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Research Article

Fast and Sensitive Detection of Oligosaccharides Using Desalting Paper Spray Mass Spectrometry (DPS-MS)

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ABSTRACT: Conventional mass spectrometry (MS)-based analytical methods for small carbohydrate fragments (oligosaccharides, degree of polymerization 2−12) are time-consuming due to the need for an offline sample pretreatment such as desalting. Herein, we report a new paper spray ionization method, named desalting paper spray (DPS), which employs a piece of triangular filter paper for both sample desalting and ionization. Unlike regular paper spray ionization (PSI) and nanoelectrospray ionization (nanoESI), DPS-MS allows fast and sensitive detection of oligosaccharides in biological samples having complex matrices (e.g., Tris, PBS, HEPES buffers, or urine). When an oligosaccharide sample is loaded onto the filter paper substrate (10 × 5 mm, height × base) made mostly of cellulose, oligosaccharides are adsorbed on the paper via hydrophilic interactions with cellulose. Salts and buffers can be washed away using an ACN/H_2O (90/10 *v*/*v*) solution, while oligosaccharides can be eluted from the paper using a solution of ACN/H_2O/formic acid (FA) (10/90/1 *v*/*v*/*v*) and directly spray-ionized from the tip of the paper. Various saccharides at trace levels (e.g., 50 fmol) in nonvolatile buffer can be quickly analyzed by DPS-MS (<5 min per sample). DPS-MS is also applicable for direct detection of oligosaccharides from glycosyltransferase (GT) reactions, a challenging task that typically requires a radioactive assay. Quantitative analysis of acceptor and product oligosaccharides shows increased product with increased GT enzymes used for the reaction, a result in line with the radioactivity assay. This work suggests that DPS-MS has potential for rapid oligosaccharide analysis from biological samples.

INTRODUCTION

Carbohydrates play an indispensable role in the physiology of humans and plants. For example, carbohydrates represent the storage energy in cells (starch and glycogen); they provide structural strength to plants and play a role in signaling.[1] The enzymes responsible for the synthesis of all carbohydrates on earth are called glycosyltransferases (GTs). Biochemical characterization of GTs is a challenge due to several factors: (1) GT proteins are present in small amounts in cells, (2) GTs are difficult to purify, and (3) their transfer reaction products are difficult to detect and characterize, as these products are produced in very small quantities. In general, detection of transferase activity typically requires the use of sensitive assays that utilize radioactive nucleotide diphosphate (NDP) sugars, the activated state of monosaccharides, which serve as the sugar donors during the transferase reaction catalyzed by GTs within the cell.[2] Thus, developing a strategy to produce GTs *in vitro* in combination with a rapid and sensitive detection method (as an alternative to radioactive precursors) to monitor transferase reaction products (i.e., oligosaccharides) would tremendously benefit the field of glycomics and open the possibility to design high-throughput analytical methods for GT activities.

Paper spray ionization (PSI) introduced by Ouyang, Cooks, and co-workers[3] allows rapid and direct analysis with minimal or no purification steps. PSI[4] has become one of the most widely used ambient ionization mass spectrometry methods for a variety of compounds, including drugs,[3,5] peptides, proteins,[6,7] reaction intermediates,[8] food,[9] metabolites,[10] and environmental pollutants.[11] PSI uses a triangular paper, onto which a sample is loaded and subsequently dried. Analyte ions are generated by applying a high voltage (3−5 kV) to the paper substrate moistened with a small volume of spray solvent (e.g., 10 μL). In comparison with traditional electrospray ionization, no sample pretreatment is needed during PSI, which makes the method a superior tool for analyzing real-world biological samples such as blood and urine. PSI-MS has been previously used for carbohydrate investigation, including fructose and sucrose detection in beverages[12] and protein glycosylation.[13] However, oligosaccharide analysis from complex matrices using PSI-MS is still lacking, probably because the conventional PSI solvent (MeOH or ACN/H_2O 50/50 *v*/*v*) cannot elute target oligosaccharides from the paper due to their strong hydrophilic interaction with the fibrous paper.[5,7]

Traditionally, electrospray ionization mass spectrometry (ESI-MS) and matrix-assisted laser desorption-ionization

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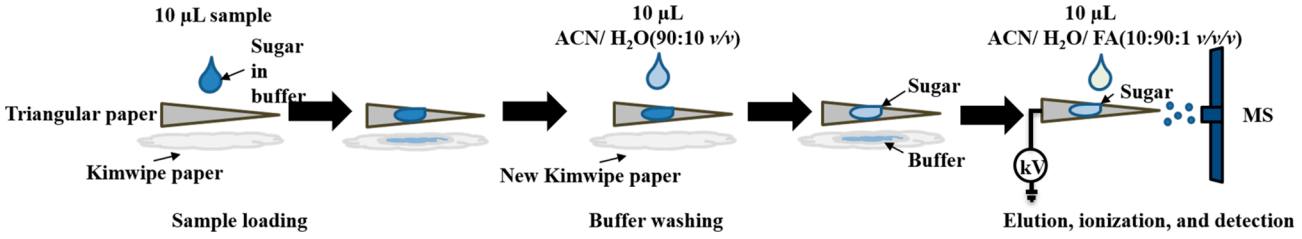
Scheme 1. Schematic Presentation of the Workflow and Setup of DPS-MS method



mass spectrometry (MALDI-MS) are often used to characterize N-linked glycans[14−17] and plant cell wall polymers.[18] Accurate molecular mass, along with tandem mass analysis, provides unprecedented capability for glycan structure characterization and sugar sequence assignment.[19−24] However, because of signal suppression by more readily ionized compounds (e.g., salts and other solutes), it is still challenging to detect and analyze oligosaccharides in complex biological mixtures.[25] Thus, efficient ionization and detection of these carbohydrates require enrichment and desalting steps. Traditional desalting methods include reversed-phase liquid chromatography (RPLC),[26] size-exclusion chromatography (SEC),[27] anion-exchange chromatography (AEC),[28−30] lectin affinity chromatography,[31,32] hydrophilic interaction liquid chromatography (HILIC),[33−36] and graphite affinity chromatography.[33,37−41] All these desalting methods are time-consuming and have limitations such as low enrichment efficiency and nonspecific binding of oligosaccharides due to their high polarities. For example, although low limits of detection (LOD) of 100 pmol and 50 fmol were reported for ESI-MS and MALDI-MS methods, respectively,[42,43] those methods took time ranging from 50 min to 2 h. Additionally, derivatization is needed prior to MALDI-MS analysis due to the low ionization efficiency of oligosaccharides, which is also time-consuming. Thus, developing a fast and sensitive MS detection method for analyzing oligosaccharides in complex matrices is still needed.

A cellulose hydrophilic interaction solid-phase extraction (SPE) microtip has been applied for *N*-glycan purification and enrichment.[31] In this method, glycans are first adsorbed on the cellulose-based SPE cartridge by hydrogen bonding. Salts are washed away with an organic solvent containing only 10% water. By adjusting the water content of the solvent from 10 to 90% (*v*/*v*), adsorbed glycans can be eluted from the cellulose substrate. Herein, we present a new approach, termed as desalting paper spray mass spectrometry (DPS-MS), that takes use of the same triangular paper substrate for both carbohydrate desalting and spray ionization, for fast and sensitive analysis of various oligosaccharide types in complex media including GT reactions. Samples are directly deposited on paper, and oligosaccharides are retained through hydrogen bonding. Unbound salts and other chemicals in the reaction mixture are washed away with ACN/H_2O (90/10 *v*/*v*) solution. The adsorbed, relatively pure, oligosaccharides are eluted from the paper using high water content (90%) and low ACN content (10%). The released oligosaccharides are ionized through the application of a high voltage for spray ionization. This new analytical method is not only fast (ca. <5 min per sample) and straightforward but also highly sensitive (e.g., LOD of 1.1 nM for β-cyclodextrin). This method was successfully used to detect various oligosaccharides from nonvolatile buffers (e.g., Tris, phosphate, and HEPES buffers). To test the validity of this method for biological samples, we produced two well-characterized *Arabidopsis* GTs (AtXXT1 and AtGUX1) through an *in vitro* coupled Transcription/Translation System (TNT) and used the produced GTs in glycosyl transfer reactions. Our DPS-MS method could directly detect products generated by these GTs despite the high salt concentration and proteins (from the *in vitro* transcription/translation system). Furthermore, quantitative analysis by DPS-MS is feasible. For instance, the amount of acceptor sugars in GT reaction control samples was measured with a small deviation (6.5∼12.6%) from theoretical values. The combination of *in vitro* transcription/translation synthesis of GTs from plasmid DNA and a DPS-MS detection method provides great potential for on-site, high-throughput characterization of GT activities and for carbohydrate diagnostics in general.

EXPERIMENTAL SECTION

Materials. Maltoheptaose, β-cyclodextrin, glucose tetrasaccharide, 3α,6α-mannopentaose, uridine diphosphate (UDP)−glucuronic acid, synthetic urine, manganese(II) chloride, magnesium chloride, detergents, and DOWEX 1X8-100 resin (Cl) were obtained from Sigma-Aldrich (St. Louis, MO, United States). HPLC-grade acetonitrile, Tris, HEPES, and potassium phosphate buffers (pH 7.4), formic acid, scintillation liquid, Immobilon membranes, and SuperSignal West Femto Maximum Sensitivity Substrate were from Fisher Scientific (Waltham, MA, United States). Deionized water was from EMD Millipore (Burlington, MA, United States). Glutathione S-transferase (GST) was from GenScript (Piscataway, NJ, United States). Whatman-grade 42 and 2.5 μm chromatography paper (Whatman International Ltd. Maidstone, England) used in our DPS-MS method and anti-GST antibody were purchased from GE Healthcare Life Sciences (Pittsburgh, PA, United States). UDP−[^{14}C]xylose (7.141 GBq/mmol) and UDP−[^{14}C]glucuronic acid (9.213 GBq/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA, United States). UDP−xylose was purchased from CarboSource (University of Georgia, Athens, United States). Secondary goat antirabbit antibodies conjugated to peroxidase were purchased from Santa Cruz Biotechnology (Dallas, TX, United States). The protein expression kit (TNT Quick Coupled Transcription/Translation System) was from Promega (Madison, WI, United States). Cellohexaose and xylohexaose were purchased from Megazyme (Bray, Ireland). Tween 20 and X-ray film were from Research Products International Corp.

Apparatus. A high-resolution Q Exactive Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA, USA) was used throughout this study. The commercial ESI ion source was removed to accommodate DPS, PS, and nanoESI. Data analysis was acquired by Thermo Xcalibur (3.0.63).

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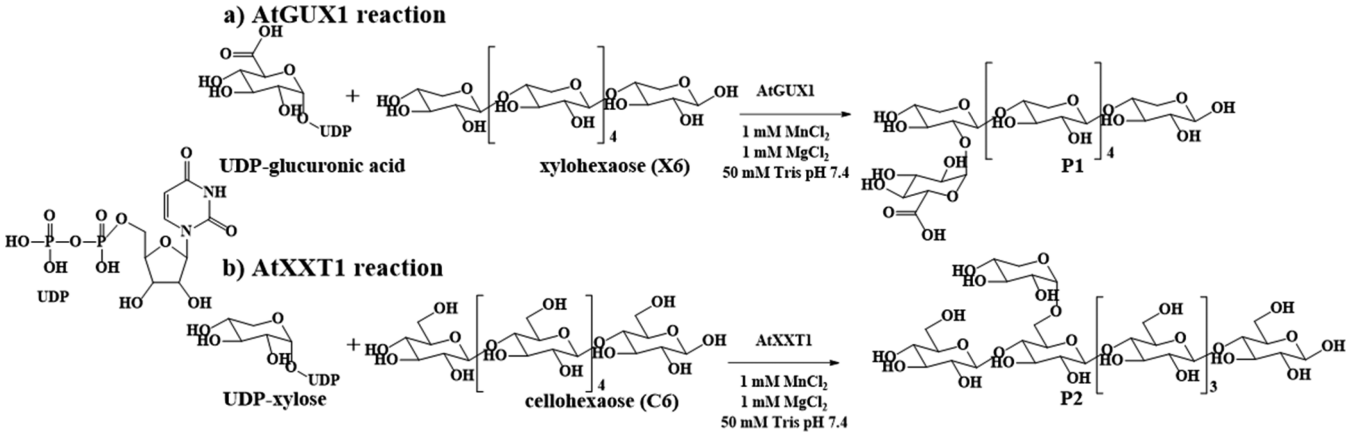
Scheme 2. Equations for Glycosyltransferase Reactions Catalyzed by (a) AtGUX1 and (b) AtXXT1



DPS-MS Method. The filter paper was cut into a triangle (10 × 5 mm, height × base) after sequential sonication-assisted cleaning in acetone, methanol, and methanol/water (50/50 *v*/*v*, 15 min each). As shown in Scheme 1, a 10 μL sample solution was dropped onto the paper triangle that was placed on top of a Kimwipe to facilitate the absorption by capillarity. Desalting was achieved by loading 10 μL of ACN/H_2O solution (90/10 *v*/*v*) onto the paper placed on top of another Kimwipe to wick the eluent containing salts and other chemicals. The paper triangle was then held in front of the MS inlet (8 mm away) using a high-voltage cable alligator clip, and 10 μL of ACN/H_2O/FA solution (10/90/1 *v*/*v*/*v*) was added directly onto the paper triangle to elute the target compounds for ionization upon application of a high voltage (3.5 kV) to the wetted paper. Paper spray optimization was guided by the previous works of Ouyang, Cooks, and co-workers.[44] Experimental conditions were optimized including: the paper tip to MS inlet distance (8 mm); sample loading volume (10 μL); spray voltage (3.5 kV); washing solvent (10 μL of H_2O/ACN, 10/90 *v*/*v*); and spray solvent (10 μL of H_2O/ACN/FA, 90/10/1 *v*/*v*/*v*) (see Figure S1, Supporting Information).

nanoESI-MS Method. A pulled fused-silica capillary with a conical tip (o.d. ≈ 15 μm) was produced using a laser puller (model P1000, Sutter Instrument Inc., USA). The sample solution was infused into the pulled fused-silica capillary at a flow rate of 2 μL/min. The capillary was placed in front of mass spectrometer, and high voltage (3.5 kV) was applied on the sample for ionization.

PSI-MS Method. Filter paper was cut into a triangle (10 × 5 mm) after sequential sonication-assisted cleaning in acetone, methanol, and methanol/water (50/50 *v*/*v*, 15 min each). Sample solution (10 μL) was dropped onto the paper triangle. After totally drying, the paper triangle was then held in front of the MS inlet (8 mm away), and 10 μL of ACN/H_2O/FA solution (50/50/1 *v*/*v*) was added directly onto the paper triangle to elute target compounds for ionization upon application of a high voltage (3.5 kV) to the wetted paper.

GT Fusion Protein Production. *Arabidopsis* glucuronic acid substitution of xylan 1 (AtGUX1, At3g18660, GT8 family) and *Arabidopsis* xyloglucan-xylosyltransferase (AtXXT1, At3g62720, GT34 family) were produced as fusion proteins tagged with GST at the C-terminus from 1 μg of plasmid DNA containing *AtXXT1* or *AtGUX1* genes and a TNT quick coupled transcription/translation system for 3 h at 28 °C according to the manufacturer's recommendations. The production of AtGUX1−GST and AtXXT1−GST fusion proteins was monitored by Western blotting of TNT quick coupled transcription/translation reaction products. Samples (4 μL) were mixed with Laemmli buffer and separated on 10% SDS−polyacrylamide gels before transfer onto Immobilon membranes using the Mini-PROTEAN Tetra Cell system (Bio-Rad) according to the manufacturer's recommendations. Membranes were blocked for 1 h with 5% (*w*/*v*) fat-free dry milk in 10 mM phosphate buffer containing 137 mM NaCl and 2.7 mM KCl, pH 7.2 (PBS). The primary antibody (anti-GST) was applied at 1/5000 dilution in PBS, pH 7.2, containing 5% (*w*/*v*) fat-free milk for 1 h, and unbound antibody was washed with PBS containing 0.05% Tween 20. After four washes (15 min each), membranes were incubated for 1 h with the secondary antibody goat antirabbit conjugated to peroxidase (1/20 000 dilution in PBS, pH 7.2, containing 5% fat-free milk). Excess secondary antibody was removed by several washes of the membrane with PBS containing 0.05% (*v*/*v*) Tween 20. Antibody binding was detected using SuperSignal West Femto Maximum Sensitivity Substrate and exposure to X-ray film. Known quantities of GST standard (5−25 ng) were used to estimate the amount of fusion proteins produced.

Nonradioactive Reactions for AtGUX1 (Scheme 2a) and AtXXT1 (Scheme 2b). Enzymatic reactions were carried out in 50 μL of reaction solution. For AtGUX1, the reaction mixture contained 25 μg of xylohexaose (X6, acceptor), 5 mM UDP−glucuronic acid (donor), 1 mM $MnCl_2$, and 1 mM $MgCl_2$ in 50 mM Tris-HCl buffer, pH 7.4. For AtXXT1, the reaction mixture contained 25 μg of cellohexaose (C6, acceptor), 5 mM UDP−xylose (donor), 1 mM $MnCl_2$, and 1 mM $MgCl_2$ in 50 mM Tris-HCl buffer, pH 7.4. Transfer reactions were carried out for 3 h at 28 °C after different amounts of expressed GST-tagged proteins were added (36−216 ng, estimated via Western blotting). The reactions were stopped by adding 0.3 mL of water and then freeze-dried. For DPS-MS analysis, three reactions were combined, freeze-dried, and then resuspended in 500 μL of aqueous solvent.

Radioactive Reactions for AtXXT1. Each reaction mixture contained 25 μg of cellohexaose (C6, acceptor), UDP−[^{14}C]xylose (60 000 cpm, 7.141 GBq/mmol), cold UDP−xylose (3 μM), 1 mM $MnCl_2$, and 1 mM $MgCl_2$ in 50 mM Tris-HCl buffer, pH 7.4. The reactions were carried out for 3 h at 28 °C in the presence of various amounts of expressed fusion proteins (0−216 ng, estimated via Western

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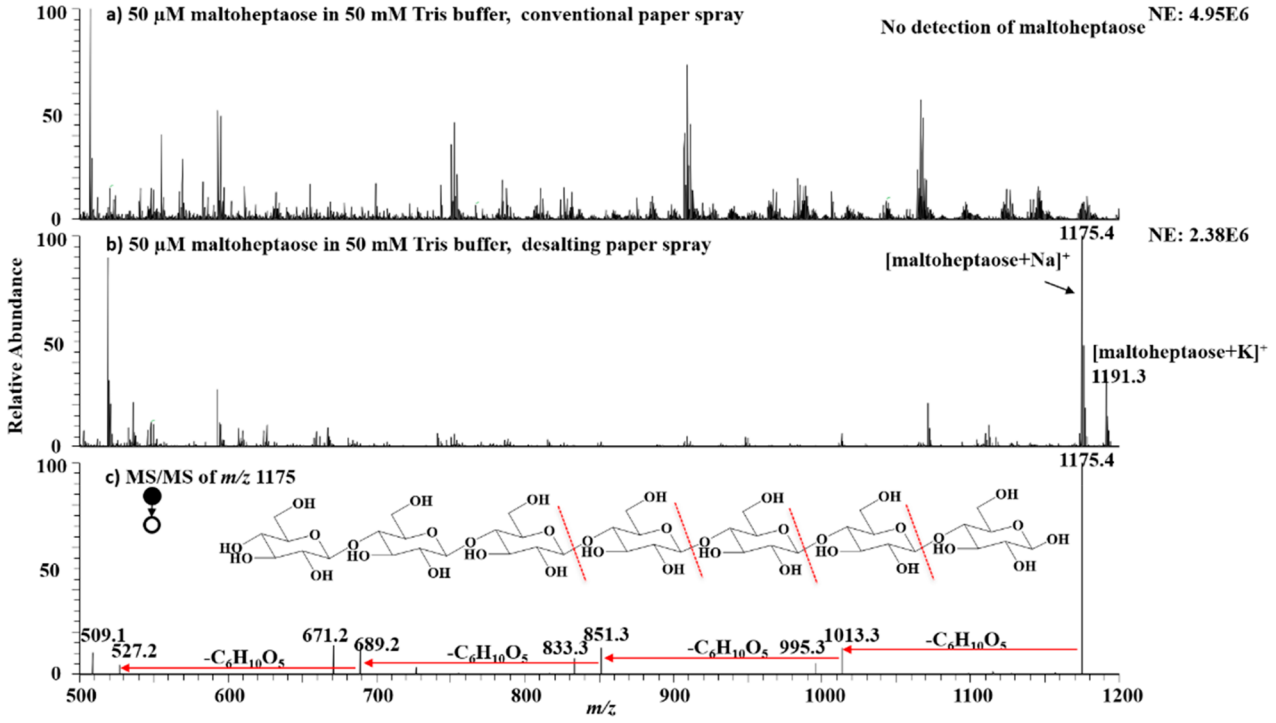

Figure 1. MS spectra of 50 μM maltoheptaose in 50 mM Tris buffer (pH 7) acquired by (a) conventional paper spray and (b) desalting paper spray. (c) Collusion-induced dissociation (CID) MS/MS analysis of the [maltoheptaose + Na]$^+$ ion at m/z 1175.

blotting analysis). The reactions were stopped by adding 0.3 mL of water, and sufficient ion-exchange resin (DOWEX 1X8-100 resin (Cl) 1:1 (v/v) was added to remove unused UDP−[^{14}C]xylose. The incorporation of [^{14}C]xylose onto cellohexaose was measured (as cpm) after resuspension in 5 mL of liquid scintillation solution using a LS 6500 multipurpose scintillation counter (Beckman). Using specific radioactivity, the amount of products formed was estimated (as nM). All reactions were performed in duplicate.

Radioactive Reactions for AtGUX1. Each reaction mixture contained 25 μg of xylohexaose (X6, acceptor), UDP−[^{14}C]glucuronic acid (80 000 cpm, 9.213 GBq/mmol), cold UDP−glucuronic acid (3 μM), 1 mM $MnCl_2$, and 1 mM $MgCl_2$ in 50 mM Tris-HCl buffer, pH 7.4. Reactions were carried out for 3 h at 28 °C in the presence of various amounts of expressed fusion proteins (0−216 ng, estimated via Western blotting analysis). Reactions were stopped by adding 0.3 M acetic acid. [^{14}C]glucuronic-acid-containing xylohexaose was separated from free UDP−[^{14}C]glucuronic acid by paper chromatography, as described earlier.[45] [^{14}C]glucuronic-acid-containing xylohexaose stayed at the origin, and [^{14}C]-radiolabel was measured (as cpm) after cutting the origin of paper chromatography and submersion in 5 mL of liquid scintillation solution as described for AtXXT1. All assays were performed in duplicate.

RESULTS AND DISCUSSION

In most biological samples, oligosaccharides are present among a mixture of various other chemicals, including salts and proteins. Therefore, the removal of these contaminants is necessary for efficient MS analysis. First, we sought to test hydrophilic interaction bonding between oligosaccharides and cotton fibers as the SPE stationary phase. In these tests, we used 50 μM maltoheptaose in 50 mM Tris-HCl buffer (pH 7) using the Manfred method.[46] Our results showed that maltoheptaose could adsorb onto cotton, and the addition of the ACN/H_2O mixture (83/17 v/v) allowed removal of the buffer. Maltoheptaose was then eluted with H_2O/ACN/FA (80/20/0.1 $v/v/v$) and collected for nanoESI-MS analysis. Although we could successfully detect the protonated maltoheptaose ion at m/z 1153 with a mass error of 0.40 ppm (Figure S2, measured m/z 1153.38806, theoretical m/z 1153.38760), the protocol was time-consuming, as the eluted maltoheptaose needed to be loaded to a nanoESI emitter. To speed up the analysis, we replaced nanoESI with DPS. The rationale being that the substrate for paper spray ionization mainly contains the same composition of cellulose as the SPE stationary phase and could serve as both a desalting media and a spray emitter for oligosaccharide ionization. In this protocol, we first examined tested DPS's capability to analyze trace oligosaccharides in a complex matrix solution, using 50 μM maltoheptaose in 50 mM Tris-HCl buffer (pH 7) as a test sample. Due to significant signal suppression from salt (buffer), both conventional PSI (Figure 1a, following the PSI-MS procedure described in the Experimental Section) and nanoESI (data not shown) failed to detect maltoheptaose from the sample, without desalting. By contrast, using our DPS-MS method, sodium and potassium adduct ions of maltoheptaose at m/z 1175 (measured m/z 1175.37197, theoretical m/z 1175.36955, mass error 2.06 ppm) and m/z 1191 (measured m/z 1191.34469, theoretical m/z 1191.34349; mass error 1.01 ppm) were observed (Figure 1b). The identity of the maltoheptaose peak was further confirmed through collusion-induced dissociation (CID) analysis of the ion at m/z 1175, which produced fragment ions of m/z 1013, 851, 689, and 527 by consecutive losses of hexose residues ($C_6H_{10}O_5$, 162 Da), consistent with its structure (Figure 1c). In this DPS-MS experiment, as the spray solvent of H_2O/ACN/FA (90/10/1 $v/v/v$) contained 90% water, there was a time delay of about 0.3 min to get a stable EIC current for m/z 1175.4

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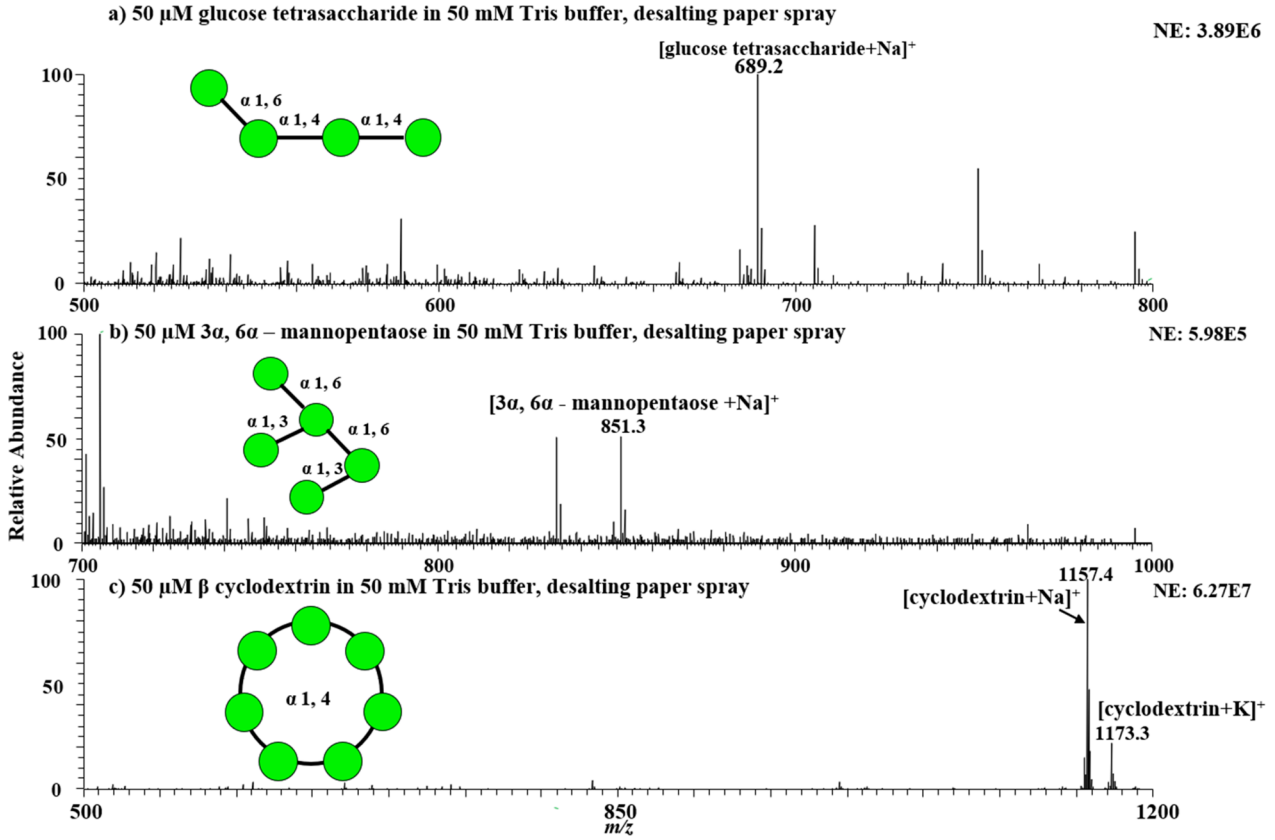

Figure 2. MS spectra of 50 μM oligosaccharide in 50 mM Tris-HCl buffer (pH 7) acquired by desalting paper spray of (a) glucose tetrasaccharide, (b) 3α,6α-mannopentaose, and (c) β-cyclodextrin. The green circle represents one monosaccharide ring (i.e., glucose or mannose).

(Figure S3, Supporting Information). The delay might be caused by the electrophoretic migration of the oligosaccharide sample to the paper tip for spray. The signal was then stabilized for about 0.8 min (0.3−1.1 min) before declining (Figure S3, Supporting Information).

Next, we sought to evaluate the detection sensitivity of the DPS-MS method. For this experiment, we used samples containing a constant concentration of Tris buffer (50 mM), while the concentration of maltoheptaose was adjusted from 500 to 5 nM such that, the Tris-HCl concentration was 1×10^5 to 1×10^7 times higher than the maltoheptaose concentration. We could detect the sodium adduct ion of maltoheptaose at m/z 1175 in samples containing 500 nM maltoheptaose in 50 mM Tris-HCl buffer (Figure S4a, Supporting Information). Importantly, when the concentration of maltoheptaose was further lowered to 5 nM (Tris-HCl/maltoheptaose ratio was 1×10^7), the analysis of 10 μL of this 5 nM solution (total amount 50 fmol) showed clear detection of the ion at m/z 1175 (Figure S4b, Supporting Information). Our data indicate that, despite the increase by 4 orders of the magnitude of the ratio of Tris-HCl to maltoheptaose and the reduced intensity of m/z 1175 by around 300 times (Figure 1b (2.38E6) vs Figure S4b (9.04E3)), the signal at m/z 1175 corresponding to the sodium adduct ion of maltoheptaose was still detectable. In other words, the DPS method appears to have a good desalting capability. In addition, compared to traditional MS methods, DPS-MS requires much less time (<5 min per sample) to analyze trace amounts of oligosaccharides in matrix samples. For example, although LODs of 100 pmol and 50 fmol were reported for ESI-MS and MALDI-MS methods, respectively,[29,30] those methods are time-consuming (ca., 50 to 120 min).

In another set of experiments, we sought to determine if the DPS-MS method could tolerate various buffers and matrix. Thus, we dissolved maltoheptaose (50 μM) in 50 mM PBS buffer, 50 mM HEPES, or 50 mM synthetic urine and used them to test the efficiency of the DPS-MS method compared to conventional PSI-MS (Figure S5, more experimental details in the Experimental Section). When HEPES and PBS buffers were tested, desalting was achieved using 10 μL of ACN/H_2O solution (80/20 v/v) instead of 10 μL of ACN/H_2O solution (90/10 v/v) for a better signal-to-noise ratio (S/N). While conventional PSI failed to detect maltoheptaose signal (top panels in Figure S5a−c), the DPS-MS method successfully detected a maltoheptaose signal at m/z 1175.4 with a mass error of <1.5 ppm (bottom panels in Figure S5a−c).

Glucose tetrasaccharide, 3α,6α-mannopentaose, and β-cyclodextrin (all at 50 μM in 50 mM Tris-HCl buffer, pH 7) were also tested using the DPS-MS method. Our data show that DPS-MS could detect all these oligosaccharides, as shown in Figure 2, namely, [tetrasaccharide + Na]$^+$ at m/z 689.21234 with a mass error of 1.83 ppm (theoretical m/z 689.21108), [3α,6α-mannopentaose + Na]$^+$ at m/z 851.26567 with a mass error of 2.08 ppm (theoretical m/z 851.26390), and [β-cyclodextrin + Na]$^+$ at m/z 1157.35962 with a mass error of 0.55 ppm (theoretical m/z 1157.35898).

Furthermore, DPS-MS proved to be quite sensitive, since trace amounts of oligosaccharides could be detected, as depicted in MS spectrum acquired using 10 μL of 5 nM β-cyclodextrin (50 fmol) in 50 mM Tris-HCl buffer, pH 7 (buffer/β-cyclodextrin ratio 1×10^7) (Figure 3c). Conven-

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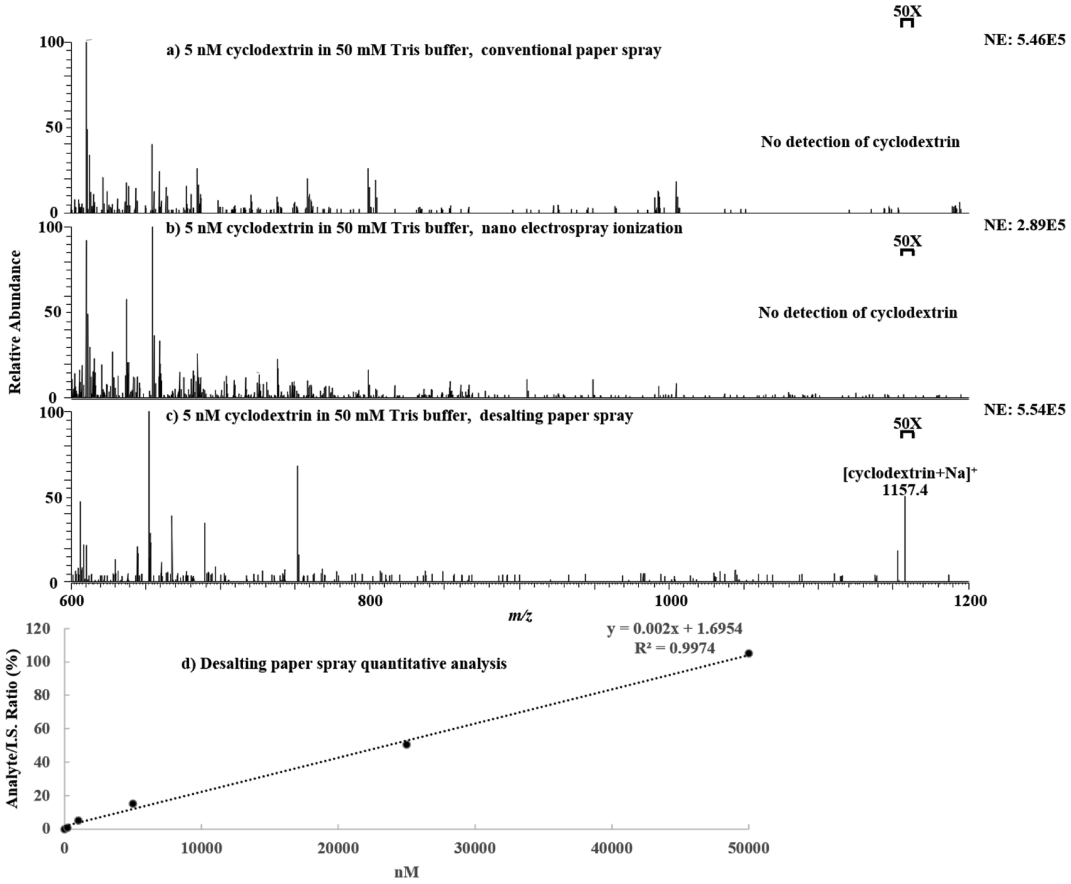

Figure 3. MS spectra of 5 nM cyclodextrin in 50 mM Tris buffer acquired by (a) PSI, (b) nanoESI, and (c) DPS; (d) shows a linear relationship of β-cyclodextrin signal vs concentration (200 nM−50 μM β-cyclodextrin in 50 mM Tris-HCl buffer, pH 7) using 5 μM maltoheptaose as an I.S. Spectra in (a−c) were acquired using only 10 μL of solution.

tional PSI-MS and nanoESI-MS could not tolerate signal suppression issues from the buffer and failed to detect β-cyclodextrin at this concentration (5 nM) (Figure 3a,b). Similarly, 10 μL of 5 nM glucose tetrasaccharide (Figure S6c) and 3α,6α-mannopentaose (50 fmol each) (Figure S7c) in 50 mM Tris-HCl buffer, pH 7, (Tris-HCl/analyte ratio 1×10^7) were also successfully detected using DPS-MS. Again, no oligosaccharide signal was detected by conventional PSI-MS and nanoESI-MS (Figures S6a,b and S7a,b).

To determine whether DPS-MS could be quantitative, we doped an internal standard (I.S.) with various concentrations of β-cyclodextrin solution, and the signal intensity ratio of β-cyclodextrin to the internal standard was plotted against β-cyclodextrin concentration. As shown in Figure 3d, the plot was linear over β-cyclodextrin concentrations ranging from (0.2 to 50 μM) with a correlation coefficient higher than 0.99 (unweighted linear curve fitting was used for plotting). The excellent linearity of the plot indicated the DPS-MS method can be used for quantitative analysis. The limit of detection (LOD) for β-cyclodextrin was calculated and found to be 1.1 nM (i.e., 1.3 ng/mL; see Table S1); in other words, our DPS-MS detection method could handle analysis of oligosaccharide as low as 11 fmol in nonvolatile buffers in a few minutes. In this experiment, to achieve a reproducible quantitative analysis result, we adopted paper substrates with the same size and loaded the same volume (10 μL) of sample onto the same location of the paper substrate each time. In addition, we fixed the paper substrate position (relative to MS inlet) using a high-voltage cable alligator clip.

Our data showed that the DPS-MS method was successful in detecting and quantifying relatively pure standard oligosaccharides under different buffer conditions. However, these buffers do not represent the complex mixture of biological and enzyme reactions that usually include buffers and other ions as well as some organelles. To further test the validity of a DPS-MS method in analyzing more complex mixtures from biological samples, we used the method to detect products generated by *in vitro* glycosyltransferase (GT) reactions. In GT reactions, sugars are transferred from UDP-activated sugars onto the acceptor (mostly oligosaccharides), producing new oligosaccharides (products). In this study, two well-characterized GTs were used, namely, AtGUX1[47] (Scheme 2a) and AtXXT1[48] (Scheme 2b). Currently, GTs are heterologously produced in organisms (expression systems) such as bacteria, yeast, or eukaryotic cells (from plants or animals)[49−53] and then partially purified for testing their activity using radioactive assays. However, these expression systems are time-consuming and cannot be adapted for fast analysis. To overcome these limitations, we opted for an *in vitro* Coupled Transcription/Translation System to produces GT proteins for enzyme activity testing. To demonstrate the validity of this approach, we used two well-characterized *Arabidopsis* GTs, namely, AtGUX1 or AtXXT1. These two GTs are membrane proteins. To improve their solubility, GTs were synthesized as fusion proteins with a GST tag at their C-terminal ends. Transferase

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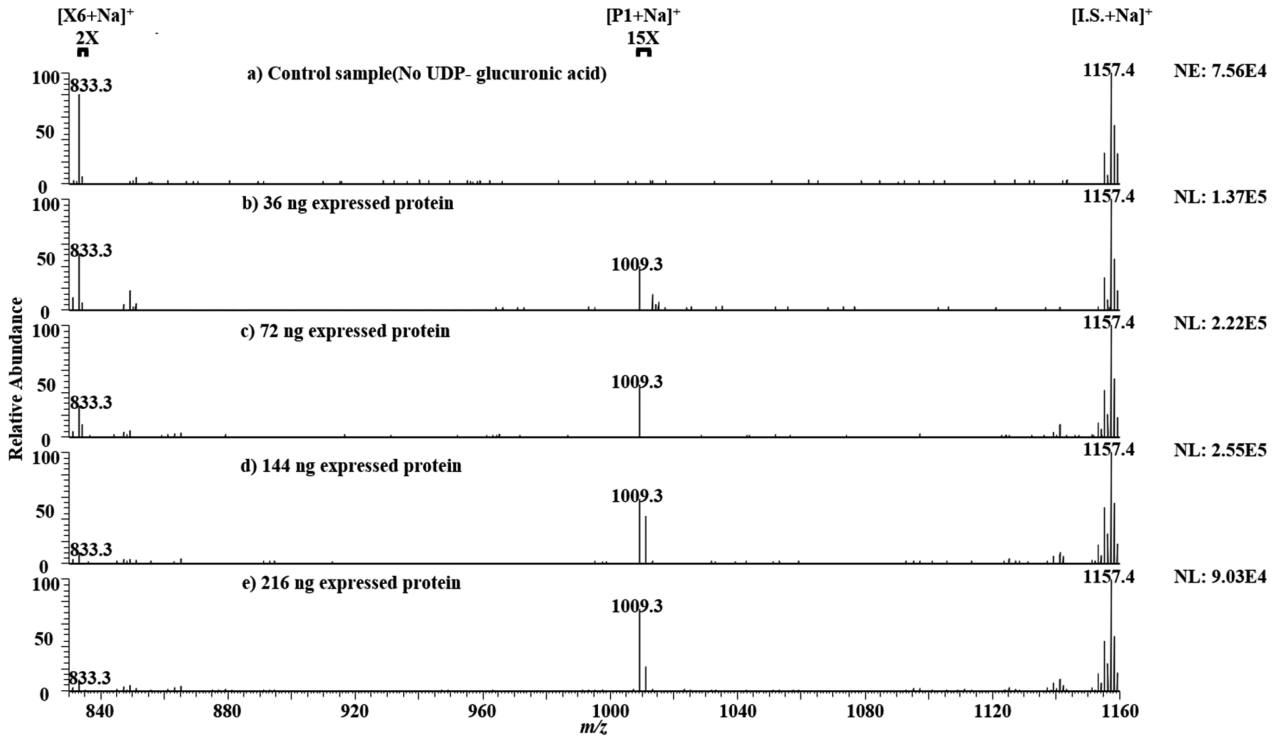

Figure 4. DPS-MS analysis of AtGUX1−GST reactions. MS spectra from (a) control sample (no UDP−glucuronic acid) and reaction samples catalyzed by (b) 36 ng of AtGUX1 protein, (c) 72 ng of AtGUX1 protein, (d) 144 ng of AtGUX1 protein, and (e) 216 ng of AtGUX1 protein.

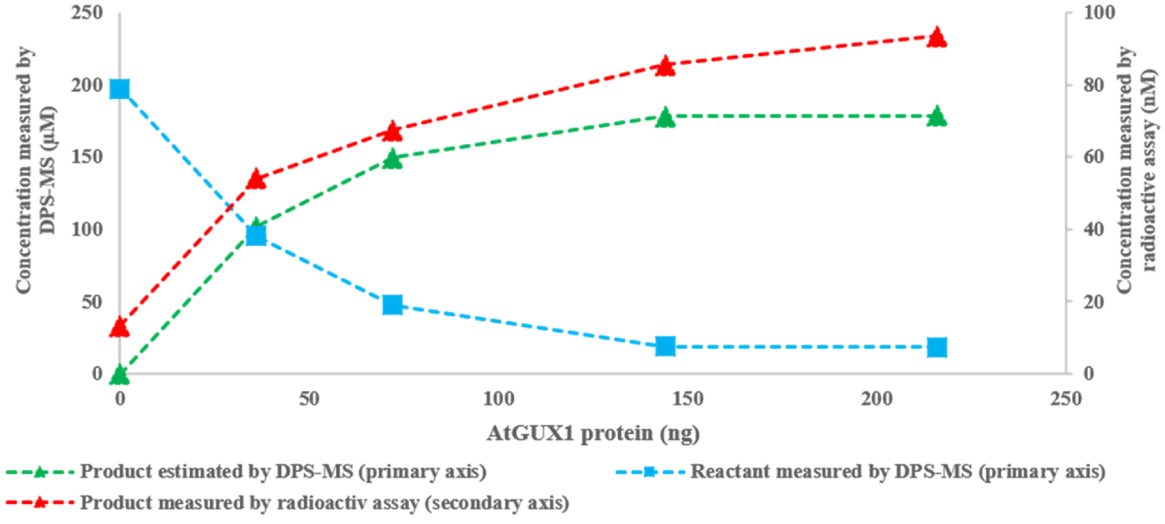

Figure 5. Estimation of the acceptor X6 and the product (**P1**) concentrations using DPS-MS and radioactive assay, as a function of the amount of AtGUX1 protein used in the enzymatic reactions.

activity of these GTs was monitored via both radioactive assays and the DPS-MS detection method. In addition, to determine the lower limit of detection (for both the radioactive assay and DPS-MS method, five different amounts (36 to 216 ng) of AtGUX1−GST or AtXXT1−GST were added to UDP-activated monosaccharides (UDP−glucuronic acid for AtGUX1 reaction and UDP−xylose for AtXXT1 reaction) and acceptor oligosaccharide (X6 for AtGUX1 and C6 for AtXXT1) mixture (50 μL) containing 50 mM Tris-HCl (pH 7.4), 1 mM $MgCl_2$, and 1 mM $MnCl_2$ to promote the enzymatic reactions. After reacting, each reaction mixture was subsequently analyzed by DPS-MS. One control sample without added UDP−xylose was also tested. For the AtGUX1 reaction control sample, no donor UDP−glucuronic acid was added. For the AtXXT1 reaction control sample, no donor (UDP−xylose for AtXXT1) was included.

As shown in Figure 4a, acceptor X6, m/z 833.3 (mass error < 2.5 ppm), was observed, with no glycosyl transfer reaction product **P1** (Scheme 2a) detected in the control reaction. However, with both donor (UDP−glucuronic acid) and acceptor (X6) substrates present in the reaction mixture, a new oligosaccharide product, m/z 1009.3 (mass error < 3.0 ppm), was detected, and its intensity increased with increased AtGUX1−GST fusion protein added to the reaction (Figure 4b−e). Furthermore, DPS-MS was used to quantify the amount of remaining X6 acceptor, using pure X6 alone as a

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standard. Increasing the concentration of the standard X6 relative to the internal standard, β-cyclodextrin (m/z 1157.4), gave excellent linearity between the X6 signal and concentration (Figure S8 and Table S2, RSD < 6%). The concentration of the remaining X6 in the enzymatic reaction thereby could be quantified (Figure 5, square dots). The concentration of the glycosyl transfer reaction product was estimated based on X6 consumption (Figure 5, green triangular dots) assuming a 1:1 stoichiometric reaction ratio between X6 and product. The measured concentration of X6 in the control sample was 197.1 μM, compared to a theoretical concentration of 185 μM, giving a measurement error of only 6.5%. The small measurement error validated the quantitative ability of the DPS-MS method. Traditionally, product analysis of GT-catalyzed reactions requires the use of radioactive assays using [^{14}C]-labeled sugar donors, which are expensive and present a safety hazard. For comparison, a radioactive assay was also performed under similar experimental conditions except that less UDP−sugar donor was used. As indicated in Figure 5 (red triangular dots), radioactive assays showed a similar trend, namely an increase in the amount of the product (**P1**) with the increased amounts of AtGUX1−GST enzyme, which is in agreement with DPS-MS result (green triangular dots).

Similar analysis was conducted for glycosyl transfer reaction catalyzed by AtXXT1−GST fusion protein produced by an *in vitro* Coupled Transcription/Translation system. AtXXT1 uses C6 oligosaccharide as the acceptor and UDP−xylose as the donor (Scheme 2b). The remaining C6 acceptor sugar was quantified based on the acquired standard calibration curve (Figure S10 and Table S3, RSD < 3%). The measurement C6 concentration in the control sample was 131.9 μM, close to the expected theoretical concentration of 151 μM (12.6% error). For the reaction samples, it was found that the intensity of acceptor (C6) decreased with the increased amount of fusion protein added to the enzymatic reaction, while the formed oligosaccharide product (**P2**, m/z 1145.4, mass error < 3 ppm) increased (Figure S11). Again, DPS-MS and the radioactivity assay gave a similar trend of product formation (Figure S11, i.e., an increase in the amount of the resulting product (**P2**) with using an increased amount of AtXXT1−GST enzyme), which confirms the robustness and reproducibility of the DPS-MS method.

CONCLUSIONS

As reported herein, DPS-MS integrates purification and ionization together for the detection of oligosaccharides. This approach is not only fast (<5 min), as demonstrated by detection of 50 fmol of oligosaccharides in different nonvolatile buffers and an LOD of 1.1 nM for β-cyclodextrin in 50 mM Tris buffer (pH 7). By using our approach, we successfully quantified standard oligosaccharides (β-cyclodextrin, X6, and C6) as well as oligosaccharide products from GT reactions. We showed that DPS-MS detection is also accurate, as it measured the amount of acceptor in GT reaction control sample with acceptable error (6.5∼12.6%). In addition, DPS-MS detection of the GT reaction product was in good agreement with radioactive assay results. Although the LOD of DPS-MS is ∼100 times higher compared to the radioactive assay (11 femtomol for DPS-MS vs 0.1 femtomol for radioactive assay), the DPS-MS method is fast as well as reliable and can be adapted to future rapid analysis of GT activity. This work demonstrated that a high-throughput pipeline combining *in vitro* synthesis of GTs and DPS-MS detection is possible and opens new doors for GT functional genomics.[48]

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jasms.0c00310.

Additional MS spectra, experiment setup, and data are included (PDF)

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Notes

The authors declare no competing financial interest.

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