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ENHANCED SENSITIVITY OF LC-MS ANALYSIS OF PERMETHYLATED N-GLYCANS THROUGH ONLINE PURIFICATION

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Abstract

Aberrant glycosylation of proteins and lipids has been implicated in many human diseases, thus prompting the need for reliable analytical methods that permit reliable quantification of glycans originating from biological specimens. MS of permethylated glycans is currently employed to monitor disease related aberrant glycosylation of proteins and lipids. However, enhancing the sensitivity of this type of analysis is still needed. Here, analysis of permethylated glycans at enhanced sensitivity is attained through miniaturized solid-phase permethylation as well as on-line solid-phase purification. Solid-phase permethylation method was miniaturized by reducing the amount of sodium hydroxide beads (one-third the original amount) packed in microspin columns. The efficiency of glycan permethylation was not adversely affected by this reduction. On-line solid-phase purification of permethylated N-glycans derived from model glycoproteins, such as fetuin, α -1 acid glycoprotein and ribonuclease B, offered more sensitive and reproducible results than off-line liquid-liquid and solid-phase extractions. On-line solid-phase purification method described here permitted a seventy-five percent increase in signal intensities of permethylated glycans relative to off-line purification methods. This is mainly due to the minimized sample handling associated with an on-line cleaning procedure. The efficiency and utility of on-line solid-phase purification was also demonstrated for N-glycans derived from human blood serum. On-line solid-phase purification permitted the detection of 66 N-glycan structures, while only 58 glycan structures were detected in the case of samples purified through liquid-liquid extraction. The intensities of the 58 structures that were detected in both cases were seventy-five percent higher for samples that were purified through the described method.

Keywords

Glycans; Glycoproteins; Permethylation; LC-MS; LC-MSMS

Introduction

Glycosylation is considered to be one of the most common and structurally diverse posttranslational modifications of proteins. The multiple glycosylation sites of proteins and their associated microheterogeneity are deemed to be the source of heterogeneity of glycosylation patterns and the difficulties associated with resolving fine structural differences in large biopolymers. The glycoforms of a protein, or the complexity and

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structural variation associated with a glycoprotein, ultimately define the function and activity of a glycoprotein [1–3].

Key biochemical processes, such as protein folding, stability, and localization are defined by the glycosylation of proteins [4]. Additionally, cellular communication, such as cell-cell, cell-matrix, protein-protein, and sugar-sugar interactions are controlled through specific interactions between a glycan and its target protein(s) [2, 5–10]. Modulating the glycosylation of proteins by various site occupancy changes on the polypeptide chain or in the variation of the oligosaccharide structures occupying a particular size on a protein modulates the biological activity of glycoproteins, and abnormal glycosylations of glycoconjugates have been identified in many mammalian diseases, including hereditary disorders, immune deficiencies, cardiovascular disease, and cancer [2, 6, 7]. The focus of many biomedical research initiatives has turned to developing analytical tools that allow the subtle monitoring of these biologically significant glycosylation changes so as to aid in the diagnosis and prognosis of these diseases and to further understand them at the molecular level so as to assist in development of effective drugs able to cure [2, 5–11].

With this goal in mind, glycan analysis is an essential first step to better understanding the biological roles of glycoproteins. However, glycans have enormous structural variation and frequently exist at low concentrations, rendering glycan analysis a challenge. Comprehensive structural analysis of glycans is only achieved through the use of integrated analytical methodologies at high sensitivity [3, 12, 13]. Over the past decade, mass spectrometry (MS) has become a powerful tool for the structural elucidation of glycan structures in various biological molecules [3, 12, 13]. MS analysis of native glycans is routinely employed in these studies, but MS analysis of permethylated glycans offers several advantages, including enhanced structural determination through enhancing sensitivity and improving the interpretation of tandem MS data, and stabilized and efficient ionization of sialic acid residues. Moreover, permethylation of glycan structures allows for the simultaneous analysis of both acidic glycans (sialic acids) and neutral glycans in positive MS mode, which allows for the quantitative comparison of the abundances of both acidic and neutral glycans. The enhanced sensitivity observed when analyzing permethylated glycans is mainly due to the enhanced ionization efficiency of glycans as a result of derivatization. This has prompted the recent widespread use of MS profiles of permethylated glycans for the analysis and characterization of protein glycosylation [4, 14–21].

Ciucanu and Kerek method of permethylation of glycans is currently employed routinely in many laboratories due to its simplicity and cleaner end products [22]. This is a modification of a previous method described by Hakomori [23] and it involves the reaction of glycans with methyl iodide in the presence of sodium hydroxide beads prepared in dimethyl sulfoxide. The efficiency of this permethylation approach was recently enhanced through the addition of traces of water, but it has remained to be less satisfactory for the analysis of small amounts (low picomole to femtomole quantities) of glycans [24]. This inability to permethylate trace levels of glycan is mainly attributed to the peeling reactions and oxidative degradations that are commonly associated with the high pH resulting from the solubilization of sodium hydroxide beads when the reaction is deemed complete. These limitations have been recently alleviated through the development of solid-phase permethylation method which involves the packing of sodium hydroxide in small columns [18, 26–28].

A major advantage of solid-phase derivatization is the separation of excess reagent from samples, resulting in better sample recovery, especially when working with small amounts of sample [25]. Solid-phase permethylation, which yields highly efficient permethylation of small amounts of glycans derived from glycoproteins found in biological samples, has been

very effective in enhancing MS analysis of small quantities [18, 26–28]. This permethylation approach has been recently utilized for the characterization of sulfated glycan structures [29, 30]. Solid-phase permethylation was recently employed in conjunction with non-specific proteolytic digestion of glycoproteins to release and permethylate O-glycans. This new method allowed, for the first time, the MS analysis of O-glycans at a level of sensitivity comparable to that routinely attained for N-glycans [31].

In this study, the potential of miniaturizing solid-phase permethylation and on-line purification of solid-phase permethylated N-glycans derived from model glycoproteins and human blood serum was explored as a means to minimize the loss of already minute amounts of sample and yield more sensitive results during MS analysis. The solid-phase permethylation approach was reduced by using one third the amount of sodium hydroxide beads that are conventionally used. Additionally, solid-phase permethylated N-glycans derived from model glycoproteins and human blood serum were on-line purified after permethylation using an LC-MS setup that is routinely used in proteomics which employ C18 trapping columns for purification of samples prior to LC-MS/MS analysis. Additionally, the viability of this setup and the ability to use it for glycomics analyses potentially permits high throughput analysis.

Experimental

Materials

Model glycoproteins, including ribonuclease B (RNase B) from bovine pancreas, fetuin from bovine serum, α_1 -acid glycoprotein (AGP) from human serum, human blood serum (BS), ammonium-borane complex, sodium hydroxide beads, dimethyl sulfoxide, methyl iodide, trifluoroacetic acid, chloroform, and formic acid were purchased from Sigma-Aldrich (St. Louis, MO). N-Glycosidase purified from *Flavobacterium meningosepticum* (PNGase F) was acquired from New England Biolabs Inc. (Ipswich, MA). HPLC grade methanol, isopropanol, and acetic acid were procured from Fisher Scientific (Pittsburgh, PA). Acetonitrile was obtained from JT Baker (Phillipsburg, NJ). HPLC grade water and sodium hydroxide were purchased from Mallinckrodt Chemicals (Phillipsburg, NJ).

Release and reduction of N-glycans from model glycoproteins

A 10- μ g aliquot of fetuin was reconstituted in 10 μ L G7 buffer (50 mM sodium phosphate buffer, pH 7.5) that has been diluted ten times in HPLC. A 25- μ g aliquot of AGP and a 25- μ g aliquot of RNase B were also prepared in a similar fashion. A 1- μ L aliquot of PNGase F stock solution was added to 9 μ L HPLC water, while a 1- μ L aliquot of this diluted PNGase F solution (50 units) was added to each glycoprotein samples prior to incubation overnight at 37° C in a water bath (Thermo Scientific, Pittsburgh, PA). Next, samples were dried under vacuum (Labconco, Kansas City, MO). The reducing end of N-glycans was reduced to alditols by adding a 30- μ L aliquot of ammonium-borane complex aqueous solution (10 μ g/ μ L concentration) to each dried sample prior to incubation at 60° C for one hour. Each of the samples was then dried and resuspended in a 300- μ L aliquot of an HPLC grade methanol to form volatile methyl borate. This salt was removed through repeated addition of methanol and dring, until methyl borate white powder is no longer visible in the samples.

Release, reduction, and purification of human blood serum N-glycans

Five blood serum samples were prepared by adding a 20- μ L aliquot of phosphate buffer saline (50 mM sodium phosphate, 100 M sodium chloride, pH 7.5) to a 5- μ L aliquot of blood serum. These samples were then subjected to thermal denaturation through incubation at 60° C for 45 minutes in a water bath. Samples were then cooled to room temperature prior to the addition of 2.4- μ L aliquots of PNGase F (120 units) and incubation at 37°C overnight.

The samples were then subjected to solid-phase purification with charcoal microspin columns (Harvard Apparatus, Holliston, MA). The charcoal spin columns were initially washed with 400 μ l of 100% ACN and 400 μ l of 85% aqueous ACN solution containing 0.1% TFA. This was repeated three times. The columns were then conditioned with a 400- μ l aliquot of 5% aqueous ACN solution containing 0.1% TFA twice. Next, the PNGase F treated samples were applied to the columns after the addition of a 350- μ l aliquot of 5% ACN with 0.1% TFA to each sample. Spin columns with bound glycans were then washed with 400 μ l of 5% ACN aqueous solution containing 0.1% TFA three times. N-Glycans bound to the charcoal spin columns were finally eluted with 400 μ l of 40% aqueous ACN solution containing 0.1% TFA. This elution step was repeated three times and all elunets were collected in one tube the content of which was finally dried under vacuum.

The purified N-glycans were then resuspended in 10- μ L aliquots of 10 μ g/ μ L aqueous ammonium-borane complex solution. Next, samples were placed in a 60° C water bath for 1 hour to reduce the reducing ends of glycans. Reduced glycan samples were then dried under vacuum. A 100- μ L aliquot of HPLC grade methanol was added and sample was dried under vacuum. This step was repeated until all methyl borated formed as a result of the addition of methanol evaporated.

Miniaturized Solid-Phase Permethylation

Released N-glycans were permethylated according to previously published procedure with modification [18, 26–28]. Briefly, empty spin columns (Harvard Apparatus, Holliston, MA) were filled with sodium hydroxide beads suspended in ACN to 1 cm depth. This was one-third the amount previously described [18, 26–28]. The columns were then placed in eppendorf tubes and the tubes were centrifuged (Thermo Scientific, Pittsburgh, PA) at 1.6K rpm. The spin columns were next conditioned with 200- μ L aliquots of dimethyl sulfoxide (DMSO) and centrifuged again at 1.6K rpm. This step was repeated three times.

Initially, N-glycans from fetuin were used to determine the optimum amount of methyl iodide required to attain efficient permethylation under the reduced amount of sodium hydroxide beads. Samples were divided into groups of 5 and each group had one volume of methyl iodide added to it, including 5 μ L, 7.5 μ L, 10 μ L, and 15 μ L. Each of these samples were resuspended in 7.5 μ L of DMSO, and 0.3 μ L water prior to loading to the sodium hydroxide columns. The spin columns prepared as described above were transferred to a clean 2-mL eppendorf tubes and the resuspended samples were added to the columns and allowed to sit for thirty minutes. The columns were centrifuged at 1.6K rpm for 1 minute. A second aliquot of methyl iodide was added to the mixture in the tube prior to re-applying to the spin columns. The solution was then allowed to sit for another fifteen minutes prior to centrifugation again at 1.6K rpm for 1 minute. The content of the spin column was then washed with a 50- μ L aliquot of ACN to ensure efficient elution of all permethylated glycans. The permethylated samples were then vacuum dried and resuspended in enough aqueous solution to have no more than 20% organic content of the solution. One-third of the permethylated glycans were subjected to online purification as described below, while the other 2 one-thirds were subjected to off-line solid-phase and liquid-liquid extractions (SPE and LLE, respectively) as described next. Dried N-glycans derived from RNase B, AGP, and BS were permethylated using the reduced amount of sodium hydroxide beads and optimum methyl iodide determined from fetuin experiments. The samples were then divided into two halves which were subjected to purification by on-line SPE and LLE.

Liquid-liquid Extraction of Permethylated N-glycans

A 400- μ L aliquot of 500 mM sodium chloride solution and a 400- μ L aliquot of chloroform were used for LLE of permethylated glycans. Samples were mixed thoroughly in a vortex

mixer (Labnet, Edison, NJ) and centrifuged at 10K rpm. The top (aqueous) layer was discarded and another 400- μ L aliquot of water was added to the chloroform. Samples were mixed, centrifuged and the top (aqueous) layer was again discarded. This step was repeated once more before drying samples under vacuum.

Off-line solid-phase purification of permethylated N-glycans

Off-line SPE was performed using C18 cartridges (Harvard Apparatus, Holliston, MA) which were initially washed twice with 400- μ L aliquot of 100% ACN. The cartridges were also washed twice with 400- μ L aliquot of 80% aqueous ACN solution containing 0.1% TFA. These washes were accomplished by centrifugation at 1.6K rpm. The cartridges were then conditioned twice with 400- μ L aliquot of 5% aqueous ACN solution containing 0.1% TFA prior to the application of samples.

At the end of the derivatization, permethylated glycans are present in DMSO solution which does not allow efficient trapping on the C18 cartridges; therefore, permethylated samples were mixed with a 200- μ L aliquot of 5% aqueous ACN solution containing 0.1% TFA prior to loading to the conditioned cartridges. The columns were then centrifuged at 1.6K rpm and the excess solution was discarded. The samples were then washed three times with 400 μ L of 5% aqueous ACN solution containing 0.1% TFA prior to eluting the permethylated glycans using two 200- μ L aliquots of 80% aqueous ACN solution containing 0.1% TFA. Finally, the eluted glycans were resuspended in enough 2% aqueous ACN solution containing 0.1% formic acid (FA) to make the equivalent of 1 μ g/ μ L glycoprotein solutions which were subsequently subjected to LC-MS/MS analysis. The equivalence of N-glycans derived from 0.1 μ g of each of the model glycoproteins were subjected to LC-MS/MS analysis. The equivalence of N-glycans derived from 0.5 μ L blood serum were also subjected to LC-MS/MS analysis.

On-line purification of permethylated N-glycans and LC-MS and LC-MSMS

Upon complete permethylation, the permethylated glycans are present in 7.5 μ L DMSO. In order to be able to effectively retain the permethylated glycan on the C18 trapping column configured as part of LC-MS system, the total percentage of DMSO should not exceed 20%. Accordingly, samples are resuspended in a 2% aqueous ACN solution containing 0.1% FA solution to make 20% aqueous DMSO/ACN solution with final glycoprotein concentration equivalent to 1 μ g/ μ L. Next, samples were initially injected onto an Acclaim® PepMap100 C18 nano-trap column (Dionex, Sunnyvale, CA). The samples were then washed using mobile phase A, consisting of 98% HPLC grade water, 2% ACN, and 0.1% formic acid. The washing was performed for 10 min at a flow rate of 3 μ L/min. prior to loading to a C₁₈ column. The equivalence of N-glycans derived from 0.1 μ g of the model glycoproteins was injected. The equivalent of N-glycans derived from 0.5 μ L human blood serum was injected. Permethylated glycans purified by the C18 trapping columns were then injected onto a nano Acclaim® PepMap100 C18 column (75 μ m ID $\times$ 15 cm) which is connected to the nano source of the orbitrap mass spectrometer. Permethylated glycans were separated using a reversed-phase gradient of solvent B (100 % acetonitrile with 0.1% formic acid) increasing from 20% to 38% over 1 minute, from 38%–45% over the next 32 minutes, and from 45%–90% over 3 minutes at 300 nL/min flow rate. The mass spectrometer was operated in an automated data-dependent acquisition mode, switching between MS scan and CID tandem MS. In this mode, ions were subjected to an initial full MS scan from m/z 500 to 2000 in the Orbitrap at 15,000 mass resolutions. Subsequently, the precursor ions were automatically isolated using the data-dependent acquisition mode with a 3 m/z isolation width. The eight most intense ions in the survey scan (starting with the most intense) were sequentially subjected to CID tandem MS in the ion trap at 35% normalized collision energy with activation Q and activation time set to 0.25 and 15 ms, respectively. The total cycle (9 scans)

is continuously repeated for the entire LC-MS run under data-dependent conditions with dynamic exclusion set to 60 seconds.

Results and Discussion

Reducing the number of steps involved in the preparation of any samples, which inherently reduces the surface areas that come into contact with any samples, substantially minimize sample losses and subsequently enhance analytical sensitivity. Upon complete permethylation, high amount of sodium hydroxide and unreacted methyl iodide are present in the final reaction mixture. These adversely influence the MS ionization efficiency of the permethylated glycans, thus rendering their analysis quite impossible. Therefore, permethylated glycans are purified prior to MS analysis to remove such interfering species. LLE and SPE are the most common methods employed to purify permethylated glycans, since this type of derivatization of glycans produces highly hydrophobic species that are easily partition in the organic layer of LLE or retained onto the reversed-phase solid support of SPE. These purification methods involve several steps and are time consuming. The former jeopardizes efficient sample recovery, while the latter limit throughput. Therefore, this study is aimed at enhancing the sensitivity of MS analysis of permethylated N-glycans derived from glycoproteins through miniaturizing solid-phase permethylation in conjunction with on-line SP purification using an LC-MS/MS setup that is routinely employed in proteomics. Miniaturizing solid-phase permethylation reduces the amount of sodium hydroxide presents at the end of permethylation, while on-line SP purification minimize sample losses associated with the other purification procedures (SPE and LLE).

The optimum amount of methyl iodide needed to efficiently permethylate N-glycans derived from glycoproteins when a reduced amount of sodium hydroxide beads is used in solid-phase permethylation, was initially investigated. Fetuin N-glycans were permethylated using the miniaturized solid-phase permethylation and varied amount of methyl iodide (5–15 μ l). The intensities of the different N-glycans derived from fetuin were then compared between the different amounts tested. For each amount five separate permethylation were performed followed by LC-MS/MS analysis of each sample. Figure 1a demonstrates that increasing the amount of methyl iodide utilized yielded an increase in the intensity of permethylated N-glycans derived from fetuin. Optimum amount of methyl iodide needed to sustain efficient permethylation of N-glycans at reduced sodium hydroxide beads appeared to be 15 μ l (Figure 1a) with limited variation as suggested by the standard deviation bars for the 5 analysis at each methyl iodide amount. This volume of methyl iodide was deemed optimum and was used for the permethylation of all samples described below. Additionally, the data shown in Figure 1a suggest that reducing the amount of hydroxide beads does not appear to substantially diminish the permethylation efficiency as long as enough methyl iodide is used (Figure 1a).

Next, the efficiency of online SP purification was compared to SPE and LLE for N-glycans derived from fetuin. Figure 1b illustrates the differences in intensity of the 3 different purification approaches investigated here (namely on-line SP purification, off-line SPE, and LLE). On-line SP purification employing the C18 trapping columns yielded the most intense signals out of the three purification procedures. The MS signals observed for all permethylated glycans derived from fetuin and purified on line were at least 75% and 95% higher than those observed for LLE and off-line SPE, respectively. These results were expected since sample losses to the surfaces of devices being used take place prior to MS measurements. Sample losses during sample preparation (SPE, LLE, dialysis, lyophilization, etc.) may easily become a bottleneck of the entire analysis. Working at reduced scale also introduces other problems such as contamination (dust, solvent, reagent impurities, etc.). Accordingly, minimizing sample handling and transfer steps during analysis are critical. In

high-sensitivity work, reducing column diameters, solvent flow-rates and the overall surface area that a glycan sample may encounter during analysis can significantly enhance analytical sensitivity. Therefore, the ability to perform on-line SP purification of permethylated glycans is invaluable to minimizing sample losses and, consequently enhancing analysis sensitivity. Additionally, the miniaturized permethylation approach we employed gives results comparable to those attained utilizing the established permethylation approach, demonstrating that smaller amounts of sample can be used to obtain accurate, reproducible results. Reducing the amount of sodium hydroxide not only permitted the permethylation of small amount of glycans, but also made on-line SP purification attainable. By reducing the amount of sodium hydroxide beads, the final amount of sodium hydroxide present in the reaction mixture at the end of permethylation was also reduced. This ensured that efficient on-line trapping and cleaning using the same time commonly employed in proteomics (10 min) possible.

Since the results for fetuin were very promising (Figure 1), the potential of using the described methods for other model glycoprotein with different types of glycans was explored. The on-line SP purification step also offered higher signal for the high-mannose N-glycans derived from RNase B. Figure 2 depicts the glycomic profile of Man 5 through Man 9, comparing off-line SP extraction (Figure 2a) to on-line SP purification (Figure 2b). The equivalence of N-glycans derived from 0.1- μ g of RNase was analyzed in both cases. The relative peak intensities of on-line SP purification exhibited 80% increase relative to SPE (compare Figure 2a to 2b). This increase was observed for all glycans derived from RNase B. This increase was consistent for all five samples that were prepared separately and subjected to LC-MS/MS analysis with on-line purification (RSD < 5%, data not shown).

The on-line purification technique was also effective in enhancing the analytical sensitivity of permethylated focusylated and sialylated glycans, as illustrated in Figure 3 for sialylated N-glycans derived from human α_1 -acid glycoprotein. A 0.1- μ g aliquot of the sample was loaded on to the nano C₁₈ trap column and the peak intensities of the permethylated sialylated glycans reflect the enhanced sensitivity when on-line SP purification was employed. Using an on-line SP purification approach clearly provides higher sensitivity. On-line SPE permitted again a 75% increase in the MS signals of all glycans derived from AGP relative to off-line PSE.

Moreover, miniaturized permethylation and on-line SPE were also very effective for real application, such as pooled human blood serum Figures 4 and 5. Representatives of the N-glycans derived from human blood serum are shown in Figure 4 to depict the difference between LLE (Figure 4a) and online PSE (Figure 4b). On the other hand, Table 1 summarizes the intensities of 66 permethylated N-glycan structures observed in the LC-MS analyses for 5 samples separately prepared (N=5) and subjected to on-line SP purification and another five that were purified by LLE prior to LC-MS analysis. The same amount was subjected to LC-MS/MS. As illustrated in Figure 4 and summarized in Table 1, sialylated, high mannose, branched and hybrid N-glycans derived from human blood serum also demonstrated an enhanced sensitivity upon utilizing both miniaturized permethylation and on-line SPE. The peak intensities of these heterogeneous N-glycans show three-fold or more increase in abundance in the case of on-line SPE relative to LLE (Figure 5). These results are from 0.5 μ l being loaded on to the column which is very minute amount. Therefore, online SP purification also appears to enhance the MS signal of permethylated N-glycans derived from blood serum. This enhancement is achieved by reducing the amount of salts and unreacted reagents as well as sample losses. Moreover, sample purified through LLE allowed the detection of only 58 N-glycans by LC-MS/MS analysis, while 66 structures were detected for on-line SP purified samples. These structures endogenously exist at low abundance [19–21]. This increase in the number of detected glycans is attributed

to the 3 folds enhanced MS sensitivity attained through online SP purification. Additionally, on-line SP purification is allowing the utility of LCMS/MS setup that is routinely utilized for proteomics which permits high throughput analysis. Samples were loaded to the autosampler and were consecutively analyzed.

Conclusions

Enhanced sensitivity of analytes was observed through on-line purification due to the minimization of sample handling and less overall surface interaction with the sample. Also, reducing the amounts of sodium hydroxide beads in conjunction with on-line purification allowed enhanced MS sensitivity. Previously, a purification step was necessary before LC-MS/MS of permethylated N-glycans. On-line purification on both model glycoproteins and pooled human blood serum prior to LC-MS/MS has shown to be rapid and reproducible across a range of complex N-glycan structures. The described online purification permits the utility of conventional proteomics LC-MS/MS platform for the analysis of permethylated N-glycans. Thus, the described approach employs proteomics platform for the glycomics analysis of real samples such as human blood serum samples.

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Abbreviations

(CID)	Collision induced dissociation
(ESI)	electrospray ionization
(HPLC)	high-performance liquid chromatography
(MS)	mass spectrometry
(MALDI)	matrix-assisted laser desorption ionization

REFERENCES

1. Bertozzi CR, Kiessling LL. Chemical Glycobiology. *Science*. 2001; 291:2357–2364. [PubMed: 11269316]
2. Dwek RA. Glycobiology: Toward Understanding the Function of Sugars. *Chem Rev*. 1996; 96(2): 683–720. [PubMed: 11848770]
3. Mechref Y, Novotny MV. Structural investigations of glycoconjugates at high sensitivity. *Chem Rev*. 2002; 102(2):321–69. [PubMed: 11841246]
4. Viseux N, Hronowski X, Delaney J, Domon B. Qualitative and quantitative analysis of the glycosylation pattern of recombinant proteins. *Anal Chem*. 2001; 73(20):4755–62. [PubMed: 11681448]
5. Clark GF, Dell A, Morris HR, Patankar M, Oehninger S, Seppala M. Viewing AIDS from a glycobiological perspective: potential linkages to the human fetoembryonic defence system hypothesis. *Mol Hum Reprod*. 1997; 3(1):5–13. [PubMed: 9239703]
6. Dennis JW, Granovsky M, Warren CE. Protein glycosylation in development and disease. *Bioassays*. 1999; 21:412–421.
7. Lowe JB, Marth JD. A genetic approach to Mammalian glycan function. *Ann. Rev. Biochem*. 2003; 72:643–691. [PubMed: 12676797]
8. Pochee E, Litynska A, Amoresano A, Casbarra A. Glycosylation profile of integrin alpha 3 beta 1 changes with melanoma progression. *Biochim Biophys Acta*. 2003; 1643(1-3):113–23. [PubMed: 14654234]

9. Rudd PM, Elliott T, Cresswell P, Wilson IA, Dwek RA. Glycosylation and the immune system. *Science*. 2001; 291(5512):2370–6. [PubMed: 11269318]
10. Zachara NE, Hart GW. The emerging significance of O-GlcNAc in cellular regulation. *Chem Rev*. 2002; 102(2):431–8. [PubMed: 11841249]
11. Hakomori S. Glycosylation defining cancer malignancy: New wine in an old bottle. *Proc. Nat. Acad. Sci. U.S.A.* 2002; 16:10232–10233.
12. Mechref Y, Novotny MV. Miniaturized Separation Techniques in Glycomic Investigations. *J. Chromatogr. B*. 2006; 841:65–78.
13. Novotny MV, Mechref Y. New Hyphenated Methodologies in High - Sensitivity glycoprotein Analysis. *J. Sep. Sci.* 2005; 28:1956–1968. [PubMed: 16276785]
14. An HJ, Dodds ED, Lebrilla CB. A novel solution for glycoproteomics: rapid method for the determination of glycosylation site and oligosaccharide heterogeneity in glycoproteins. *Am. Biotechnol. Lab.* 2007; 25:28–29.
15. An HJ, Miyamoto S, Lancaster KS, Kirmiz C, Li B, Lam KS, Leiserowitz GS, Lebrilla CB. Profiling of Glycans in Serum for the Discovery of Potential Biomarkers for Ovarian Cancer. *J. Proteome Res.* 2006; 5:1626–1635. [PubMed: 16823970]
16. Goetz JA, Mechref Y, Kang P, Jeng M-H, Novotny MV. Glycomic Profiling of Invasive and Non-invasive Breast Cancer Cells. *Glycoconjugate J.* 2009; 26:117–131.
17. Goldman R, Ransom H, Varghese R, Goldman L, Bascug G, Loffredo C, Abdel-Hamid M, Gouda I, Abo-Elkhir S, Kyselova Z, Mechref Y, Novotny MV. Detection of Hepatocellular Carcinoma using Glycomic Analysis. *Clin. Cancer Res.* 2009; 15:1808–1813. [PubMed: 19223512]
18. Kang P, Mechref Y, Kyselova Z, Goetz JA, Novotny MV. Comparative Glycomic Mapping (C-GlycoMAP) through Quantitative Permethylated and Stable-isotope Labeling. *Anal. Chem.* 2007; 79:6064–6073. [PubMed: 17630715]
19. Kyselova Z, Mechref Y, Bataineh MM, Dobrolecki LE, Hickey RJ, Vinson J, Sweeney CJ, Novotny MV. Alterations in the Serum Glycome Due to Metastatic Prostate Cancer. *J. Proteome Res.* 2007; 6(5):1822–1832. [PubMed: 17432893]
20. Kyselova Z, Mechref Y, Kang P, Goetz JA, Dobrolecki LE, Sledge G, Schnaper L, Hickey RJ, Malkas LH, Novotny MV. Breast Cancer Diagnosis/Prognosis through Quantitative Measurements of Serum Glycan Profiles. *Clin. Chem.* 2007 in press.
21. Mechref Y, Hussein A, Bekesova S, Pungpapong V, Zhang M, Dobrolecki LE, Hickey RJ, Hammoud ZT, Novotny MV. Quantitative Serum Glycomics of Esophageal Adenocarcinoma, and Other Esophageal Disease Onsets. *J. Proteome Res.* 2009; 8:2656–2666.
22. Ciucanu I, Kerek F. A simple and rapid method for the permethylation of carbohydrates. *Carbohydrate Research*. 1984; 131(2):209–217.
23. Hakomori S. A Rapid Permethylated Glycolipid, and Polysaccharide Catalyzed by Methylsulfinyl Carbanion in Dimethyl Sulfoxide. *J. Biochem (Tokyo)*. 1964; 55:205–8. [PubMed: 14135466]
24. Ciucanu I, Costello CE. Elimination of oxidative degradation during the per-O-methylation of carbohydrates. *J Am Chem Soc.* 2003; 125(52):16213–9. [PubMed: 14692762]
25. Rosenfeld JM. Solid-phase analytical derivatization: enhancement of sensitivity and selectivity of analysis. *J Chromatogr A.* 1999; 843(1-2):19–27. [PubMed: 10399851]
26. Kang P, Mechref Y, Novotny MV. High-throughput solid-phase permethylation of glycans prior to mass spectrometry. *Rapid Commun Mass Spectrom.* 2008; 22:721–734. [PubMed: 18265433]
27. Kang P, Mechref YK, I. Novotny MV. Solid-phase permethylation of glycans for mass spectrometric analysis. *Rapid Commun Mass Spectrom.* 2005; 19(23):3421–8. [PubMed: 16252310]
28. Mechref Y, Kang P, Novotny MV. Solid-phase permethylation for glycomic analysis. *Methods Mol. Biol.* 2009; 534:53–64. [PubMed: 19277536]
29. Lei M, Mechref Y, Novotny MV. Structural Analysis of Sulfated Glycans by Sequential Double-Permethylated Using Methyl Iodide and Deuteromethyl Iodide. *J. Amer. Soc. Mass Spectrom.* 2009; 20:1660–1671. [PubMed: 19546010]

30. Lei M, Novotny MV, Mechref Y. Sequential Enrichment of Sulfated Glycans by Strong Anion-exchange Chromatography prior to Mass Spectrometric Measurements. *J. Amer. Soc. Mass Spectrom.* 2010; 21:348–357. [PubMed: 20022260]
31. Goetz JA, Novotny MV, Mechref Y. Highly Sensitive Enzy/Chemical Release of O-Glycans. *Anal. Chem.* 2009 in press.

Figure 1.

Bar graphs of On-line SP purified permethylated N-glycans derived from fetuin. (A) bar graphs of the intensities of fetuin permethylated N-glycans using different amounts of methyl iodide. (B) bar graphs of the intensities of fetuin permethylated N-glycans purified by LLE, off-line C18 SPE and on line C18 SPE. Symbols, solid squares, N-acetylglucosamine, solid circles, mannose, light circles, galactose, diamond, N-Acetylneuraminic acid, solid triangles, fucose. The y-axis labels represent the composition of glycan structures (GlcNAcManGalFucNeuAc), while the error bars are representing the standard deviation associated with the measurements.

Figure 2. Extracted ion chromatograms of RNase B permethylated N-glycans purified through (A) off-line C18 solid-phase extraction and (B) on-line C18 nano-trap column. The insets are zoomed traces. Symbols: as in Figure 1.

Figure 3. Extracted ion chromatograms of AGP permethylated N-glycans purified using on-line C18 SPE. The inset is a zoomed trace and spectrum representing each of the extracted ion peaks. Symbols: as in Figure 1.

Figure 4.

Extracted ion chromatograms of 11 permethylated N-glycans derived from human blood serum and purified (A) off line using C18 SPE and (B) on-line using C18 SPE. The inset is a zoomed trace. Symbols: as in Figure 1.

Figure 5.

Bar graph of the intensities of the 11 permethylated N-glycans derived from blood serum, which are depicted in Figure 4. The y-axis labels represent the composition of glycan structures (GlcNAcManGalFucNeuAc), while the error bars are representing the standard deviation associated with the measurements.

Intensities of N-glycans derived from human blood serum, permethylated and purified on- and off-line. Identification of glycan structures was based on tandem MS data and mass accuracy (< 2ppm). In the case of low abundant peaks that were subjected to tandem MS identification is based on mass accuracy.

Table 1

GlcNAc	Man	Gal	Fuc	NeuNAc	Structures	[M]	On-line [E+5] ^d	LLE [E+5] ^d
2	5	0	0	0		1572.825	75 ± 8	29 ± 5
2	6	0	0	0		1776.925	39 ± 3	12 ± 3
2	7	0	0	0		1981.024	53 ± 6	3 ± 1
2	8	0	0	0		2185.124	19 ± 2	5 ± 2
2	9	0	0	0		2389.224	48 ± 3	11 ± 1
2	10	0	0	0		2593.324	6 ± 1	2 ± 0.5
3	3	0	1	0		1583.841	9 ± 2	3 ± 1
3	4	1	0	0		1817.951	25 ± 2	11 ± 1
3	5	1	0	0		2022.051	60 ± 5	31.9±5

GlcNAc	Man	Gal	Fuc	NeuNAc	Structures	[M]	On-line [E+S] ^d	LLE [E+S] ^d
3	7	1	0	0		2430.251	7 ± 0.1	4 ± 0.3
3	7	1	0	1		2791.424	304 ± 40	6 ± 3
3	3	3	0	0		2022.051	63 ± 5	32 ± 5
4	3	1	0	0		1858.978	107 ± 31	29 ± 10
4	3	2	0	0		2063.077	82 ± 5	26 ± 2
4	3	2	1	0		2237.167	914 ± 8	287 ± 10
4	3	2	0	1		2424.251	340 ± 20	85.8 ± 13
4	3	2	1	1		2598.34	333 ± 30	87 ± 10
4	3	2	0	2		2785.425	1430 ± 92	381 ± 70
4	3	2	1	2		2959.514	335 ± 28	71.3 ± 15
4	4	2	0	2		2989.525	9.1 ± 4	2.6 ± 0.6
4	5	2	0	2		3193.624	6.7 ± 5	1.4 ± 1
4	3	3	1	0		2441.266	57.3 ± 3	13.7 ± 2
4	3	1	1	1		2394.241	61.5 ± 4	16.9 ± 6

GlcNAc	Man	Gal	Fuc	NeuNAc	Structures	[M]	On-line [E+S] ^d	LLE [E+S] ^d
4	3	1	0	1		2220.151	12.1±2	3.6±0.5
4	3	0	0	0		1654.878	32.0±0.4	10.3±3
4	3	0	1	0		1828.967	1755±149	622 ± 20
5	3	2	0	0		2308.204	24 ± 2	6 ± 1
5	3	2	1	0		2482.293	122 ± 12	32 ± 5
5	3	2	0	1		2669.377	18.5 ±0.4	5 ± 1
5	3	3	1	0		2686.393	4.2 ±0.4	1 ± 0.1
5	5	2	0	0		2716.403	4.4 ± 0.4	1 ± 0.3
5	3	3	0	1		2873.477	21 ± 2	3 ± 1
5	4	2	0	1		2873.477	21 ± 3	5.7±1
5	6	2	0	0		2920.503	22 ± 6	2 ± 0

GlcNAc	Man	Gal	Fuc	NeuNAc	Structures	[M]	On-line [E+S] ^d	LLE [E+S] ^d
5	3	3	1	1		3047.566	16 ± 1	3.0 ± 0.1
5	7	2	0	0		3124.603	5 ± 1	ND
5	3	3	0	2		3234.651	102 ± 7	18 ± 2
5	4	2	0	2		3234.651	102 ± 7	18 ± 3
5	3	3	1	2		3408.74	26 ± 3	4 ± 1
5	3	3	0	3		3595.825	193 ± 1	22 ± 1
5	3	3	1	3		3769.914	160 ± 15	15 ± 2
5	3	3	0	4		3956.998	0.4±0.01	ND
5	3	3	2	3		3944.003	7 ± 1	1 ± 0.1
5	3	3	0	2		3234.651	7 ± 1	18 ± 2
5	3	2	1	1		2843.467	121±20	1 ± 0.02

GlcNAc	Man	Gal	Fuc	NeuNAc	Structures	[M]	On-line [E+S] ^d	LLE [E+S] ^d
5	3	4	0	0		2716.403	3.1±2	0.3 ± 0.03
5	3	3	1	0		2686.393	4.5 ± 0	0.7±0.03
5	3	2	2	0		2656.382	5 ± 0.2	2 ± 0.2
5	3	1	1	1		2639.367	18 ± 1	3 ± 0.2
5	3	1	1	0		2278.193	337 ± 20	57 ± 7
5	3	1	0	0		2104.104	41 ± 3	44 ± 4
5	3	0	1	0		2074.093	299 ± 20	60 ± 7
5	3	0	0	0		1900.004	14.8±1	37 ± 4
6	3	4	0	1		3322.703	9 ± 0.1	2 ± 0.1
6	3	4	1	1		3496.793	4 ± 0.2	0.3±0

GlcNAc	Man	Gal	Fuc	NeuNAc	Structures	[M]	On-line [E+S] ^d	LLE [E+S] ^d
6	3	4	0	2		3683.877	27 ± 1	4 ± 1
6	3	4	1	2		3857.966	5 ± 0.1	0.5±0.2
6	3	4	0	3		4045.051	20 ± 1	2 ± 0.2
6	3	4	1	3		4219.14	8 ± 0.2	0.4 ± 0.1
6	3	4	0	4		4406.224	27 ± 2	1.7 ± 1
6	3	4	2	3		4393.229	11 ± 1	ND
6	3	4	3	3		4567.318	4 ± 1	ND
6	3	4	1	4		4580.314	4 ± 1	ND
7	3	5	0	2		4133.103	3 ± 0.3	ND

GlcNAc	Man	Gal	Fuc	NeuNAc	Structures	[M]	On-line [E+S] ^d	LLE [E+S] ^d
7	3	5	1	3		4668.366	0.8 ± 0.1	ND
7	3	5	0	4		4855.451	2 ± 0.2	ND

^d values are presented as the average of 5 analyses ± standard deviation of the 5 analyses