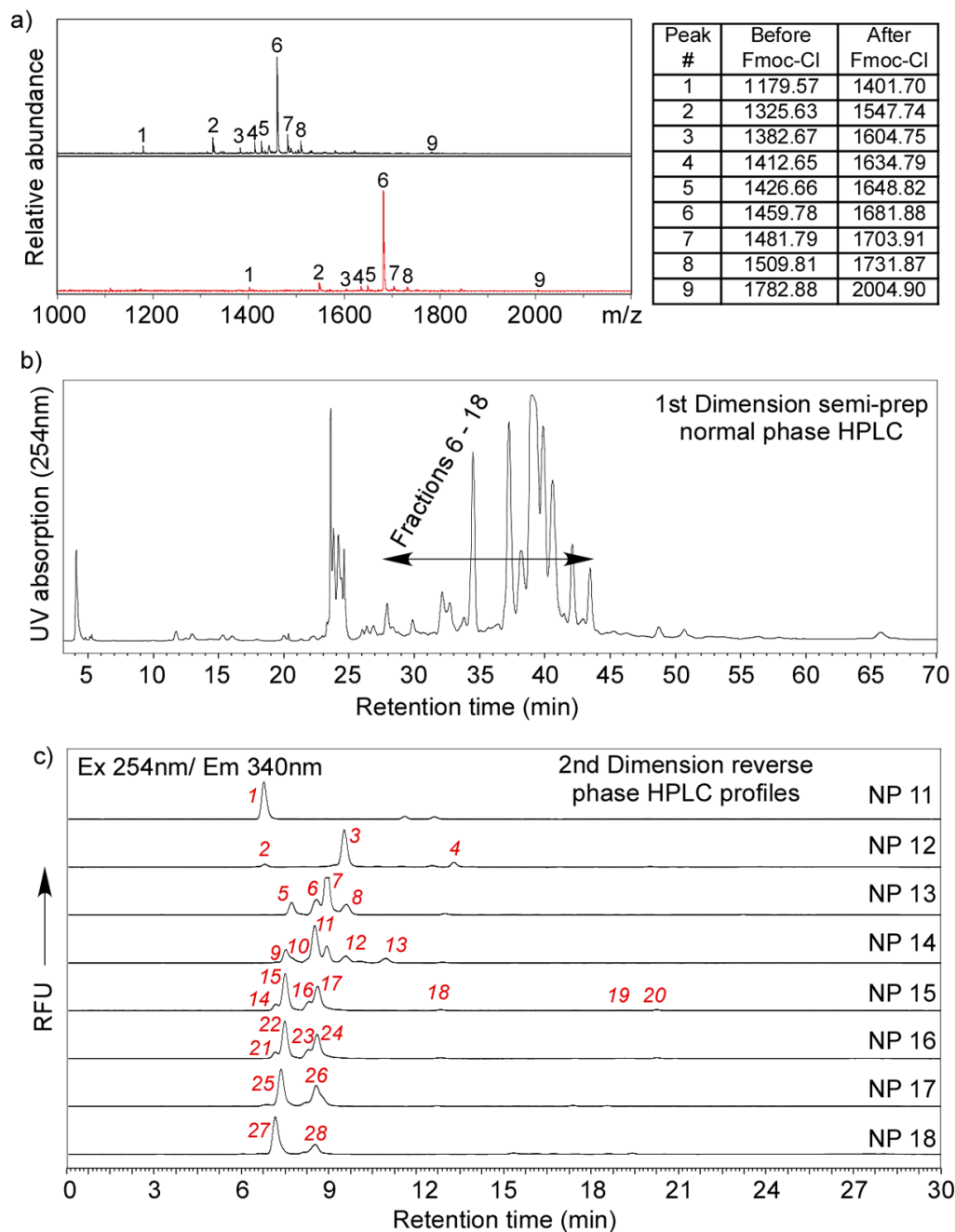


Figure 5. Generation of a tagged glycan library from horseradish peroxidase (HRP): a) MALDI-TOF spectra of Pronase treated HRP before (top) and after (bottom) FmocCl treatment, where the minor component (Hex)₃(HexNAc)₂(Xyl)₁(Fuc)₁-Asn-Arg was shown to be the dominant peak; b) The first dimensional normal phase HPLC; c) The second dimensional C18 reverse phase HPLC with all of the 2D fractions marked. The scale of the Y-axis of each profile was individually adjusted to show the minor peaks. The relative abundance was calculated based on re-quantification of each 2D fraction.

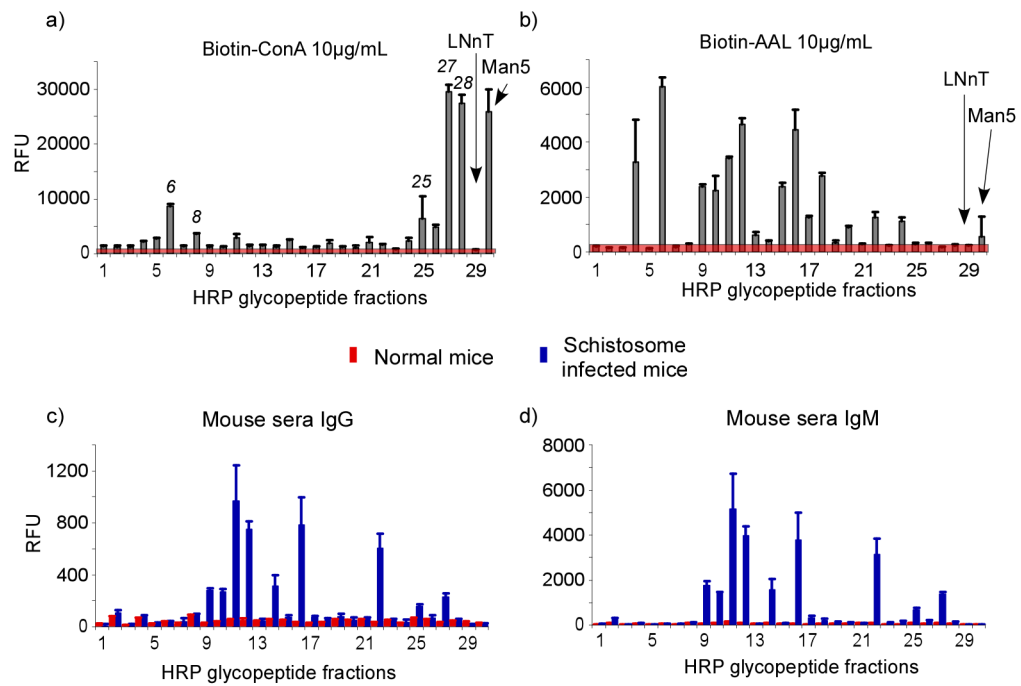


Figure 6.

The lectin and sera binding to the 2D-HPLC separation of Fmoc-glycopeptides from HRP. The average RFU of 6 replicates on the microarray was plotted against each fraction and the standard deviation was incorporated as the error bar. a) Binding of biotinylated ConA (10 µg/mL) was detected by cyanine5-streptavidin; b) Binding of biotinylated AAL (10 µg/mL) was detected by cyanine5-streptavidin; c) Binding of normal (red) and *Schistosoma*-infected (blue) mouse serum (1:20 dilution) was detected by Alexa568 labeled goat anti-mouse IgG; d) Binding of normal (red) and *Schistosoma*-infected (blue) mouse serum (1:20 dilution) was detected by Alexa488 labeled goat anti-mouse IgM.

Table 1

a) The masses (Calculated and Found) for Fmoc-protected glycoamino acids and glycopeptides as 2D-HPLC fractions obtained from horseradish peroxidase, their proposed compositions and relative abundances. All the masses are sodium adducts ($[M+Na]^+$) except fractions 1 & 2, which are proton adducts ($[M+H]^+$). Green = fucosylated structures, Yellow = xylosylated structures, White = fucosylated and xylosylated structures, Blue = high mannose structures; b) The relative abundances of fucosylated, xylosylated, both fucosylated and xylosylated, and high-mannose glycan structures from HRP.

a)				
2D fraction No.	Mass (Found)	Mass (Calc.)	Proposed composition	Relative abundance (%)
1	1681.75	1681.55	Hex3HexNAc2Fuc1 Xyl1 -NR	1.8
2	1681.68	1681.55	Hex3HexNAc2Fuc1 Xyl1 -NR	0.3
3	1401.54	1401.37	Hex3HexNAc2Xyl1-N	3.0
4	1646.72	1646.49	Hex3HexNAc2Fuc1 Xyl1 -NV	0.4
5	1458.60	1458.39	Hex3HexNAc2Xyl1-GN	1.2
6	1415.59	1415.43	Hex3HexNAc2Fuc1-N	0.9
7	1401.53	1401.37	Hex3HexNAc2Xyl1-N	3.9
8	1486.65	1486.46	Hex3HexNAc2Fuc1-NA	0.4
9	1634.63	1634.46	Hex3HexNAc2Fuc1 Xyl1 -NS	5.3
10	1648.67	1648.47	Hex3HexNAc2Fuc1Xyl1-NT	6.3
11	1547.63	1547.43	Hex3HexNAc2Fuc1Xyl1-N	32.0
12	1731.74	1731.55	Hex3HexNAc2Fuc1Xyl1-LNA	6.2
13	1849.82	1849.57	Hex3HexNAc3Fuc1Xyl1-NV	0.7
14	1634.53	1634.46	Hex3HexNAc2Fuc1Xyl1-NS	3.8
15	1603.86	1604.45	Hex3HexNAc2Fuc1Xyl1-GN	0.5
16	1648.63	1648.47	Hex3HexNAc2Fuc1Xyl1-NT	8.1
17	1547.59	1547.43	Hex3HexNAc2Fuc1 Xyl1 -N	4.4
18	1731.75	1731.55	Hex3HexNAc2Fuc1Xyl1-LNA	1.8
19	1804.71	1804.56	Hex3HexNAc2Fuc1 Xyl 1 -SVNA	0.4
20	1802.74	1802.55	Hex3HexNAc2Fuc1Xyl1-SPNA	1.2
21	1675.63	1675.49	Hex3HexNAc2Fuc1Xyl1-NQ	0.7
22	1634.62	1634.46	Hex3HexNAc2Fuc1 Xyh -NS	5.7
23	1807.69	1807.53	Hex3HexNAc3Fuc1 Xyl1 -NG	1.2
24	1750.68	1750.51	Hex3HexNAc3Fuc1Xyl1-N	4.6
25	1842.63	1842.56	Hex6HexNAc2-NS	1.2
26	1912.71	1912.56	Hex4HexNAc3Fuc1 Xyl1 -N	0.9
27	2004.70	2004.61	Hex7HexNAc2-NS	2.4
28	1917.72	1917.58	Hex7HexNAc2-N	1.0

b)	
N-glycan Category	Molar Percentage(%)
Fucosylated only	1.3
Xylosylated only	8.1
Both fucosylated and xylosylated	86.1

b)	
N-glycan Category	Molar Percentage(%)
High-mannose	4.5