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Automated Annotation and Quantitation of Glycan by LC-ESI-MS analysis using MultiGlycan-ESI Computational Tool

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Abstract

Rationale—LC-MS is currently considered to be a conventional glycomics analysis strategy due to the high sensitivity and ability to handle complex biological samples. Interpretation of LC-MS data is a major bottleneck in high-throughput glycomics LC-MS based analysis. The complexity of LC-MS data associated with biological samples prompts the needs to develop computational tools capable of facilitating automated data annotation and quantitation.

Methods—An LC-MS based automated data annotation and quantitation software, MultiGlycan-ESI, was developed and utilized for glycan quantitation. Data generated by the software from LC-MS analysis of permethylated N-glycans derived from fetuin were initially validated by manual integration to assess the performance of the software. The performance of MultiGlycan-ESI was then assessed for the quantitation of permethylated fetuin N-glycans analyzed at different concentrations or spiked with permethylated N-glycans derived from human blood serum.

Results—The relative abundance differences between data generated by the software and those generated by manual integration were less than 5%, indicating the reliability of MultiGlycan-ESI in quantitation of permethylated glycans analyzed by LC-MS. Automated quantitation resulted in a linear relationship for all six N-glycans derived from 50 ng to 400 ng fetuin with correlation coefficients (R^2) greater than 0.93. Spiking of permethylated fetuin N-glycans at different concentrations in permethylated N-glycan samples derived from a 0.02 μ L of HBS also exhibited linear agreement with R^2 values greater than 0.9.

Conclusion—With a variety of options, including mass accuracy, merged adducts, and filtering criteria, MultiGlycan-ESI allows automated annotation and quantitation of LC-ESI-MS N-glycan data. The software allows the reliable quantitation of glycans LC-MS data. The software is reliable for automated glycan quantitation, thus facilitating rapid and reliable high-throughput glycomics studies.

Keywords

MultiGlycan-ESI; Glycans; Glycoproteins; Permethylation; LC-MS; Quantitation

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Introduction

Glycosylation is one of the most common post-translational modifications of proteins. Developing qualitative and quantitative glycomics is of great importance to understand the biological roles of glycans in many processes, including the development and progression of human diseases. With the development of matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) techniques, mass spectrometry (MS) is currently considered to be one of the most powerful tools in quantitative glycomics. LC-ESI-MS permits liquid chromatographic separation prior to MS detection, thus minimizing ion suppression and resulting in enhanced glycan detection.

Quantitative glycomics methods are classified into two categories: label free and isotopic labeling. Label free quantitation method does not involve derivatization and purification. Glycan derivatization, on the other hand, offers advantages, including increased MS ionization efficiency,^[1–4] improved tandem MS interpretation facilitating structural information^[5–7], enhanced detection by fluorescence or UV detectors^[8–10]. These advantages and others outweigh the additional preparation steps. Also, isotopic labels can be introduced to N-glycan through derivatization strategy. Isotopic labeling methods allow simultaneous analysis of several samples, which may overcome the variation that might be contributed to instrument instability, injection variation and different ionization environment. Recently, stable isotopic labeling of glycans is commonly attained by metabolic,^[11–13] reductive amination,^[2, 14–16] and permethylation^[17, 18] derivatization methods.

Regardless of the different quantitation approaches applied for glycomics study, manual evaluation of large sets of data is challenging and time consuming. Thus, developing software tools for automated glycan annotation and quantitation is required for high-throughput glycomics. Cartoonist^[19] and GlycoWorkBench^[20] are early developed glycan annotation tools, which assign glycan peaks by matching experimental masses to theoretical masses of MALDI-MS. The annotation of glycan peaks only depends on the glycan mass. GlycReSoft^[21] is a recently developed software tool that enables annotation and quantitation of glycan analyzed by LC-ESI-MS. The de-isotoped LC-ESI-MS data generated by Decon2LS^[22] are annotated by a default or a user-defined glycan library and reports the intensity of each glycan. However, GlycReSoft only process Decon2LS processed data. GlycReSoft does not account for ESI formation of several ion adducts and charge state ions.

Recently, we developed an open-source LC-ESI-MS data annotation software, MultiGlycan-ESI^[23]. The de-isotoping algorithm (Decon2LS) is included in the written code, thus eliminating the need to preprocess the data. Similar to GlycReSoft, default glycan list or user-defined list can be used for automated annotation. The output of MultiGlycan-ESI is the summation of the abundances of all charges and adducts associated with each glycan structure.

In this study, the performance of an updated version, MultiGlycan-ESI 1.0, was evaluated using permethylated N-glycans derived from fetuin and human blood serum. The software generated results were initially compared to manual integration results to evaluate the

reliability of the software in quantitative glycomics. The performance of MultiGlycan-ESI was further demonstrated using different concentrations of permethylated N-glycans derived from fetuin. Different amounts of permethylated fetuin glycans were also spiked in permethylated glycan samples derived from human blood serum (HBS) prior to LC-MS analyses was also utilized to evaluate the performance of the software.

2. Experimental

2.1 Materials

The model glycoprotein fetuin and HBS were purchased from Sigma-Aldrich (St. Louis, MO). Borane-ammonia complex, sodium hydroxide beads, dimethyl sulfoxide (DMSO), iodomethane, and MS-grade formic acid were also purchased from Sigma-Aldrich (St. Louis, MO). Empty microspin columns were obtained from Harvard Apparatus (Holliston, MA). Formic acid and HPLC-grade solvents, including acetonitrile, methanol and isopropanol, were procured from Fisher Scientific (Pittsburgh, PA). HPLC grade water was acquired from Mallinckrodt Chemicals (Phillipsburg, NJ). N-Glycosidase peptide purified from *Flavobacterium meningosepticum* (PNGase F) was obtained from New England Biolabs Inc. (Ipswich, MA).

2.2 N-glycans released from a model glycoprotein fetuin and HBS

N-glycans were first released from model glycoprotein fetuin and HBS. Briefly, a 10- μ L aliquot of 20 mM ammonium bicarbonate was added to a 10- μ L aliquot of HBS and fetuin stock solution (1 μ g/ μ L). The glycoproteins were mixed and denatured at 80°C for 1h. Then, 1.2 μ L of 10 times diluted PNGase F was added to glycoprotein mixture prior to incubation at 37°C in a water bath for 18h.

2.3 Dialysis to remove salts

An in-house built dialysis device was utilized to remove the salts from the digested samples. The dialysis device consisted of a dialysis membrane that was stabilized between two 12-well templates, each sample was dropped on top of the membrane in each well; the lower template was attached to a chamber, where circulated deionized water was supplied to remove salts and impurities that are smaller than 500 Da. Cellulose Ester (CE) membrane with molecular weight cut off (MWCO) of 500–1000Da was used. The samples were dialyzed for 18 h to remove all impurity of molecular weight less than 1000 Da. The purified samples were then collected and dried prior to reduction and permethylation.

2.4 Reduction

Borane-ammonia complex was used for the reduction of the purified samples. A 10- μ g/ μ L fresh borane-ammonium complex solution was prepared. A 10- μ L aliquot of this solution was then added to each sample and incubated at 65°C for 1h. A 10- μ L aliquot of aqueous acetic acid solution (5% v/v) was then added to each sample to neutralize the excess borane ammonia. Next, the samples were dried under vacuum. Methanol was applied to each dried sample to remove the borate salts. This process was repeated several times until no white solid was observed in the samples.

2.5 Permethylation

The reduced and cleaned samples were then permethylated. This process was performed according to our previously published protocol^[4, 24, 25]. Briefly, the sodium hydroxide beads-filled spin columns were first prepared. Then, DMSO was applied to the spin column to wash it. This process was repeated twice to ensure the removal of all impurities related to the beads and column. The reduced and dried samples were resuspended in 30 μ L DMSO, 1.2 μ L water and 20 μ L iodomethane. The reaction mixture was then applied to the sodium hydroxide beads-filled spin column. The mixture was allowed to react with the beads for 25 minute and then another fresh 20- μ L aliquot of iodomethane was applied to the spin column. The reaction was allowed to proceed for another 15 minute prior to centrifugation. A 50- μ L aliquot of acetonitrile was applied to the column to wash out all the reaction mixture. Finally, the samples were dried under vacuum.

2.6 NanoLC-ESI-MS

The permethylated samples were resuspended into 20% ACN with 0.1% formic acid and subjected to nano-LC-ESI. Dionex nano-LC system was used for separation. Samples were on-line purified with Acclaim[®] PepMap100 C₁₈ nano-trap column (Thermo Scientific, Waltham, MA)^[26]. The separation of glycans was performed on Acclaim[®] PepMap RSLC (75 μ m $\times$ 15 cm, 2 μ m, 100 Å) column (Thermo Scientific, Waltham, MA). The separation was attained at a flow rate of 350 nL/minute over 32 minute with a gradient from 38% solvent B to 55% solvent B. Solvent B was 100% ACN with 0.1% formic acid while solvent A consisted of 98% water and 2% acetonitrile with 0.1% formic acid. The separated N-glycans were infused to Velos LTQ Orbitrap hybrid mass spectrometer (Thermo Scientific, San Jose, CA) through a nano-ESI source. The capillary temperature was kept at 300°C while capillary voltage was maintained at 1.5 kV thus allowing desolvation of LC separated sample. The mass spectrometer was operated in an automated data-dependent acquisition mode with the eight most abundant ions were subjected to CID MS/MS scan. The m/z full scan range was set to 500–2000 m/z . Tandem MS conditions were set to 0.250 Q-value, 20 ms activation time, and 35% normalized collision energy.

2.7 Data processing and quantitation using MultiGlycan-ESI

The generated raw files were processed with MultiGlycan-ESI software. MultiGlycan-ESI provides a built-in N-glycan list (Default list). Also, users can compose their own glycan list file in CSV format and use it for glycan annotation. The software matches the LC-ESI-MS ions with the default or user-defined glycan list. The THRASH algorithm was utilized to basic peak processing and de-isotope isotopic distributions in MultiGlycan-ESI. In this study, reduced and permethylated N-glycan masses were selected to be searched. The mass tolerance was set to 5 ppm. The search retention time window was set to 5 minute. Different charge states and different adducts, including ammonium and sodium, were defined to be merged. The minimum peak width of an LC peak was set to 0.2 minute while the minimum abundance was set to 10,000.

3. Results and Discussion

The development of a data analysis tool is of great importance to achieve high-throughput quantitative glycomics. Here, a recently developed software tool, MultiGlycan-ESI, was utilized in quantitative glycomics. Figure 1 summarizes the workflow to achieve automated quantitative glycomics employing MultiGlycan-ESI. Briefly, N-glycans were released from glycoproteins and then reduced. The reduced glycans were then permethylated and subjected to nanoLC-ESI-MS analysis. The LC-MS raw data was uploaded to MultiGlycan-ESI. The whole scan range was searched to identify isotopic distributions of N-glycan peaks. User-defined glycan list was uploaded for searching reduced and permethylated N-glycans. Different adducts, including proton ions, ammonium ions and sodium ions, were selected for processing. Mass tolerance of peak searching was defined to be 5 ppm, which was in alignment with the high-mass accuracy of an Orbitrap instrument used in this study. Different charge states and adducts of the same glycan were merged. The criteria of merged LC peak were minimum peak width of 0.2 minute, maximum peak width of 5 minute, and minimum abundance of 10,000. The total ion intensity of different charge states were reported in the output file. A default peak processing parameter was utilized. Two csv files are resulting from data processing: one csv file provides a list of annotated glycan structures and merged intensity, and a second csv file includes a full list of annotated glycans are uploaded into elution profile viewer (an internal function of the MultiGlycan-ESI software) to generate extracted ion chromatograms.

The performance of MultiGlycan-ESI was initially evaluated by comparing the peak intensities generated by the software to the peak area generated manually using Xcalibur (Thermo Scientific), referred to here as manual integration. The LC-MS data acquired from three injections of permethylated N-glycans derived from 500 ng fetuin were utilized for comparison. In the case of manual integration, the peak areas of all adducts and charge states of a glycan structure were added up. In the case of MultiGlycan-ESI, the intensities of the first monoisotopic peaks of all adducts and charge states were added. The comparison results are summarized in Table 1. The absolute intensities calculated using the two integration methods were not comparable because different quantitation scales were used in each case. However, the relative intensities of N-glycans calculated from the two integration methods were comparable within less than 5% differences, indicating a good agreement among the software integration and the manual integration. Thus suggesting, the software is reliable for automated quantitation of LC-MS data.

One advantage of utilizing MultiGlycan-ESI is the ability to automatically merge the different adducts and charge states corresponding to the same glycan. Manually adding the intensities of all adducts for each structure is laborious as most of the glycans have multiple m/z values generated by ESI. The necessity of adding all adducts for quantitation was demonstrated in Supplementary Figure 1 (Figure S1). As illustrated in Figure S1, the RSD of 15 injections can be as high as 67% for sodium adducts and ammonium adducts. When all adducts were added, %RSD of the 15 injections dropped to less than 25%. Moreover, the relative intensity distribution of one adduct is different from the distribution of the summation, which indicates that for reliable quantitation all adducts and charges states needs to be accounted for.

Quantitative glycomics utilizing MultiGlycan-ESI

MultiGlycan-ESI was also utilized to assess the reproducibility of injection and on-line purification of permethylated glycans. Same amount of glycans derived from fetuin were injected at different injection volumes. Three injections were made for each injection volume. Figure 2 depicts the comparison of five groups of different injection volumes as well as the average of five groups. Concentrations of 500 ng/ μ L, 250 ng/ μ L, 166.7 ng/ μ L, 125 ng/ μ L and 100 ng/ μ L were used to achieve a final injection amount of 500 ng in each case. The injection volumes were 1 μ L, 2 μ L, 3 μ L, 4 μ L and 5 μ L. For each group, the %RSD was determined to be less than 15% (Table S1). The RSD of all 15 injections is less than 25% (Table S1). As the LC-MS RSD can be as high as 15%^[27], the variation contributed by sample handling and injection is less than 10%, which is an analytically acceptable value.

Label-free quantitation of N-glycans derived from fetuin

MultiGlycan-ESI was also employed to assess the data generated for LC-MS analyses of different concentrations of permethylated N-glycans derived from fetuin. The two orders of magnitude dynamic range of six N-glycan derived from fetuin was employed. Triplicate injections of four different amounts spanning from 50 ng to 400 ng were made. All six N-glycans were detected even at the lowest injection amount. The intensities of N-glycans were calculated using MultiGlycan-ESI as described above. The relationship of injected fetuin amount to observed intensity is shown in Figure 3. Figure 3a illustrates the relationship of concentrations to the intensities of the two low-abundance glycans derived from fetuin. Both demonstrated a good linear response with the increase of glycan amount. Correlation coefficients (R^2) of HexNAc4Hex5NeuAc1 and HexNAc5Hex6NeuAc1 structures are 0.998 and 0.988, respectively. The four relatively high-abundance N-glycans derived from fetuin were shown on Figure 3b. The linearity of the plots generated for the high-abundance glycans is less than that of the low abundance structures. Such discrepancy might be due to detector saturation. R^2 is higher than 0.93 for all six N-glycan derived from fetuin, indicating a good linear response between glycan amount and intensity.

Label-free quantitation of N-glycans derived from fetuin and mixed with N-glycans derived from human blood serum

Different amount of N-glycans derived from fetuin were spiked into permethylated N-glycans derived from HBS to test the efficiency of MultiGlycan-ESI in the quantitation of glycans present in complex biological samples. Six different amounts (0–200ng) of fetuin samples were added to N-glycans derived from 0.2 μ L HBS samples. Triplicate injections were made for each amount. Figure 4 shows the linear relationship of concentration to the intensity. HexNAc5Hex6NeuAc3 and HexNAc5Hex6NeuAc4 show good linear response, with R^2 of 0.974 and 0.976, respectively. Due to the contribution of the “zero” point, the overall linearity (R^2) of HexNAc5Hex6NeuAc2 is 0.795 (Figure 4c). A 200-ng aliquot of the fetuin sample contains less than 10% of the HexNAc5Hex6NeuAc2 concentration in HBS. Thus, the contribution of the spiked fetuin glycans may be outweighed by the instability of ESI. As it is shown in Figure 4d, there is no obvious increase of the intensity with the increase of spiked fetuin amount for the other three N-glycan structures. This also

can be explained as the spiked amount of glycans derived from fetuin is lower than 20% of the glycan in HBS. Similarly, a change of intensity is outweighed by the ESI variation.

To further confirm this assumption, same amount of fetuin was spiked in permethylated N-glycans derived from 0.002 μ L HBS. In this case, all six glycans exhibit a linear relationship with R^2 higher than 0.94, as shown in Figure 5. For each structure, the spiked fetuin amount is higher than 20% of HBS sample amount. Thus, linear relationships for all six glycans were observed.

Conclusion

Due to the complexity of glycomics LC-MS data, bioinformatics are in demand. Currently, most of the glycomics software processing tools only enable the automated annotation of MALDI-MS data. The completely automated annotation and quantitation of LC-ESI MS data are still lacking. MultiGlycan-ESI is a recently developed glycan LC-ESI data quantitation tool. MultiGlycan-ESI provides a user-friendly interface and allows the summation of all different adducts and charge states of a glycan. The output file provides the structure information as well as the intensities associated with all detected structures. Extracted ion chromatograms can be generated when the full results are loaded to the built-in elution profile viewer. The software proved to be a reliable tool for the quantitation of N-glycans derived from simple and complex samples. MultiGlycan-ESI is a reliable tool for quantitative glycomics and is highly effective in high-throughput LC-MS glycomics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Workflow of MultiGlycan-ESI based automatic quantitation strategy. a) Permethylated N-glycan preparation workflow; b) Software annotation and quantitation method.

Figure 2.

Bar graph of six permethylated N-glycans derived from fetuin. Same amount of fetuin was injected at different injection volume. Triplicate injections were made for each group.

Figure 3.

A plot of the linear relationship of the permethylated N-glycans derived from fetuin.
Triplicate injections of four groups from 50 ng-400 ng of fetuin were made.

Figure 4.

A plot of the permethylated N-glycans derived from fetuin spiked into 0.2 μ L HBS.

Triplicate injections of five groups from 0 ng-200 ng fetuin spiked into HBS were made.

Figure 5.

A plot of the permethylated N-glycans derived from fetuin spiked into 0.002 μ L HBS. Triplicate injections of five groups from 0 ng-200 ng fetuin spiked into HBS were made.

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Table 1
Integrated areas of permethylated N-glycans derived from fetuin, calculated by manual or MultiGlycan-ESI integration

N-glycan structure	Intensity Manual	Intensity MultiGlycan ESI	Relative intensity of Manual integration	Relative intensity of MultiGlycan ESI	Difference (%)
	(2.60±0.05)e8	(4.45±0.1)e7	0.57%	0.58%	1.71%
	(8.65±0.2)e9	(1.41±0.02)e9	19.02%	18.43%	3.13%
	(2.20±0.3)e7	(3.72±0.6)e7	0.05%	0.05%	0.61%
	(1.74± 0.005)e9	(3.08± 0.03)e9	3.83%	4.01%	4.57%
	(2.62±0.06)e10	(4.40±0.06)e10	57.57%	57.40%	0.30%
	(8.62±0.5)e9	(1.50±0.03)e9	18.95%	19.53%	2.97%