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Targeted glycomics by selected reaction monitoring for highly sensitive glycan compositional analysis

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Abstract

The development of glycomics increasingly requires the detection and quantification of large numbers of glycans, which is only partially achieved by current glycomics approaches. Taking advantage of selected reaction monitoring to enhance both sensitivity and selectivity, we report here a strategy termed targeted glycomics that enables highly sensitive and consistent identification and quantification of diverse glycans across multiple samples at the same time. In this proof-of-principle study, we validated the method by analyzing globally *N*-glycans expressed in different systems; single proteins, cancer cells and serum samples. A dynamic range of three orders of magnitude was obtained for the detection of all five glycans released from ribonuclease B. The limit of detection of 80 attomole for Man₉GlcNAc₂ demonstrated the excellent sensitivity of the method. The capability of the strategy to identify diverse glycans was demonstrated by identification and detection of 162 different glycans and isomers from pancreatic cancer cells. The sensitivity of the method was illustrated further by the ability to detect 8 glycans from 250 cancer cells and 5 glycans released from 100 cancer cells. In serum obtained from rabbits fed control diet or diet enriched with 2% cholesterol, differences to 42 glycans were accurately measured and this indicates that this strategy might find use in studies of biomarker discovery and validation.

Keywords

N--glycan; quantitative glycomics; glycosylation; carbohydrate; LC-MRM; LC-SRM

1 Introduction

Glycomics is a critical facet of post-genome science [1, 2] because accumulating evidence has revealed that glycans play diverse roles in cell growth and development [3, 4], tumour growth and metastasis [5, 6], anticoagulation [7], immune recognition and response [8], cell-cell communication [9], and microbial pathogenesis [10]. The development of glycomics requires large-scale analysis of the structure and function of diverse glycans, and ultimately of whole glycomes. However, global analysis of large numbers of glycans such as the complete set of glycans expressed by specific cells, tissues or organisms has faced inherent challenges including complex nontemplate-based biosynthesis of glycans, large heterogeneity and diversity of glycan structures, large dynamic range of abundance, and

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relatively low response to mass spectrometer [1, 11, 12]. Therefore, the development of glycomics requires highly sensitive techniques for the fast and consistent detection and quantification of large numbers of glycans across multiple samples, which is only partially achieved by current glycomics approaches.

Three main types of techniques have been developed for identification and quantification of glycans including fluorophore-assisted methods, microarrays, and mass spectrometry (MS)-based techniques [13-15]. However, the currently available methods have limitations in detection of complex glycan samples. Fluorophore-assisted methods require the separation of fluorescently-labeled glycans and thus are limited to analysis of samples containing small numbers of glycans. Microarrays use affinity ligands such as lectins to recognize the glycan targets and thus are limited by the availability and specificity of ligands. MS-based techniques including matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and electrospray ionization MS (ESI-MS) are powerful approaches in the study of glycomics, but each has its limitations [15, 16]. MALDI-MS has been widely used in glycan analysis due to its low cost, simplicity and ease of operation, but its application to the quantitative analysis of glycans is limited. The development in LC-MS enables separation and analysis of glycans, but it is difficult to identify glycans with low abundance because of limited sensitivity when using full scan MS as a detection method. Furthermore, *N*-glycans released by enzymes have reducing ends that are equilibrium between cyclic hemiacetals and open ring aldehydes, which can lead to peak splitting due to the separation of anomeric forms of the reducing end residue. The most common derivatization for LC-MS analysis of glycans is reductive amination. It may be used to attach a stable isotope tag and increase volatility and stability of glycans for MS analysis as well.

Recently, a targeted proteomic strategy was introduced for quantification of proteins in complex samples [17-19]. The key concept of targeted proteomics is to apply selected reaction monitoring (SRM) to detect a set of ion transitions, which can significantly enhance the signal-to-noise for the detection of low abundant components. Targeted proteomics has advantages in both specificity and sensitivity over other quantitative proteomics [20]. However, because the SRM transitions have to be selected this strategy is only applicable to the detection of those proteins that have available information on the proteotypic peptides and fragmentation profiles of the peptides. Unlike a proteome, a glycome is composed of a limited number of glycan compositions, especially when only the compositions are used to build the SRM transition for glycans. Moreover, glycans usually produce conserved fragment ions at m/z 204 and/or 366. The limited number of total glycan compositions (excluding isomeric structures) and conserved fragment ions allows one to generate assays for the global identification and quantification of glycans in complex samples. We describe here a targeted glycomics strategy enabling highly sensitive and consistent identification and quantification of large numbers of glycans across multiple samples. The strategy was demonstrated by detection and quantification of global *N*-glycans expressed in three different systems; single proteins, cancer cells and whole serum samples.

2 Materials and methods

2.1 Materials

Ribonuclease B (RNase B) was purchased from Sigma-Aldrich (Oakville, ON, Canada). Oligomannose 5 (Man-5), oligomannose 6 (Man-6), and oligomannose 9 (Man₉GlcNAc₂) standards were obtained from ProZyme (Hayward, CA). Peptide *N*-glycosidase F (PNGase F) was purchased from Roche Diagnostics (Indianapolis, IN). Aniline-2,3,4,5,6-d₅ was obtained from CDN Isotopes (Pointe-Claire, QC, Canada), while acetonitrile (ACN) and trifluoroacetic acid (TFA) were from Burdick & Jackson (Muskegon, MI). Non-porous graphitic carbon (PGC) cartridges were obtained from Alltech Associates, Inc. (Deerfield,

IL). Avicel PH-101 was purchased from Sigma-Aldrich (Oakville, ON, Canada). Blood was collected at three-week intervals over a twelve week period (5 time points) from rabbits fed either a normal diet or a diet supplemented with 2% cholesterol, and the serum was separated by centrifugation and stored at -20°C until use. Other chemicals and materials were purchased from Sigma-Aldrich (Oakville, ON, Canada), unless otherwise stated. All chemicals were of analytical reagent grade and used as received. Distilled water was deionized on a Millipore Milli-Q water reagent system (Billerica, MA).

2.2 Enzymatic release of *N*-glycans

N-glycans were prepared from RNase B and rabbit sera using similar procedures as described previously [21]. Briefly, 50 μg of RNase B or 10 μL of serum samples was dissolved in 90 μL of 50 mM sodium phosphate (pH 7.5) containing 0.1% sodium dodecyl sulfate (SDS) and 40 mM dithiothreitol (DTT). The samples were heated at 100°C for 5 min. After cooling, 10 μL of 10% NP-40 was added. After mixing the samples for 30 s and standing at room temperature for 5 min, 10 units of PNGase F were added, followed by incubation at 37°C for 24 h.

The cells used in this study were human pancreatic cancer cell line BxPC3 obtained from the American Type Culture Collection, ATCC (Manassas, VA), and cultured in RPMI medium (Invitrogen, Carlsbad, CA). Foetal bovine serum (Roche, Mississauga, ON) at 10% of the final volume was added to the medium beforehand. Cells were grown at 37°C , 5% CO_2 in 75 cm^2 flask (BD Falcon, Mississauga, ON). Cells were cultured until they were approximately 80% confluent and harvested by using 0.25% Trypsin-EDTA (Invitrogen, Carlsbad, CA). To prepare *N*-glycans from pancreatic cancer cells, the cancer cells were washed five times with 1 $\times$ PBS buffer. Briefly, approximate 0.5 million pancreatic cancer cells in 1.0 mL medium were spun at $1,000 \times g$ for 5 min. The cell pellet was first washed four times with 1.0 mL cold 1 $\times$ PBS, pelleted by centrifugation at $1,000 \times g$ for 5 min each. The cell debris was then washed with the same buffer, but centrifuged at $13,000 \times g$ for 2 min. Cells were lysed using traditional freeze/thaw techniques. The cell pellet was resuspended in 0.5 mL of 50 mM ammonium bicarbonate (pH 7.8) and placed in a dry ice/ethanol bath for freezing and then thawing the materials at room temperature. This freeze/thaw process was repeated three times. The lysated solution was centrifuged at $13,000 \times g$ for 10 min and the pellet was then resuspended in 20 μL of 50 mM ammonium bicarbonate (pH 7.8). In order to enhance release of *N*-glycans from membrane proteins, cancer cells were first subjected to trypsin digestion. The digestion was carried out with a 1:20 protease-to-protein ratio (by weight) and the solution was subjected to microwave-assisted digestion at 58°C for 30 min at the power of 50 W using CEM Discover® System (CEM, Matthews, NC). The temperature in the sample was controlled with a fiber-optics-based temperature probe located in a reference solution. The *N*-glycans were then released using PNGase F digestion.

2.3 *N*-glycan purification

PNGase F-released glycans were purified by using a non-PGC cartridge as described previously [22, 23]. 500 μL of H_2O was added into each PNGase F-digested sample. After sonication for 20 min, the samples were centrifuged at $15,000 \times g$ for 10 min. PGC cartridges were conditioned using 3.0 mL of 80% ACN with 0.1% TFA, followed by 3.0 mL of water. The supernatant of sample solution was loaded, and subsequently washed with 3.0 mL of water three times to remove salts. Glycans were eluted with 1.5 mL of 25% ACN with 0.1% TFA. The fraction was dried in a centrifugal evaporator apparatus.

2.4 Derivatization of *N*-glycans with aniline or aniline-2,3,4,5,6-d₅

25 μ L of aniline or aniline-2,3,4,5,6-d₅ and 25 μ L of 1 M sodium cyanoborohydride, freshly prepared in dimethyl sulfoxide (DMSO) : acetic acid (7:3, v/v), were added to the dried glycans. The reaction mixture was incubated in a closed tube for 3 h in a heated oven at 65 °C, then 400 μ L of 1-butanol/ethanol/H₂O (4:1:1, v/v) was added to the reaction mixture and aniline-labeled glycans were purified using a cellulose column [24]. The cellulose column was prepared by loading 30 mg cellulose (Avicel PH-101) into an 1 mL filtration tube. Prior to purification, the column was conditioned by using 1 mL of water for 6 times and 1 mL of ethanol : H₂O (50:50, v/v) for 3 times. After being dried under vacuum, the column was rinsed with 1 mL of 1-butanol/ethanol/H₂O (4:1:1, v/v). The reaction mixture was loaded onto the column and washed 5-times with 1 mL of 1-butanol/ethanol/H₂O (4:1:1, v/v). Labeled glycans were eluted by 3 mL of ethanol : H₂O (50:50, v/v), and dried for further analysis.

2.5 Construction of SRM transitions

The choice of the transitions is critical for the success of targeted glycomics. We select the glycan sets based on the previous experiments, scientific literature and/or internet resources, such as the CFG (Consortium of Functional Glycomics) glycan database or the KEGG (Kyoto Encyclopedia of Genes and Genomes) glycan database. At this stage, only the composition is used to define each glycan transition and the differentiation of glycan isomers relies on their LC retention times.

It is well-known that multiple charged ions can be produced in ESI-MS for an analyte. We found that the charge state of the most intense ion for each glycan depends on their molecular weights. The charge state of the most intense ion for the glycans with different sizes was experimentally determined as follows; singly charged ions for the glycans with molecular weights less than (1300 ~ 1600 Da), doubly charged ions for the glycans (1300 ~ 1600 Da) - (2400 ~ 2800 Da), triply charged ions for the glycans (2400 ~ 2800 Da) - (3800 ~ 4400 Da), and quadruply charged ions for the glycans with molecular weights of >4400 Da. Glycans whose molecular weights lie in the range of each bracket were capable of carrying both lower and higher charges. For the aniline-labeled glycans, the m/z values of parent ions were calculated according to the formulation: $m/z = (\text{molecular weight of the glycan} + 77.13 \text{ or } 82.13 + n)/n$, where n is the charge state. The addition of 77.13 or 82.13 is due to derivatization of *N*-glycans with aniline or aniline-d₅. The tandem mass spectrometry experiments revealed that aniline-labeled *N*-glycans produced three conserved fragment ions, ions at m/z 299, 366 and 502 for aniline-labeled *N*-glycans; ions at m/z 304, 366 and 507 for the *N*-glycans labeled with aniline-d₅. Therefore, ions at m/z 299, 366 and 502 for aniline-labeled *N*-glycans and ions m/z 304, 366 and 507 for the *N*-glycans labeled with aniline-d₅ were used as Q3 transitions for the analysis of aniline-labeled and aniline-d₅ labeled glycans, respectively. Ideally, all possible *N*-glycans that could be present in pancreatic cancer cells are expected for generation of the SRM transition. However, because the exact number of glycoforms expressed by pancreatic cancer cells have not been reported, we generated the glycan set for the pancreatic cancer cells by including all *N*-glycan compositions that have been reported for human samples. By searching CFG glycan database, we obtained a total of 187 *N*-glycan compositions from 3406 different *N*-glycan structures that have ever been detected in human samples. The human samples refer to all samples pertinent to human, including serum, plasma, different tissues (*i. e.* liver, kidney, spleen, and colon.), milk, urine, and various cells. Among these 187 *N*-glycan compositions, 15 pairs have mass difference of 1 Da, which makes it difficult to differentiate them, because the most abundant ion of a glycan is often multiply charged in ESI processing. Therefore, only one glycan composition was selected to construct the SRM transitions for these 15 pairs, or a total of 172 glycan compositions were used to determine Q1 transitions

for human samples. The *N*-glycans that were enzymatically released from cancer cells were split into two aliquots and derivatized with aniline and aniline- d_5 , respectively. Q1 transition of each glycan composition was calculated based on molecular weight of glycan after aniline or aniline- d_5 labeling and its charge states. The glycan set for serum samples was established in combination of the CFG glycan database and a previous paper [25]. A total of 62 compositions were chosen for construction of SRM transitions for the analysis of rabbit sera.

2.6 HPLC-MS/MS analysis

Samples were analyzed using a 4000 Q-Trap (Applied Biosystems/MDS Sciex, Concord, ON, Canada) coupled to an UltiMate 3000 HPLC (Dionex, Sunnyvale, CA). Chromatographic separations were performed on a Hypercarb PGC column (0.32×100 mm; particle size, $5\mu\text{m}$; pore size, $250\text{\AA}$) (Thermo Scientific, San Jose, CA) at a flow rate of $6\mu\text{L min}^{-1}$ with a gradient as follows: 15% B for 5 min, increasing to 70% B over 35 min, then increasing to 90% B over 5 min, and held at 90% B for 5 min. Mobile phase A consisted of 0.1% formic acid in water (v/v). Mobile phase B consisted of 0.1% formic acid in 90% ACN in water (v/v).

The SRM acquisition was in positive-ion mode with Q1 and Q3 at unit resolution. The dwell time was determined based on the concentration of glycans in the samples, usually from 10 to 100 ms per transition. Collision energies were set according to m/z values of precursor ions, typically 45 V for m/z 750, 55 V for m/z 750~950 and 65 V for m/z 950. Other settings: spray voltage, 5500 V; curtain gas, 10 units; collision gas, 8 units; ion source gas flow rates 1 and 2 at 5 and 0 units, respectively; declustering potential, 50 V; and temperature 50°C .

3 Results

3.1 Generation of SRM assays

A workflow describing the process of the targeted glycomics for identification and quantification of glycans is shown in Figure 1. This strategy consists of the following stages; preparation of glycans, derivatization of glycans, selection of a glycan set, determination of SRM transitions, identification of glycans by LC-SRM, further validation of glycan identified, and quantification of glycans.

The glycans were prepared from glycoproteins by enzymatic approaches and subsequent purification using PGC cartridges. After purification, the glycans were isotopically labeled with aniline or aniline- d_5 through reductive amination, because it is robust, easy to implement and quantitate [26, 27].

Depending on the specific sample type, a targeted glycan set containing different glycans was selected for developing SRM transitions. There are two major issues that hinder the construction of specific SRM transitions for large numbers of glycans, the presence of diverse isomers and availability of MS/MS data of glycans. The complexity of glycomes is greatly increased by the presence of diverse isomers and highly branched structures. Meanwhile, the technical challenges in separation and identification of glycan isomers resulted in the limitation of the availability of MS/MS data for each specific glycan. Furthermore, the selection of fragment ions only specific to an individual glycan isomer might be difficult, sometimes impossible even when MS/MS spectra are available. To circumvent these difficulties, we adopted two approaches to the generation of SRM transitions for large numbers of glycans. First, we utilized glycan compositions to define Q1 transitions of SRM. Typically, the parent ions or Q1 transitions (m/z) were selected based on the molecular weights of the glycan compositions of interest and their corresponding charge states. Second, we chose three conserved fragment ions that are suitable for all glycan

composition structures as Q3 transitions. The limited number of total glycan compositions and conserved fragment ions enable opportunities for the global identification and quantification of glycans in complex samples.

Although it is impossible to differentiate structural isomers with these SRM transitions, the limitations were overcome by coupling a LC system, e.g. graphitized carbon chromatography, with MS/MS. For example, the appearance of multiple peaks with different retention times but the same SRM transition indicates the existence of isomeric glycan structures and the retention times can be then used to identify these isomers. Furthermore, SRM-triggered MS/MS experiments were conducted to acquire additional fragmentation information of the glycan isomers that were separated by graphitized carbon LC.

3.2 Analysis of *N*-glycans released from RNase B

To demonstrate the proof of principle, we first applied the strategy to the analysis of *N*-glycans released from RNase B. Aniline was chosen as the derivative reagent because stable isotopic aniline is inexpensive and available [28]. Most importantly however, aniline-derivatization offers good detection sensitivity and superior separation resolution. Among the reagents that have been used for *N*-glycan derivatization, it has been reported that procaine-derivatization led to a 1000-fold increase in sensitivity of glycan detection [29]. Our results showed aniline and procaine labeled glycans yielded similar sensitivity upon the detection by LC-SRM. Furthermore, aniline derivatization resulted in much better separation than that of procaine derivatization (Figure 2, Figure S1).

Tandem mass spectrometry experiments were performed on light/heavy isotope-labeled *N*-glycans. The MS/MS spectra for high mannose type *N*-glycan were shown in Figure 2. As expected, the aniline-labeled high mannose structures gave rise to a series of isotope-tagged fragment ions, *i.e.* m/z 299.0, 502.2, 664.2, 826.1, 988.0, and non isotope-tagged fragment ions, *i.e.* m/z 366.0, 528.1, 690.1, 852.1, 1014.2, respectively (Figure 2a). In Figure 2b, the non isotope-tagged fragment ions show the same m/z as that in Figure 2a; whereas the isotope-tagged fragment ions show an increase of 5 Da for each fragment ion, respectively. Similar fragmentation patterns were observed for the sialylated and non-sialylated complex glycoforms (Figure S2a, S2b, S2c and S2d). The conserved fragment ions (m/z 299, 366, and 502 for aniline labeled glycans; m/z 304, 366, and 507 for aniline- d_5 labeled glycans) were used as Q3 transitions. The use of these conserved fragment ions as Q3 transitions avoids the predetermining fragmentation profiles of glycans for analysis, which is useful for the fact that the availability of fragmentation profiles for the large number of glycans is limited, especially for glycan isomers. In addition, these three fragment ions usually show relatively high intensity under appropriate MS conditions, which ensures the high sensitivity of LC-SRM analysis. Table S1 listed SRM transitions for identification and detection of glycan compositions and their isomers from RNase B.

Figure 3 shows the chromatograms for the analysis of *N*-glycans released from RNase B. The total 17 peaks were observed in five extraction ion chromatograms, which suggested 17 high mannose isomers were separated and detected. To confirm the detection of these isomers, MS/MS experiments were conducted for Man₅₋₉GlcNAc₂. The extracted MS/MS spectra revealed that all isomers of each glycan produced similar fragment ions. To further validate the results, we analysed commercially available standards, Man-5 and Man-6, which were obtained from porcine thyroglobulin (Figure S3). The LC-SRM analysis of Man-5 indicated the presence of three isomers showing the same retention times as that of Man-5 released from RNase B. Similarly, Man-6 standard showed four isomer peaks, three of which had comparable retention times to that of the isomers of Man₆ from RNase B. In addition, our results on Man_{5, 7, 8} GlcNAc₂ isomers are well supported by two recent studies on isomer characterization of these three glycans [30, 31], although other comprehensive

characterization methods might be needed for further validation. The ability of this strategy to separate and detect 17 isomers for high mannose type glycans is a result of the enhanced separation performance that resulted from the aniline-derivatization and the increased sensitivity due to the SRM assay. It is worth mentioning that the declustering potential (potential between the orifice plate and the skimmer) should be optimized as such high enough to reduce the chemical noise but low enough to avoid in-source fragmentation. A declustering potential of 50 V was used for this study.

The effect of using internal standards on the reproducibility of LC-SRM analysis was examined by analysing 6 replicates of the mixture of aniline labeled and aniline-d₅ labeled RNase B glycans. For peak areas of aniline or aniline-d₅ labeled glycans the coefficients of variation (CV) varied from 9.5 to 20.5%. In contrast, the CV was reduced to less than 5% when peak area ratios of aniline to aniline-d₅ labeled glycans were used for data analysis. The linearity of detection was determined using serial dilutions of the *N*-glycans released from 100 ng RNase B and it was found that there was a linear dynamic range over 3 orders of magnitude (up to 1000-fold dilution) for the isomers with the highest intensity. Since the absolute amounts of glycans released from RNase B were unknown, a Man₉GlcNAc₂ standard with known amount was used to examine the sensitivity of the method. When 5 µL of 1.6×10^{-11} M aniline-labeled Man₉GlcNAc₂ was injected, a peak corresponding to Man₉GlcNAc₂ was obtained with an intensity approximately equal to three times of noise. It suggested that the limit of detection is 80 attomole for analysis of Man₉GlcNAc₂ (Figure 4).

3.3 Analysis of *N*-glycans expressed in pancreatic cancer cells

In order to demonstrate that targeted glycomics is capable of identifying a wide range of glycans in a specific sample, we applied the method to pancreatic cancer cells. A glycan is considered to be present in the sample, only if the extracted ion chromatograms of all corresponding ion transitions for the same glycan showed peaks with the same retention time for both aniline and aniline-d₅ labeled glycans. A total of 98 different glycan compositions were identified and the number of identified glycans increased to 162 after including glycan isomers (Table 1). Although assignment of glycan isomers is challenging due to lack of isomer standards, a retention time library of glycan isomers could be generated by using the relative retention time of each isomers compared to that of the internal standard [32]. Because conserved fragment ions were used as Q3 transitions for all glycans, the selectivity of SRM is mainly dependant on the resolution of Q1. If two glycans have only 1 Da in their mass difference, the resolution of Q1 is insufficient to completely differentiate these glycan compositions. In pancreatic cancer cell line, we detected 10 glycans (noted in Table 1) from such 15 pairs of glycan compositions with 1 Da in mass difference. The accurate assignment of them requires additional experimental data, such as MS/MS spectra of glycan standards and/or retention times of glycan standards. The data indicated that the high mannose glycans (Man₅₋₉GlcNAc₂) presented with significantly higher ion intensities than the other types of glycans from pancreatic cancer cells. Although no reports on the global analysis of *N*-glycans from pancreatic cancer cells are available to allow for direct comparisons, our findings are in agreement with the results from the glycomic studies of four different types of cancer cells; histocytic lymphoma cells [33], human colon carcinoma cells [34], human breast MCF-7 carcinoma, and T-lymphoblastoid CEM cells [35]. The targeted glycomics by SRM was sensitive enough to enable us to detect 8 glycans released from 250 cells and even 5 glycans from 100 cells (Figure 5). It should be noted that the starting cell number for PNGase F digestion was 1,000 for the sensitive study and the certain volume of glycan samples corresponding to 250 or 100 cells was injected into chromatographic column for LC-SRM analysis.

3.4 Analysis of *N*-glycans released from rabbit serum samples

We further examined the targeted glycomics strategy with the analysis of *N*-glycans released from serum samples. Serum samples were collected at three-week intervals over a twelve-week period (5 time points) from rabbits fed either a normal diet (5 rabbits in control group) or a diet supplemented with 2% cholesterol (5 rabbits in cholesterol-treated group). Based on the CFG glycan database and a previously published paper [25], 62 different glycan compositions were targeted in this study (Table S3). We first analyzed *N*-glycans released from pooled serum samples to identify the presence of glycans. The pooled serum sample was prepared by mixing equal amounts of serum from 5 control and 5 cholesterol-treated rabbits. A total of 42 different compositions and 92 different *N*-glycans including isomers were detected (Table 2). The *N*-glycans from the pooled serum sample were then permethylated and analyzed by using MALDI-TOF for validation (Figure S4a). The results confirmed the identification of glycan compositions, although it did not identify the glycan isomers. In addition, we also compared the distributions of *N*-glycans between rabbit serum and human serum (Figure S4b). As expected, the glycans from rabbit serum have a distribution similar to that of human serum, with Hex₅NeuAc₂HexNAc₄ showing the highest relative intensity.

We then used the aniline-d₅ derivatives of the glycans from pooled serum as isotope internal standards to quantify the 42 glycan compositions in each serum sample. The isotope-labeled internal standards were mixed with each sample prior to LC-SRM analysis. However, this spiking strategy while improving the reproducibility of LC-SRM analysis could not reduce the variability in glycan preparation and derivatization. Thus, we used maltoheptaose as an additional internal standard by adding 25 ng maltoheptaose into 10 μL of a serum sample before PNGase F digestion. Therefore, the relative amount of each glycan can be calculated based on the following equation:

$$\text{Relative amount} = \frac{A_s}{A_{IS}} \times \frac{A_{MI}}{A_{MS}}$$

A_s is the peak area of a glycan in a sample (labeled with aniline); A_{IS} is the peak area of the corresponding internal standard (labeled with aniline-d₅); A_{MI} is the peak area of aniline-labeled maltoheptaose; and A_{MS} is the peak area of aniline-d₅-labeled maltoheptaose.

To examine its reproducibility for analysis of large numbers of glycans, one serum sample was analyzed three times. The CVs ranged from 2.1% to 15.0% with a mean value of 6.5%, good reproducibility especially for analysis of complex samples. Replicate analyses of 42 glycans in a serum sample yielded a correlation coefficient of 0.97 (Figure 6). Figure 7 shows results of quantification of 42 glycans in all 10 serum samples by monitoring the ratio of its peak intensity to that of the corresponding isotope internal standard peak rather than the relative intensity to the maximum peak intensity. The influence of a diet enriched with 2% cholesterol on the abundance of some specific glycans could be clearly observed, including Hex₃HexNAc₄, Hex₃dHex₁HexNAc₄, Hex₃dHex₁HexNAc₅, and Hex₄dHex₁HexNAc₅. The amounts of both Hex₃HexNAc₄ and Hex₃dHex₁HexNAc₄ were relatively constant for serum samples collected from the control rabbits, but their amounts increased with longer ingestion periods of the 2% cholesterol diet (Fig. 7, group 7 and 9). While the amounts of both Hex₃dHex₁HexNAc₅, and Hex₄dHex₁HexNAc₅ in the serum samples from the control rabbits, collected from week 0 and week 3, were significantly higher than those in week 6, week 9 and week 12, their amounts remained relatively constant or increased in the samples collected from the treated rabbit during the course of treatment (Fig. 7 group 16 and 22). In addition, three of four altered glycans contained

fucose which might imply that the 2% cholesterol treatment has a larger impact on the abundance of fucose glycans. Although the sample number is not sufficient for us to draw the final conclusion on the effect of treatment of 2% cholesterol on the rabbit serum glycome, the results demonstrated the capacity of targeted glycomics to determine the profile change of large number of glycans in complex samples. Actually it was recently discovered that a calorically restricted diet could affect expression of two fucosylated *N*-glycans of mouse serum glycoproteins, caused by the increased expression and activity of α -1,6-fucosyltransferase (FUT8) in the mice liver [36].

4 Discussion

Here, we developed a targeted glycomics strategy to fulfill the requirement of large-scale analysis of diverse glycans. Targeted glycomics integrates technical advances in glycan derivatization, glycan separation by graphitized carbon chromatography and SRM. After being enzymatically released from glycoproteins, free glycans were derivatized by reductive amination using aniline or aniline- d_5 . The introduction of isotopic label into glycans generates the isotopically labeled internal standards that can minimize the variability of sample preparation and LC-SRM analysis.

Taking advantages of SRM in sensitivity and specificity, targeted glycomics is able to identify and quantify *N*-glycans. The identification of global glycan expression in a specific biological sample was achieved based on the generation of a glycan set that contains all possible glycan compositions. The sensitivity of the method was demonstrated by the detection of Man₉GlcNAc₂ at attomole levels and the glycans released from as few as 100 cells. Because hundreds of ion transitions can be monitored simultaneously in a single SRM experiment, targeted glycomics have the ability to detect large numbers of glycans in a single analysis. The potential of the method was advanced by coupling graphitized carbon chromatography to SRM, because glycan isomers could be separated prior to MS analysis. Therefore, targeted glycomics circumvents shortcomings of previous glycomics approaches in sensitivity and throughput, enabling highly sensitive identification and quantification of large numbers of glycans.

Although the use of conserved fragment ions as Q3 transitions avoids the requirement of fragmentation profiles of glycans of interest and then improves the capability of the method to cover more glycans and glycan isomers, it, on the other hand, reduces the selectivity of the method compared to the use of specific fragment ions as Q3 transitions. Therefore, the use of LC-SRM under the current strategy is difficult to differentiate glycan compositions with mass difference in 1 Da.

Targeted glycomics is not limited to analysis of *N*-glycans, and it can also be applied to analysis of *O*-glycans, lipid-linked glycans, or other specific types of glycans, such as sialylated glycans, sulfated glycans and acetated glycans. Targeted glycomics shows a promising perspective in quantitative analysis of diverse glycans expressed in various systems, thereby boosting the development of glycomics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Raman R, Raguram S, Venkataraman G, Paulson JC, et al. Glycomics: an integrated systems approach to structure-function relationships of glycans. *Nat Methods*. 2005; 2:817–824. [PubMed: 16278650]
2. Shriver Z, Raguram S, Sasisekharan R. Glycomics: a pathway to a class of new and improved therapeutics. *Nat Rev Drug Discov*. 2004; 3:863–873. [PubMed: 15459677]
3. Inatani M, Irie F, Plump AS, Tessier-Lavigne M, et al. Mammalian brain morphogenesis and midline axon guidance require heparan sulfate. *Science*. 2003; 302:1044–1046. [PubMed: 14605369]
4. Haltiwanger RS, Lowe JB. Role of glycosylation in development. *Annu Rev Biochem*. 2004; 73:491–537. [PubMed: 15189151]
5. Sasisekharan R, Shriver Z, Venkataraman G, Narayanasami U. Roles of heparan-sulphate glycosaminoglycans in cancer. *Nat Rev Cancer*. 2002; 2:521–528. [PubMed: 12094238]
6. Dube DH, Bertozzi CR. Glycans in cancer and inflammation--potential for therapeutics and diagnostics. *Nat Rev Drug Discov*. 2005; 4:477–488. [PubMed: 15931257]
7. Casu B, Guerrini M, Torri G. Structural and conformational aspects of the anticoagulant and anti-thrombotic activity of heparin and dermatan sulfate. *Curr Pharm Des*. 2004; 10:939–949. [PubMed: 15078125]
8. Rudd PM, Elliott T, Cresswell P, Wilson IA, et al. Glycosylation and the immune system. *Science*. 2001; 291:2370–2376. [PubMed: 11269318]
9. Parker RB, Kohler JJ. Regulation of intracellular signaling by extracellular glycan remodeling. *ACS Chem Biol*. 2010; 5:35–46. [PubMed: 19968325]
10. Miller SI, Ernst RK, Bader MW. LPS, TLR4 and infectious disease diversity. *Nat Rev Microbiol*. 2005; 3:36–46. [PubMed: 15608698]
11. Krishnamoorthy L, Mahal LK. Glycomic analysis: an array of technologies. *ACS Chem Biol*. 2009; 4:715–732. [PubMed: 19728746]
12. Turnbull JE, Field RA. Emerging glycomics technologies. *Nat Chem Biol*. 2007; 3:74–77. [PubMed: 17235338]
13. Domann PJ, Pardos-Pardos AC, Fernandes DL, Spencer DI, et al. Separation-based glycoproteomics approaches using fluorescent labels. *Proteomics*. 2007; (1):70–76. [PubMed: 17893855]
14. Oyelaran O, Gildersleeve JC. Glycan arrays: recent advances and future challenges. *Curr Opin Chem Biol*. 2009; 13:406–413. [PubMed: 19625207]
15. North SJ, Hitchen PG, Haslam SM, Dell A. Mass spectrometry in the analysis of N-linked and O-linked glycans. *Curr Opin Struct Biol*. 2009; 19:498–506. [PubMed: 19577919]
16. Mechref Y, Novotny MV. Structural investigations of glycoconjugates at high sensitivity. *Chem Rev*. 2002; 102:321–369. [PubMed: 11841246]
17. Domon B, Aebersold R. Options and considerations when selecting a quantitative proteomics strategy. *Nat Biotechnol*. 2010; 28:710–721. [PubMed: 20622845]
18. Gerber SA, Rush J, Stemman O, Kirschner MW, Gygi SP. Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. *Proc Natl Acad Sci USA*. 2003; 100:6940–6945. [PubMed: 12771378]
19. Anderson L, Hunter CL. Quantitative mass spectrometric multiple reaction monitoring assays for major plasma proteins. *Mol Cell Proteomics*. 2006; 5:573–588. [PubMed: 16332733]
20. Lange V, Picotti P, Domon B, Aebersold R. Selected reaction monitoring for quantitative proteomics: a tutorial. *Mol Syst Biol*. 2008; 4:222. [PubMed: 18854821]
21. Liu X, Afonso L, Altman E, Johnson S, et al. O-acetylation of sialic acids in N-glycans of Atlantic salmon (*Salmo salar*) serum is altered by handling stress. *Proteomics*. 2008; 8:2849–2857. [PubMed: 18655054]
22. Packer NH, Lawson MA, Jardine DR, Redmond JW. A general approach to desalting oligosaccharides released from glycoproteins. *Glycoconj J*. 1998; 15:737–747. [PubMed: 9870349]

23. Kirmiz C, Li B, An HJ, Clowers BH, et al. A serum glycomics approach to breast cancer biomarkers. *Mol Cell Proteomics*. 2007; 6:43–55. [PubMed: 16847285]
24. Shimizu Y, Nakata M, Kuroda Y, Tsutsumi F, et al. Rapid and simple preparation of N-linked oligosaccharides by cellulose-column chromatography. *Carbohydr Res*. 2001; 332:381–388. [PubMed: 11438095]
25. Furukawa J, Shinohara Y, Kuramoto H, Miura Y, et al. Comprehensive approach to structural and functional glycomics based on chemoselective glycoblotting and sequential tag conversion. *Anal Chem*. 2008; 80:1094–1101. [PubMed: 18205388]
26. Xia B, Kawar ZS, Ju T, Alvarez RA, et al. Versatile fluorescent derivatization of glycans for glycomic analysis. *Nat Methods*. 2005; 2:845–850. [PubMed: 16278655]
27. Pabst M, Kolarich D, Pörtl G, Dalik T, et al. Comparison of fluorescent labels for oligosaccharides and introduction of a new postlabeling purification method. *Anal Biochem*. 2009; 384:263–273. [PubMed: 18940176]
28. Xia B, Feasley CL, Sachdev GP, Smith DF, et al. Glycan reductive isotope labeling for quantitative glycomics. *Anal Biochem*. 2009; 387:162–170. [PubMed: 19454239]
29. Yoshino K, Takao T, Murata H, Shimonishi Y. Use of the derivatizing agent 4-aminobenzoic acid 2-(diethylamino)ethyl ester for high-sensitivity detection of oligosaccharides by electrospray ionization mass spectrometry. *Anal Chem*. 1995; 67:4028–4031. [PubMed: 8633763]
30. Prien JM, Ashline DJ, Lapadula AJ, Zhang H, et al. The high mannose glycans from bovine ribonuclease B isomer characterization by ion trap MS. *J Am Soc Mass Spectrom*. 2009; 20:539–556. [PubMed: 19181540]
31. Prien JM, Prater BD, Cockrill SL. A multi-method approach toward de novo glycan characterization: a Man-5 case study. *Glycobiology*. 2010; 20:629–647. [PubMed: 20110246]
32. Pabst M, Bondili JS, Stadlmann J, Mach L, et al. Mass + retention time = structure: a strategy for the analysis of N-glycans by carbon LC-ESI-MS and its application to fibrin N-glycans. *Anal Chem*. 2007; 79:5051–5057. [PubMed: 17539604]
33. Naka R, Kamoda S, Ishizuka A, Kinoshita M, et al. Analysis of total N-glycans in cell membrane fractions of cancer cells using a combination of serotonin affinity chromatography and normal phase chromatography. *J Proteome Res*. 2006; 5:88–97. [PubMed: 16396498]
34. Vercoutter-Edouart AS, Slomianny MC, Dekeyser-Beseme O, Haeuw JF, et al. Glycoproteomics and glycomics investigation of membrane N-glycosylproteins from human colon carcinoma cells. *Proteomics*. 2008; 8:3236–3256. [PubMed: 18651673]
35. Lattová E, Tomanek B, Bartusik D, Perreault H. N-glycomic changes in human breast carcinoma MCF-7 and T-lymphoblastoid cells after treatment with herceptin and herceptin/Lipoplex. *J Proteome Res*. 2010; 9:1533–1540. [PubMed: 20063903]
36. Vanhooren V, Dewaele S, Kuro-O M, Taniguchi N, et al. Alteration in N-glycomics during mouse aging: a role for FUT8. *Aging Cell*. 2011; 10:1056–1066. [PubMed: 21951615]

Abbreviations

Neu5Ac	N-acetylneuraminic acid
Fuc	fucose
Hex	hexose
HexNAc	N-acetylhexosamine
PNGase F	Peptide-N-Glycosidase F

Figure 1.

Outline of targeted glycomics by LC-SRM workflow. Targeted glycomics is composed of six stages, including preparation of glycans, derivatization of glycans, selection of a glycan set, determination of SRM transitions, identification of glycans by LC-SRM, further validation of glycan identified, and quantification of glycans.

Figure 2.

The MS/MS spectra of Man₅GlcNAc₂ labeled with aniline (a) or aniline-d₅ (b). The total *N*-glycans released from 50 μg RNase B were split into two aliquots for aniline or aniline-d₅ derivatization.

Figure 3.
The extracted ion chromatograms of LC-SRM showing separation and detection of high mannose glycans ($\text{Man}_{5-9}\text{GlcNAc}_2$) released from 50 μg RNase B.

Figure 4.

The extracted ion chromatogram demonstrating the limit of detection of 80 attomole on column for targeted glycomics analysis of Man₉GlcNAc₂. The concentration of Man₉GlcNAc₂ was 1.6×10^{-11} M, and injection volume was 5 μ L.

Figure 5.

The extracted ion chromatograms of the glycans from 250 cancer cells (a) and 100 cancer cells (b). Peak 1: Hex₈HexNAc₂; Peak 2: Hex₉HexNAc₂; Peak 3: Hex₇HexNAc₂; Peak 4: Hex₆HexNAc₂; Peak 5: Hex₅dHex₁HexNAc₄; Peak 6: Hex₅HexNAc₂; Peak 7: Hex₅NeuAc₁HexNAc₄; Peak 8: Hex₅dHex₁NeuAc₁HexNAc₄.

Figure 6.

Plot of relative amounts of 42 glycans between the replicate analyses of the same sample. The replicate analyses were conducted by using four aliquots of 10 μ L serum sample. Four aliquots were divided into two pairs and each pair was subjected to enzymatic release of N-glycans, derivatization of N-glycans with aniline or aniline- d_5 , and LC-SRM analysis. The relative amounts of 42 glycans from one analysis were plotted versus corresponding relative amounts of those glycans from the other analysis.

Figure 7.

Bar graphs of the ion intensity ratios of 42 glycan compositions to their corresponding isotope internal standards. Rabbits (n=5) in control group were fed with a normal diet, and rabbits (n=5) in treated group were fed with a diet supplemented with 2% cholesterol. T-1 to T-5 and C-1 to C-5 correspond to sera collected every three weeks up to 12 weeks from the same rabbit. The glycan compositions were summarized in Table 2. Triplicate analyses were conducted for each serum sample and standard deviation was indicated by the error bar. The glycans with changes in abundance upon the treatment of 2% cholesterol diet were highlighted by circles, including Hex₃HexNAc₄, Hex₃dHex₁HexNAc₄, Hex₃dHex₁HexNAc₅ and Hex₄dHex₁HexNAc₅.

Table 1
Identification and detection of *N*-glycans and isomers from pancreatic cancer cells

Composition	MW	Aniline labeled	Charge state	Isomer number
Hex ₃ HexNAc ₂	910.82	987.95	1	1
Hex ₄ HexNAc ₂	1072.96	1150.09	1	2
Hex ₅ HexNAc ₂	1235.10	1312.23	1	3
Hex ₃ dHex ₁ HexNAc ₃	1260.15	1337.28	1	2
Hex ₄ HexNAc ₃	1276.15	1353.28	1	2
Hex ₃ HexNAc ₄	1317.21	1394.34	1	1
Hex ₅ dHex ₁ HexNAc ₂	1381.24	1458.37	2	2
Hex ₆ HexNAc ₂	1397.24	1474.37	2	3
Hex ₅ HexNAc ₃	1438.29	1515.42	2	2
Hex ₃ dHex ₁ HexNAc ₄	1463.35	1540.48	2	1
Hex ₄ HexNAc ₄	1479.35	1556.48	2	3
Hex ₇ HexNAc ₂	1559.38	1636.51	2	2
Hex ₅ dHex ₁ HexNAc ₃	1584.44	1661.57	2	2
Hex ₆ HexNAc ₃	1600.44	1677.57	2	1
Hex ₄ dHex ₁ HexNAc ₄	1625.49	1702.62	2	1
Hex ₅ HexNAc ₄	1641.49	1718.62	2	1
Hex ₃ dHex ₁ HexNAc ₅	1666.54	1743.67	2	1
Hex ₄ dHex ₁ NeuAc ₁ HexNAc ₃ , or Hex ₄ dHex ₃ HexNAc ₃ *	1713.55	1790.68	2	3
	1714.58	1791.71		
Hex ₈ HexNAc ₂	1721.53	1798.66	2	2
Hex ₃ HexNAc ₆	1723.60	1800.73	2	2
Hex ₆ dHex ₁ HexNAc ₃	1746.58	1823.71	2	2
Hex ₃ dHex ₁ NeuAc ₁ HexNAc ₄	1754.61	1831.74	2	2
Hex ₄ NeuAc ₁ HexNAc ₄ , or Hex ₄ dHex ₂ HexNAc ₄ *	1770.61	1847.74	2	2
	1771.63	1848.76		
Hex ₅ dHex ₁ HexNAc ₄	1787.63	1864.76	2	2
Hex ₄ dHex ₁ HexNAc ₅	1828.68	1905.81	2	2
Hex ₅ HexNAc ₅	1844.68	1921.81	2	2
Hex ₃ dHex ₁ HexNAc ₆	1869.74	1946.87	2	2
Hex ₅ dHex ₃ HexNAc ₃	1876.72	1953.85	2	1
Hex ₉ HexNAc ₂	1883.67	1960.80	2	1
Hex ₄ HexNAc ₆	1885.74	1962.87	2	1
Hex ₆ NeuAc ₁ HexNAc ₃ , or Hex ₆ dHex ₂ HexNAc ₃ *	1891.69	1973.82	2	3
	1892.72	1974.85		
Hex ₃ NeuAc ₂ HexNAc ₄	1899.72	1976.85	2	2
Hex ₄ dHex ₁ NeuAc ₁ HexNAc ₄	1916.75	1993.88	2	2
Hex ₃ HexNAc ₇	1926.79	2003.92	2	2

Composition	MW	Aniline labeled	Charge state	Isomer number
Hex ₅ NeuAc ₁ HexNAc ₄ , or Hex ₅ dHex ₂ HexNAc ₄ [*]	1932.75	2009.88	3	3
	1933.77	2010.90		
Hex ₆ dHex ₁ HexNAc ₄	1949.77	2026.90	2	2
Hex ₆ HexNAc ₅	2006.83	2083.96	2	2
Hex ₃ dHex ₂ HexNAc ₆	2015.88	2093.01	2	2
Hex ₄ dHex ₁ HexNAc ₆	2031.88	2109.01	3	1
Hex ₆ dHex ₁ NeuAc ₁ HexNAc ₃	2037.84	2114.97	2	2
Hex ₁₀ HexNAc ₂	2045.81	2122.94	3	1
Hex ₅ HexNAc ₆	2047.88	2125.01	2	1
Hex ₄ dHex ₂ NeuAc ₁ HexNAc ₄	2062.89	2140.02	3	2
Hex ₅ dHex ₁ NeuAc ₁ HexNAc ₄ , or Hex ₅ dHex ₃ HexNAc ₄ [*]	2078.89	2156.02	3	2
	2079.92	2157.05		
Hex ₄ HexNAc ₇	2088.93	2166.06	2	1
Hex ₆ dHex ₂ HexNAc ₄ , or Hex ₆ NeuAc ₁ HexNAc ₄ [*]	2095.92	2173.05	3	3
	2094.89	2172.02		
Hex ₄ dHex ₁ NeuAc ₁ HexNAc ₅ , or Hex ₄ dHex ₃ HexNAc ₅ [*]	2119.94	2202.07	2	2
	2120.97	2203.10		
Hex ₃ HexNAc ₈	2129.99	2207.12	3	2
Hex ₅ NeuAc ₁ HexNAc ₅ , or Hex ₅ dHex ₂ HexNAc ₅ [*]	2135.94	2213.07	2	1
	2136.97	2214.10		
Hex ₆ dHex ₁ HexNAc ₅	2152.97	2230.10	2	1
Hex ₃ dHex ₁ NeuAc ₁ HexNAc ₆ , or Hex ₃ dHex ₃ HexNAc ₆ [*]	2161.00	2238.13	2	1
	2162.02	2239.15		
Hex ₅ dHex ₁ HexNAc ₆	2194.02	2271.15	2	1
Hex ₆ HexNAc ₆	2210.02	2287.15	3	2
Hex ₅ HexNAc ₄ NeuAc ₂	2224.01	2301.14	2	2
Hex ₅ dHex ₄ HexNAc ₄	2226.06	2303.19	2	1
Hex ₄ dHex ₁ HexNAc ₇	2235.07	2312.20	2	1
Hex ₆ dHex ₁ NeuAc ₁ HexNAc ₄	2241.03	2318.16	2	2
Hex ₃ dHex ₁ HexNAc ₈	2276.13	2353.26	2	1
Hex ₆ NeuAc ₁ HexNAc ₅ , or Hex ₆ dHex ₂ HexNAc ₅ [*]	2298.09	2375.22	2	3
	2299.11	2376.24		
Hex ₇ dHex ₁ HexNAc ₅	2315.11	2392.24	2	1
Hex ₅ dHex ₂ HexNAc ₆	2340.16	2417.29	2	2
Hex ₆ dHex ₁ HexNAc ₆	2356.16	2433.29	2	2
Hex ₅ dHex ₁ NeuAc ₂ HexNAc ₄	2370.15	2447.28	3	1
Hex ₄ dHex ₁ NeuAc ₂ HexNAc ₅	2411.20	2488.33	2	3
Hex ₅ HexNAc ₅ NeuAc ₂	2427.20	2504.33	2	2
Hex ₃ dHex ₁ NeuAc ₂ HexNAc ₆	2452.25	2529.38	2	1
Hex ₅ HexNAc ₈	2454.27	2531.40	2	2

Composition	MW	Aniline labeled	Charge state	Isomer number
Hex ₈ dHex ₁ HexNAc ₅	2477.25	2554.38	2	1
Hex ₃ dHex ₁ HexNAc ₉	2479.32	2556.45	2	1
Hex ₄ HexNAc ₉	2495.32	2572.45	2	1
Hex ₇ dHex ₁ HexNAc ₆	2518.31	2595.44	2	2
Hex ₆ dHex ₁ HexNAc ₇	2559.36	2636.49	2	3
Hex ₆ NeuAc ₂ HexNAc ₅	2589.34	2666.47	2	2
Hex ₄ dHex ₁ HexNAc ₉	2641.47	2718.60	3	2
Hex ₆ dHex ₁ NeuAc ₁ HexNAc ₆	2647.42	2724.55	3	1
Hex ₇ dHex ₂ HexNAc ₆	2664.45	2741.58	3	1
Hex ₇ NeuAc ₂ HexNAc ₅	2751.49	2828.62	3	1
Hex ₆ HexNAc ₆ NeuAc ₂	2792.54	2869.67	2	1
Hex ₆ dHex ₄ HexNAc ₆	2794.59	2871.72	2	1
Hex ₅ NeuAc ₄ HexNAc ₄	2806.52	2883.65	2	2
Hex ₇ dHex ₁ NeuAc ₁ HexNAc ₆	2809.56	2886.69	3	2
Hex ₄ dHex ₁ NeuAc ₂ HexNAc ₇	2817.59	2894.72	3	1
Hex ₅ dHex ₂ NeuAc ₁ HexNAc ₇	2834.62	2911.75	3	2
Hex ₇ dHex ₁ NeuAc ₂ HexNAc ₅	2897.63	2974.76	3	1
Hex ₅ dHex ₁ NeuAc ₂ HexNAc ₇	2979.73	3056.86	3	2
Hex ₇ dHex ₄ HexNAc ₆	2956.73	3033.86	3	2
Hex ₆ dHex ₇ HexNAc ₅	3029.82	3106.95	3	1
Hex ₉ HexNAc ₅ NeuAc ₂	3075.77	3152.90	3	1
Hex ₁₁ dHex ₁ HexNAc ₆	3166.87	3244.00	3	2
Hex ₈ dHex ₁ NeuAc ₁ HexNAc ₇	3174.90	3252.03	3	1
Hex ₆ dHex ₁ NeuAc ₃ HexNAc ₆	3229.94	3307.07	3	1
Hex ₇ dHex ₁ NeuAc ₂ HexNAc ₇	3304.02	3381.15	3	1
Hex ₇ dHex ₁ NeuAc ₃ HexNAc ₆	3392.08	3469.21	4	2
Hex ₉ dHex ₁ NeuAc ₂ HexNAc ₆	3425.11	3502.24	4	1
Hex ₇ NeuAc ₄ HexNAc ₆	3537.20	3614.33	3	1
Hex ₇ dHex ₁ NeuGc ₄ HexNAc ₆	3747.33	3824.46	3	1
Hex ₈ dHex ₁ NeuAc ₄ HexNAc ₇	4048.68	4125.81	3	1
Hex ₇ dHex ₁ NeuAc ₄ HexNAc ₉	4292.92	4370.05	4	1

*
Two glycan compositions having only 1Da in mass difference

Table 2
Identification and detection of N-glycans and isomers from rabbit sera

No#	Composition	MW	Aniline labeled	Charge state	Isomer number
1	Hex ₃ HexNAc ₂	910.82	987.95	1	1
2	Hex ₄ HexNAc ₂	1072.96	1150.09	1	2
3	Hex ₃ HexNAc ₃	1114.01	1191.14	1	2
4	Hex ₅ HexNAc ₂	1235.10	1312.23	1	2
5	Hex ₃ dHex ₁ HexNAc ₃	1260.15	1337.28	1	2
6	Hex ₄ HexNAc ₃	1276.15	1353.28	1	3
7	Hex ₃ HexNAc ₄	1317.21	1394.34	1	1
8	Hex ₆ HexNAc ₂	1397.24	1474.37	2	3
9	Hex ₃ dHex ₁ HexNAc ₄	1463.35	1540.48	2	1
10	Hex ₄ HexNAc ₄	1479.35	1556.48	2	1
11	Hex ₃ HexNAc ₅	1520.40	1597.53	2	1
12	Hex ₇ HexNAc ₂	1559.38	1636.51	2	2
13	Hex ₄ NeuAc ₁ HexNAc ₃	1567.41	1644.54	2	3
14	Hex ₄ dHex ₁ HexNAc ₄	1625.49	1702.62	2	2
15	Hex ₅ HexNAc ₄	1641.49	1718.62	2	2
16	Hex ₃ dHex ₁ HexNAc ₅	1666.54	1743.67	2	1
17	Hex ₄ HexNAc ₅	1682.54	1759.67	2	4
18	Hex ₈ HexNAc ₂	1721.53	1798.66	2	2
19	Hex ₅ NeuAc ₁ HexNAc ₃	1729.55	1806.68	2	4
20	Hex ₄ NeuAc ₁ HexNAc ₄	1770.61	1847.74	2	3
21	Hex ₅ dHex ₁ HexNAc ₄	1787.63	1864.76	2	5
22	Hex ₄ dHex ₁ HexNAc ₅	1828.68	1905.81	2	2
23	Hex ₉ HexNAc ₂	1883.67	1960.80	2	1
24	Hex ₆ NeuAc ₁ HexNAc ₃	1891.69	1968.82	2	2
25	Hex ₄ dHex ₁ NeuAc ₁ HexNAc ₄	1916.75	1993.88	2	2
26	Hex ₅ NeuAc ₁ HexNAc ₄	1932.75	2009.88	2	3

No#	Composition	MW	Aniline labeled	Charge state	Isomer number
27	Hex ₄ NeuAc ₁ HexNAc ₅	1973.80	2050.93	2	3
28	Hex ₆ HexNAc ₅	2006.83	2083.96	2	2
29	Hex ₅ dHex ₁ NeuAc ₁ HexNAc ₄	2078.89	2156.02	2	2
30	Hex ₆ dHex ₂ HexNAc ₄	2095.92	2173.05	2	3
31	Hex ₄ dHex ₁ NeuAc ₁ HexNAc ₅	2119.94	2197.07	2	3
32	Hex ₅ NeuAc ₁ HexNAc ₅	2135.94	2213.07	2	2
33	Hex ₆ HexNAc ₆	2210.02	2287.15	2	1
34	Hex ₅ NeuAc ₂ HexNAc ₄	2224.01	2301.14	2	2
35	Hex ₅ dHex ₁ NeuAc ₁ HexNAc ₅	2282.09	2359.22	2	2
36	Hex ₆ NeuAc ₁ HexNAc ₅	2298.09	2375.22	2	3
37	Hex ₅ dHex ₁ NeuAc ₂ HexNAc ₅	2573.34	2650.47	3	1
38	Hex ₅ dHex ₁ NeuAc ₂ HexNAc ₆	2776.54	2853.67	2	2
39	Hex ₆ NeuAc ₃ HexNAc ₅	2880.60	2957.73	2	1
40	Hex ₆ dHex ₁ NeuAc ₃ HexNAc ₅	3026.74	3103.87	2	5
41	Hex ₇ dHex ₁ NeuAc ₄ HexNAc ₆	3683.34	3760.47	3	1
42	Hex ₈ dHex ₁ NeuAc ₄ HexNAc ₇	4048.68	4125.81	3	2