Published in final edited form as:

Anal Bioanal Chem. 2017 July ; 409(18): 4437–4447. doi:10.1007/s00216-017-0387-6.

Development of a hydrophilic interaction liquid chromatography coupled with matrix-assisted laser desorption/ionization-mass spectrometric imaging platform for N-glycan relative quantitation using stable-isotope labeled hydrazide reagents

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Abstract

In this work, the capability of newly developed hydrophilic interaction liquid chromatography (HILIC) coupled with matrix-assisted laser desorption/ionization-mass spectrometric imaging (MALDI-MSI) platform for quantitative analysis of N-glycans has been demonstrated. As a proof-of-principle experiment, heavy and light stable-isotope labeled hydrazide reagents labeled maltodextrin ladder were used to demonstrate the feasibility of the HILIC-MALDI-MSI platform for reliable quantitative analysis of N-glycans. MALDI-MSI analysis by an Orbitrap mass spectrometer enabled high-resolution and high-sensitivity detection of N-glycans eluted from HILIC column, allowing the re-construction of LC chromatograms as well as accurate mass measurements for structural inference. MALDI-MSI analysis of the collected LC traces showed that the chromatographic resolution was preserved. The N-glycans released from human serum was used to demonstrate the utility of this novel platform in quantitative analysis of N-glycans from a complex sample. Benefiting from the minimized ion suppression provided by HILIC separation, comparison between MALDI-MS and the newly developed platform HILIC-MALDI-MSI revealed that HILIC-MALDI-MSI provided higher N-glycan coverage as well as better quantitation accuracy in the quantitative analysis of N-glycans released from human serum.

Keywords

Glycomics; N-Glycans; MALDI imaging; Quantitation; HILIC; Hydrazide reagents

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Electronic supplementary material The online version of this article (doi:10.1007/s00216-017-0387-6) contains supplementary material, which is available to authorized users.

Conflict of interest The authors declare that they have no conflict of interest.

Introduction

Glycosylation, the most diverse and complex form of protein post-translational modifications (PTMs), has been proven to play essential role in many key biological processes including cell adhesion, molecular trafficking and clearance, receptor activation, signal transduction and endocytosis [1]. In fact, glycosylation has been widely detected in different kinds of proteins such as growth factors, cytokines, immune receptors, and enzymes [2]. It is estimated that over 70% of all human proteins are glycosylated [3]. Aberrant glycosylation has been implicated in various diseases, most strikingly in a class of diverse diseases collectively referred to as congenital disorders of glycosylation [4]. Furthermore, it has been well established that glycosylation pattern alters significantly in cancer cells [5, 6]. Thus, accurate and reliable glycan structure characterization and quantitation is crucial for glycan bio-marker discovery and understanding the roles of glycosylation in biological processes.

Electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) have become very powerful and widely used analytical tools for structural and quantitative analysis of N-glycans. As a complementary ionization method to ESI, MALDI generates only singly charged ions which simplifies the spectrum and the signal of a specific analyte is “focused” and “enhanced” on these singly charged ions. As MALDI is more tolerant to salts and other contaminants when proper matrix or matrix additives is applied [7–10], less sample cleanup is needed, thus increasing sample recovery rate and decreasing sample preparation time. However, MALDI-MS analyses of complex glycan samples are often hampered by the complexity of the sample due to ion suppression effect [11], leading to low glycan coverage, poor MS/MS fragmentation quality, and inaccurate quantitation. One possible solution is to couple offline liquid chromatography (LC) separation with MALDI-MS to decrease the complexity of the analyte mixtures by chromatographic separation before MS analysis, which could provide improved sensitivity and dynamic range. In addition, decoupling the separation from MS analysis provides the opportunity to independently optimize LC separation performance and MS performance. Another benefit for LC coupled to MALDI is that non-volatile salts such as ion pairing agent could be used to improve the chromatographic separation capability without negative impact on the MS signal.

In fact, the powerful analytical potential of both commercial and in-house built devices for LC-MALDI-MS coupling has been demonstrated for both proteomics and glycomics studies [12–17]. Due to the strong retention of polar compounds, hydrophilic interaction liquid chromatography (HILIC) has been widely used for the separation of both native [18–21] and derivatized [22–28] glycans in glycomics studies. Offline coupling of HILIC with automated MALDI-MS analysis has been established for glycan structure characterization [16, 17]. However, one disadvantage is that discrete spotting of the eluate on the MALDI plate causes loss of chromatographic resolution. To overcome this weakness, our lab [29, 30] has previously developed LC-MALDI-MSI platform to demonstrate its ability for enhanced proteomics and peptidomics studies. In contrast to the offline LC-MALDI-MS coupling schemes, continuous real-time LC flow is collected on a MALDI plate and subject to MSI analysis after applying matrix. In this way, chromatographic resolution from LC separation

dimension could be almost fully preserved, which leads to minimized ion suppression and enhanced signal. Comparable LC chromatograms can be re-constructed, allowing visualization of separation of analytes. Herein, taking advantage of the powerful separation ability of HILIC for glycans, we developed a novel HILIC-MALDI-MSI platform to demonstrate its capability for carbohydrate analysis or glycomics study.

The present study is the first demonstration of HILIC-MALDI-MSI as a novel platform for relative quantitative analysis of N-glycans using stable-isotope labeled hydrazide reagents. Maltodextrin ladder was used to demonstrate the feasibility of this approach for accurate relative quantitative analysis. To demonstrate the practical utility of the developed approach in complex biological samples, we conducted relative quantitative analysis of the N-glycans enzymatically released from human serum. The data revealed that the approach is reproducible and reliable. A comparison between the commonly used MALDI-MS platform and the newly developed HILIC-MALDI-MSI platform revealed that HILIC-MALDI-MSI outperformed MALDI-MS analysis in both N-glycan coverage and quantitation accuracy. Collectively, our study suggests that HILIC-MALDI-MSI assisted by the hydrazide labeling reagent is a promising new approach for quantitative N-glycan profiling with significant potential in glycan biomarker discovery and comparative glycomic studies.

Experimental section

Chemicals and materials

Acetic acid, acetonitrile, ammonium acetate, and ammonium bicarbonate were obtained from Fisher Scientific (Pittsburgh, PA). Formic acid (FA), *N,N*-dimethylaniline (DMA), maltotriose, maltodextrin, and ribonuclease B (RNase B) from bovine pancreas were purchased from Sigma-Aldrich (St. Louis, MO). 2,5-Dihydroxybenzoic acid (99%, DHB) was from Acros Organics (Geel, Belgium). Dithiothreitol (DTT) and PNGase F were from Promega (Madison, WI). Microcon filters YM-30 were purchased from Merck Millipore (Billerica, MA). Human serum powder, LC/MS grade isopropanol (IPA), acetonitrile (ACN), methanol (MeOH), and water were from Fisher Scientific (Pittsburgh, PA). The hydrazide labeling reagents, light tag 2-hydrazino-4,6-bis-(diethylamino)-*s*-triazine (HDEAT) and heavy tag 2-hydrazino-4,6-bis-(d₁₀-diethylamino)-*s*-triazine (d₂₀-HDEAT), were synthesized according to a protocol described previously [31].

Release of N-glycans

The filter-aided N-glycan separation (FANGS) strategy was modified based on the previous protocol [32]. Briefly, 100 µg of human plasma or RNase B dissolved in 100 µL digestion buffer (50 mM ammonium bicarbonate) was loaded onto a 30-kDa molecular weight cutoff filter. Then 100 µL of 20 mM DTT in digestion buffer was added to the filter. Filters were incubated in water bath alternating between 100 °C (boiling water) and 25 °C (room temperature) for 15 s each for four cycles (2 min total time) to denature protein. Then the samples were concentrated onto the filter by centrifugation at 14,000×g for 40 min. The samples were washed with 100 µL of digestion buffer by centrifugation at 14,000×g for 20 min three times to remove small molecular weight contaminants. After that, the filter was transferred to a fresh collection vial, and 4 µL of PNGase F (1 IUB milli-unit/mL) and 96 µL

of digestion buffer were added. The reaction was incubated at 37 °C for 18 h to enzymatically cleave N-glycans. N-Glycans were eluted by centrifugation at 14,000×g for 20 min. To ensure complete elution of N-glycans from the filter, two more washes with 100 µL digestion buffer were collected. Purified N-glycans were dried down in SpeedVac and re-dissolved in 100 µL 50% MeOH 0.1% formic acid and incubated in 37 °C water bath for 1 h to allow deamidation. The samples were then dried down again and stored at –20 °C before further derivatization.

Derivatization of glycans

To each reaction vial, 230 µg of HDEAT and d₂₀-HDEAT was aliquoted. Glycan samples (100 µg maltotriose, 100 µg maltodextrin, and human serum N-glycans from 100 µg protein) were reconstituted in 200 µL 1% acetic acid and 70% IPA solution and transferred to the reaction vial. Then the reaction was incubated at 37 °C water bath for 2 h. To quench the reaction, the labeled samples were dried down in SpeedVac. The light and heavy labeled maltotriose were mixed at different ratios of 5:1, 4:1, 3:1, 2:1, 1:1, 1:2, 1:3, 1:4, and 1:5 for the following MALDI-MS analysis. The heavy and light labeled maltodextrin and human serum N-glycans were mixed at a ratio of 1:1 and re-dissolved in HILIC gradient initial condition for HILIC-MALDI-MSI analysis without further purification. The heavy and light labeled human serum N-glycans were also subject to MALDI-MS analysis.

Nanoflow hydrophilic interaction liquid chromatography

The LC separation was performed with a homemade HILIC column on a Waters nanoACQUITY UPLC system. Samples were loaded and separated on a 75 µm × 40 cm homemade capillary column packed with 3 µm poly(2-hydroxyethyl aspartamide) (PHEA) (Poly LC, Columbia, MD). Mobile phases A and B were 10 mM ammonium acetate in water (pH 4.7) and 100% ACN, respectively. The flow rate was set at 0.3 µL/min. After dissolved in the initial gradient condition (25% A for labeled human serum N-glycans, 10% A for labeled maltodextrin), 1 µL sample was injected. The gradient for labeled human serum N-glycans was from 25% A to 80% A over 55 min. The gradient for labeled maltodextrin was set as follows: 10–35% A (0–20 min), 35–40% A (20–21 min), 40–40% A (21–30 min), 40–45% A (30–31 min), 45–45% A (31–40 min), 45–55% A (40–41 min), 55–55% A (41–46 min), 55–70% A (46–47 min), and 70–70% A (47–55 min).

HILIC-MALDI interface and matrix application

The LC flow was collected directly on a ground stainless steel MALDI plate, which was fixed on a syringe pump (Pump 11 Elite, Harvard Apparatus, Holliston, MA, USA) and moved along with the pump. The tip of the column directly touches the MALDI plate to deposit LC traces with an angle of 45°. Compared to the original capillary, the pooled tip with a smaller diameter at the very distal end could minimize the effluent diffusion. The LC eluent from 15 to 40 min eluent was collected for labeled human serum N-glycans. After that, matrix (100 mg/mL DHB, 2% DMA in 50% ACN) was sprayed on the dried traces using a TM-Sprayer from HTX Technologies (Carrboro, NC). The parameters were carefully adjusted and set as follows: matrix flow rate 0.25 mL/min; nozzle velocity 1000 mm/min; temperature 85 °C; gas pressure 10 psi; line spacing 3 mm; number of layers 2; dry time 1 min.

Data acquisition and processing

The MS images of collected LC traces were acquired with a MALDI-Orbitrap mass spectrometer (Thermo Scientific, Waltham, MA, USA) in positive ion mode. The instrument methods were set using Xcalibur (Thermo Scientific, Waltham, MA, USA). Labeled maltodextrin was acquired in a mass range of m/z 500–4000, and labeled human serum N-glycans were acquired in the mass range of m/z 1000–3500 with a resolution of 60,000. A laser energy of 30 μ J was used in all spectra collection. The LC traces to be imaged and the raster step size were controlled using the LTQ Tune software (Thermo Scientific, Waltham, MA, USA). To generate images, the spectra were collected at 150 μ m intervals in both the x and y dimensions. The images of analyte ions were extracted centered at the m/z with a window of 10 ppm using ImageQuest (Thermo Scientific, Waltham, MA, USA). ImageQuest enables construction of MS images by reconstituting the x and y coordinates of the spectra in the acquired image file with their original locations within the LC traces. Images were assigned with an intensity-based color scale for optimal visualization. The same intensity scales were used for the MS images of different analytes from the same image file. For each analyte, the averaged intensity of the monoisotopic peak in the extracted ion image was used for relative quantitation. The composition of human serum N-glycans was identified by matching the accurate mass (<5 ppm) with the established human serum N-glycan library [33, 34]. LC chromatograms were re-constructed using Excel and Origin based on the extracted ion intensities information exported using Xcalibur. Namely, the x coordinate of each MS spectrum was converted into the retention time, while the MS intensities at the same x coordinate but different y coordinate were summed up. In this way, the actual peak intensity of a specific ion can be plotted against the retention time.

Results and discussion

Isotopic hydrazide labeling reagent

Zhang group [31] has synthesized the hydrazino-*s*-triazine-based labeling reagents, and previous studies [35, 36] showed that the hydrazide labeling reagent HDEAT enabled a 40-fold detection enhancement in ESI mode. The labeling reagent contains three basic tertiary amine and is thus readily protonated; the labeled high-mannose N-glycans released from RNase B are almost exclusively observed as protonated species, as shown in the Electronic Supplementary Material (ESM) Fig. S1A using a MALDI source. While for the N-glycans released from human serum, which includes all kinds of high-mannose, complex, fucosylated and sialylated glycans, sodiated peaks dominated in the mass spectrum as shown in Fig. S1B (see ESM), probably due to the sodium ions brought by glass bottles used for human serum storage. In our study, the intensity of the most abundant peak was used for relative quantitation. Stable-isotope labeling appears to be one of the most popular and effective strategies in the relative quantification of N-glycans. An identifiable mass difference was generated by incorporation of different isotopic species onto chemically similar analytes of interest, allowing simultaneous MS analysis of multiple samples. By introducing 20 deuterium atoms into light tag HDEAT, a heavy tag d_{20} -HDEAT was synthesized. With a 20-Da mass shift between the light and heavy tag, the possible isotopic distribution can be avoided for a glycan with molecular weight up to 10,000 Da, ensuring quantitation precision, simplifying data processing as well as accurate glycan identification.

The structure information of the isotopic hydrazide labeling reagent and its reaction scheme with N-glycans is shown in Fig. 1b, c. The overall workflow including labeling with heavy and light tags, HILIC separation, matrix applying, MALDI imaging, and MS1-based quantitation is shown in Fig. 1a.

Method validation of isotopic labeling strategy for quantitation in MALDI mode

The hydrazide labeling efficiency was evaluated using maltotriose as standards with different labeling ratio. As we could see from Fig. S2 (see ESM), when the labeling reagent vs. maltotriose molar ratio reaches 20, the labeling efficiency reaches above 90%. The labeling reaction almost went to complete when the labeling ratio was increased to 50. The labeling efficiency was determined based on the sum of the monoisotopic peak of proton and sodium and potassium adducts of labeled and unlabeled standard maltotriose. Previously, the reliability and accuracy of quantitation of the isotopic labeling reagent HDEAT and d₂₀-HDEAT has been demonstrated in ESI mode [37]. In our study, maltotriose and high-mannose N-glycans released from RNase B were used to evaluate their feasibility and reliability in MALDI mode. Our results show that accurate quantitation can be achieved.

Even though the synthesized d₂₀-HDEAT achieved rather high purity above 90% benefiting from the commercially available high deuterated (99.6%) d₁₁-diethylamine, there is still 8.2% of d₁₉-HDEAT in the final product [37]. Thus, the intensity of d₂₀-HDEAT labeled glycan peak needs to be corrected to ensure accurate quantitation. As shown in the example of labeled maltodextrin₍₃₎ in Fig. S3 (see ESM), instead of being labeled exclusively by d₂₀-HDEAT, part of the samples was labeled by d₁₉-HDEAT. On the other hand, the isotopic peak of d₁₉-HDEAT labeled glycan overlaps with the monoisotopic peak of d₂₀-HDEAT labeled glycan at resolution of 60,000 used in our study. Taking these two aspects into consideration, we can get the equation for the corrected peak intensity of heavy labeled glycan, as shown in Eqs. (1) and (2). The correction factor varies from each N-glycan; thus, we calculated the factor based on the N-glycan composition with resolution set at 60,000 using the isotope distribution calculation tools on the website <http://www.chemcalc.org/>.

$$\begin{aligned} \text{Intensity of heavy labeled glycan monoisotopic peak} &= \text{Intensity of } d_{20} \\ &- \text{HDEAT labeled glycan monoisotopic peak} + \text{Correction factor} \\ &\times \text{Intensity of } d_{19} - \text{HDEAT labeled monoisotopic peak} \end{aligned} \quad (1)$$

$$\begin{aligned} \text{Correction factor} &= 1 - \frac{\text{Abundance of } 2^{\text{nd}} \text{ isotopic peak of } d_{19} - \text{HDEAT labeled monoisotopic peak}}{\text{Abundance of } 1^{\text{st}} \text{ isotopic peak of } d_{19} - \text{HDEAT labeled monoisotopic peak}} \end{aligned} \quad (2)$$

Light and heavy labeled maltotriose was used to evaluate the quantification accuracy of the isotopic quantification in different ratio. A total of nine samples were prepared in different ratios: 5:1, 4:1, 3:1, 2:1, 1:1, 1:2, 1:3, 1:4, and 1:5. The quantitation errors were well within 10% when the light/heavy (L/H) ratio varied from 1:1 to 1:5. To verify that there was no essential difference between the light and heavy tags, the accuracy of a reverse labeling,

namely light/heavy ratio from 5:1 to 1:1, was also evaluated. The results showed that comparable coefficients of variations within 10% were obtained. Figure 2a plots experimental $\log_2(L/H)$ vs. theoretical $\log_2(L/H)$ ratio, and a weighted least squares regression was calculated. One example of the mass spectrum of light and heavy labeled maltotriose at the ratio of 5:1 and 1:5 is shown in Fig. 2b. The linear range of quantification spans a 5-fold change in glycan abundance in both directions. Labeling accuracy and efficiency were also evaluated using the high-mannose N-glycans released from RNase B. The mass spectrum of heavy and light labeled (ratio 1:1) N-glycans released from RNase B is shown in Fig. 3a. A percentage difference within 15% compared with the theoretical ratio was achieved, as shown in Fig. 3b using three technical replicates. When labeling the two samples in parallel with light and heavy tags, the labeling efficiency difference plays a vital role in the accuracy of quantitation. Thus, we evaluated the labeling efficiency in two parallel labeling experiments. The results in Fig. 3c showed pretty consistent labeling efficiency for the same N-glycan with a maximum difference of 4% and an average difference of 3.4% (95% confidence interval, 3.2–3.7%).

Re-construction of HILIC chromatograms from MALDI-MSI results

Maltodextrin ladder, a mixture of oligomers, was used for the methodology evaluation. MALDI-Orbitrap mass spectrometer was used to image the collected LC effluent trace. On the basis of a HILIC pre-run, a time span from 14 to 35 min was set as the effluent and imaging data collection window, which covered an effective distance of 7.6 cm on the MALDI sample plate. The relative coordinates for each spectrum taken along the sample trace were recorded during acquisition and were converted to LC retention times. The MALDI plate mounted on a syringe pump moved at a speed of 3.3 mm/min. Thus, a 1.5-min HILIC peak, a typical peak width of LC separation achieved using our homemade column, can generate a length of 4.95 mm ($3.3 \text{ mm/min} \times 1.5 \text{ min}$) trace on the plate. As the raster increment was set at 0.15 mm for both x and y coordinates, up to 33 ($4.95 \text{ mm} \div 0.15 \text{ mm}$) data points could be collected for each chromatographic peak imprinted on the MALDI plate, which was sufficient for re-constructing LC chromatograms. Fig. S4 in the ESM shows a typical reconstructed peak in our study, which contains 35 data points across the analyte peak. A complete re-construction of eight extracted maltodextrin peaks and the base peak imaging results are shown in Fig. 4. From the figure, we can see the spatial distribution of the extracted ion images on the collected LC trace on the MALDI plate that correlates well with the reconstructed peak in terms of retention time. The quantitative results are shown in Table S1 (see ESM).

HILIC-MALDI-MSI quantitative analysis of isotopic labeled maltodextrin standards

To verify the feasibility of isotope relative quantitative analysis on the newly developed HILIC-MALDI-MSI platform, maltodextrin was selected as model analytes. Maltodextrin standards at a 1:1 ratio were labeled with HDEAT and d_{20} -HDEAT, respectively, and combined for HILIC-MALDI-MSI analysis. The chromatograms were re-constructed based on the imaging results shown in Fig. 4b, which shows that a baseline separation of maltodextrin oligomers ($n = 2-8$) was achieved on the homemade HILIC column (Fig. 4a). Retention time shift has often been an issue for deuterium encoded tag in reversed phase LC runs due to isotopic effect, which may negatively affect the quantitation accuracy. The

extracted ion chromatograms shown in Fig. S5 (see ESM) indicated that negligible retention time shift (<5 s) was observed on our homemade HILIC column. The almost identical image distribution and retention time of the re-constructed peak based on the extracted ion images of the light and heavy labeled N-glycans shown in Fig. 5a, b also support this conclusion. The previous study in our lab [38] using CE-MALDI-MSI for quantitation of peptides using isotopic formaldehyde labeling has shown the peak pair ratios of the extracted ion intensity from MALDI-MS imaging can achieve reliable and accurate quantitation results (with CV $<15\%$). Hence, the intensity of the monoisotopic peak from extracted ion image was used for quantitation, and the results shows that a good quantitation accuracy was achieved, which was detailed in Table S1 (see ESM). An example of the peak pair intensity ratios from the extracted ion images of isotopically labeled maltodextrin (degree of polymerization = 4) is shown in Fig. 5c.

Improved quantitative N-glycan profiling on HILIC-MALDI-MSI platform

To demonstrate the feasibility of the currently developed approach in complex sample, N-glycans enzymatically cleaved from the human serum were labeled by HDEAT and d_{20} -HDEAT respectively and mixed at a 1:1 ratio and subjected to HILIC-MALDI-MSI analysis. A total of 35 N-glycans were detected in protonated and sodiated form, with sodiated peak dominating the spectra as detailed in Table S2 in the ESM. Base peak images from MSI results in Fig. 6 show that the signal of the detected N-glycans spread out along the entire trace of the MALDI plate with a span of 9.0 cm, significantly reducing ion suppression, which would help to produce enhanced ion signals for targeted analytes. The extracted ion images of two high-mannose and two sialylated N-glycans are also shown in Fig. 6. The spatial separation of the N-glycans on the MALDI plate confirms our previous observation that the temporal resolution of the LC separation was well preserved. MALDI-MS analysis was used as a comparison to evaluate the capability of HILIC-MALDI-MSI platform for quantitative analysis of N-glycans in complex sample. MALDI-MS analysis was conducted using the same amount of sample. As shown in Fig. 7a, although three N-glycans (H5N4S2, H5N4F1S2, and H7N6) show a slightly smaller quantitation error on MALDI-MS platform, probably due to the “sweet spots” being sampled, an improved overall quantitation accuracy was achieved and five additional N-glycans were detected on the HILIC-MALDI-MSI platform. A further examination of the intensity of each analyte ion revealed that the peak intensity was ~ 10 -fold higher on the HILIC-MALDI-MSI platform, as shown in Fig. 7b, due to less ion suppression after separation.

These results indicated that the newly developed HILIC-MALDI-MSI platform provided improved performance for quantitative profiling of N-glycans. In fact, initially quantitative analysis was viewed as irreproducible and implausible in MALDI mode because crystallization does not yield a uniform distribution of the analyte and the measured ion intensity may vary for a given amount of analyte loaded onto the MALDI plate [39]. The intensity of an analyte extracted from MALDI mass spectrum is highly dependent on the spot that is irradiated by the laser. The presence of so-called “sweet spots” is the main cause, which often results in poor shot-to-shot reproducibility and poor quantitation accuracy. Various approaches including matrix-comatrix system [40, 41] or other improved sample preparation methods [42, 43] have been developed to overcome this issue. In our study,

instead of irradiating just a couple of spots of the crystallized sample, mass spectrometric imaging irradiates almost the entire samples via step-wise rastering in an area on the MALDI plate, which functions as “scanning” or “mapping” every spot in the whole area. In this way, the variations caused by “sweet spot” can be reduced. Furthermore, numerous studies [44–46] have discussed that suppression effects can also distort the quantitation accuracy when complex biological samples are analyzed due to different analytes’ charge competition, basicity, hydrophobicity, and relative abundances. To this end, benefiting from mass spectrometric imaging analysis, the HILIC chromatographic resolution used in our study could be well preserved, largely reducing the ion suppression effects, thus improving quantitation accuracy.

Isobaric tags, such as aminoxy TMT [47] and QUANTITY [48], have been extensively developed and applied for quantitative glycomics in ESI mode, with reporter ions readily generated in an automated data dependent acquisition (DDA) fashion. However, tandem MS experiments in MALDI source often require prior knowledge of the analytes to construct an inclusion list of precursor ions and far less automated compared to the tandem MS analysis in ESI source, which makes MS1-based quantitation more desirable in the MALDI source. In the present study, isotopic tags HDEAT and d20-HDEAT with 20 Da mass difference were utilized, which largely facilitated the MS1 ion intensity-based quantitation. With various effective approaches being developed such as MALDI-MS, LC-MALDI-MS, and LC-ESI-MS for quantitative glycomics study, our current developed approach enriches the toolbox by pushing MALDI-based quantitative glycomics one step forward via the incorporation of mass spectrometric imaging to thoroughly sample the analytes on a typical MALDI plate. Benefiting from the almost 10-fold intensity improvement, increased N-glycan quantitation accuracy and coverage using the HILIC-MALDI-MSI platform has been demonstrated compared to conventional MALDI-MS in the present study, but still there are many facets of this platform worth being further explored. For example, how will it perform during the characterization and quantitation of glycan isomers when a porous graphitic carbon (PGC) column is used? To what extent can the glycan separation, especially for glycan isomer, benefit from using high concentration of volatile salts or even non-volatile salts such as ion pairing agent? Moreover, a systematic performance comparison with LC-ESI will be quite interesting to see how they complement with each other, in terms of glycan separation efficiency, glycan coverage, glycan isomer characterization, and required efforts in sample preparation. These additional in-depth evaluations will help to further promote the HILIC-MALDI-MSI platform as a viable alternative that can be included in the rapidly expanding quantitative glycomics toolbox and to address the various challenges facing deep glycoproteomics and quantitative glycomics research.

Conclusion

Our study explored the utility of a novel HILIC-MALDI-MSI platform for quantitative glycomics analysis for the first time. MALDI-MSI analysis of the collected LC traces showed that the chromatographic resolution was preserved, making the most use of HILIC separation to minimize ion suppression. Accurate and reliable relative quantitation with stable-isotope labeled hydrazide reagents was demonstrated using a variety of samples ranging from maltooctose, maltodextrin standards, and N-glycans released from RNase B to

more complex human serum sample. Compared to direct MALDI-MS, HILIC-MALDI-MSI provided higher N-glycan coverage as well as better quantitation accuracy due to unbiased sampling of all the spots by mass spectrometric imaging and minimized ion suppression after HILIC separation. Overall, the HILIC-MALDI-MSI system utilizing hydrazide labeling reagents is robust with highly reproducible results, providing great potential in comparative glycomics studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This research was supported in part by the National Institutes of Health (NIH) grants R21AG055377, R01 DK071801, and R56 MH110215. The Orbitrap instruments were purchased through the support of an NIH shared instrument grant (NIH-NCRR S10RR029531) and Office of the Vice Chancellor for Research and Graduate Education at the University of Wisconsin-Madison. LL acknowledges a Vilas Distinguished Achievement Professorship with funding provided by the Wisconsin Alumni Research Foundation and University of Wisconsin-Madison School of Pharmacy.

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Fig. 1.

(A) Overall workflow for quantitative analysis of N-glycans using duplex HDEAT labeling reagents and LC-MALDI-MSI platform. Light and heavy labeled samples were mixed together and separated by HILIC; HILIC traces were collected on MALDI plate and subjected to MSI analysis; the intensity of the analyte peak was extracted from the imaging area and used for quantitation. **(B)** The chemical structures of light and heavy version of HDEAT. **(C)** Labeling reaction, hydrazide group of labeling reagent react with aldehyde group in glycans to form stable hydrazone product

Fig. 2.

(A) A plot of theoretical $\log_2(H/L)$ vs. experimental $\log_2(H/L)$ for the labeling ratios at 5:1, 4:1, 3:1, 2:1, 1:1 and reverse labeling at ratios of 1:2, 1:3, 1:4, 1:5 using maltotriose as standards. A weighted linear least squares regression is plotted for the data. (B) The mass spectra of heavy and light labeled maltotriose at the ratios of 5:1 and 1:5, respectively; the ion peak shown here is in protonated form. Note: The experiments were conducted using MALDI-MS

Fig. 3. (A) Mass spectrum of heavy and light labeled (ratio 1:1) N-glycans released from RNase B (*pink shaded peaks* are light labeled and *blue shaded peaks* are heavy isotope labeled). (B) Experimental ratios of heavy and light labeled RNase B N-glycans. The *error bars* stand for standard deviations in three technical replicates. (C) Labeling efficiency comparison of the heavy and light labeled RNase B N-glycans in the two parallel reaction vials. (*H*hexose, *N* *N*-acetylhexoseamine). Note: The experiments were conducted using MALDI-MS

Fig. 4. (A) Re-constructed extracted ion chromatograms of heavy and light labeled maltodextrin labeled at 1:1 ratio based on MSI results. (B) HILIC traces base peak images for heavy and light labeled maltodextrin labeled at 1:1 ratio. Note: The image area of heavy and light labeled maltodextrin overlapped with each other

Fig. 5.

(A) Extracted ion images of heavy and light labeled maltodextrin₍₄₎ at 1:1 ratio based on MSI results. (B) Reconstructed LC peak of heavy and light labeled maltodextrin (DP = 4) based on HILIC-MALDI-MSI results. (C) Mass spectrum of heavy and light labeled maltodextrin (DP = 4) in protonated form

Fig. 6.

HILIC traces base peak images for heavy and light labeled N-glycans released from human serum and extracted ion imaging results of four representative light labeled N-glycans. All the ions are in sodiated form. Note: Heavy and light labeled N-glycans overlapped with each other; the extracted images presented here are light labeled N-glycans

Fig. 7.

(A) Quantitation accuracy and N-glycan coverage comparison between HILIC-MALDI-MSI and MALDI-MS using 1:1 labeled N-glycans released from human serum. The *pink shaded part* represents the N-glycans only identified and quantified on the HILIC-MALDI-MSI platform. Quantitation accuracy was represented as the percentage difference between experimental ratio and theoretical ratio. The *error bar* stands for standard deviations of measured ratios from three experimental replicates. (B) Average intensity difference between these two platforms