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Mass Spectrometric Analysis of Sialylated Glycans Using Solid Phase Labeling of Sialc Acids

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Abstract

The analysis of sialylated glycans is critical for understanding the role of sialic acid in normal biological processes as well as in disease. However, the labile nature of sialic acid typically renders routine analysis of this monosaccharide by mass spectrometric methods has been difficult. To overcome this difficulty we pursued derivatization methodologies, extending established acetohydrazide approaches to aniline-based methods, and finally to optimized p-toluidine derivatization. This new quantitative glycoform profiling method using MALDI-TOF in positive ion mode was validated by first comparing N-glycans isolated from fetuin and serum and was then exploited to analyze the effects of increased metabolic flux through the sialic acid pathway in SW1990 pancreatic cancer cells by using a co-labeling strategy with light and heavy toluidine. The latter results established that metabolic flux, in a complementary manner to the more well-known impact of sialyltransferase expression, can critically modulate the sialylation of specific glycans while leaving others virtually unchanged.

Keywords

Sialic acid; Amidation; Sialylated Glycan Quantitation; Mass Spectrometry; Metabolic Flux

Introduction

Glycosylation is one of the most abundant post-translational modifications on proteins. Glycans are important for protein folding, maintaining their structural stability, and they play important roles in protein-ligand interactions and the resulting biological functions. In addition, several glycoproteins are clinically used to diagnose diseases, such as Cancer Antigen 125 (CA-125), more commonly carcinoembryonic antigen (CEA) and prostate specific antigen (PSA). Virtually all therapeutic proteins, such as widely used monoclonal antibodies and erythropoietin, are glycosylated. Glycosylation in general, and the presence or absence of sialic acid in particular, affects the serum half life and pharmacokinetics of this emerging class of therapeutic glycoproteins^{1, 2}. Sialic acids are normally terminal monosaccharide residues in glycans and are generally attached to penultimate galactose or N-acetylgalactosamine (GalNAc) residues with α -2,3 or α -2,6 linkages with negatively charged at the physiological pH. It has been shown that higher expression of sialyltransferases or higher number of glycan branches available for sialylation are responsible for the elevated sialylation associated with disease development³. Therefore, it is important to study sialylated glycans for diagnosis, prognosis and therapeutic purposes⁴.

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Mass spectrometry is the emerging technology for analyzing quantitative glycan structures. However, identification and accurate quantification of glycans, especially sialylated glycans, is challenging. This is due to the fact that sialylated glycans have negative charges that have decreased ionization efficiency compared to neutral glycans. The labile nature of sialic acid also makes the analysis of glycans challenging due to the loss of sialic acids within glycans during MS analysis before the glycan ions reach the detector^{5, 6}. There have been a few methods adopted to modify sialic acids in neutralizing and making glycan MS amicable. Permethylolation is also a widely used method to stabilize the acidic component of glycans by modifying hydroxyl, amino, carbonyl and carboxylic moieties of glycans with methyl groups^{7, 8}. This methyl incorporation makes the glycan ionization more efficient, and also prevents the loss of sialic acids^{7, 8}. Other approaches are also reported to modify sialic acids including esterification⁹, amidation¹⁰, and oxime formation reactions¹¹.

Accurate quantitation of glycans is generally performed by isotopic labeling of glycans using permethylation or reductive amination at the reducing ends. Permethylolation is used for quantitation of glycans by differentially labeling samples using light and heavy isotopes of methyl group ($^{13}\text{CH}_3$, $^{12}\text{CDH}_2$, $^{12}\text{CD}_2\text{H}$, or $^{12}\text{CD}_3$), resulting in a mass shift of 1–3 Da per site^{12, 13}. Isotope labeling with permethylation reagents makes variable mass shifts for glycans depending on the number of permethylation sites available. Orlando et al developed the quantitative isobaric labeling (QUIBL) technique using permethylation for nominal mass shift by labeling one sample with $^{13}\text{CH}_3\text{I}$ and other with $^{12}\text{CH}_2\text{DI}$ ¹⁴. QUIBL results in a mass shift of 0.002922 Da per methylation site that can be quantified by a high resolution mass spectrometer. For the permethylation approach to be used for quantitation, the efficiency of labeling needs to be high. Errors can be introduced to quantitation with even a small difference in labeling efficiency of two samples because large numbers of sites have to be permethylated. In the case of the most abundant glycan in human serum, the bi-antennary sialylated oligosaccharide ($\text{GlcNAc}_4\text{Man}_3\text{Gal}_2\text{NeuNAc}$)₂, there are a total of 39 sites that have to be modified. Hence, a difference of 0.2% in labeling efficiency will result in 7.8% overall quantitative error. Glycan quantitation with MS can also be achieved by isotopic glycan labeling at the reducing ends. Quantification can also be achieved by isotopically labeling the reducing end of a glycan. Because each glycan only has one reducing end, an end-labeling strategy will result in the same mass shift for all glycans. However, acidic glycans are required to be neutralized in a separate derivatization reaction, which could introduce additional errors. Isotopic aniline tags were used for quantitation of N-glycans known as Glycan Reductive Isotopic Labeling (GRIL) and similarly tetraplex labeling was used for quantitation of plasma from four different specimens^{15, 16}. Walker et al used hydrophobic hydrazide labeling at the reducing end to quantify glycans¹⁷. Recently, Tandem Mass Tags (TMT) were developed using hydrazide or aminooxy reagents, and it was determined that aminooxy tags are better compared to hydrazide tags for quantitative glycomics¹⁸. Recently, isobaric labeling on the glycan reducing end has been demonstrated on glycan quantitation for standard glycoprotein gp120 (our unpublished results). Similar to iTRAQ reagents for quantitative analysis of peptides, the labeled glycans show same mass peak in mass spectrum while the relative abundance is characterized by tandem mass spectrometry through reporter ions (e.g., 114 vs, 115)¹⁹. Another glycan quantitative method, Isotopic Detection of Amino sugars With Glutamine (IDAWG), which was developed by Orlando et al²⁰, is a metabolic labeling technique that incorporates 1 Da mass shift per HexNAc and NeuAc into glycans. IDAWG is similar to SILAC technique used for peptide quantitation²¹. In IDAWG, cells grown in light and stable isotopically labeled heavy glutamine culture are mixed in 1:1 ratio for quantitative glycan analysis. IDAWG has an advantage of negating quantitation errors introduced by sample preparation/handling. However, this approach can only be used in a cell system and cannot be used on tissue or serum samples from human. Sialic acids on glycans also have to be modified separately to prevent in-source fragmentation with IDAWG technique.

In this study, we developed a strategy to stabilize and label sialylated glycans with stable isotopic tags in a single process for quantitative MS analysis. The derivatization of sialylated glycans was performed via amidation with p-toluidine to stabilize the sialic acid glycans. p-Toluidine also neutralizes negatively charged sialic acids and renders derivatized N-glycans more hydrophobic for mass spectrometry detection. Proteins were first conjugated to solid support by reduction amination. The sialic acid groups on conjugated proteins were then modified by adding p-toluidine in the presence of N-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC). Glycans were then released from proteins on solid support and analyzed by mass spectrometry in positive mode. When light and stable isotopic heavy p-toluidine reagents were used in derivatization reaction, seven mass unit differences between light and heavy tags for each sialic acid are effectively resolved in the mass spectrum, allowing the identification of number of sialic acids in the glycan structures or relative quantitation of sialylated glycans from different samples. The method was applied to the identification of sialylated N-glycans from fetuin and human serum and also used for the quantitative analysis of sialylated glycans from pancreatic cancer cells, SW1990, to study N-glycan sialylation after the cells were incubated with a ManNAc analog to increase metabolic flux through the sialic acid biosynthetic pathway.

Materials and Methods

Materials

Bovine fetuin, human serum, p-toluidine, aniline, dimethylaniline (DMA), 2,5-dihydroxybenzoic acid (DHB), and EDC were purchased from Sigma Aldrich (St Louis MO) p-toluidine-d9 was purchased from CDN isotopes (Pointe-Claire, Quebec Canada). Acetohydrazide was purchased from Tokyo Chemical Industry Co. Ltd (Tokyo, Japan). PNGase F was obtained from New England Biolabs (Ipswich, MA). Amino link coupling resin was purchased from Thermo Scientific (Waltham, MA).

Protein Binding to Solid Support

Proteins were immobilized to amino link beads via reductive amination. Briefly, AminoLink resin (200 μ L) was loaded onto snap-cap spin-column, centrifuged at 2000 g for 1 minute. Resin was washed with 450 μ L of pH 10 buffer (Sodium citrate 100 mM and sodium carbonate 50 mM) followed by centrifugation. The washing step was repeated twice. Proteins dissolved in pH 10 buffer were loaded onto prepared AminoLink resin in snap-cap spin-column in 1mg/200 μ L beads ratio. The volume was adjusted to 450 μ L using pH 10 buffer. Sample-resin mixture was incubated at room temperature overnight. The mixture was centrifuged at 2000 g to remove any unbound protein. Resin was rinsed by 1 $\times$ PBS buffer (Sigma-Aldrich; pH 7.4; 450 μ L) three times. PBS buffer in the presence of 50 mM sodium cyanoborohydride (450 μ L) was added to resin (spin-column capped during each incubation step). After a four hour incubation, supernatant was removed via centrifugation (2000 g) and 450 μ L of 1 M Tris-HCl (pH 7.6) in the presence of 50 mM sodium cyanoborohydride was added to block un-reacted aldehyde sites of resin. The blocking process was terminated after 1 hour, followed by washing of resin with PBS twice, 1M NaCl 1.5M twice and water three times.

Derivatization of Sialic Acids on Proteins Conjugated to Solid Support and Release of N-glycans

Proteins conjugated on solid support were mixed with 450 μ L of 1M p-toluidine and 40 μ L of EDC at pH 4.5 adjusted using HCl. After incubation for 4 hr beads were washed twice with 500 μ L of 1M NaCl and water followed by 50 mM ammonium bicarbonate, three times for each solution, and treated with 100 units of PNGase F in 0.3 mL of 50 mM ammonium bicarbonate overnight at 37°C to release N-glycans from proteins. N-glycans released in the

supernatant were subsequently collected. The N-glycans released from glycoproteins were further purified and concentrated over Carbohydrate columns (Extract-Clean SPE Carbo 150 mg; Grace Division Discovery Science; Deerfield, IL) and eluted in 0.1% TFA 50% acetonitrile/water following the manufacturer's instruction (Grace Division Discovery Sciences, Milwaukee, WI). Glycans were then dried in Savant Speed-Vac (Thermo Scientific, Asheville, NC),

Mass Spectrometric Analysis of Glycans

Glycans were resuspended in 20 μ L of water. Sample 1.5 μ L was mixed 1.5 μ L of matrix on a 384-well μ Focus MALDI plate (Hudson Surface Technology, Fort Lee, NJ). DHB matrix solution was prepared by dissolving 100 mg of DHB in 1 mL of a 1:1 solution of water and ACN followed by addition of 40 μ L of dimethylaniline. Glycans were analyzed by Shimadzu AXIMA Resonance Mass Spectrometer (Shimadzu, Columbia, MD) in positive mode.

Results and Discussion

Solid Phase Labeling of Sialylated Glycans

The derivatization of sialylated glycans was successfully performed on protein glycans conjugated to the solid phase. Labeled sialylated glycans were sequentially released from solid support for mass spectrometry analysis (Figure 1). First, proteins were conjugated to solid support by reduction amination. Second, the carboxylic groups of sialic acids were modified by p-toluidine in the presence of EDC while glycans were still attached to the protein on solid support. Third, N-glycans were then released from proteins by PNGase F treatment. Fourth, the released glycans were analyzed using mass spectrometry in positive mode. When light and stable isotopic heavy p-toluidine reagents were used to derivatize sialic acids, seven mass unit differences between light and heavy tags for each sialic acid could be effectively resolved in the mass spectrum. The mass difference allowed the identification of the number of sialic acids in the glycan structures when 1:1 mix of light and heavy reagents were used for glycan labeling. The light and heavy reagents were also applied to different samples for the relative quantification of sialylated glycans.

Fetuin was used as a model glycoprotein to develop method for analysis of the sialylated glycans. Fetuin consists of following previously reported sialylated glycans GlcNAc₄Man₃Gal₂NeuNAc₁, GlcNAc₄Man₃Gal₂NeuNAc₂, GlcNAc₅Man₃Gal₃NeuNAc₂, GlcNAc₅Man₃Gal₃NeuNAc₃, and GlcNAc₅Man₃Gal₃NeuNAc₄⁹. Sialic acid loss was observed when 10 μ L of bovine unmodified fetuin N-glycans were analyzed using mass spectrometry with DHB/Dimethylaniline matrix (supplemental data 1). To stabilize and prevent sialic acid loss, it was necessary to modify sialic acids. Thus, amidation was used to modify the sialic acid in presence of EDC. Previously, Toyoda et al used acetohydrazide in presence of EDC to amidate sialic acid²². Van Cott and coworkers used one pot method, which included acetohydrazide to modify sialic acids which are still attached to the protein to study N-glycans on coagulation Factor IX²³. In the previous study, authors suggest that the reaction conditions used in amidation reaction may lead to protein loss due to precipitation and buffer exchange. To prevent protein loss, in the currently study, proteins were linked to solid support prior to the amidation reaction, which also aids in removal of impurities and changed reaction conditions with minimal sample loss. Once 10 μ g of fetuin was conjugated on beads, amidation was used to modify sialic acids followed by releasing of N-glycans using PNGase F. Finally, glycans were analyzed using mass spectrometry (Figure 1). We compared the amidation of sialylated glycans with acetohydrazide and aniline. It was observed that the signal intensity of four fetuin sialylated glycans modified by acetohydrazide was not similar to the previously published data with permethylated fetuin⁹

(supplemental data 1). However, sialylated glycans modified by aniline provided satisfactory results with similar sialylated glycan patterns as previously reported⁹ (Supplemental data 1). Aniline makes the glycan more hydrophobic than acetohydrazide and hence aids the ionization of sialylated glycans enhancing their detection in MALDI. However, the mass of modified sialic acid with aniline is only 1.0744 Da apart from mass of Hexose + HexNAc, resulting in the overlap of peaks. This overlap was difficult to resolve by mass spectrometry for quantitative analysis. Hence, p-toluidine was used to prevent overlap between different glycans. p-Toluidine modification produced similar results as the Aniline modification and all the previously reported fetuin sialylated glycans were observed (Figure 2). Relative intensities of all the sialylated glycans were similar to previously observed by esterified or permethylated fetuin glycans with tri-antennary tri-sialylated glycan, which is the most abundant glycan^{9, 12} (Figure 2). Therefore, the loss of sialic acid was prevented with p-toluidine modification and detection of all the glycans was possible.

Detection of sialylated glycans from serum and determination of number of sialic acids in each glycan

We then applied the above method to analyze N-glycans from serum. In this case, we took two equal aliquots 1.6 μ L of human serum and followed the protocol described above except the fact that one aliquot was labeled with heavy p-toluidine (D9) and other aliquot was labeled with light p-toluidine. Glycans from each aliquot were analyzed with MS individually. If the m/z peak was observed in light labeled spectrum and absent in heavy labeled spectrum, then that m/z value was considered a sialylated glycan peak (Figure 3A). If a peak observed in heavy labeled spectrum was absent in light labeled spectrum, and then such a peak was confirmed as signal from the same sialylated glycan (Figure 3B). Serum glycans were also analyzed from each aliquot when mixed in 1:1 ratio (Figure 3C). The differences between the peaks were used to determine the number of sialic acid present in the specific glycan (Figure 3C). A difference of 7.06 Da between a light and heavy sialylated peak meant that one sialic acid had been modified by light and heavy p-toluidine thereby indicating the presence of one sialic acid on the glycan. A difference of 14.121 Da indicated that the glycan contained two modified sialic acids. Difference of a peak pair of differentially labeled peak pairs was searched for m/z values of multiple of 7 light-heavy ion pairs (Figure 3D). A total of 45 N-glycans were identified from serum proteins and 21 sialylated N-glycan structures were successfully identified (Table 1). The sialylated glycan structures observed in our analysis were similar to results previously reported in serum²⁴. The 1:1 ratio observed in Figure 3D also indicated the method could be used for quantitative analysis sialylated glycans.

Quantitative Analysis of Sialylated N-glycans from Cells Treated with ManNAc: Changes of N-Glycan Sialylation Driven by Metabolic Flux

It has been well established that the extent of glycan sialylation depends on sialyltransferases, the biosynthetic enzymes responsible for adding a sialic acid to an underlying galactose or GalNAc residue. However, we recently showed that N-glycan sialylation also depends on metabolic flux through the sialic acid biosynthetic pathway²⁵. In the previous study we identified glycopeptides that were affected by flux changes in flux but did not analyze the structures of the glycans themselves. To fill this void, in the current study we profiled N-glycans in SW1990 treated with 1,3,4-O-Bu₃ManNAc as previously described²⁵. Protein extract from 1,3,4-O-Bu₃ManNAc treated SW1990 cells and untreated control cells were conjugated to solid support and labeled with isotopic p-toluidine as described above. The mass spectrometric analysis of labeled glycans identified 87 N-glycans based on accurate mass (supplement table 1). Sialic acid compositional assignment was determined based on the differences of heavy and light labeled glycans. Twenty one heavy and light glycan peaks were identified with a mass shift of 7 or a multiple of 7, confirming

that the presence of 21 sialylated glycans (Figure 4A and 4B). Qualitative analysis of N-glycans from cells with and without ManNAc treatment using isotopic labeling of sialylated N-glycans with 1:1 combined samples identified and quantified 14 sialylated glycans (Table 2). Seven other sialylated N-glycans could not be quantified due to low signal to noise ratio when the two samples were combined for MS analysis. The quantitative results showed that while the most N-glycans showed minor or no obvious differences between ManNAc treated and untreated cells (Figure 4C), six sialylated N-glycan compositions exhibited a significant increase in abundance, specifically the mono-sialylated tetra-antennary N-glycan (Table 2). There was no significant decrease in sialylated N-glycans when cells were treated with 1,3,4-O-Bu₃ManNAc, indicating metabolic flux contributed to the overall extent of sialylation on the proteins.

Advantages and Limitations of Quantitative Analysis of Sialylated Glycans Using Solid Phase Labeling of Sialic Acids

Aberrant glycosylation has been previously reported in various diseases including, as an outstanding example, increased sialylation often linked to cancer progression due to increased expression of sialyltransferases in cancer^{26, 27}, which leads to an increase in sialylation of the proteins²⁸. In particular, ST6Gal-1 links sialic acid to galactose also shows over expression in different cancers^{26, 29–31}. ST6Gal-1 has been implicated in increased invasiveness of tumor cells and cell motility^{28, 32}. However, the glycan structural bases for these glycosyltransferase changes and the mechanism through which they alter cell function have not been well characterized due to the lack of robust method for the quantitative analysis of sialylated glycans.

Here, we reported a novel chemical derivatization strategy that stabilizes the sialylated glycans and prevents loss of sialic acid. Specifically, the labeling of sialic acids with p-toluidine makes glycans hydrophobic and allows retention on C18 columns and improves ionization of glycans³³. The isotopic labeling with the 7 Da difference per sialic acid in the same sialylated glycan structure from two different samples is substantial enough to provide non-overlapping pairs compared to other low mass shift techniques and hence improves quantitation efficiency. The difference between the pair also helps definitively determine the number of sialic acids present in the glycan. Using solid-phase sialic acid labeling, the sample is first bound to the beads, and all the subsequent steps result in minimum sample loss. Sialic acid labeling is performed at protein level without processing and removal of proteins. The labeled samples are combined and processed through the rest of sample preparation steps, which reduces the errors due to sample handling in all the subsequent steps such as PNGase F digestion and sample clean up. Along with sialic acids on glycans, aspartic acids and glutamic acids on proteins are also modified and can be used for peptide/protein quantitation from the same specimens upon releasing peptides from solid support using proteolysis. A similar approach for protein quantitation has been previously reported in which aniline and benzoic acid was used to quantitation³⁴. The limitation of the sialylated glycan labeling method is that only sialylated glycans are quantified. However, the reducing ends of glycans retain activity and can be used to quantify global glycans using reductive amidation, hydrazone or oxime formation. Another limitation of the method is that HPLC elution of heavy and light glycans is not at the same time due to heavy deuterium labeling; however, the use of deuterium can be replaced by C13 chromatography in p-toluidine.

Conclusion

In this paper we describe a method for solid-phase sialic acid labeling. p-Toluidine was successfully used to modify the acid component of sialylated glycans on solid phase with a reliable and robust method. The modification provides stability to the sialylated glycan and

prevents loss of sialic acid during MS analysis. A deuterium reagent, d9-p-toluidine was used reliably for the qualitative and relative quantitative analysis of sialylated glycans by MS. The applicative nature of this developed method has also been explored from the qualitative and relative quantitative analysis of N-glycans isolated from human serum and pancreatic cells. The results demonstrate the utility of this approach for detailed and comparative investigations of sialylated glycans from different samples. In future work this method will be applied to the quantitative analysis of O-linked glycans derived from complicated biological specimens in order to obtain aberrant glycosylation associated with major diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Scheme strategy for the solid-phase labeling of sialic acid and quantitative analysis of sialylated glycans by mass spectrometry. Proteins are conjugated to solid support. Bound proteins are labeled with light or heavy p-toluidine. N-glycans are released from the proteins using PNGase F. N-glycans are analyzed using MALDI-MS

Figure 2.

Mass Spectrum from Shimadzu AXIMA Resonance MALDI Mass spectrometer of N-glycans from bovine fetuin. Sialic acids of N-glycans were amidated with p-toluidine in presence of EDC. Glycans were analyzed in DHB/DMA matrix by positive ion mode. 1000 shots were acquired. Glycoworkbench was used for cartoons of the most plausible structure based on accurate mass. Blue square represents GlcNAc, green circle represents mannose, yellow circle represents galactose, red triangle represents fucose and pink diamond represents p-toluidine modified sialic acid.

Figure 3. MALDI-MS spectrum of N-glycans from human serum. Two equal aliquots of serum proteins were bound to beads and one aliquot was labeled with light p-toluidine, second aliquot was labeled with heavy p-toluidine D9. N-glycans were released using PNGase F. The mixture of glycans was subjected to MALDI-MS analysis. A) Light labeled monosialylated N-glycan. B) Heavy labeled mono sialylated N-glycan. C) MS spectrum of 1:1 mixture of light and heavy labeled monosialylated glycan. (D) Mass spectrum of serum N-glycans, difference between the peak pairs from light and heavy labeled sialic acids represents number of sialic acids present in the N- glycan structure. Glycoworkbench was used for cartoons of the most plausible structure based on accurate mass and difference between the doublets. Blue square represents GlcNAc, green circle represents mannose, yellow circle represents galactose, red triangle represents fucose and pink diamond represents p-toluidine modified sialic acid.

Figure 4.

N-Linked glycans of proteins from pancreatic cancer cell line SW1990 treated with 1,3,4-O-Bu₃ManNAc. A) Mass spectrum of N-glycans from SW1990 cells treated with 1,3,4-O-Bu₃ManNAc and labeled with heavy p-toluidine. B) Mass spectrum of N-glycans from SW1990 cells not treated with 1,3,4-O-Bu₃ManNAc as control and labeled with light p-toluidine. C) Mass Spectrum of N-glycans from SW1990 cells treated with and without 1,3,4-O-Bu₃ManNAc and labeled with heavy and light p-toluidine respectively and then mixed in 1:1 ratio. Glycoworkbench was used for cartoons of the most plausible structure based on accurate mass and difference between the doublets. Blue square represents GlcNAc, green circle represents mannose, yellow circle represents galactose, red triangle represents fucose and pink diamond represents p-toluidine modified sialic acid, and white diamonds represents sialic acid modified with heavy p-toluidine.

N-glycan compositions identified from serum. Two equal aliquots of serum proteins were bound to beads and one aliquot was labeled with light p-toluidine, second aliquot was labeled with heavy p-toluidine. PNGase F released glycans were mixed and analyzed using MALDI. The number of sialic acids present on the glycans was identified by calculating the difference between light and heavy labeled serum samples.

Table 1

Core	Fucose	HexNAc	Hexose	Sialic Acid	Theoretical Mass	Observed Mass
Core + Na	0	1	0	0	1136.423	1136.18
Core + Na	1	0	1	0	1241.438	1242.20
Core + Na	0	0	2	0	1257.433	1257.46
Core + Na	1	1	0	0	1282.481	1280.44
Core + Na	0	1	1	0	1298.475	1298.40
Core + Na	0	2	0	0	1339.518	1339.36
Core + Na	0	0	3	0	1419.486	1419.20
Core + Na	1	1	1	0	1444.533	1442.17
Core + Na	0	1	2	0	1460.528	1457.19
Core + Na	1	2	0	0	1485.576	1485.12
Core + Na	0	2	1	0	1501.571	1501.09
Core + Na	0	3	0	0	1542.613	1541.79
Core + Na	1	0	3	0	1565.544	1561.91
Core + Na	0	0	4	0	1581.538	1580.89
Core + Na	1	2	1	0	1647.629	1646.85
Core + Na	0	2	2	0	1663.624	1662.80
Core + Na	0	1	1	1	1678.741	1677.81
Core + Na	1	3	0	0	1688.671	1688.78
Core + Na	0	0	5	0	1743.591	1742.65
Core + Na	1	2	2	0	1809.682	1808.55
Core + Na	1	1	1	1	1824.799	1822.34
Core + Na	1	3	1	0	1850.724	1850.32
Core + Na	0	2	1	1	1881.836	1881.42
Core + Na	0	0	6	0	1905.644	1904.35
Core + Na	1	0	3	1	1945.809	1942.84
Core + Na	1	3	2	0	2012.777	2011.17
Core + Na	1	2	1	1	2027.894	2026.16

Core	Fucose	HexNAc	Hexose	Sialic Acid	Theoretical Mass	Observed Mass
Core + Na	0	2	2	1	2043.889	2043.12
Core + Na	1	2	2	1	2189.947	2189.38
Core + Na	1	4	2	0	2215.872	2215.74
Core + Na	0	3	2	1	2246.85	2247.12
Core + Na	0	7	0	0	2354.995	2354.49
Core + Na	1	3	2	1	2393.042	2393.41
Core + Na	0	2	2	2	2424.154	2424.43
Core + Na	1	2	2	2	2570.212	2570.26
Core + Na	1	3	2	2	2773.308	2773.26
Core + Na	0	3	3	2	2789.303	2788.29
Core + Na	1	3	3	2	2935.361	2935.23
Core + Na	0	4	4	2	3154.451	3154.69
Core + Na	0	3	3	3	3169.568	3169.30
Core + Na	1	3	3	3	3315.626	3315.28
Core + Na	0	4	4	3	3534.716	3534.28
Core + Na	1	4	4	3	3680.774	3680.18
Core + Na	0	4	4	4	3914.982	3914.23
Core + Na	1	4	4	4	4061.040	4061.23

Observed mass is the mass of glycan labeled with light p-toluidine
Core represents core structure of N-glycan which is 2 HexNAc and 3 Hexose

Relative Quantitation of Sialylated N-glycans Identified from SW1990 Cells with and without 1,3,4-O-Bu₃ManNAc Treatment. Glycans from cells without treatment were labeled with light p-toluidine, glycans from cells with treatment were labeled with heavy p-toluidine. PNGase F released glycans were analyzed using MALDI. The number of sialic acids present on the glycans was identified by calculating the difference between light and heavy labeled serum samples and quantified based on their intensities.

Table 2

Core + Na	Fucose	HexNAc	Hexose	Sialic Acid	[M+Na] ⁺	H/L	Stdev
Core +Na	0	2	2	1	2043.89	1.29	0.44
Core +Na	1	2	2	1	2189.95	1.09	0.17
Core +Na	2	2	2	1	2336.01	1.10	0.13
Core +Na	1	3	2	1	2393.04	1.95	0.11
Core +Na	2	3	2	1	2539.10	1.31	0.10
Core +Na	1	3	3	1	2555.10	1.31	0.45
Core +Na	1	2	2	2	2570.21	1.02	0.13
Core +Na	3	3	2	1	2685.16	1.00	0.17
Core +Na	2	3	3	1	2701.15	1.03	0.23
Core +Na	1	4	3	1	2758.19	1.66	0.31
Core +Na	1	3	2	2	2773.31	1.70	0.18
Core +Na	2	4	3	1	2904.25	2.04	0.50
Core +Na	1	3	3	2	2935.36	0.98	0.21
Core +Na	3	4	3	1	3050.31	1.39	0.30

H: Heavy p-toluidine labeled sialylated N-glycans from 1,3,4-O-Bu₃ManNAc treated cells
L: Light p-toluidine labeled sialylated N-glycans from untreated (control) cells
Core represents core structure of N-glycan which is 2 HexNAc and 3 Hexose