Published in final edited form as:

Anal Chim Acta. 2019 February 07; 1048: 105–114. doi:10.1016/j.aca.2018.10.015.

Sensitive and robust MALDI-TOF-MS glycomics analysis enabled by Girard's reagent T on-target derivatization (GTOD) of reducing glycans

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Abstract

Sensitive glycomics analysis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) is of great importance but significantly hampered by their low ionization efficiency and labile sialic acid moieties. Chemical derivatization offers a viable way to improve both the ionization efficiency and analytical sensitivity of the glycans in MS analysis by altering their hydrophobicity or charge property. Here we employed Girard's reagent T (GT) for on-target derivatization (GTOD) of reducing glycan under mild acid condition to form stable hydrazones at room temperature, allowing rapid and sensitive identification of neutral and sialylated glycans in positive-ion mode as only permanently positive charged molecular ions without multiple ion adducts by MALDI-TOF-MS. The MS signal intensities of lactose, sialylated N-glycans derived from bovine fetuin and neutral N-glycans derived from RNaseB and ovalbumin were boosted by 7.44, 9.13, 12.96 and 13.47 folds on average ($n = 3$), respectively. More importantly, after GTOD strategy, unwanted desialylation of sialylated glycans during MS was suppressed. The detection limit of the assay is desirable since the nanogram of N-glycans derived from 0.16 μg ovalbumin could be detected. The assay demonstrated good stability (RSD = 2.95%, within 10 days), reliable reproducibility (RSD = 2.96%, $n = 7$) and a desirable linear dynamic range from 78 nmol/mL to 10 $\mu\text{mol/mL}$. The strategy has been successfully applied to MS analysis of reducing glycans from human milks, neutral and sialylated O-, N-glycans from glycoproteins, and reducing glycans derived from glycosphingolipids, presenting neater $[M]^+$ signals which allow detection of more low-abundance glycans and assignation of Neu5Ac vs. Neu5Gc or fucose vs. hexose in glycans due to the absence of the ambiguous interpretation from multiple peaks (ion adducts $[M+Na]^+$ and $[M+K]^+$). Moreover, the GTOD assay prevents desialylation during

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Notes

The authors declare no competing financial interest.

Appendix B. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.aca.2018.10.015>.

MALDI-TOF-MS profiling and enables distinct linkage-specific characterization of terminal sialic acids of N-glycans derived from human serum protein when combines with an esterification.

GRAPHICAL ABSTRACT

Keywords

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS); Reducing glycan; Derivatization; Girard's reagent T (GT); Sialic acids; Hydrazide

1. Introduction

Glycans including free reducing glycans or constituents of glycoproteins, glycolipids and proteoglycans, play fundamental roles in many biological processes including extracellular or intracellular interaction [1], communication [2], signaling [3], receptor interaction [4–6] and disease development [7–10]. Glycomics is the systematic study of glycan structures, including monosaccharide compositions, glycan sequence, branching pattern and linkage specifications, which are essential to the functions of glycoconjugates [11–14]. Nevertheless, glycomics analysis is inherently challenging due to their structure complexity and heterogeneity, low natural abundance and the lack of amplification methods [5,14,15].

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has become widely used for high throughput profiling of glycans with high heterogeneity and low abundance [14,16–21]. MALDI-TOF-MS generates dominantly singly charged ions and detects a large mass range. Sample preparation is simple and high throughput analysis can be carried out easily and quickly [14,22,23]. However, MALDI-MS analysis of underivatized glycans is limited by their low ionization efficiency.

For purpose of improving MS detection sensitivity, many chemical derivatizations of free glycans have been developed to improve the detection sensitivity during MS analysis [24–26], including widely used permethylation [27], reductive amination with various type of hydrophobic aromatic amines [26], Michael's addition with 1-phenyl-3-methyl-5-pyrazolone (PMP) [28] and acid catalyzed condensation with hydrazides or hydroxylamines to form hydrazones or oximes [29–32]. These derivatizations are usually applied to significant amounts of samples (μ g to mg), often under relatively strong reaction conditions involving heating with acid or base. Lengthy and tedious clean-up procedures are often required to remove the excess labeling reagents.

To overcome these limitations, derivatizations of reducing sugar with suitable tags directly on target plate has been exploited to simplify and increase the sensitivity of MALDI-MS analysis of glycans. 3-Aminoquinoline/*p*-coumaric acid [33] and phenylhydrazine [34–36] have been employed to on-target derivatization of the reducing glycans through acid catalyzed condensation reaction. However, the consistency of these derivatizations of various samples for MALDI-MS analysis has not been well-established, possibly due to the instability of Schiff-base formation. Furthermore, sialic acids are functionally important terminal components of many glycans but a significant portion of sialic acids falls off during MALDI-MS analysis [37]. Many derivatizations including methylation [38] and amidation [39] have been developed to stabilize sialic acids but none of these methods are compatible with on-target derivatization. Therefore, developing a robust on-target derivatization of reducing glycans including neutral and particularly sialylated glycans for highly-sensitive MALDI-TOF-MS analysis is greatly needed.

Positively charged reagents including Girard's reagent T/P (GT, GP) and other cationic tags [40,41] have been employed as MS detection enhancers to analyze glycan after acid catalyzed non-reductive condensation [42–44]. The yields are high and only $[M]^+$ ions were observed in MS analysis, avoiding multiple ion adducts such as the $[M+Na]^+$, $[M+NH_4]^+$ and $[M+K]^+$, which simplifies the MS data interpretation and increases the detection sensitivity [40]. Moreover, the positively charged tags can promote the ionization efficiency of the tagged glycans [42,44], and thus further boost the detection sensitivity of glycans in MS analysis. However, these derivatizations were carried out independently from MS analysis using μ g even mg amount of glycan samples, often with undesired loss of labile sialic acid due to heating [43]. Nevertheless, many reports suggest that hydrazides react with glycan reducing end quickly under room temperature or even at 4 °C [45–47]. We therefore explored Girard's reagent T (GT) for on-target derivatization (GTOD) under neutral or mild acid conditions at room temperature to facilitate MALDI-TOF-MS analysis neutral and sialylated glycans in positive-ion mode, as shown in Scheme 1.

In our GTOD strategy, glycans, GT and matrix (DHB) were sequentially spotted on the MALDI target and gently mixed. The spotted glycans were efficiently derivatized on-target with GT in the presence of DHB matrix under room temperature over normal drying time. After drying, MALDI-MS analysis of the derivatized glycans are directly carried out. Only $[M]^+$ type molecular ions were observed, greatly improving spectral quality and subsequent MS data analysis. This strategy has been applied to a variety of neutral and sialylated glycans including those released from glycoproteins and glycosphingolipids (GSLs) and proved to be an effective and reproducible procedure for highly sensitive glycomics analysis by MALDI-TOF-MS at micro-scale level. Additionally, when combined with an esterification [48], the assay can be used to characterize linkage-specificity of glycan terminal sialic acids by MALDI-TOF-MS.

2. Experimental section

2.1. Chemicals

Girard's reagent T (GT), phenylhydrazine, 2,5-dihydroxybenzoic acid (DHB), lactose (Lac), ribonuclease B (RNase B), ovalbumin (chicken egg white albumin), bovine fetuin, 3'-

sialyllactose (3'-SL, α 2,3-linked sialic acid), 6'-sialyllactose (6'-SL, α 2,6-linked sialic acid), 1-ethyl-3-(3-(dimethylamino)propyl)-carbodiimide (EDC) hydrochloride 1-hydroxybenzotriazole (HOBt) hydrate, DL-dithiothreitol (DTT), sodium dodecyl sulfate (SDS), β -cyclodextrin and peptide-N-glycosidase F (PNGase F; supplied glycerol free) were obtained from Sigma (St. Louis, MO, USA). HPLC-grade solvents (acetonitrile, ethanol) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Water was obtained through a Milli-Q water purification system (Millipore, Bedford, MA, USA). Nonporous graphitized carbon (150 mg/4 mL) solid phase extraction (SPE) columns were obtained from Alltech Associates (Deerfield, IL, USA). Sep-Pak C18 (3 cc) solid phase extraction (SPE) cartridges were obtained from Water Corporation (Milford, Massachusetts, USA). Human serum was obtained from a healthy volunteer in Xi'an Jiaotong Hospital. The research investigations were performed after obtained the informed consent and in accordance with an institutional review board protocol approved by the Human Research Ethic Committee at the Jiaotong Hospital. All other chemicals were purchased from Aladdin Inc. (Shanghai, China).

2.2. Preparation of free oligosaccharides from human milks

This procedure was conducted according to a previously reported paper [49]. Human milk was from a healthy volunteer within 20 days after giving birth. 0.5 mL of milk was centrifuged at 4 °C and 13000 rpm for 30 min. The clear solution in the middle layer containing oligosaccharides was collected and loaded onto a graphitized carbon SPE column pre-washed with 5 mL of acetonitrile (ACN) and equilibrated with 5 mL of water. After a wash of the graphitized carbon column with 10 mL of water to remove salts, glycans were eluted with 2 mL of 25% ACN containing 0.1% tri-fluoroacetic acid (TFA) [50]. The elutes were dried under a stream of nitrogen gas and dissolved in 50 μ L water for further derivatization or analysis by MS.

2.3. Preparation of reducing N-glycans from glycoprotein by PNGase F digestion

This procedure was based on previously reported papers [42]. 100 μ L human serum or human milk was dialyzed in an 8000-12,000 KDa dialysis membrane against fresh double distilled water for 3 days at 4 °C. The dialysate was dried to afford protein using a vacuum evaporator. 100-500 μ g of protein sample was dissolved in 200 μ L of protein denaturation solution and denatured. After the sample was cooled down, the denatured sample was subjected to the PNGase F (10 μ L, 30-60 U/mL) digestion at 37 °C for 24 h. Then sample was subjected to a glycan purification process using C18 SPE cartridge combined with graphitized carbon SPE column [50]. Briefly, the sample solution was loaded onto a C18 cartridge pre-washed with 5 mL of ACN and equilibrated with 5 mL of water. The target glycans were eluted with 4 mL of water, and the eluted residue was collected. Then the elutes were loaded onto a graphitized carbon SPE column and purified as described above. The purified glycans were dried under a stream of nitrogen gas and dissolved in 50 μ L of water for further use.

2.4. Preparation of reducing O-glycans from glycoproteins

This procedure was operated according to reference [51]. Briefly, 500 μ g porcine stomach mucin (PSM) was incubated in 500 μ L of 26–28% ammonium hydroxide at 45 °C for 24 h. After reaction was completed, the mixture was purified by C18 SPE cartridge combined

with graphitized carbon SPE column as described in section 2.2 and 2.3. The purified glycans were dried and dissolved in 50 μ L of water for further use.

2.5. Preparation of GSLs-glycan from glycosphingolipids (GSLs)

The GSLs-glycan were released from glycosphingolipids according to the procedure reported in Ref. [52]. Briefly, 100 μ g of glycosphingolipids were treated with ozone in 2 mL chloroform/methanol (1:1) about 3 min in a white tube until blue color occurs. To this solution, 1/3 volume of 100 mM ammonium bicarbonate buffer (pH = 8.0) was added and the mixture is heated at 65 $^{\circ}$ C for 16 h without a cap. The resulting mixture was lyophilized and purified by C18 and graphitized carbon SPE columns as described in section 2.2 and 2.3. The purified glycans were dried and dissolved in 50 μ L of water for further use.

2.6. GT on-target derivatization (GTOD) of reducing glycans

Glycans were dissolved in double distilled water. DHB matrix solution was prepared by dissolving 20 mg of DHB in 1 mL of 1:1 (v/v) acetonitrile/0.1% TFA mixture. GT was dissolved in a solvent of water/methanol/glacial acetic acid mixture (6:1:3, v/v/v) at 0.05 M as the glycan-labeling solution. Briefly, 0.5 μ L DHB matrix solution, 0.5 μ L glycan solution and 0.5 μ L GT solution were deposited sequentially and gently mixed on MALDI target. After the deposited sample was naturally dried at room temperature, the on-target labeling reaction is completed. Then the target is directly inserted for MALDI-TOF-MS analysis. Linkage specific derivatization of sialylated glycans was achieved by one-pot on-target derivatization with GT combined with a linkage specific derivatization via the ethyl esterification [48]. EDC and HOBt, respectively, were dissolved in ethanol at 0.25 M as the esterification buffer. Then 0.5 μ L DHB mixture, 0.5 μ L glycans liquor, 0.5 μ L esterification buffer and 0.5 μ L GT glycans-labeling solution were sequentially deposited and gently mixed on MALDI target. After the spotted mixture was dried at room temperature, the labeled glycans could be submitted to linkage-specific characterization of glycans terminal sialic acids by MALDI-TOF-MS.

2.7. MALDI-TOF-MS conditions

MS signal was obtained on an AXIMA instrument (Shimadzu, Tokyo, Japan) according to the conditions described previously [23,45]. The matrix was DHB (20 mg/mL, in 50% ACN containing 0.01% TFA). The sample in water was loaded onto target with GT labeling buffer and DHB matrix solution. The target was directly subjected into MALDI-TOF-MS profiling after drying in ambient air. The MS data acquisition was carried out in positive-ion polarities, using reflectron time-of-flight (TOF) mode. The instrument was equipped with a 337 nm nitrogen laser capable of operating at repetition rates of 5 Hz and the 90-110 mV power level. The acceleration potential was set to be 20 kV. All data was acquired in the low-mass range (m/z 200-4000). For each sample, 200 laser shots per point were irradiated and acquired. For the post-source decay (PSD) experiments, the PSD fragmentation conditions were adjusted in reflectron positive-ion mode with ion accelerating voltage of 20 kV to get the enhanced intensity and (signal/noise) S/N ratios of ionized peaks in spectra.

3. Results and discussions

3.1. GTOD improves detection sensitivity of reducing glycans in MALDI-TOF-MS analysis

We first tested GTOD on reducing glycans under room temperature using maltodextrin (glucose polymers), followed by direct MALDI-TOF-MS analysis. As shown in Fig. 1, after GTOD, reducing glycan such as G12 was labeled with GT through oximation to form corresponding hydrazone product G12-GT, leading to mass increments of m/z 114 addition per sugar observed in MS. All maltodextrin oligosaccharides with different DPs (degree of polymerizations) were efficiently derivatized and only $[M]^+$ type molecular ion peaks with mass increments of m/z 114 addition were observed in MS profiles (Fig. 1B) comparatively to the underivatized group (Fig. 1A, mainly $[M+Na]^+$ peaks), correspondingly. Remarkable increase in S/N (signal/noise) ratios of $[M]^+$ peaks was observed compared to $[M+Na]^+$ and $[M+K]^+$ peaks of underivatized sample (Fig. 1A). Significantly higher DP oligosaccharides can be detected after GTOD than those without derivatization.

3.2. Effect of molar ratios of GT to glycans on reaction efficiency of GTOD by MALDI-TOF-MS

To determine the amount of labeling reagent required for efficient derivatization, we applied GTOD on lactose (Lac) and 3'-sialyllactose (3'-SL) using different molar ratios of GT/glycan (1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1 and 10:1) and measured the increase of MS peak intensity after derivation (Fig. S1). β -Cyclodextrin, which is a non-reducing sugar and can not be derivatized, was used as an internal standard for relative quantification. It is shown that a molar ratio of GT to glycans larger than 7:1 is sufficient to achieve significant amplification of MS peak intensities. While it is impossible to determine concentrations of each glycan sample, the universal GTOD protocol described in experimental section provides sufficient amount of GT for derivatizations.

3.3. GTOD suppresses desialylation of sialylated glycans in MALDI-TOF-MS analysis

Fig. 2 shows MALDI-MS profiles of Lac (Fig. 2A) and sialylated N-glycans released from bovine fetuin (Fig. 2B) before and after GTOD at the same concentration. MS profiles annotated with $[M]^+$ ion peaks after GTOD show obvious increase in the S/N ratios and detection sensitivity, especially when compared to the β -cyclodextrin internal standard. After GTOD labeling, S/N ratios of lactose-GT conjugates ionized at m/z 456 ($S/N = 69$, Fig. 2A, lower layer) as well as one random H5N4S2-GT conjugates ionized at m/z 2336 ($S/N = 49$, Fig. 2B, lower layer) were enhanced about 11 and 10 folds over those of native glycans (Fig. 2A, upper layer, lactose, m/z 365, $S/N = 6$; Fig. 2B, upper layer, H5N4S2, m/z 2245, $S/N = 5$), respectively (H, hexose; N, N-acetylhexosamine; S, Neu5Ac). The formation of $[M]^+$ ions and the suppression of other metal ion adduct helps preventing possible misinterpretation from multiple metal molecular ion peaks and improving the detection sensitivity and S/N ratios, consistent with our previous work [42]. Moreover, before GTOD, peak at m/z 1662.93 was assigned to desialylated product of the sialylated glycans (m/z 1954.31) during ionization. However, after GTOD, no corresponding desialylation peak was observed, suggesting that GTOD stabilize sialylated glycans and prevent desialylation during MALDI-MS analysis (Fig. 2B). Although detailed mechanism is not fully understood, the stabilization of sialylated glycans after GTOD is likely due to the

neutralization of negative charge, the enhancement of ionization efficiency and the increase of ion abundance by cationic GT tag [53–58].

3.4. Evaluation of GTOD assay for reducing glycans MALDI-TOF-MS analysis

Compared with the underivatized reducing glycans, the MS signal intensities after GTOD of four groups of glycan samples (lactose; sialylated N-glycans derived from bovine fetuin; neutral N-glycans derived from RNaseB and ovalbumin, respectively) in Fig. 2 and S2-3 were boosted by 7.44, 9.13, 12.96 and 13.47 folds on average ($n = 3$, Fig. S4), based on quantification using β -cyclodextrin peaks as an internal standard. Importantly, desialylation of sialylated glycans during MALDI-TOF-MS was suppressed and greater MS signals were observed (Fig. 2B and S2-3), presumably due to the permanent positive charge promoting more efficient ionization and ion abundance [53–58].

To assess the minimal detection limitation using GTOD strategy, equal amounts of N-glycans released from chicken ovalbumin was treated without or with GTOD and analyzed by MALDI-TOF-MS in parallel. As shown in Fig. S5, the S/N ratios of native glycans as well as glycan-GT [M]⁺ peaks decreased with decreasing amount of original glycoprotein used for glycan release. Equal amount of N-glycans after GTOD derivatization demonstrated higher and neater MS signals over that of the underivatized ones, correspondingly. Major N-glycans derived from as little as 0.16 μ g ovalbumin can be successfully detected after GTOD, whereas major peaks in same amount of N-glycan before GTOD could hardly be monitored. Additionally, a total of 17 N-glycans were detected before GTOD (Fig. S5A, upper layer). In contrast, 20 N-glycans identified with evidently improved signal intensity were achieved after GTOD (Fig. S5B, upper layer) when equal amount of sample used under same experimental conditions. This result suggests that GTOD greatly enhance glycan detection sensitivity, which is of great importance for highly sensitive identification of glycans derived from microscale biological specimen.

When different concentrations of lactose (0.078, 0.156, 0.312, 0.625, 1.25, 2.5, 5.0, 8.0 and 10.0 μ M/mL) were treated with GTOD and analyzed by MALDI-TOF-MS, a linear concentration dependence of MS ion relative abundance normalized based on β -cyclodextrin internal standard was observed (Fig. S6, $y = 1.4687x + 0.2215$, $R^2 = 0.9992$), suggesting the potential of GTOD for quantitative or semi-quantitative analysis of glycans which don't require high quantification accuracy. When the analysis is repeated multiple times, the relative abundance is highly reproducible (Table S1, RSD = 2.96%, $n = 7$), suggesting a robust derivatization by GTOD. Furthermore, when GTOD treated lactose was stored at room temperature for a period of time (1, 2, 3, 4, 5, 7, 10 and 14 d) and then submitted to MALDI-MS analysis, no significant deterioration of MS peaks was observed, suggesting the glycan-GT derivatives are rather stable (RSD 2.95% within 10 days, $n = 3$) after GTOD (Table S2).

We also compared the GTOD approach with a previous method described by Erika et al. [34], which employed a hydrazide reagent phenylhydrazine (PHN) for on-target derivatization of reducing glycans to improve MS analysis of reducing sugars and simplify the MALDI-MS procedure (Fig. 3). While both GT and PHN on-target derivatization improved MS spectra quality over un-derivatized glycans, in our hands, the GTOD approach

provides stronger signals and simpler MS profile than PHN on-target derivatization, presumably due to the permanent positive charge installed.

3.5. Application of GTOD to a variety of reducing glycans derived from biological specimens

We then applied the GTOD approach to a variety of glycans from biological specimens, including free oligosaccharides, N-, O-glycans released from glycoproteins and glycans derived from GSLs. Released and purified reducing glycans were processed with or without GTOD and analyzed by MALDI-TOF-MS (Fig. 4 and S7). All MS profiles generated with GTOD show greatly enhanced mass signals and neater pattern than MS profiles without GTOD, resulting more glycan compositions detected. In GTOD-MALDI-MS profiles of human milk N-glycans (Fig. 4A) and free oligosaccharides (Fig. 4B), porcine brain (Fig. 4C) and liver (Fig. 4D) GSLs-glycans, 37, 19, 13 and 10 different compositions were identified, whereas only 29, 11, 7 and 9 compositions were identified in the MS profiles without GTOD treatment. The compositions assigned based on MS data are listed in Tables S4–8, which are consistent with previous reports [14,52,59,60]. The compositions identified only with GTOD (labeled with blue characters) were all assigned to low-abundance glycans. This brings significant advantage in the analysis of minor components of a glycome, which are often overlooked due to less sensitive MS detection.

Interestingly and importantly, many of these compositions identified only with GTOD are sialylated glycans, suggesting that sialylation is significantly stabilized by the positive charge introduced by GTOD. It is well known that sialylated glycans are prone to desialylation during MALDI-TOF-MS analysis and often need to be chemically stabilized before MS analysis [23,44,48,61]. The stabilization of sialic acid moieties by GTOD provides a one-pot procedure for MALDI-TOF-MS analysis of sialylated glycans. Furthermore, the 16 Da mass difference between Neu5Ac and Neu5Gc cannot be discriminated from the 16 Da mass difference between $[M+Na]^+$ and $[M+K]^+$ adducts under normal resolution, which often leads to misinterpretation of Neu5Gc. Since GTOD-MALDI-MS only exhibits the $[M]^+$ ions without the interference by $[M+Na]^+$ and $[M+K]^+$ ions, it provides a simple and unique solution for the discrimination of Neu5Ac (S) and Neu5Gc (G), as shown in Fig. 4D. Peaks signals (Fig. 4D) at m/z 1112.12 and 1128.23 of in GTOD-MALDI-MS profile were unambiguously assigned to H3N1S1 and H3N1G1 (H, hexose; N, N-acetylhexosamine; F, fucose). Similarly, peaks signals at m/z 1403.23, 1419.32 and 1435.12 (Fig. 4D) were assigned to H3N1S2, H3N1S1G1 and H3N1G2. These compositions were confirmed by MS/MS analysis in Figs. S8–9.

Besides discriminating Neu5Ac and Neu5Gc, the exhibition of only $[M]^+$ ions by GTOD also helps discrimination of other compositions that can potentially be confused by $[M+Na]^+$ and $[M+K]^+$ in MS profiles of underivatized glycans, such as fucose vs. hexose. For instance, as shown in Fig. 4A, three groups of peaks of m/z 1576.74 and 1593.04, 1738.29 and 1754.88, 1779.87 and 1795.19 with m/z difference of 16 were assigned to six compositions as N4H3F1 and N4H4, N4H4F1 and N4H5, N5H3F1 and N5H4 definitively, as presented in Table S4, whereas in the profile of underivatized glycans, three compositions (N4H3F1, N4H4F1 and N5H3F1) as $[M+Na]^+$ and $[M+K]^+$ adducts could be wrongly

assigned. The same phenomena was also observed in Fig. 4D and Table S7, where m/z 1258.23 and 1274.47 with difference ($\Delta m = 16$) were undoubtedly assigned to be two compositions H3N1F1S1 and H4N1S1. Whereas, in the MS profile of underivatized glycans, corresponding m/z 1167.08 and 1183.09 ($\Delta m = 16$) could be mis-interpreted as a single composition H3N1F1S1 as $[M+Na]^+$ and $[M+K]^+$ adducts.

3.6. Linkage-specific analysis of glycan terminal sialic acids using GTODE

Due to the pathological importance of the glycan terminal sialic acids, it is of great necessity to develop high-throughput method for their linkage-specific analysis. While sialylated glycans are stabilized by GTOD and can be successfully profiled by MALDI-MS, it is very challenging to assign specific linkages by MS/MS due to quick desialylation, as illustrated in Fig. S10. Fortunately, a linkage-specific assignation of terminal sialic acid could be achieved by combining our GTOD strategy with a mild esterification [48], termed as GTODE which converts α 2,6-linked sialic acid to ethyl ester and α 2,3-linked sialic acid to lactone under mild room temperature reaction conditions.

As shown in Fig. 5A and B, after GTOD, both 3'-SL and 6'-SL presented only $[M]^+$ peak at m/z 747 without desialylations (Fig. 5A and B, middle panels) whereas desialylations are abundant without GTOD (Fig. 5A and B, upper panels). While this is significant in preventing desialylation, 3'-SL and 6'-SL cannot be discriminated. However, when GTOD was combined with esterification (GTODE) (Fig. 5A and B, lower panels), distinct $[M]^+$ ions at m/z 729 and 775 were produced from 3'-SL and 6'-SL respectively, suggesting lactone formation for 3'-SL-GT with m/z reduction ($- \Delta m = 18$) and ethyl ester formation for 6'-SL-GT with m/z addition ($+ \Delta m = 28$). These features strongly suggest the potential of using GTODE for distinguishing glycan terminal α 2,3-linked sialic acid from α 2,6-linked sialic acid. We therefore applied GTODE to sialic acid linkage specification analysis of N-glycans released from human serum glycoprotein (HSG).

As presented in Fig. 5C, when treated with GTOD (middle panel) and GTODE (lower panel), cleaner and stronger MALDI-MS profiles and signals of HSG N-glycans were obtained in contrast to that without treatment (upper panel) when detected at the same concentration and MS conditions. Compared with the GTOD-MALDI-MS profile of HSG N-glycans (Fig. 5C, middle panel), m/z values of neutral N-glycans in the GTODE-MALDI-MS profile (Fig. 5C, lower panel) did not change, such as peaks at m/z 1348, 1511, 1576, 1738 and 1901. However, an α 2,3-linked sialic acid terminal of N-glycans would yield an 18 Da mass loss and an α 2,6-linked sialic acid terminals would yield a 28 Da mass increase. Therefore, the mass changes from sialylated peaks in GTOD-MALDI-MS profile to sialylated peaks in GTODE-MALDI-MS profile can be calculated as $n_2 \times 28 - n_1 \times 18$, where n_1 is the number of α 2,3-linked sialic acid and n_2 is the number of α 2,6-linked sialic acid. For example, peaks at m/z 2045 and 2191 in GTOD-MALDI-MS profile were shifted to m/z 2072 and 2219 in GTODE-MALDI-MS profile, suggesting that these two peaks are α 2,6-sialylated glycans. Similarly, all other peaks, including di and tri-sialylated glycans, in the GTODE-MALDI-MS profile can be compared to the GTOD-MALDI-MS profile to provide important information about the terminal sialic acid linkages. Through GTOD strategy, sialic acids residues at HSG N-glycans were stabilized but the linkages specificity could not be

determined. After GTODE, not only the sialic acids residues at HSG N-glycans were further stabilized but also the linkage specificity could be well characterized. From HSG N-glycans, we were able to assign specific linkages to 14 glycans with terminal sialic acids moieties within the 1000-3500 Da mass detection range, consistent with data reported in previous works [48,62–64]. Therefore, GTODE allows a one-step profiling of a mixture of glycans and the linkage specific characterization α 2,3- and α 2,6-linked terminal sialic acids with high fidelity, which will be suitable for high-throughput and robust glycomics analysis of reducing glycans derived from diverse biological specimens.

4. Conclusions

We have developed a novel approach termed GTOD for on-target derivatization and highly sensitive analysis of neutral and sialylated reducing glycans by MALDI-TOF-MS in positive-ion mode. Through the introduction of Girard's reagent T with a permanent positive charge, the MALDI-MS spectra resolution and peak intensities are greatly improved. Unwanted desialylation during MALDI-MS analysis was significantly suppressed. Importantly, the GTOD-MALDI-MS profiles presents only $[M]^+$ peak without interference from other ion adducts such as $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$. This results in much cleaner mass spectra and greatly simplified m/z assignment and data interpretation. Specifically, peaks with 16 Da difference, which are often assigned to $[M+Na]^+$ and $[M+K]^+$ adducts, can now be unambiguously assigned, such as glycans with Neu5Ac or Neu5Gc sialylation and fucosylation.

Moreover, the GTOD assay prevents desialylation during MALDI-TOF-MS profiling, allowing for distinct linkage-specific characterization of terminal sialic acids of glycans. When the GTOD approach is combined with esterification, termed GTODE, linkage specific characterization of terminal sialic acids with modifications of α 2,3- and/or α 2,6-linkages was achieved based on the clean $[M]^+$ profile and distinct sialic acid esterification patterns of different linkages. The sialic acids linkage specificities of N-glycans released from glycoprotein in human serum have been successfully determined.

In summary, the GTOD and GTODE methods are simple, fast, robust and highly reproducible. With all the merits described above, we believe these methods will make significant contribution to glycomics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was funded by National Natural Science Foundation (Nos. 31370804, 21375103, 31670808 and 31300678), the Scientific Research Program Fund for Shannxi Province key laboratory (14JS101) and Shaanxi basic research program of natural science (2016JQ3018). This work was also partially supported by NIH Common Fund Glycoscience (U01GM116254).

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HIGHLIGHTS

- GTOD greatly increase MALDI-MS peak intensities of free reducing glycans.
- Only $[M]^+$ peaks were observed without interference from diverse ion adducts.
- GTOD greatly suppresses unwanted desialylation of glycans during MALDI-MS analysis.
- Neu5Ac vs. Neu5Gc or fucose vs. hexose in glycans can be assigned unambiguously.
- Terminal sialic acids with $\alpha 2,3$ - and $\alpha 2,6$ -linkages could be assigned through GTODE.

Fig. 1. MALDI-TOF-MS spectra of maltodextrins without (A) or with (B) GTOD treatment. Both spectra were acquired under the same concentration and MS conditions. (DP: degree of polymerization; G1: saccharide with DP of 1; G1-GT: G1 labeled with GT).

Fig. 2.

Positive-ion mode spectra of lactose (A) and sialylated N-glycans released from bovine fetuin (B) before and after GTOD by MALDI-TOF-MS. Lac, lactose; Structure formulas: green circle, mannose; blue square, N-acetylglucosamine; yellow circle, galactose; purple diamond, N-acetylneuraminic acid (Neu5Ac). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig. 3. Positive-ion spectra of N-glycans released from ribonuclease B (RNase B) before (A) or after on-target derivatization with phenylhydrazine (PHN) (B) or after GTOD (C) by MALDI-TOF-MS at the same concentration and MS conditions.

Fig. 4.

Positive-ion MS profiles of a variety of reducing glycans derived from biological specimens with or without GTOD at the same concentration. (A) MS profiles of N-glycans released from glycoproteins in human milk before and after GTOD; (B) MS profiles of free oligosaccharides from human milk before and after GTOD; (C) MS profiles of GSLs-glycan released from porcine brain glycosphingolipids before and after GTOD; (D) MS profiles of GSLs-glycan released from porcine liver glycosphingolipids before and after GTOD. Abbreviations used are hexose (H), N-acetylhexosamine (N), fucose (F), Neu5Ac (S) and Neu5Gc (G) with unspecified linkage. * indicates the presence of $[M+K]^+$ ion peak. Blue characters numbered peaks were low abundance glycans observed after GTOD only. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig. 5.

Positive-ion MS profiles of glycans before and after GTOD or GTODE. (A) MS profiles of 3'-sialyllactose (3'-SL) before, after GTOD or GTODE; (B) MS profiles of 6'-sialyllactose (6'-SL) before, after GTOD or GTODE; (C) MS profiles of N-glycan derived from human serum glycoproteins before, after GTOD or GTODE. 3'-sialyllactose (3'-SL), 6'-sialyllactose (6'-SL). HSG N-glycans, human serum glycoprotein N-glycans; Structure formulas: yellow square, N-acetylgalactosamine; blue circle, glucose; yellow circle, galactose; red triangle, fucose; purple diamond, sialic acid (Neu5Ac). * means the $[M+K]^+$ or $[M+2Na]^+$ ionized peaks. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Scheme 1.

The strategy for GT on-target derivatization (GTOD) of reducing glycans and subsequent positive-mode MALDI-TOF-MS analysis. (A) The chemical reaction of free reducing glycans with GT form stable hydrazones under room temperature. (B) The schematic presentation of GTOD.