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The N-Glycome of Human Plasma

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Abstract

N-linked glycans isolated from human plasma proteins have been profiled and sequenced by mass spectrometry using an ion trap instrument (ITMSⁿ). The released glycans were prepared as reduced, methylated analogs and directly infused into a chip-based nanoelectrospray ionization system and analyzed by ITMSⁿ. The resulting mass profiles (MS¹) of IgG-depleted and non-depleted plasma samples were contrasted and these results were again compared with recent literature reports. Before depletion approximately 50 independent glycan ions were detected, this more than doubled to 106 after depletion. The mass range profiled was 1K–5KDa which included many doubly and triply charged ions that were resolved by higher MS resolution. Selected ions in the depleted sample were disassembled to define their detailed structure providing a high performance sequencing result. The simplicity of this non-chromatographic, direct infusion and gas-phase structural characterization compares most favorably with the latest reports using alternative instrumentation and adjunct techniques.

Keywords

glycosylation; ion trap mass spectrometry; human plasma; carbohydrate sequencing

Introduction

Structural changes in glycosylation that are influenced by disease states have been of great interest, especially with respect to oncogenesis and tumor progression,^{1–3} and many reports have focused on such biomarkers for cancer related diagnosis.^{4–8} We have reported such changes in murine cancer cells, including metastatic and non-metastatic astrocytomas.⁹ The development of a simple non-invasive test that allows for detection and classification of malignancy would be the logical follow-up to this finding, and such biomarkers could monitor the kinetics and recurrence of disease as appropriate therapies are introduced. Given that molecular glycosylation has the propensity to possess more chemical information than any other comparable oligomer of equal weight, and structural variations due to tissue and temporal factors are well recognized,^{10–11} this glycomic monitor of tissue metabolism is well positioned to become a superb disease detector. Thus, we have focused on the plasma glycome to represent that non-invasive monitor of malignancy. The need for a detailed characterization, (linkage, branching, stereo and structural isomers), for every monomer, and documenting each intervening linkage brings enormous analytical challenge. Current protocols remain a cottage industry of techniques, where no existing approach is comprehensive. The complexity of the glycoproteome presents a challenge not seen in

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Supporting Information Available. Additional mass spectral data is available in supporting information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

genomic and proteomic analyses, but the proliferation of reports attributing biological function to oligosaccharide epitopes continues. Such structures exhibit exacting domains, but yet intriguing overall heterogeneity. Functional attributes may be temporal by modulation of an existing structure or *de novo* with development introducing tissue specific and environmental variations. Because structural specificity of these interactions is the basis of form and function, a detailed knowledge of such components remains the chemical end-game to functional understanding. Human plasma is more accessible than murine and represents a recognized goal, thus this report summarizes our approach for an N-glycome analysis of human plasma. Moreover, earlier reports^{12–15} have provided comparative targets to contrast and compare these analytical findings. Specifically, the technology of ITMSⁿ provides for repetitive steps of ion isolation and collision induced dissociation (CID), exposing the details of glycan linkage, branching, and isomeric resolution.^{16–22} Using standard methods of sample preparation we profiled the serum glycans between 1K – 5KD and established a table of ion compositions and abundance. This was followed with a detailed structural characterization of several ions that were unique to the IgG-depleted sample. Previous reports on the plasma glycome have utilized chromatographic methods in order to reduce sample complexity, however, the results presented here strongly suggest that direct infusion nanoESI and ITMSⁿ alone are well-suited to resolve these complex serum samples, using methods previously reported and summarized²³ in a wide cross-section of samples.

Experimental Section

Materials

All materials were of high purity and used as received. Ammonium bicarbonate, trifluoroacetic acid (TFA), sodium dodecyl sulfate (SDS), sodium hydroxide (NaOH), iodomethane, sodium borohydride (NaBH₄), HPLC grade dimethylsulfoxide (DMSO), Trizma hydrochloride, and sequencing grade trypsin were obtained from Sigma-Aldrich (St. Louis, MO). HPLC grade methanol, acetonitrile, dichloromethane, water, Alltech C18 solid-phase extraction (SPE) columns and porous graphitized carbon (PGC) were obtained from Fisher Scientific (Fairlawn, NJ). All water used was 18 mΩ and purified using a Barnstead NanoPure system (Dubuque, IA). PNGase F (glycerol free) was obtained from New England Biolabs (Ipswich, MA). Slide-A-Lyzer 7K MWCO cassettes, Coomassie Plus Bradford Protein Assay, Protein A/G Binding Buffer, IgG Elution Buffer, and Protein A/G Plus Agarose were obtained from Pierce Biotechnology (Rockford, IL).

Plasma Sample Preparation

A single sample of citrated plasma was obtained from a healthy male volunteer according to HUPO standard methods.²⁴ The sample were purified by dialysis and protein concentration determined using a Coomassie Plus Bradford Assay (Pierce, Rockford, IL). Immunoglobulin G (IgG) was depleted using a Protein A/G Plus Agarose (Pierce, Rockford, IL), following instructions from the supplier. Briefly, a column was packed with 0.75 mL of resin slurry which had a binding capacity of 5.25 mg of IgG. The column was equilibrated with 5 mL of Pierce Protein A/G Binding Buffer and then the sample applied (3 mg of protein). Non-bound proteins were eluted using 3 mL aliquots of Pierce Protein A/G Binding Buffer and 5 fractions were collected. Bound IgG was eluted using 1 mL aliquots of IgG Elution Buffer where 100 μL of neutralization buffer (1 M Tris-HCl, pH 8.5) was immediately added, and 5 fractions were collected. Protein concentration in all fractions was determined using a Coomassie Plus Bradford assay (Pierce, Rockford, IL).

N-Glycan Release & Sample Preparation

N-linked glycans from the IgG-depleted and non-depleted samples were released using PNGase F. For each 50 μ L sample, 50 μ L of 40 mM ammonium bicarbonate and 2 μ L of PNGase F were added; samples were heated at 37°C for 24 hr. Glycans were purified using a C18 SPE column, previously wetted with 5 mL of methanol and equilibrated with 5 mL of 5% acetonitrile/95% water in 0.1% TFA. Glycans were added and eluted with 6 mL of 5% acetonitrile/95% water in 0.1% TFA. Samples were dried and then desalted using a PGC column. The column was prewashed with 5 mL of water, the sample applied and washed again with 6 mL of water. The glycans were eluted with 6 mL of 25% acetonitrile/75% water in 0.1% TFA. The sample was dried and the glycans reduced with NaBH₄ (10 mg/mL in 0.01 M NaOH) and left overnight at room temperature. The resulting reaction mixture was quenched by the addition of 20% acetic acid while in an ice bath, followed by the addition of 3 mL of ethanol before drying. Borate esters were removed by repeated addition and drying of a 1% acetic acid methanol solution, followed by desalting using a PGC column. The flow through glycans were methylated.²⁵ The respective samples were dissolved in 1 mL DMSO, followed by addition of powdered NaOH. After vortexing to produce a suspension, 100 μ L iodomethane was added and the reaction allowed to proceed for 1 hr while being vortexed. The reaction was stopped by addition of water and the methylated oligosaccharides were extracted two times with dichloromethane. The extracts were combined and washed until the water was pH 7; the dichloromethane was evaporated under a nitrogen stream. To simplify structural representation, cartoons have been specifically drawn to represent exacting details.²⁶

Mass spectrometry and Data Interpretation

High and low resolution mass spectra were obtained using an ITMSⁿ (LTQ-Orbitrap and LTQ, respectively, Thermo Fisher, Waltham, MA) equipped with a chip-based nanoESI interface, (Advion-Triversa, Ithaca, NY). Signal averaging was accomplished by adjusting the number of microscans within each scan, which ranged between 5 and 15. Collision parameters were left at default values with normalized collision energy set to 30%; activation Q was set at 0.25, and activation time for 30 ms. Higher resolution Orbitrap spectra were obtained using a nanoelectrospray ion source. Spectra were manually interpreted and possible glycan compositions were determined using the ExPASy GlycoMod tool (<http://ca.expasy.org/tools/glycomod/>) with the following parameter limits: (i) monosaccharide residues: Hex $\leq$ 9, HexNAc $\geq$ 2, NeuAc $\leq$ antennary HexNAc, NeuAc $\leq$ antennary Hex, Fuc $\leq$ 5, (ii) monoisotopic mass tolerance $\pm$ 0.5 Da, (iii) ions existed as [M + Na]⁺, and (iv) on the basis of enzymatic release all returned compositions were assumed to have a core structure of Man₃GlcNAc₂. Using these delimiters compositions were established that satisfied all profiled ions.

Results and Discussion

The N-glycome of human plasma provided 53 ions using direct infusion ITMSⁿ. All ions were confirmed to be glycans by MS² analysis, and the most abundant corresponded to IgG glycans,^{20,26,27} (Fig. 1A). The series m/z 831.6, 925.6, 1039.6, 1054.6, 1142.2, 1235.7, and 1416.2 suggested them to be G1 and G2 analogs with variations in fucosyl and neuraminyl residues. Structural confirmation of the G2 ion, m/z 1142.2 ion, (H5N4F1) was shown by authentic by ITMSⁿ which resolved into a three isomer set, (Fig. 2). These isomers were comparable to earlier studies in this laboratory²⁰ although the serum derived samples exhibited greater background noise. Isomers are frequently missed following a single collision (MS²) which is inadequate to identify structural details. Resolution of isomers is also dependent on sample derivatization (reduction and methylation) which fixes components of structure to precise positions in a glycan array. These positions are then

confirmed by MSⁿ disassembly. Isomers appear to be common features of glycosylation even from a purified glycoprotein,²⁰ and as such, their type and abundance must represent a more exacting biological signature.

A structural comparison of this m/z 1142.2 ion and its three isomers isolated from purified IgG²⁰ and the same isomer set derived from serum provided an assessment of serum complexity (background) that limited disassembly of minor ions. However, a library spectral comparison of each step in the disassembly pathway indicated major fragments to be comparable even though background ions from serum were prominent. Clearly, improved or additional prefractionation would affect these results. To apply this consideration, and structurally detect lower abundance glycans, the plasma was IgG-depleted, and then prepared for glycan analysis, (Fig. 1B). The resulting mass profiles provided 82 additional ions, 106 of which were N-glycans. A high-resolution profile was also obtained and all 106 ion peaks were observed along with 10 additional compositions, a consequence of resolving multiply charged ions (Table 1). A large number of sialylated and fucosylated structures were also observed (60 and 63, respectively). Neutral oligosaccharides have been reported to suppress ion signals of anionic species and separation via HPLC was necessary¹³. However, methylation should avoid polarity complications and expectedly, the acidic and neutral species appeared together unperturbed in all profiles. The utility of an LC microfluidic chip has also been applied and reported,¹³ but this adjunct approach failed to identify as many glycan compositions as detailed here, and equally as important, a detailed characterization of structures was not considered.

The mass assignments and relative abundance between runs appears reproducible, especially considering sample derivatization and small 50 μ L size. This was briefly evaluated by reinjection of four independent runs where ions and abundance were cataloged for fourteen prominent ions, (Table S1).

In the IgG depleted plasma sample isomers were readily detected for the ions at m/z 1221.6, 1279.6, and 1502.8, (Fig. 1B), with additional differences possible for neuraminyl linkage, (Fig. S1–S4). Notably, Knezevic *et al.*,¹² reported only 3 isomers for m/z 1279.6, where the differences were in neuraminyl linkages, but using ITMSⁿ we have been able to confirm 4 unique isomers without considering such linkages. A single structure was observed for m/z 1491.6, not including those possible for the neuraminyl linkage. For m/z 1371.6, a core structure with 3 (NeuAc-Hex-HexNAc) antennae and 1 (Hex-HexNAc) antenna was confirmed, but because of low ion signal the exact location of the asialo-antenna could not be determined. Interestingly, a structure with antennal fucosylation was observed for m/z 1502.8, a composition not reported previously in humans according to the Consortium for Functional Glycomics database.²⁸ Given the low ion current for this ion it was not possible to interrogate every aspect of structure.

An obstacle in glycan sequencing when using collisional activation is directly related to the linkage lability of the neuraminyl and HexNAc linkages, as these moieties are easily ruptured missing an opportunity to capture inter-residue structural details. A partial resolution to this problem can be approached by determining the former linkage position in the products, an exposed hydroxyl group (scar) on the non-reducing side of the oligomer. In practice, the usual approach is to isolate the scarred (open hydroxyl) of LacNAc fragments (m/z 472, Fig. 2A, 3C, S3A), the fragment previously bordered by the neuraminyl and HexNAc linkages. Fragmenting and isolating this disaccharide provides a ^{3,5}X fragment across the Gal residue (loss of the 4, 5 and 6-carbons) at m/z 157. If a scar is located at the 3-position a ^{2,4}X fragment will be apparent with the elimination of the 3 and 4 carbons. If both ions are present (mixed neuraminyl linkages) then the LacNAc fragment must be associated to a specific core mannose, (Fig. 3). The MS² spectrum of m/z 1279.6 (Fig. 1B) suggested 4

isomers, with 2 having core fucosylation and 2 having antennal fucosylation. In order to specifically determine antennal structure, the ion at m/z 866.4 was isolated and fragmented (Fig. 2). The base peak at m/z 472 corresponds to an internal LacNAc, and other common internal glycosidic bond cleavages were observed (*e.g.*, m/z 694, 662, 490, 417). Several MS³ product ions (m/z 763, 560, and 315) indicate the single antenna (without fucosylation) to be at the 6-position of the core mannose. Conversely, the ions at m/z 708, 546, and 504 indicate the single antenna (without fucosylation) to be at the 3-position of the core mannose. The ion at m/z 866 was then treated as a potential marker for determining single antenna linkages. The ions at m/z 1221.6 and 1502.8 from the profile (Fig. 1B) were further examined and found to have isomers with a single antenna at the 3- and 6-positions (structures inserted).

In this study it was hoped that the prefractionation steps (enzymatic release, SPE extraction, derivatization, lipophilic extraction) might prove adequate to fully disassemble ions for a comprehensive understanding of structure. But serum is a remarkably complex liquid, and further improvements in this area will be necessary as minor peaks failed to provide the ion current necessary. Contributing to this understanding is the fragment library²¹ which is associated with search tools that confirm components of structure while the ion disassembly pathway establishes array connectivity.

Even though the derivatized sample would be expected to have low polarity, multiple charging by sodium adduction was significant at higher masses. This, of course, has the advantage of making higher molecular weight glycans accessible in the lower mass range of the instrument, but the trade-off was overlapping ions. As an example, the initial analysis of m/z 1010.1, (charge state unknown), provided a series of overlapping ions at low resolution (Fig. 3A, panel 1), which resolved into three compositions at higher resolution, (Fig. 3A, panel 2). Although structural understanding may be most direct when disassembled under these high resolution conditions, considerable insight can be gained in low resolution to suggest the putative structures, (Fig. 3A, panel 3). Disassembly of overlapping multiply charged ions can be even more challenging to evaluate, but the product ions, related patterns, and support from a fragment library can provide, in many cases, an adequate solution. However, ample ion current remains fundamental. Such an approach has been presented here, (Fig. 3B and C), with the low resolution MS² spectrum of the overlapping precursors within, m/z 1010.1, (Fig. 3A, panel one). The collision spectrum (Fig. 3B) provided the expected rupture between the core GlcNAcs which indicated a fucosylated, reduced GlcNAc fragments (m/z 490.36) and the other a neutral loss of the unfucosylated reducing terminus, (m/z 1704.91). These two ions from the same precursor (H5N4A2F1) suggest the structures presented, (Fig. 3A, panel 3). In the same manner, the two base ions in the spectrum would be expected to represent a facile loss and they are identical (with an additional sodium adduct on one). Disassembly of m/z 1622.91⁺, which corresponds to loss of neuraminyl group (H4N3A1) shows neutral losses that make it easy to determine the topology of the glycans (Fig. 3C). Additionally, the ion at m/z 764.00 (^{3.5}X) indicates the antenna is on the 6-linked mannose. Further disassembly of m/z 1315.73²⁺, which corresponds to a loss of a neuraminyl group (H5N4F1A2) suggests that two isomers are present, (Fig. S3). MS² on m/z 1040 confirms there are 4 Hex-HexNAc antennae, but ion counts were too low to determine linkages, (Fig. S5). The ability to identify several compositions in a given mass range and then disassemble each composition of interest eliminates the need for chromatography that may result in the loss of low abundance species. We have mentioned the constraint of only having enough ions to reach MS³ or MS⁴ and we are currently pursuing enrichment strategies to improve this. This is expected to be an important and never ending pursuit.

Finally, there are several aspects of these data that have not previously been reported for N-glycan analysis using electrospray ionization: (i) a large number of high molecular weight structures, especially over 4000 Da, (ii) a large number of triply charged species, and (iii) many (newly reported) compositions resolved without the need of chromatography. A brief survey of the literature shows that there are very few reports that have attempted global glycan profiling, (Table 2). In addition to these studies, Mechref *et al.*, have described the utility of MALDI-TOF MS for profiling glycans in serum without the use of chromatography and reported 134 compositions identified, however this was not verified by MS² and it is possible that some ions would fail further examination, as we described earlier. There are many reports detailing differences in glycosylation for healthy vs. disease states^{29–31} and individual proteins in plasma,^{5·8·32} but data on glycans from the entire sample are not available. Importantly, this study has identified more than double the number of glycans than previous studies and opens the door for higher performance glycan screening of complex samples.

Given the complexity of this sample it is also worth mentioning ion suppression which is generally considered a consequence of charge competition of more polar species (*e.g.*, peptides), and other non-volatile molecules from biological samples, but the exact mechanism is not well understood.^{33–35} There is a general consensus that most ion suppression effects can be reduced with two techniques: (i) sample clean-up; and, (ii) spraying with a solvent that has high organic content. Aspects of these approaches were applied in this study with modest results.^{34·35} Given the large number of glycans observed, and noting the presence of neutral and acidic species, ion suppression by direct infusion may not appear to be a major issue. However, as analysts approach the limits of detection, interceding chromatography is notorious for adsorbing and irreversibly retaining trace sample amounts and in this regard, direct infusion becomes attractive. Profile reproducibility is high (Fig. S5) and fits well with other reports that profiles are stable and reproducible over time.^{14·15}

Conclusions

This study has demonstrated that a complex glycan sample can be analyzed most effectively using direct infusion ion trap mass spectrometry on as little as 50 μ L of blood serum, (Fig. 1). These mass and abundance profiles coupled with structural details of linkage, branching and isomers brings a superior technology to characterize tissue metabolism. The profiles (ions and abundance) them self may be sufficient to define tissue processes allowing early diagnosis, prognosis, or classification of disease subtypes. When coupled with disassembly for a detailed understanding of structure, such high performance sequencing bodes well for being a superior tissue monitor. This proof-of-concept study demonstrates that rapid glycan analysis (*i.e.*, without chromatography) is possible on complex samples, and opens the door to an improved and simplified strategy for biomarker discovery. Low abundance glycans were observed and fragmented in order to determine structural isomers, including one isomer not previously reported. With this improved line of attack, biomarkers previously identified in murine tissue,⁹ now might be considered in a non-invasive manner. Although selected ions have been disassembled and sequenced, a comprehensive evaluation has not been considered. The ion composition profiles do offer a new benchmark for which others may contrast and improve (Table 1.)

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ABBREVIATIONS

LC	liquid chromatography
ITMS	ion trap mass spectrometry
CID	collision induced disassociation
IgG	Immunoglobulin G
Compositions: H	hexose
N	N-acetylhexosamine
F	fucose
A	N-acetylneuraminic acid

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Figure 1.

A. N-glycan mass profiles (MS^1) of normal (A) and IgG depleted (B) human serum samples. Analyzed by direct infusion nanoESI-positive-ion trap mass spectrometry (MS^n). Effective IgG depletion appears evident with the resulting loss of major immunoglobulin ions, (m/z 831.6, 1039.6, 1054.6, 1142.2, 1235.7, 1416.2), and the contrasting new ions, m/z 1221.6, 1279.6, 1491.3. Where H = hexose, N = NAc-hexosamine, F = fucose, and A = NAc-neuraminic acid.

B. Positive ion ESI-ion trap mass spectrum of reduced, methylated N-linked glycans from IgG-depleted human plasma, with the five most abundant unique glycans labeled.

Figure 3.

A. Zoom view of positive ion ESI-Orbitrap profile and high resolution profile mass spectrum of reduced, methylated N-linked glycans from IgG-depleted human plasma and putative structures.

B. MS² positive ion ESI-ion trap mass spectrum of m/z 1009.51 with a 1 Da window of isolation.

C. MS³ positive ion ESI-ion trap mass spectrum of m/z 1009.51 $\rightarrow$ 1622.9.

Glycan compositions for IgG-depleted plasma sample. Compositions shaded in grey present only in the IgG-depleted sample; all other compositions were observed in both samples. *Italic text* indicates doubly charged ions, normal text indicates triply charged ions. H = Hexose; N = N-Acetyl hexosamine; F = Fucose; A = N-Acetylneuramic Acid

Table 1

<i>M_{eff}</i> ¹	Composition ²	<i>M_{eff}</i> ¹	Composition ²	<i>M_{eff}</i> ¹	Composition ²	<i>M_{eff}</i> ¹	Composition ²
1433.0	H3N3	2505.6	H5N5F1	3156.9	H5N4F2A2	3881.3	H7N6F1A2
1596.1	H5N2	2535.5	H6N5	3188.9	H8N6	3954.3	H6N5F4A2
1637.0	H4N3	2546.5	H4N6F1	3188.9	H8N6	3967.3	H6N5F2A3
1678.1	H3N4	2597.4	H7N3F2	3227.8	H5N5F1A2	4005.9	H6N11
1770.2	H5N2F1	2597.4	H7N3F2	3244.3	H6N5F2A1	4068.4	H7N6A3
1794.1	H3N3A1	2621.6	H5N4F1A1	3244.3	H6N5F2A1	4101.6	H9N6F2A1
1800.1	H6N2	2621.6	H5N4F1A1	3257.9	H6N5A2	4115.4	H9N6A2
1841.2	H5N3	2627.7	H8N3F1	3257.9	H6N5A2	4119.4	H10N6F3
1852.3	H3N4F1	2679.6	H5N5F2	3263.9	H9N4A1	4129.8	H8N7F4
1881.6	H4N4	2692.5	H5N5A1	3271.0	H6N8	4156.4	H8N7A2
1944.1	H5N2F2	2709.7	H6N5F1	3274.4	H7N5F1A1	4242.4	H7N6F1A3
1998.3	H4N3A1	2761.4	H3N7F2	3292.0	H8N5F2	4428.9	H7N6A4
2004.3	H7N2	2795.5	H5N4F2A1	3331.0	H5N4F3A2	4603.6	H7N6F1A4
2045.4	H6N3	2795.5	H5N4F2A1	3346.0	H7N6A1	4766.8	H9N8F3A1
2056.3	H4N4F1	2808.7	H5N4A2	3408.0	H8N4F2A1	4777.7	H7N6F2A4
2086.3	H5N4	2808.7	H5N4A2	3421.0	H8N4A2	4878.4	H8N7A4
2096.7	H3N5F1	2814.8	H8N3A1	3431.5	H6N5F1A2		
2127.4	H4N5	2825.0	H6N4F1A1	3431.5	H6N5F1A2		
2159.5	H4N3F3	2825.0	H6N4F1A1	3462.0	H7N5A2		
2168.5	H3N6	2836.8	H4N5F2A1	3473.1	H5N6F1A2		
2172.3	H4N3F1A1	2836.8	H4N5F2A1	3490.2	H6N6F2A1		
2202.3	H5N3A1	2842.8	H7N4F2	3520.0	H7N6F1A1		
2208.3	H8N2	2866.5	H5N5F1A1	3574.1	H5N5F3A2		
2229.9	H4N4F2	2866.5	H5N5F1A1	3606.1	H8N7F1		
2243.3	H4N4A1	2883.5	H6N5F2	3607.5	H6N5F2A2		

M_{eff}^1	Composition ²	M_{eff}^1	Composition ²	M_{eff}^1	Composition ²	M_{eff}^1	Composition ²
2260.2	H5N4F1	2896.7	H6N5A1	3619.1	H6N5A3		
2290.5	H6N4	2896.7	H6N5A1	3635.5	H7N5F1A2		
2301.4	H4N5F1	2903.8	H3N9	3707.2	H7N6A2		
2345.9	H7N2F1	2937.2	H5N6A1	3717.6	H5N7F1A2		
2406.1	H6N3A1	2943.2	H8N5	3723.6	H8N6F1A1		
2412.3	H9N2	2958.1	H7N3F2A1	3739.2	H7N4F3A2		
2434.1	H5N4F2	2965.8	H4N7F2	3754.0	H9N6A1		
2447.5	H5N4A1	2982.8	H5N4F1A2	3769.2	H8N4F2A2		
2447.5	H5N4A1	2982.8	H5N4F1A2	3793.3	H6N5F1A3		
2453.6	H8N3	2984.8	H7N6	3794.6	H8N7A1		
2457.9	H3N5F1A1	3070.2	H6N5F1A1	3827.4	H8N5F3A1		
2475.3	H4N5F2	3108.2	H4N9	3874.5	H4N10F3		

Table 2

Published reports of human plasma glycans with specific comparisons of number, triple charging, and ions exceeding 4k Da.

Analysis Method	Glycans Reported	Triply Charged	$M_{\text{eff}} \geq 4000$ Da	Reference
HPLC, no MS	17	N/A	6	Knezevic <i>et al.</i> ¹²
MALDI-FTICR-MS	20	0	0	Chu <i>et al.</i> ¹³
LC, ESI-FTICR-MS	42	NR	0	Bereman <i>et al.</i> ³⁶
LC, ESI-Orbitrap-MS	12	0	0	Bereman <i>et al.</i> ³¹
CE-ESI-qTOF-MS	47	18	0	Ruhaak <i>et al.</i> ³⁷
Direct infusion IT-MS	106	59	13	this work