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Chip-based Reversed-phase Liquid Chromatography-Mass Spectrometry of Permethylated N-linked Glycans: a Potential Methodology for Cancer-biomarker Discovery

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Abstract

The study of protein glycosylation in biological fluids and tissues has substantial medical importance, as changes in glycan structures have now been associated with a number of diseases. Quantification of glycomic-profile changes is becoming increasingly important in the search for disease biomarkers. Here, we report a highly reproducible combination of a glycomic sample preparation/solid-phase derivatization of glycoprotein-derived N-linked glycans with their subsequent microchip-based separation and mass-spectrometric (MS) measurements. Following our previously-described reductive β-elimination for O-linked glycans with ammonia-borane complex to reduce N-linked structures, the N-linked alditol structures are effectively methylated in dimethylformamide medium to avoid artefacts in MS measurements. Reversed-phase microfluidic liquid chromatography (LC) of methylated N-linked oligosaccharide alditols resolved some closely related structures into regular retention increments, aiding in their structural assignments. Optimized LC gradients, together with nanospray MS, have been applied here in the quantitative measurements of N-linked glycans in blood serum, distinguishing breast cancer patients from control individuals.

Keywords

LC-MS; cancer; blood serum glycoproteins; glycans; biomarker; ammonia-borane complex; permethylation

Introduction

Biomolecular mass spectrometry (MS), namely the matrix-assisted laser desorption/ ionization (MALDI)[1] and electrospray ionization (ESI),[2] has preceded many important developments in proteomics, lipidomics, glycomics, and other "-omics" fields of the life sciences. Glycosylation, the most common protein modification (present on at least 50% of the proteins in the human proteome), participates in many critical biological functions, including immune responses, cell development, cellular differentiation and adhesion, and even host-pathogen interactions. Modern bioanalytical techniques have supported a further important area of study: aberrant changes in glycosylation. Alterations to the usual glycomic profile of humans have been associated with several different cancers, including breast,[3-5]

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prostate,[6;7] liver,[8;9] and ovarian,[10;11] and unusual glycan ratios could be recorded for these diseases.

The quest for potential glycan biomarkers now demands the development of highly-sensitive and reproducible sample preparation, separation, and MS methods to observe the often-times subtle, yet significant, changes to the overall glycomic profile. In contrast to the well-developed protocols in proteomics, where the MS analyses are relatively facile, due to the ease at which peptides and proteins ionize, glycan solutes lack a basic site that permits ionization with a comparable efficiency.[12] Increasing the sensitivity of MS measurements has thus been a key area of research in glycomics.

Methylation of glycans, originally developed in 1964 by Hakomori,[13] has been refined by many researchers over the years. The method developed by Ciucanu and Kerek[14] (later updated by Ciucanu and Costello[15]) is practiced in numerous laboratories, offering several analytical advantages. Methylation results in an increased yield of ionization, thus increasing the overall MS sensitivity by up to 20 times,[16] while methylated glycans are rendered neutral, allowing the simultaneous detection of both neutral and acidic structures in the positive-ion mode. It also stabilizes these molecules, minimizing in-source and post-source decay processes. Tandem MS analyses are also improved through more predictable fragmentation patterns and increased cross-ring cleavage reactions.[17;18]

However, the traditional slurry-based method of methylation often falls short of the requirements of many biological samples where many glycan entities are expected to be present at low-picomole to femtomole levels. The problems associated with these methods, including oxidative degradation and “peeling” reactions, when analyzing minute quantities of sample, were overcome through the development of the solid-phase methylation methods,[19;20] allowing the analysis of trace-level constituents in such samples.

Improving the overall ion yield is only one aspect in the overall analysis of glycans. When present with other sample constituents, methylated glycans may still suffer from a competitive ionization in the ion source of a mass spectrometer, hindering the observation of many trace-level analytes that may be present. Perhaps the most effective approach to minimize the adverse effects from competitive ionization is the use of a separation technique prior to the final MS analysis. Separation of glycans has been accomplished by many different methods, including porous-graphitized carbon,[10;21-31] hydrophilic interaction chromatography,[32-37] normal-phase chromatography,[38-43] and high-performance anion-exchange chromatography.[44-47] Given the advantages of methylation and the availability of new separation media, it is somewhat surprising that only a few detailed reports exist for the LC-MS analysis of methylated glycans.[48;49]

Here, we report yet another alternative, namely the development of a method for a chip-based, reversed-phase LC-MS analysis of methylated N-glycan alditols derived from the glycoproteins present in human blood serum. The enzymatically-released N-linked glycans, derived from glycoproteins in a 1-μL aliquot of human blood serum, were first reduced to alditols quickly and efficiently using a protocol based on our previously-reported method for the reductive β-elimination of O-linked oligosaccharides with ammonia-borane complex.[50] Alditols were then solid-phase methylated in dimethylformamide and subjected to a microchip-based reversed-phase LC. The method was then applied to N-linked glycan samples derived from the sera of 15 Stage IV breast cancer patients and 15 cancer-free control patients. The resulting data were analyzed by single-variable analysis of variance (ANOVA) and receiver operating characteristics (ROC) procedures to implicate certain N-linked glycans as potential biomarkers for breast cancer.

Experimental

Materials

Empty spin columns, graphitized carbon, and C_{18} microspin columns were obtained from Harvard Apparatus (Holliston, MA). Sodium hydroxide beads, model glycoproteins, trypsin (proteomics grade), methyl iodide, β-mercaptoethanol, formic acid (FA), and ammonia-borane complex were acquired from Sigma-Aldrich Co. (St. Louis, MO). Water, chloroform, sodium phosphate, dimethylformamide (DMF), and trifluoroacetic acid (TFA) were products of EMD Chemicals, Inc. (Gibbstown, NJ) while acetonitrile (ACN) and methanol originated from J.T. Baker (Phillipsburg, NJ). Sodium dodecylsulfate (SDS) was purchased from Bio-Rad Laboratories (Hercules, CA). PNGase F, used to release N-linked glycans from proteins, was acquired from Associates of Cape Cod, Inc. (East Falmouth, MA). The MALDI matrix, 2,5-dihydroxybenozic acid, was received from Alfa Aesar (Ward Hill, MA) and Nonidet P40 was purchased from Roche Diagnostics (Indianapolis, IN)

Blood Serum Samples

Blood samples were collected from cancer-free female volunteers and female patients diagnosed with late-stage breast cancer by a clinical team according to an institutional review board-approved clinical trial. The samples were age-, race-, and sex- matched. Venous blood samples were acquired during the morning fasting state, with minimal stasis, into evacuated tubes. After at least 30 min, but within 2 h, the tubes were centrifuged at 20 °C for 12 min at 1200 g. The serum samples were stored at -80 °C, in plastic vials, until needed.

N-linked Glycan Release and Purification

The N-linked glycans derived from the model glycoprotein, bovine fetuin, and human blood serum samples were liberated in a similar manner. A 1-μg/μL stock solution of bovine fetuin was prepared in a 10 mM sodium phosphate buffer (pH = 7.5). A 2-μL aliquot of the fetuin solution or a 1-μL aliquot of human blood serum (after centrifugation at 16,000 g for 10 min at 4 °C) was transferred to a clean microcentrifuge tube and a 1-μL aliquot of trypsin (1 μg/μL) was added. Tryptic digestion, used to increase solubility of the serum proteins described later in the procedure, was allowed to proceed for 1 h at room temperature. To terminate the digestion and to induce further denaturation and reduction of disulfide bonds, a 30-μL aliquot of a solution composed of 0.11%/0.11% SDS/β-mercaptoethanol in a 10 mM sodium phosphate buffer (pH = 7.5) was added and the samples were incubated at 60 °C for 1 h. After cooling the samples to room temperature, 3.6 μL of a 10% Nonident P40 solution were added, resulting in a final concentration of 1.0%. After allowing the solutions to equilibrate for 5-10 min at room temperature, a 2-μL aliquot of PNGase F (diluted 1:10) was added and the samples were incubated for 18 h at 37 °C.

The recovery and purification of the released glycans were accomplished using a two-step solid-phase extraction procedure. First, C_{18} microspin columns (Harvard Apparatus, Holliston, MA) were employed to remove proteins, peptides, and other hydrophobic components. The columns were first washed with 400 μL of an 85%/25%/0.1% ACN/water/TFA solution three times, followed by 400 μL of a 95%/5%/0.1% water/ACN/TFA solution three times, prior to the application of the sample. The centrifuge tubes were washed with 100 μL of a 95%/5%/0.1% water/ACN/TFA solution twice to minimize sample losses and each wash was added to the appropriate microspin column. The columns were then centrifuged at 1,000 rpm for 1 min and the samples were reapplied two additional times. After the final centrifugation, the media were washed twice with 100 μL of a 95%/5%/0.1% water/ACN/TFA solution to ensure a maximum recovery of the N-linked glycans, which were not retained by the stationary-phase medium. Following C_{18} purification, the glycan-

containing solutions were further purified by employing graphitized-carbon microspin columns (Harvard Apparatus, Holliston, MA). These spin columns were prepared for use as previously described for the C_{18} microspin columns and the samples were passed through the media a total of three times, each time for 1 min at 2,000 rpm. Salts were removed by washing the media with 200 μL of a 95%/5%/0.1% water/ACN/TFA solution twice. The glycans were then eluted with two 200-μL aliquots of a 25%/75%/0.1% ACN/water/TFA solution. The samples were then vacuum-centrifuged to dryness.

N-linked Glycan Reduction

After vacuum centrifugation and solvent evaporation, the N-linked glycans were reduced to alditols using a procedure based on our previously-published protocol for β-elimination of O-linked glycans.[50;51] An aqueous solution of 10 μg/μL ammonia-borane complex was prepared, a 10-μL aliquot was added to each N-glycan sample, and the samples were incubated for 1 h at 60 °C. Next, the samples were allowed to cool to room temperature and any remaining ammonia-borane complex was destroyed by the addition of three 1-μL aliquots of acetic acid, added every 30-45 min. The resulting borane salts were then removed as their methyl esters by the addition of three 100-μL aliquots of methanol, followed by vacuum centrifugation.

Spin-column Methylation

Reduced N-linked glycan samples were methylated using a static solid-phase methylation procedure. (Our previous solid-phase methylation procedures utilized flow (generated by either a syringe pump[19] or by centrifugation[20]) of the reaction mixture over the beads. In this approach, to increase the reaction time, flow was not utilized.) Briefly, spin-column reactors (Harvard Apparatus, Holliston, MA) were loaded with sodium hydroxide beads suspended in ACN. Each reactor was then thoroughly washed with DMF. The dried N-linked glycan samples were resolubilized in 100 μL of a solution composed of 70%/25%/5% DMF/methyl iodide/water, loaded onto the solid-phase reactors, and allowed to react statically for 15 min. The samples were then centrifuged at a low speed (*ca*. 600 rpm) and an additional 25-μL aliquot of methyl iodide was added to each sample. Next, each sample was reapplied to its reactor for an additional 15-min reaction period. Following a second centrifugation, the reactors were washed twice with 100-μL aliquots of ACN. The methylated N-linked glycan alditols were recovered by performing liquid/liquid extraction using 300 μL of chloroform and a repeated washing with 1 mL of 0.5 M NaCl followed by HPLC-grade water.

MALDI Analysis

To quickly optimize the N-linked glycan-reduction protocol and the subsequent DMF-based solid-phase methylation procedure, bovine fetuin-derived N-linked glycans were analyzed by MALDI-TOF MS. The samples were resuspended in 2.0 μL of an 80/20%/0.1% water/methanol solution and a 0.5-μL aliquot of each sample was spotted on a standard MALDI target and dried. A 0.5-μL aliquot of the matrix solution (10 mg/mL 2,5-DHB in a 75%/25% water/methanol solution with 1 mM sodium acetate to ensure that the glycan alditols were ionized as sodiated molecules) was added and dried under vacuum. The samples were analyzed utilizing an Applied Biosystems 4800 MALDI TOF/TOF mass spectrometer (Applied Biosystems, Inc. Framingham, MA). For the N-linked glycans derived from bovine fetuin, the instrument was operated in the positive-ion reflectron mode and 2000 laser shots were applied.

LC-tandem MS

The liquid-chromatographic analyses conducted in this study utilized an Agilent ChipCube LC-MS interface (Agilent Technologies, Inc., Palo Alto, CA) and an Agilent 1200 Series nano-pump system (Agilent Technologies, Inc., Palo Alto, CA). The LC system included two pumps: a binary capillary-flow pump, used for loading the samples, and a nano-flow gradient pump for nano-flow separations. The mobile phases were degassed with a vacuum degasser and the samples were injected via an autosampler. A liquid-chromatographic chip, featuring a 40-nL trapping column and a 75 μm × 150 mm separation column, containing C_{18} (5 μm StableBond Zorbax) particles as both the trapping and separation medium, was used. LC separations were performed using a linear gradient from 30-60% mobile phase B over 40 min at a flow rate of 250 nL/min. Mobile phase A consisted of 97%/3%/0.1% water/ACN/formic acid with 0.5 mM sodium acetate, while mobile phase B was composed of 97%/3%/0.1% ACN/water/formic acid with 0.5 mM sodium acetate. The same mobile phases were used for both the capillary and nano-flow pumps. The methylated N-linked glycan alditols were resolubilized in 4 μL of an 80%/20% water/methanol solution and a 1-μL aliquot, corresponding to the equivalent of only 0.25 μL of blood serum, was injected and loaded using the capillary-flow pump set at 20% mobile phase B. The capillary pump flow rate was set at 2 μL/min for loading and washing the samples. The total volume for the injection/wash cycle was 6 μL (1 μL of injected peptides, followed by 5 μL of 20% mobile phase B).

The separated glycans were detected and characterized using a Bruker Daltonics (Bruker Daltonik GmbH, Bremen, Germany) ultra-high capacity ion-trap mass spectrometer. Continuous electrospray ionization was accomplished by applying a spray voltage of 1750 V. Standard “enhanced” mode was used for the ordinary MS analyses, while “ultrascan” mode was used for tandem MS investigations. An *m/z* range from 300-1800 was scanned in this study.

Data Analysis

The MALDI data were analyzed using Data Explorer (version 4.6), a software tool included with the 4800 ABI operating software. Data acquired by LC-MS were exported as mzXML files using Data Analysis, a feature included with the instrument's operating software, and further processed using a modified version of ProteinQuant,[52] a software tool developed in our laboratory for the quantification of proteomic data. Briefly, the N-linked glycan solutes, many of which were observed with multiple charge states, were identified and verified using both tandem MS and retention-time data. These data were included as a text file for ProteinQuant. Normalization was performed by dividing the sum of the chromatographic peak areas for each charge state observed for a particular glycan by the sum of the total peak areas for all charge states for the observed N-linked glycans. (See Table 1 for the glycans concerned with this study.) Normalized chromatographic peak areas were statistically analyzed first by a single-factor Analysis of Variance (ANOVA) test using Microsoft Excel. Data were considered statistically significant if the resulting P-values were less than 0.05, indicating that the probability that the differences were not statistically significant was < 5%. If the P-value was less than 0.05, the data were subjected to a receiver operating characteristics statistical treatment using Origin 8 (OriginLab Corporation, Northampton, MA).

Results and Discussion

For our previous glycomic analyses, we have relied on MALDI-MS to record glycomic profiles.[3;6] For these types of analyses, the N-linked glycans were first liberated from their proteins by PNGase F, purified by C_{18} and graphite solid-phase extraction procedures,

methylated and mass-spectrally analyzed. In the work we are presenting here, we have added an extra dimension to the analysis: nano-flow LC and the several key modifications that were required to our procedure in order to generate reliable data. Following N-glycan release and purification, but prior to their methylation, N-glycans were reduced to their corresponding alditols using ammonia-borane complex to eliminate anomericity at their reducing end, resulting in a single chromatographic peak. (If non-reduced structures were subjected to nano-flow LC, the different anomeric configurations were easily separated, resulting in two well-resolved chromatographic peaks for a single structure.) Next, the N-linked glycan alditols were methylated. In our previous work, we have used DMSO as the reaction medium.[19;20] However, when we attempted to methylate N-glycan alditols in DMSO, we also observed a regeneration of the closed-ring structure.[15] Additionally, we observed a series of satellite peaks, separated by 30 Da, caused by a reaction between DMSO and methyl iodide.[53] Chromatographically, both of these side reactions would lead to multiple peaks for just a single analyte. Therefore, we have developed a methylation procedure using DMF as the reaction media to eliminate the side reactions in which DMSO may participate. After their methylation, the analytes were subjected to a chip-based, nano-flow reversed-phase LC. In this study, the separations were conducted using the Agilent ChipCube interface where all of the components (trapping and separation columns, valves and fluidic connections, and an ESI emitter) of the traditional nano-flow LC set-up were integrated onto a single microfluidic device. This configuration reduces manual connections and dead volumes, improving MS sensitivity.[54] Following nano-flow LC, methylated N-glycan alditols were subjected to ESI-based MS analyses.

Glycan Reduction using Ammonia-borane Complex

During the separation of native glycans, the α and β anomeric configurations may oftentimes complicate evaluations. First, the anomers may be resolved chromatographically into two peaks for just one glycan. Previous reports suggest that the β isomer is typically the more highly-retained analogue,[55] perhaps because this form does not suffer from a steric hindrance and is free to interact with the stationary phase more strongly. If not totally resolved, the chromatographic peak width may increase,[55] lowering the separation effectiveness. Therefore, it is plausible to eliminate anomerocity by a reduction or reductive amination.

Initially, reductive amination with a subsequent methylation was investigated in this work. However, when anthranilic acid was used as the reductive amination agent, the level of partial methylation was approximately 40%, most likely due to steric hindrance effects (data not shown). Ethanolamine was then tested and, while lower levels of partial methylation were obtained (approximately 20%), the results were still not acceptable (data not shown). A traditional reduction protocol, using 0.1-1 M NaOH and 1 M sodium borohydride was tried next. This protocol resulted in the desired partial methylation levels of less than 10%, but it required extensive desalting which resulted in substantial sample loss (data not shown).

The unsatisfactory results obtained with the above-described procedures led us to the development of a reduction method for N-linked glycans that provides both efficient methylation and minimal sample handling and transfer steps. Reduction with ammonia-borane complex was subsequently examined. The use of this reagent did not require extensive clean-up, leading to satisfactory signal intensities. It only exhibited partial methylation levels of approximately 5%. Further, the reduction yields were found to be quantitative, as judged by the corresponding MALDI-MS spectra. In these spectra, if a non-reduced analogue of a glycan was present, an ion would be seen for a species 16 Da lower than the reduced methylated mass. However, such an ion was not observed. Just a weak signal for a species 14 Da smaller, due to a partial methylation, than the signal corresponding to the reduced and completely-methylated analyte was observed. Satisfactory

reduction conditions were found with a 10-μL aliquot of 10 μg/μL ammonia-borane complex and a subsequent incubation at 60 °C for 1 h. Lower temperatures could be used, however, such as incubating at 10 °C overnight.

Optimization of DMF Solid-phase Methylation Conditions

While effective at quickly methylating glycan samples, the previously used dimethylsulfoxide (DMSO)[14] may cause two adverse side reactions.[15;53] Therefore, for a better quantitation, a modification to our previously-published spin-column methylation protocol[20] was further introduced, aiming to minimize the side reactions, as their reproducibilities on a sample-to-sample and a day-to-day basis were not known. As shown in Figure 1a, the regeneration of the closed-ring, or native glycan structure, can occur during the methylation of glycan alditols in DMSO.[15] Two distinct mass-spectral peaks for a single glycan structure, separated by 16 Da, arose from this side reaction. Moreover, these two different structures can be chromatographically resolved. The second problem results from a reaction between DMSO and methyl iodide, ultimately producing iodomethyl methyl ether.[53] This ether was reactive towards hydroxyl groups and produced a series of satellite ions separated by 30 Da (see Figure 1a). Once again, this series of structures could result in distinct chromatographic artefactual peaks. Both of these problems, as illustrated in Figure 1b, can easily be remedied by performing the methylation reaction in another suitable medium, in our case, in DMF.

Several solid-phase reaction parameters required optimization to obtain a spectrum similar to that shown in Figure 1b. In contrast to DMSO, where no or only trace levels of water are needed, higher concentrations of water were required for an efficient DMF solid-phase methylation. Higher concentrations of water were needed to solubilize the glycans in DMF, a medium possessing a lower solvent polarity index scale value (6.4) than DMSO (7.2). Therefore, increased concentrations of water elevated the total solvent polarity of the water/DMF solution and increased the solubility of glycans, resulting in more intense mass-spectral peaks and the detection of lower-abundance N-linked glycans.

To optimize the concentration of water in the DMF-reaction solution for the methylation of N-linked glycan alditols, the N-linked glycans derived from a total of 100 μg of fetuin were first methylated in DMSO to act as a "control" for the reduced N-linked glycans methylated in DMF. The fetuin-derived N-linked glycans methylated in DMSO were derivatized in a single reaction to account for any variations in the quantities side-reaction products due to performing multiple reactions times. The N-linked glycans methylated in DMSO, equivalent of 1 μg of fetuin, were added to the same amount of reduced N-linked glycans methylated in DMF prior to the liquid/liquid extraction. The relative intensities (the peak area for each N-linked glycan alditol methylated in DMF divided by the peak area of the corresponding glycan methylated in DMSO) for the reduced glycans methylated in DMF were calculated from the corresponding MALDI-MS spectra. At lower concentrations of water, 1% or less, low relative intensities (approximately 0.05, as previously defined in this section) were observed. The relative intensity increased from approximately 0.05 at 1% water to nearly 0.7 at 2.5% water. Further increases in the water concentration to 5% and 7.5% resulted in the relative intensities of 0.99 and 0.97, respectively. However, at 10% water, a decrease in the relative intensity was observed. This decrease in signal could be due to the "collapse" of the NaOH beads caused by the water or the methyl iodide may not have been as soluble as needed at higher water concentrations. All analyses were performed in triplicate. Based on these results, it was determined that 100 μL of a solution composed of 70%/5%/25% DMF/water/methyl iodide represented optimum conditions. Based on the 0.99 relative intensity of reduced glycans methylated in DMF to those derivatized in DMSO, these experiments also suggest that little, if any, sample loss occurs.

An advantage of methylation in DMSO is the fast kinetics of the reaction, as demonstrated by the finding that 12-18 samples can be solid-phase methylated in 20 min.[20] In contrast, the kinetics of methylation in DMF are significantly slower so that the "spin and recycle"[20] approach to solid-phase methylation may not be so effective and the samples must be methylated using a static approach, allowing the reaction solution to remain in contact with the NaOH beads for an extended period of time. Therefore, the reaction time also required optimization. An acceptable reaction time was deemed as a time that minimized a partial methylation and was determined by dividing the signal intensity of the partially-methylated structure by the sum of the peak intensities of the partially-methylated structure and its completely-methylated analogue. Comparable reactions were performed for 5, 10, 15, 20, 30, and 40 min. At the midpoint time (2.5, 5, 7.5, 10, 15 and 20 min, respectively), samples were centrifuged, a second 25-μL aliquot of methyl iodide was added, and the samples were reapplied to the reactors. When the reaction was allowed to proceed for only 5 min, the observed partial methylation for the biantennary-disialylated N-linked glycan derived from bovine fetuin was quite high, nearly 30% (data not shown). As the reaction time was lengthened to 10 and 15 min, the partial methylation levels fell to 20% and 15% (data not shown), respectively. When the reaction time was further increased to 30 min, the partial methylation was less than 10%, typically around 7-8% (data not shown). No further decreases in the partial-methylation level were observed by increasing the reaction time to 40 min (data not shown). Therefore, a 30-min reaction time was used in all subsequent experiments. Identical results were obtained for all N-linked glycans associated with bovine fetuin.

The overall modified method proved to be both highly sensitive and reproducible. The analyses using an aliquot as small as 100 nL of human blood serum could be performed and the resulting spectra were essentially identical to the signals that we have generated in the past using much larger starting volumes of serum. (*ca*. 10 μL), in terms of the structures detected and their normalized peak intensities. (Here we define normalized peak intensity as the intensity of each peak divided by sum of all of the intensities for all N-linked glycans) The reproducibility of the method was determined by reducing and methylating ten aliquots (1 μL each) of a single sample of human blood serum, followed by interrogation by MALDI-TOF-MS. Using the set of N-linked glycans previously reported and using the same normalization technique,[20] the average of the RSDs for all of the observed N-linked structures methylated in DMF was calculated as 9.8% To judge the spot-to-spot variation of the MALDI analysis, one sample of N-linked glycans derived from the glycoproteins in a 1 μL-aliquot of human blood serum was resuspended in 5 μL of a 50%/50% water/methanol solution and spotted 5 times (each spot was 0.5 μL). After normalization of the resulting spectra, the average of the RSDs for all N-linked structures was calculated to be 5.2%.

LC-MS Behavior of Glycans Derived from Blood-serum Samples

The investigation into the chromatographic and mass-spectrometric behavior of methylated N-glycan alditols was accomplished using a chip-based LC-MS system. In these experiments, ten replicate injections of a single sample of N-linked glycans derived from human blood serum, each consisting of the equivalent of 0.25 μL of blood serum, were run and resulted in the confident identification of approximately 20 N-linked glycans. When this pool of N-linked glycans was simplified into their specific subclasses (i.e. high-mannose, complex-type, fucosylated complex-type, etc.), distinct chromatographic trends were observed, as shown in Figure 2. This figure demonstrates that the retention time increases linearly as a function of the number of monosaccharides for a specific subclass, as consistent with a hydrophobic interaction of the heavily methylated solutes with the reversed-phase medium. When the retention times for glycans in the high-mannose subclass (blue trace in Figure 2) were plotted as a function of the *m/z* for the singly-sodiated molecule, an R^2 value

of 0.992 was obtained. Similar results were also acquired for the complex-type subclass (green trace) and the complex-fucosylated subclass (red trace). The retention time vs. *m/z* (for singly-sodiated molecules) plots for these subclasses resulted in R^2 values of 0.995 and 0.968, respectively. The ability to assign an unknown glycan to a specific subclass based on its retention time and *m/z* value could prove useful in its initial characterization and may provide insights for more targeted and detailed investigations (tandem MS analyses, enzymatic sequencing, etc.) to definitively assign its carbohydrate composition.

Chromatographically, reduced and methylated N-linked glycans behaved almost ideally, resulting in well-shaped peaks with acceptable full-width-at-half-maximum peak widths. These values ranged from 0.7 to 1.2 min for the earliest eluting structures (the Man_5 high-mannose type glycan which co-eluted with a glycan at *m/z* 937.7 possessing composition of $GlcNac_4Man_3Fuc$) and the most intense ion (a triply-sodiated biantennary-disialylated glycan), respectively. These earliest eluting N-linked glycans were detected at an average of 14.0 min, while the biantennary-disialylated structure eluted from the column at an average of 20.2 min. The latest eluting N-linked glycan in this study, a fucosylated triantennary-trisialylated glycan, exhibited a full-width-at-half-maximum peak width of 0.8 min and possessed an average retention time of 27.2 min. The peak capacity of a column is a useful figure-of-merit to determine its efficiency during a gradient elution and is defined as the number of analytes that may be separated during a chromatographic time frame. For the set of nonfucosylated, sialylated N-linked glycans, the peak capacity of the reversed-phase LC chip was calculated to be 58 using the method proposed by Dolan and co-workers.[56] LC-MS of these structures resulted in high ion counts at the chromatographic peak apex. Peak intensities ranged from approximately 2×10^8 counts (for the Man_5 glycan) to nearly 2×10^9 counts (for the triply-sodiated biantennary-disialylated glycan).

Large-scale biomedical analyses by MS require highly-reproducible retention times for the analytes of interest and a stable instrument response over the time course of these analyses. Ionization efficiency may be altered due to the constantly changing composition of the mobile phase during a gradient elution in reversed-phase LC-MS. Therefore, to ensure the observed changes between sample sets are not the result of an altered ionization efficiency, the retention time for each analyte must remain constant. Furthermore, to ensure the observed changes are indeed the result of differences in between the sample sets, the overall instrumental response for each analyte must also be stable and constant over the time the analyses are conducted. These figures-of-merit were monitored for a time period greater than 24 h for ten replicate injections, each equivalent to 0.25 μL of blood serum per injection, for a single N-linked glycan sample. Figure 3a depicts the box-and-whisker plots for the retention-time reproducibility of three important structures, discussed later, possessing statistically-significant changes between the control and disease states. In these plots, the box represents the 25^{th} and 75^{th} percentiles, while the whiskers represent outliers in the data set. This figure demonstrates that over a total analysis time greater than 24 h, the retention time reproducibility is acceptable. Generally, RSDs ranged from 1.1% (the latest eluting glycan) to 4.7% (the earliest eluting glycan). Over the time required for these analyses, glycans generally eluted within a window of approximately 30 sec, so that the ionization efficiency should not fluctuate significantly due to changes in the mobile-phase composition.

The repeatabilities of the intensity and associated normalized chromatographic peak areas were also monitored during the same set of runs. Normalized chromatographic peak areas were calculated, as previously described in the Methods section, using the modified version of ProteinQuant and plotted for the three structures of interest, as they are shown in Figure 3b. These N-linked structures, representative of the larger set, displayed very repeatable normalized chromatographic peak areas. The structure possessing an *m/z* value of 937.7

produced an average normalized chromatographic peak area of 0.034 with an RSD of 0.71%, while the biantennary-monosialylated structure produced an average normalized chromatographic peak area of 0.51 with an RSD of 0.79%. The fucosylated triantennary-trisialylated structure resulted in an average normalized chromatographic peak area of 0.085 with an RSD of 0.65%. These results indicate a high repeatability of the normalized chromatographic peak areas and any observed changes in this value are not due to changes in instrument behavior over the time course of these analyses, but rather are associated with the sample itself. Combined, these results suggest chip-based reversed-phase LC-MS of reduced and methylated N-linked glycans could be used to monitor changes in the blood-serum glycomic profiles of cancer-free patients and those suffering from late-stage breast cancer.

In this LC-ESI-MS procedure, glycans derived from blood-serum glycoproteins were analyzed and in total, 18 different N-linked glycans were confidently identified and monitored. The structures of these N-linked glycans, shown in Table 1, were determined through tandem MS data and their LC elution order. While the number of glycan structures identified through this ESI-based procedure is lower than that identified by MALDI-MS,[12] LC-ESI-MS analysis does offer several advantages. One advantage, and perhaps the most important, is the repeatability of the normalized chromatographic peak areas associated with LC-ESI measurements. As was discussed before in this publication, the relative standard deviations for the ESI-based measurements were generally less than 5% and were often-times less than 1%, making a highly-precise quantitation possible. Further, the ability to couple the separation of glycans to ion-trap instruments allows higher levels of MS^n to be performed, allowing some specific linkage information to be acquired. Finally, ESI allows investigators to quickly couple LC methods to high-resolution and high-mass accuracy instruments, such as an ion-cyclotron resonance (ICR) Fourier transform (FT) instrument or an Orbitrap.[10] These types of instruments may be required to validate unusual glycans, for example, those possessing phosphate or sulfate moieties.

LC-MS Analysis of Glycans Derived from Blood Sera from Control Patients and Stage IV Breast Cancer Patients

In this study, a total of 30 N-linked glycan alditol samples (15 cancer-free and 15 late-stage breast cancer) derived from blood serum glycoproteins were analyzed by nano-flow reversed-phase LC-MS. The glycans were grouped into their respective subgroups (high-mannose type, total fucose (including sialic acid), fucose-only (without sialic acid), total sialic acid (with fucose), and sialic acid-only (without fucose)) as a possible method to observe alterations in the glycome between the different states-of-health.[3;6] The results of this analysis, shown as the notched-boxed plots for each subclass, are depicted in Figure 4a and b. This figure shows that sum of the normalized chromatographic peak areas for the high-mannose glycan class did not exhibit significant changes between the two states-of-health and is further reflected in the P-value of 0.0633. (Only P-values less than 0.05 were considered significant in this study.) While the high-mannose N-linked glycan class was not diagnostic of the pathological condition, N-linked glycans with associated fucose units were indicative of late-stage breast cancer. When the total fucose class, including those with sialic acid, was compared, a statistically-significant increase in the sum of the normalized chromatographic peak areas was observed for this class in the late-stage breast cancer samples. This finding was further validated by the P-value of 0.00376. Similarly, when fucosylated-only N-linked glycans without sialic acid were analyzed, an increase in their relative abundance for the late-stage breast cancer samples was also observed, resulting in a P-value of 5.42×10^{-4}. Interestingly, the total sialic acid class, including those structures possessing a fucose, did not exhibit a significant change due to the pathological condition, as reflected in the P-value of 0.883. However, when the sialylated-only N-linked glycans (those

without a fucose) were considered, a decrease in their relative abundance in the late-stage breast cancer samples was observed. This particular class of N-linked glycans produced a statistically-significant P-value of 0.0183. It is interesting that the total sialic acid class of N-linked glycans, including those with fucose, appeared to be unchanged in terms of the relative abundance between the two states-of-health. However, as will be discussed later, a decrease in the relative abundance associated with late-stage breast cancer of at least two sialylated-only structures, coupled with an increase in the relative abundance of a fucosylated-sialylated N-linked glycan in the breast cancer samples accounts for this interesting result. These results are consistent with our previously published MALDI-TOF-MS study.[3]

Further investigation into the individual N-linked glycans revealed that five of the 18 N-linked glycans resulted in statistically-significant changes between the cancer-free control samples and late-stage breast cancer sample set. Of these five structures, two N-linked glycans, whose representative extracted-ion chromatograms are presented in Figure 5a and b, were markedly altered between the two health states. The normalized-chromatographic peak areas for both of these N-linked glycans, a biantennary-monosialylated structure (Figure 5a) and a fucosylated triantennary-trisialylated N-linked glycan (Figure 5b) resulted in P-values less than 0.05. From these representative chromatograms, it appears that the biantennary-monosialylated N-linked glycan is down-regulated in Stage IV breast cancer, while the fucosylated triantennary-trisialylated N-linked glycan appears to be up-regulated under such a condition. This is in good agreement with the previously published results[4] that have also implicated the N-linked fucosylated triantennary-trisialylated glycan as a potential N-linked glycan biomarker for breast cancer through a procedurally different approach. The insets provide the tandem MS spectra used to validate each structure.

To further validate the results indicated by the representative extracted-ion chromatograms, notched-box plots were constructed for the N-linked glycans derived from the 15 cancer-free and 15 Stage IV breast cancer samples (presented as Figure 6a and b). In both of these examples, the notches do not overlap, indicating that at a 95% confidence,[57] the changes observed for these structures may be considered statistically significant between the cancer-free samples and late-stage breast cancer blood-serum specimens. These plots confirm that, indeed, the biantennary-monosialylated N-linked glycan is decreased in its relative abundance in late-stage breast cancer, while the fucosylated triantennary-trisialylated N-linked structure exhibits a dramatic increase in its normalized chromatographic peak area. This larger set of samples resulted in P-values of 1.98×10^{-5} for the biantennary-monosialylated N-linked glycan and 2.82×10^{-4} for the fucosylated triantennary-trisialylated N-linked glycan.

Receiving operator characteristic (ROC) analyses were performed on these 30 blood serum samples (15 samples for each state-of-health) and area-under-the-curve (AUC) values were calculated. This test measures the sensitivity, or the true-positive rate, vs. the 1-specificity, or the false-positive rate. By convention, if the AUC value is less than 0.6, the test is considered uninformative, while AUC values between 0.6 and 0.7 are considered to be less informative tests and values between 0.7 and 0.8 are deemed moderately accurate. When $0.8< x <0.9$, the tests are thought be accurate and highly accurate tests result in AUC values greater than 0.9 and a perfectly accurate test receives an AUC value of 1.[6] Both of these N-linked glycans resulted in highly-accurate test results with an AUC value of 0.902 for the biantennary-monosialylated N-linked structure and an AUC value of 0.982 for the fucosylated triantennary-trisialylated N-linked glycan. (See Figure 7a and b for the AUC plots for the biantennary-monosialylated and fucosylated triantennary-trisialylated N-linked glycans, respectively.) These results indicate that the use of reversed-phase chromatography

coupled to ESI-MS is capable of detecting statistically-significant changes in the N-linked glycan profiles of blood serum between control and disease states.

The overall results obtained for the analysis of N-linked glycans derived from blood serum samples acquired from cancer-free individuals and patients diagnosed with late-stage breast cancer are in good agreement with previously published data. In particular, increased fucosylation is one of the common alterations associated with cancer and may be the result of increased levels of sialyl Lewis^x, a tetrasaccharide composed of a sialic acid linked α2,6 to a galactose that is linked β1,4 to a GlcNac that also possess an α1,3-linked fucose.[58] Increased levels of fucosylation have also been reported for liver,[59;60] pancreatic,[61] colorectal[62], prostate,[6] and ovarian cancers.[63] Specifically, the fucosylated triantennary-trisialylated N-linked glycan that was significantly elevated in its relative abundance in these samples has also been observed to be increased by others,[4] including our laboratory by MALDI-MS-based profiling.[3] Interestingly, this particular glycan was found to be decreased in its relative abundance in the breast cancer cell line MCF-7 after drug treatment by Herceptin or in combination with Lipoplex.[64]

Conclusions

In this study, a method was developed to reduce and methylate enzymatically-released N-glycan analytes. Because of the high concentrations of salts using traditional reduction methods (1 M sodium borohydride in dilute NaOH), glycans must be subjected to extensive clean-up procedures, leading to a potential sample loss. In the newly-developed method, ammonia-borane complex was used as an agent to promote reduction. Excess reagent could then be removed by evaporation with methanol, minimizing sample handling/transfer steps and significantly increased both the reproducibility and sensitivity of the ensuing MS measurements. The subsequent solid-phase methylation was performed statically in 70%/5%/25% DMF/water/methyl iodide for a total of 30 min. This approach resulted in a highly-sensitive method, requiring a starting volume of just 1 μL of human blood serum, of which only a 0.25-μL aliquot was injected for each LC-MS run. A total of 30 human blood serum samples (15 cancer-free and 15 Stage IV breast cancer) were then analyzed by reversed-phase LC-MS to search for potential N-linked glycan biomarkers. Statistical analyses (ANOVA and ROC) were performed, with five N-linked glycans resulting in P-values less than 0.05. Of these, a biantennary-monosialylated N-linked glycan and a fucosylated triantennary-trisialylated structure were implicated as potential indicators of late-stage breast cancer due to their highly-accurate AUC values of greater than 0.9.

Acknowledgments

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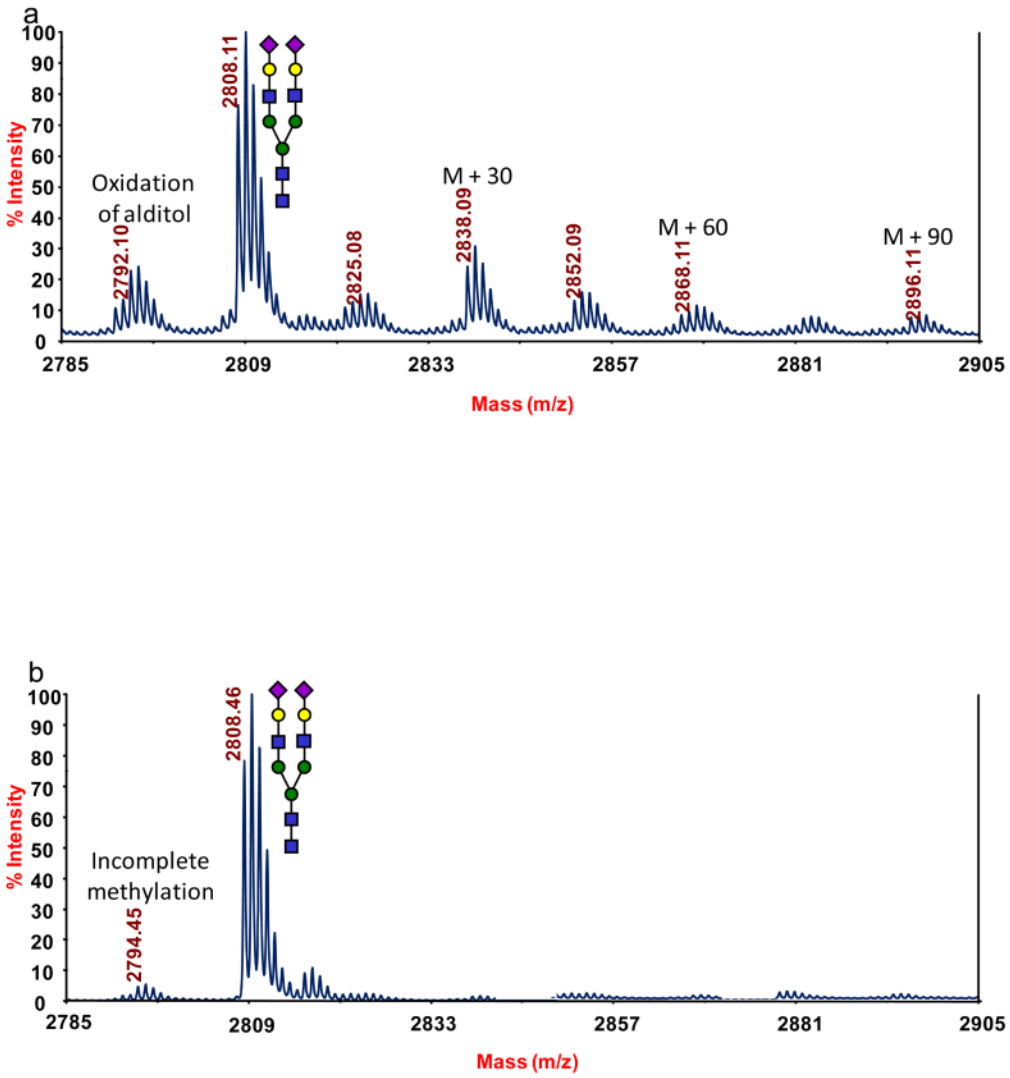

Figure 1.

A comparison of solid-phase methylation in (a) DMSO and in (b) DMF. Methylation of N-glycan alditols in DMSO resulted in the regeneration of the closed-ring structure and a reaction between DMSO and methyl iodide produced iodomethyl methyl ether that reacted with glycan hydroxyl groups, generating a series of satellite ions separated by 30 Da. These reactions were greatly reduced by performing the methylation reaction in DMF. Symbols: blue square, N-acetylgluosamine; green circle, mannose; yellow circle, galactose; purple diamond, N-acetylneuraminic acid (sialic acid); red triangle, fucose.

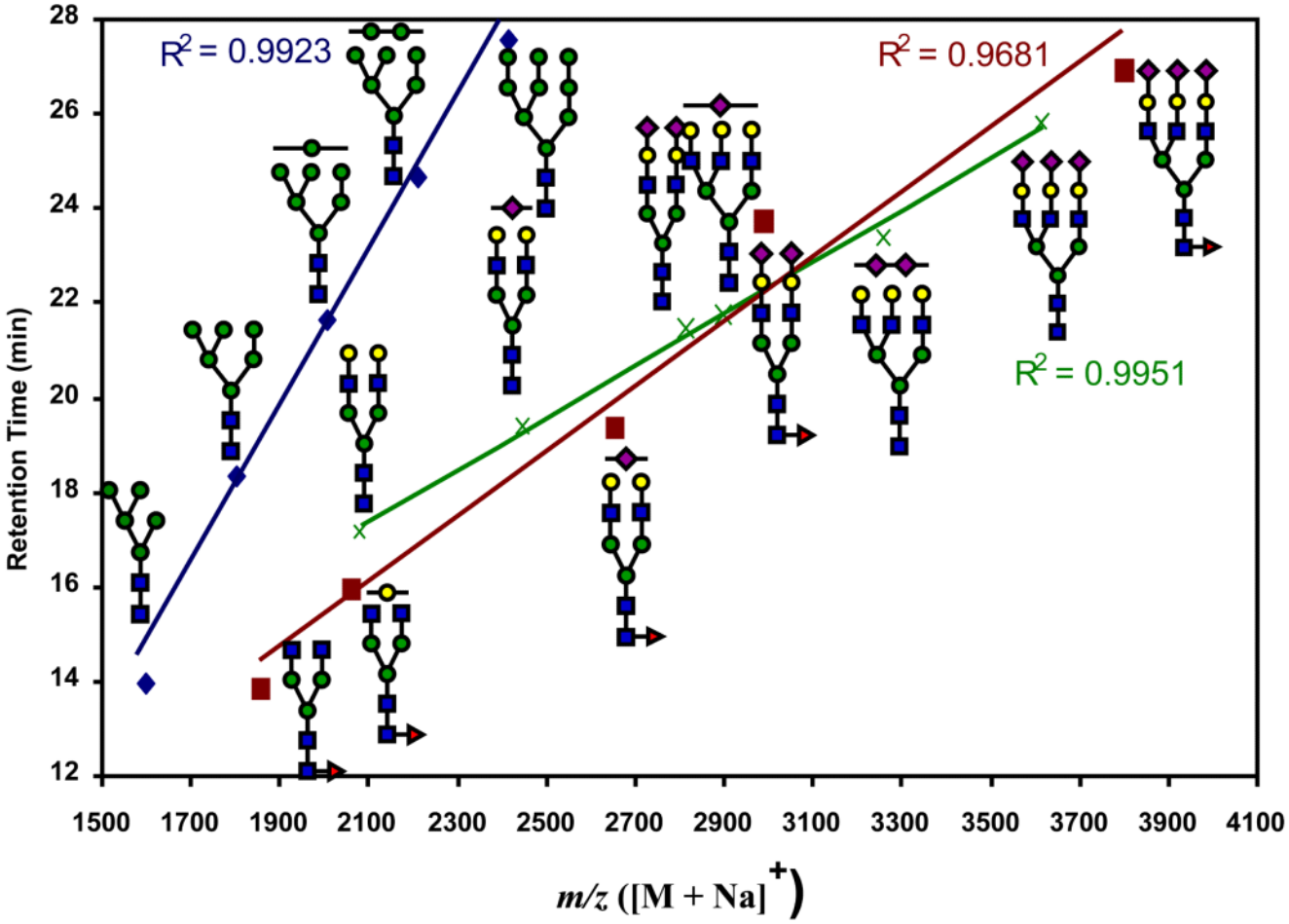

Figure 2.

The relationship of retention time vs. *m/z* for the high-mannose subclass (blue trace), the complex subclass (green trace), and the fucosylated-complex subclass (red trace) showing a linear increase in retention time as the number of monosaccharides increases within each subclass, consistent with a hydrophobic interaction with the stationary-phase medium. The symbols are the same as those used in Figure 1.

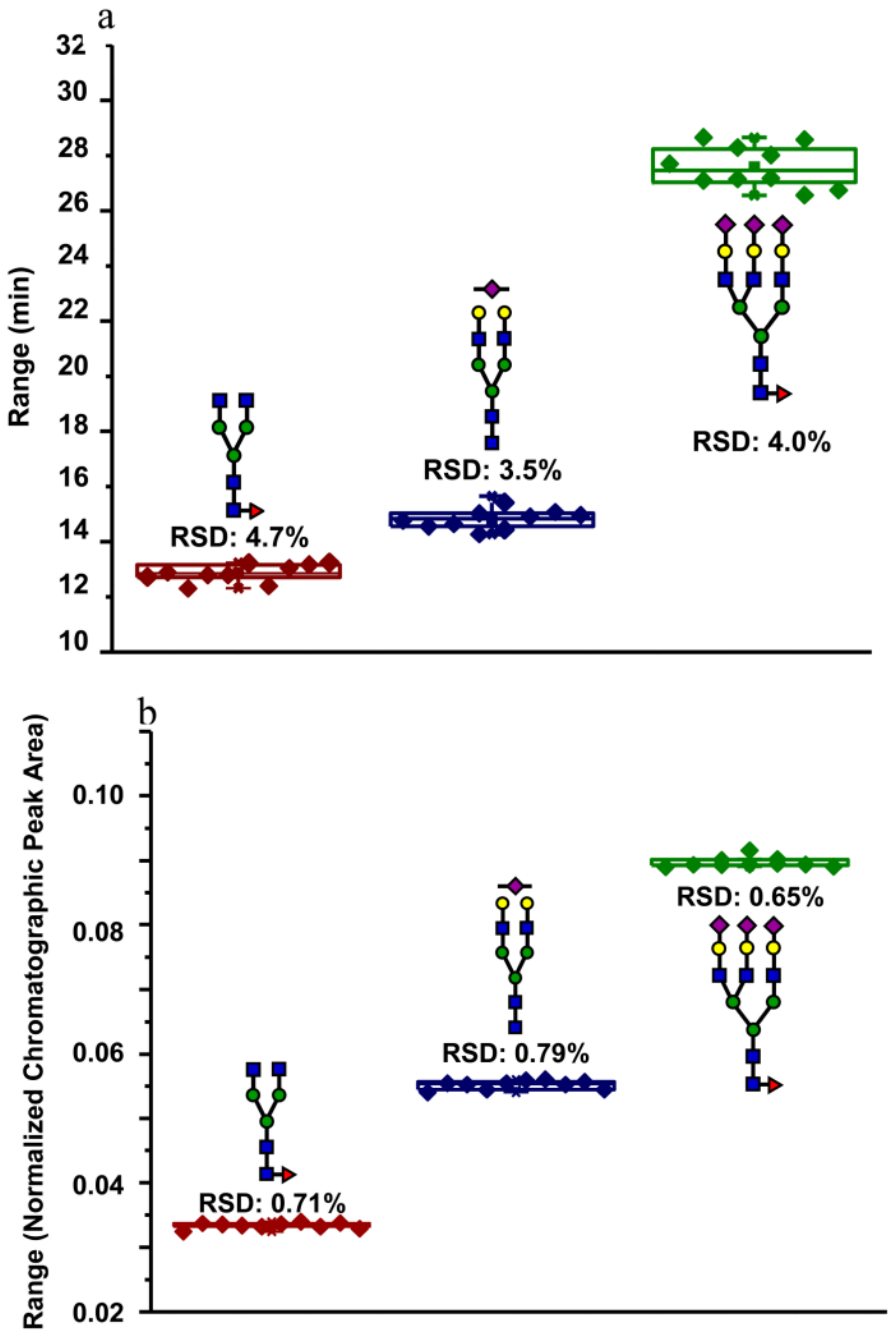

Figure 3.

(a) Retention-time repeatability of three diagnostic (reduced and methylated) N-linked glycans derived from human blood serum glycoproteins analyzed by LC-ESI-MS. A total of 10 replicate injections, each consisting of the equivalent of 0.25 μL of human blood serum, were analyzed by chip-based, reversed-phase LC-MS. Because ESI efficiency may fluctuate due to changes in mobile-phase composition, the retention time must be constant to ensure constant instrument performance. The retention time was very reproducible and ionization should not fluctuate due to mobile-phase composition changes. (b) Normalized chromatographic peak area repeatability for three diagnostic N-linked glycans derived from human blood serum glycoproteins analyzed by LC-ESI-MS. A total of 10 replicate

injections, each consisting of the equivalent of 0.25 μL of human blood serum, were analyzed by chip-based, reversed-phase LC-MS. The reproducibility of the normalized chromatographic peak area indicates that changes observed between the sample sets is due to the samples themselves, not due to changes in the performance of the instrumentation. The symbols are the same as those used in Figure 1.

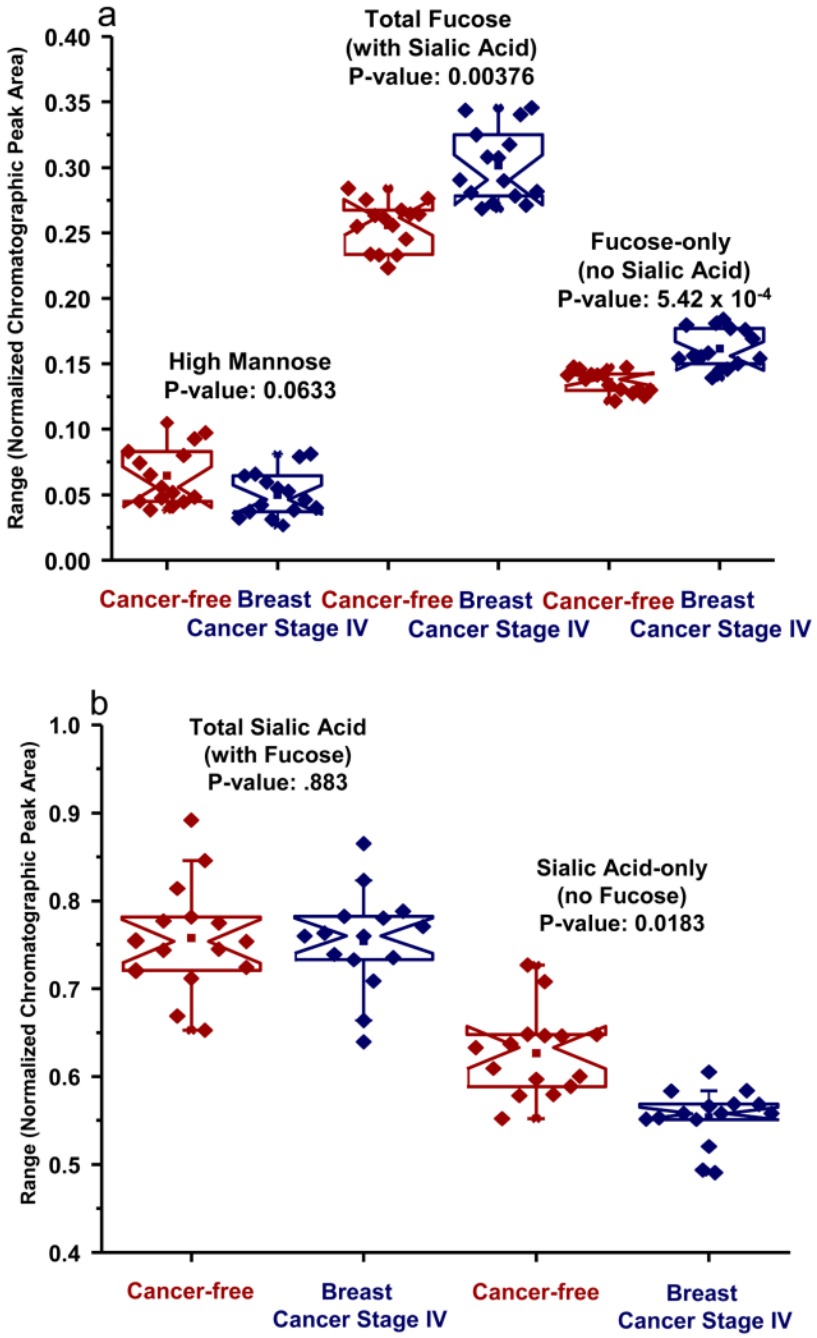

Figure 4.

Changes in the normalized peak areas for different classes of N-linked glycan classes between cancer-free and Stage IV breast cancer patients, indicating an increase in fucosylation (both with and without sialic acid) and a decrease in the relative abundance sialylated-only glycans in late-stage breast cancer. The symbols are the same as those used in Figure 1.

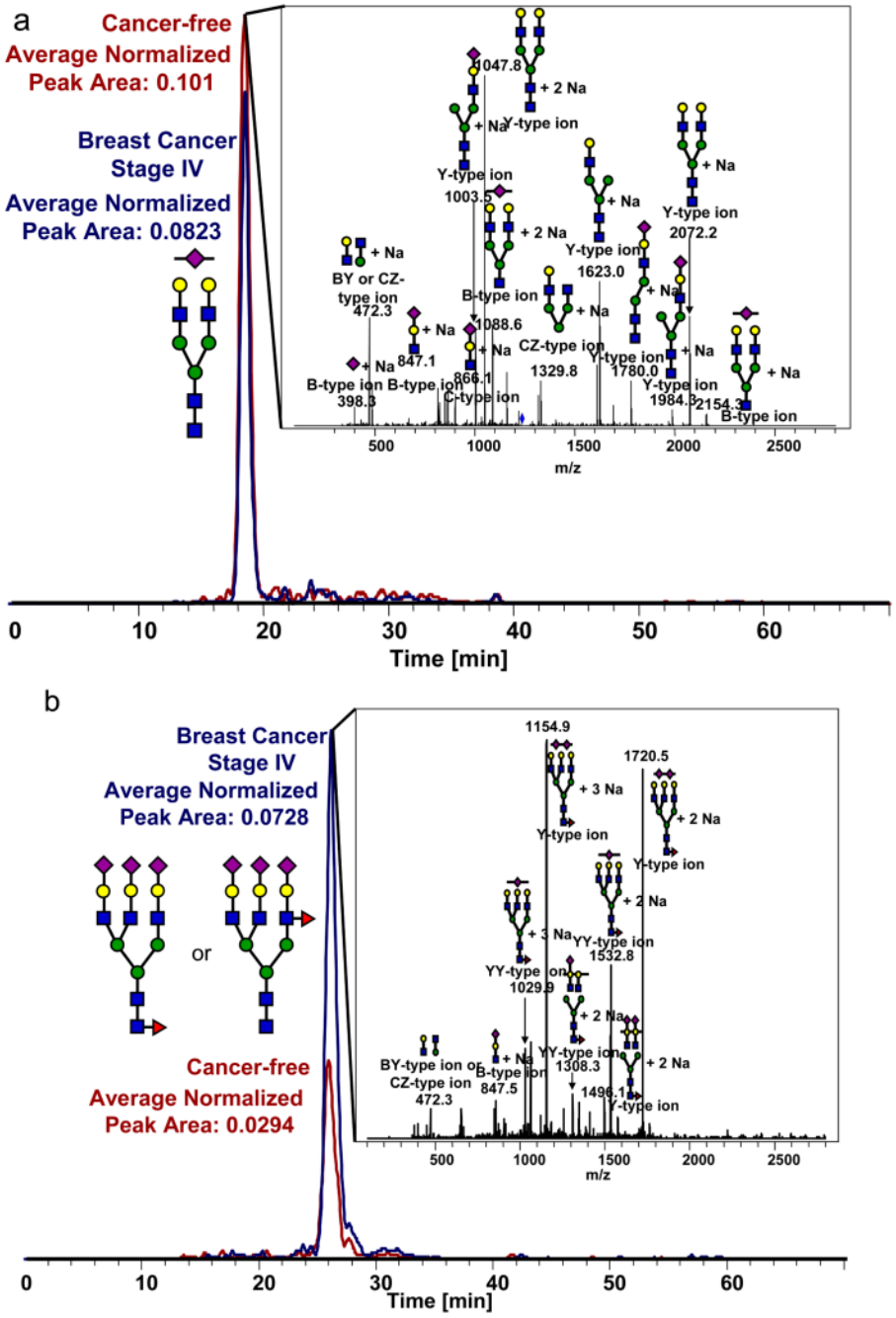

Figure 5.

Representative extracted-ion chromatograms for two statistically different N-linked glycans between cancer-free samples and Stage IV breast cancer samples derived from human blood serum glycoproteins. (a) The extracted-ion chromatogram for a N-linked biantennary-monosialylated glycan showing a decrease in relative abundance in the late-stage breast cancer sample and (b) the extracted-ion chromatogram for a N-linked fucosylated triantennary-trisialylated glycan, showing a significant increase in this N-linked glycan's relative abundance in the pathological sample. The symbols are the same as those used in Figure 1.

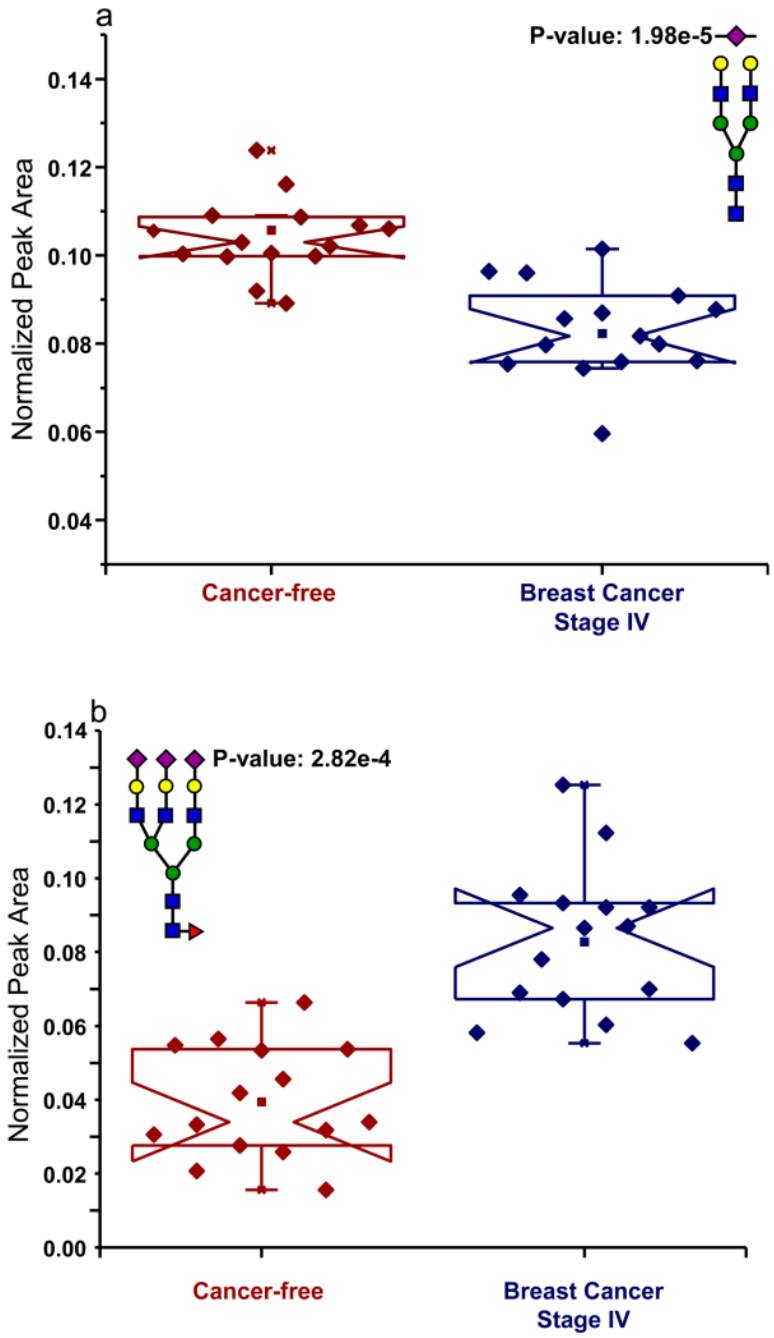

Figure 6.

Notched-box plots for 15 cancer-free blood-serum samples and blood-serum samples acquired from 15 Stage IV breast cancer patients for (a) the biantennary-monosialylated N-linked glycan derived from human blood serum glycoproteins showing the decrease in relative abundance for the larger set and (b) the N-linked fucosylated triantennary-trisialylated glycan derived from blood serum glycoproteins, showing the increase in relative abundance of this N-linked glycan for the larger set of samples. In a notched box plot, if the notches do not overlap, the differences in the sample sets may be considered significant with 95% confidence. The symbols are the same as those used in Figure 1.

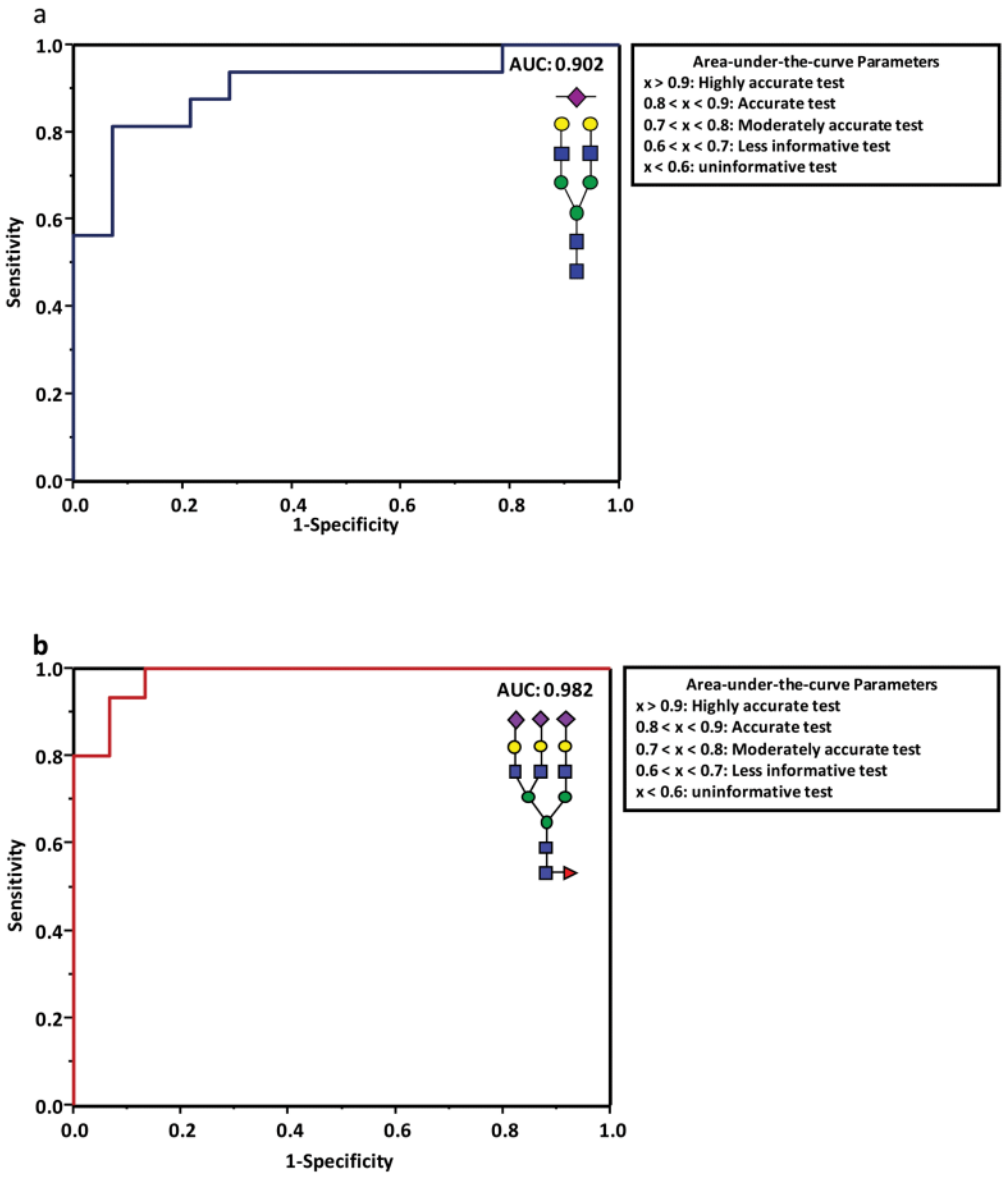

Figure 7.

The area-under-the-curve (AUC) plots for (a) the biantennary-monosialylated N-linked glycan and (b) the N-linked fucosylated triantennary-trisialylated glycan. The normalized chromatographic peak areas for both glycans resulted in highly-accurate tests, indicating their ability to accurately predict the state-of-health of the individual. The symbols are the same as those used in Figure 1.

Table 1

Glycans identified by chip-based, reversed-phase nano-flow ESI MS with charge state, P-values, and, where appropriate, area-under-the-curve (AUC) values. In this work, tests that returned an AUC of x > 0.9 were considered highly accurate, while accurate tests were those where 0.8 < x <0.9, moderately accurate tests were 0.7 <x < 0.8, less informative tests were 0.6 < x < 0.7 and x < 0.6 were uninformative tests

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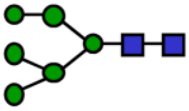
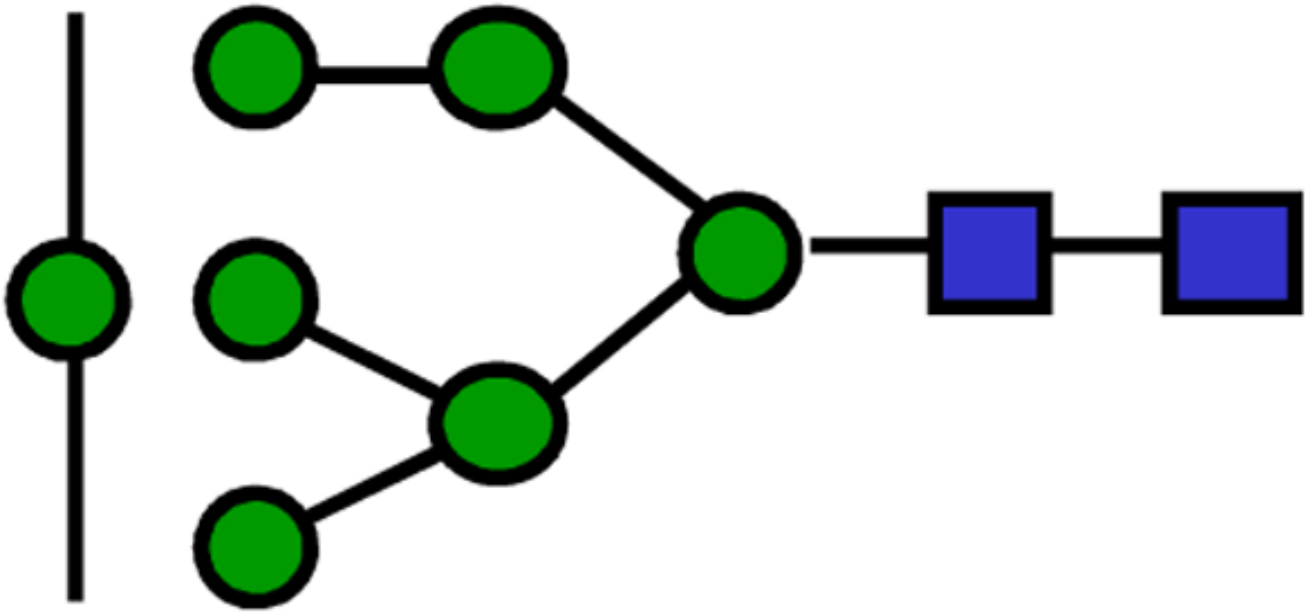
	911
	1014

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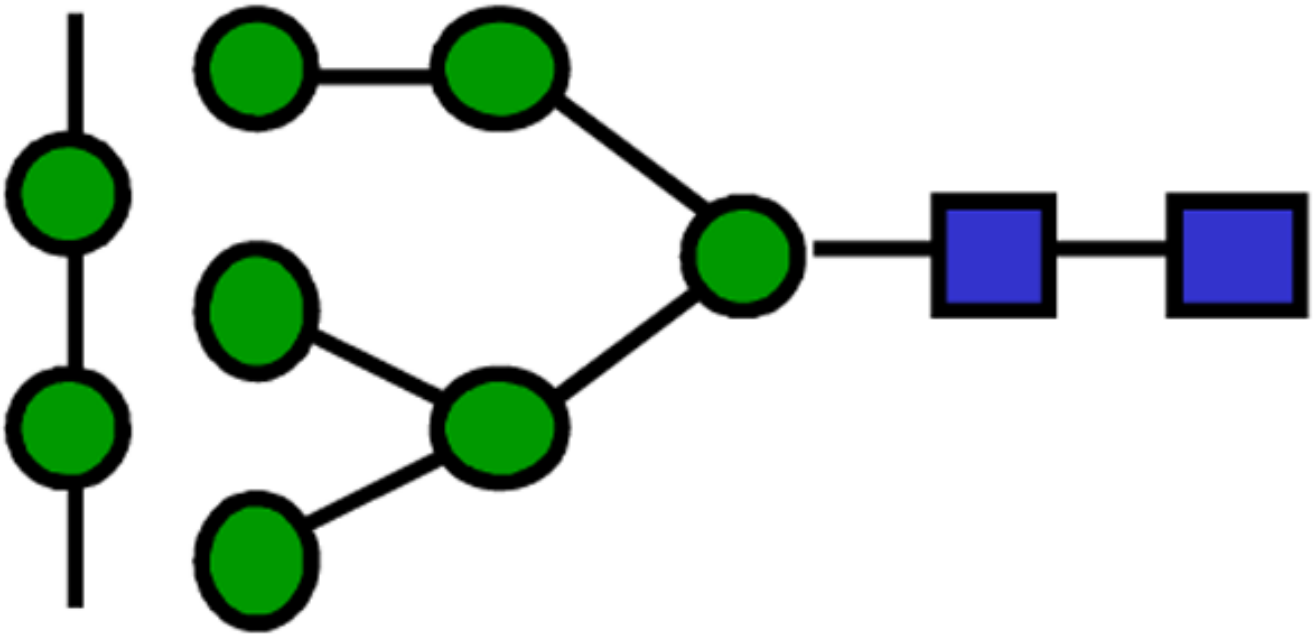
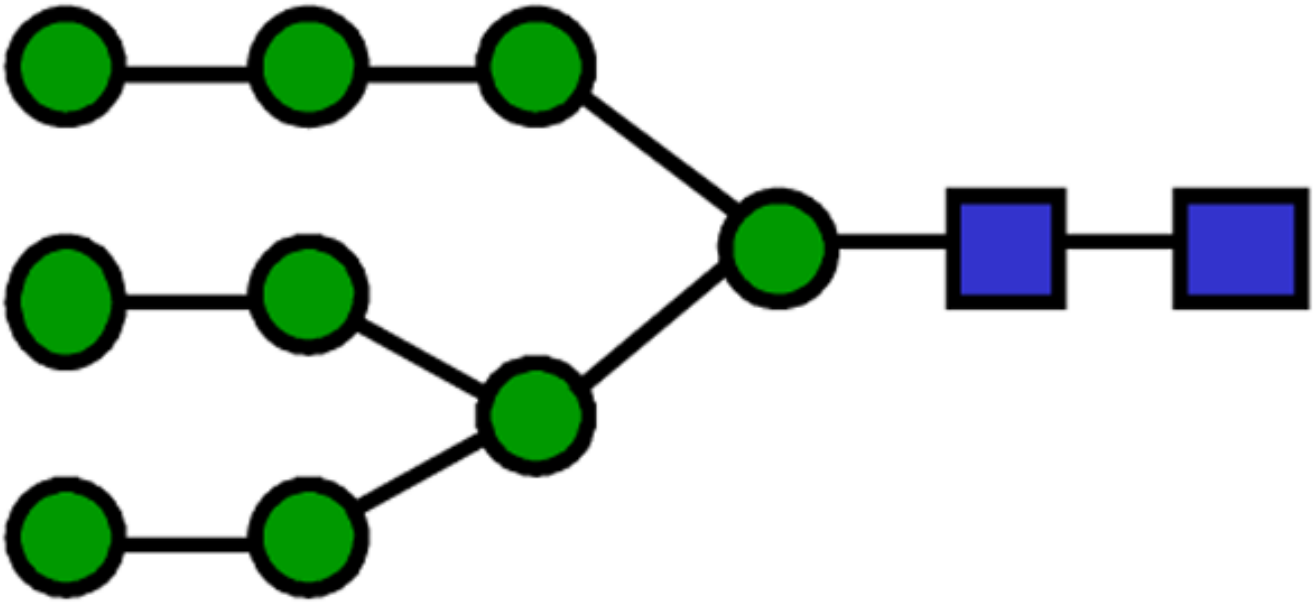
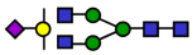
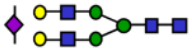
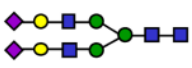
	1116
	1218 819
	1133
	1235 831
	1416 952

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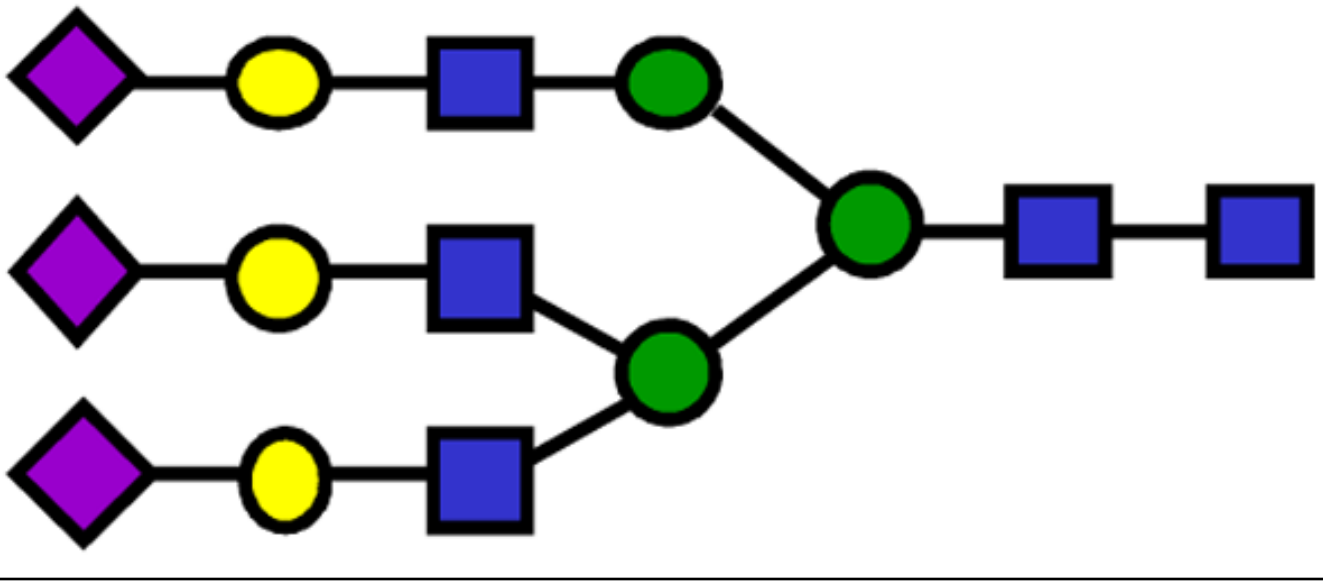
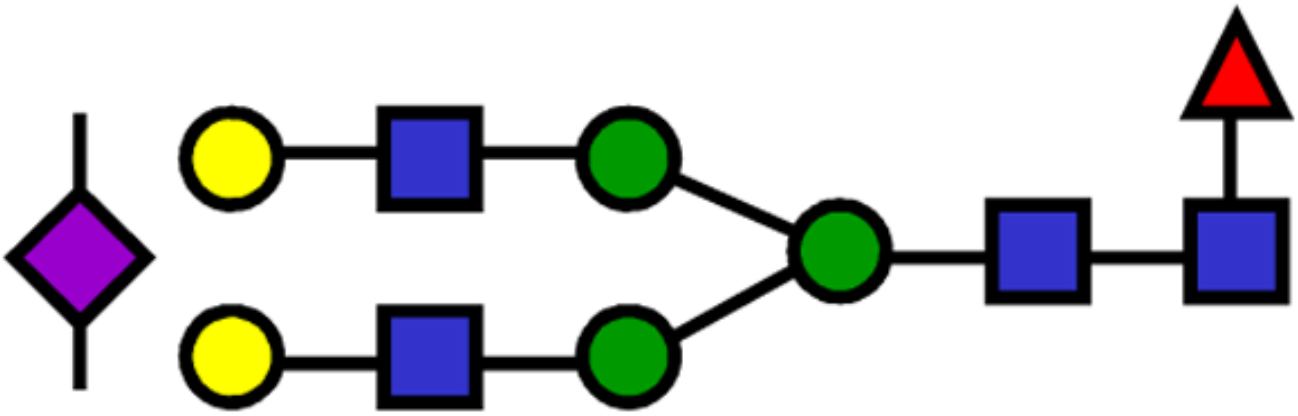
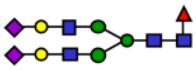
	1221 922
	1322 899
	1503 1009

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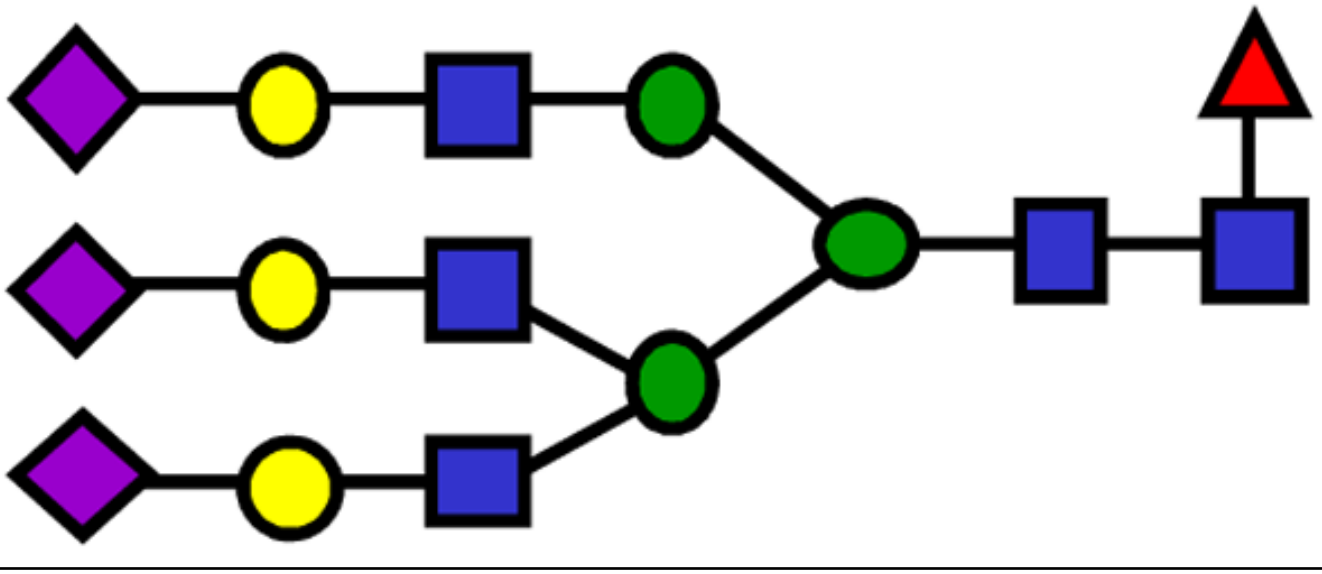
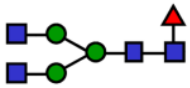
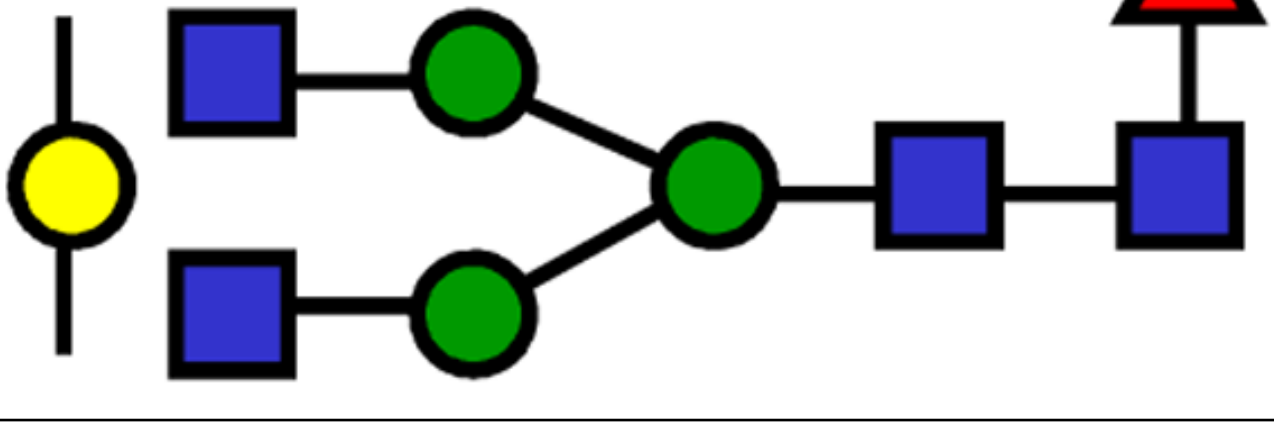
	1279 965
	937
	1039 700

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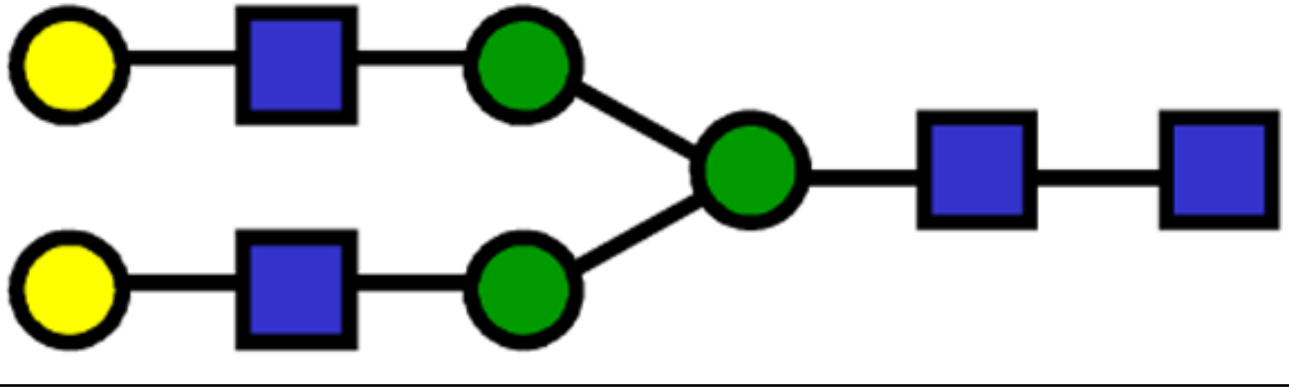
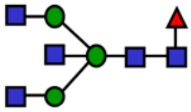
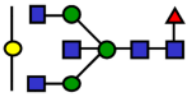
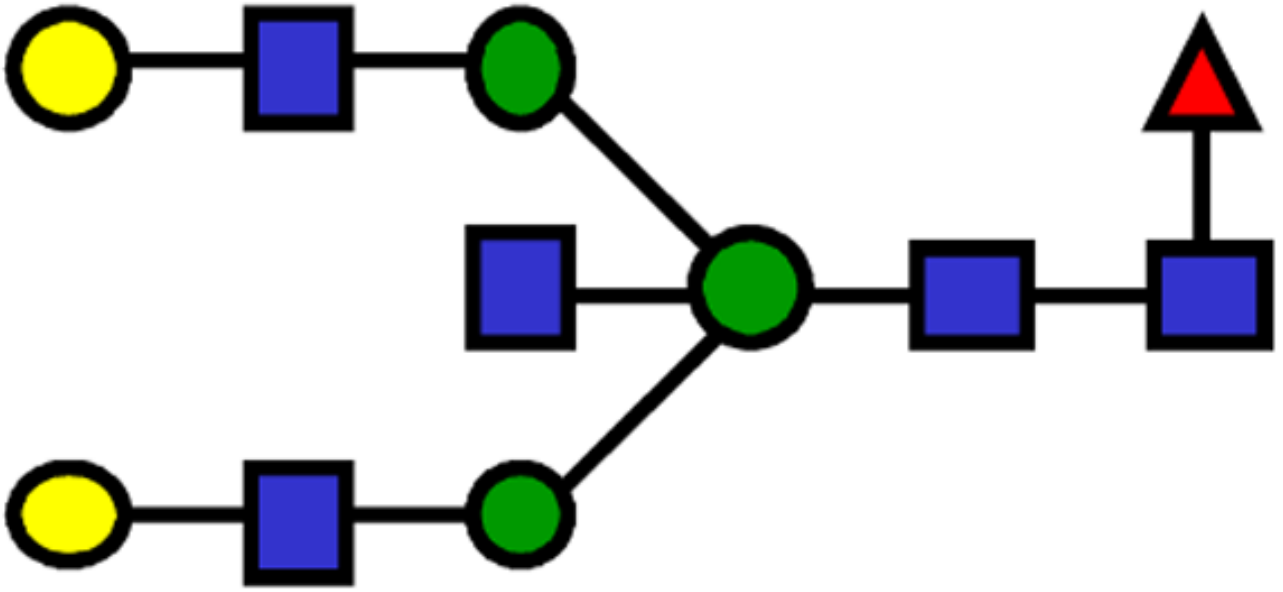
	1054 710
	1060
	1162 782
	1264 850