Flow Chemistry System for Carbohydrate Analysis by Rapid Labeling of Saccharides after Glycan Hydrolysis

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Abstract

This study demonstrates the utilization of a flow chemistry system for continuous glycan hydrolysis and saccharide labeling to assist with the existing methods in glycan structural analysis. Acidic hydrolysis of glycans could be accelerated in a flow system. Aldoses and α-ketoacid-type saccharides were effectively labeled with naphthalene-2,3-diamine (NADA) at 60 °C for 10 min to form the fluorescent naphthimidazole (NAIM) and quinoxalinone (QXO) derivatives, respectively. The NADA-labeled derivatives improved the structural determination and composition analysis for their parent saccharides by using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS), liquid chromatography mass spectrometry (LC-MS), and nuclear magnetic resonance (NMR). Furthermore, this protocol was applied to determine the SA–Gal–Glc sequence of GM3-sugar out of six possible permutations.

Introduction

Carbohydrate analysis is essential for using glycans in biological research, clinical analysis, and biotechnological production.[1] The primary structure of a glycan is defined not only by the constituent monosaccharides, but also by their linkages and branching. Often the nature and position of nonglycan substituents such as aglycan and esters (e.g., acetate, sulfate, and phosphate) need to be determined. Methods for solving the three-dimensional structures of glycans are also needed.

Various approaches for analyses of the glycan structure have been reported.[2–5] Structural and compositional analyses of glycans often require hydrolysis to release the monosaccharides, for which acidic hydrolysis is most typically used. Many hydrolysis protocols for glycan hydrolysis have been reported.[6–11] Monosaccharide analyses are usually by liquid chromatography (LC), mass spectrometry (MS), nuclear magnetic resonance (NMR), or any combination of the three techniques. Moreover, the released monosaccharides can be derivatized to facilitate detection and quantification by LC analysis.[12,13] Suitable derivatization also aids in improving ionization efficiency for MS analysis. Capillary electrophoresis mass spectrometry (CE-MS),[14–16] LC-MS,[17–19] and NMR[20,21] can be used to determine the structures of complex glycans and substantially improved

by NADA tagging.[22] We have previously explored a method using naphthalene-2,3-diamine (NADA) for the derivatization of aldoses and α-ketoacid-type saccharides (e.g., sialic acid) to their corresponding naphthimidazole (NAIM) and quinoxalinone (QXO) derivatives (**Fig. 1**).[22–24] Conversion of aldoses by reductive amination at the reducing terminals is a common practice to afford the derivatives for mass spectrometric analyses.[24] However, the high content of salts in the products needs to be removed to increase the signal level. Conjugating α-ketoacid by reductive amination is not effective due to the low reactivity and yield of two isomers. In comparison, the NAIM and QXO sugars are readily

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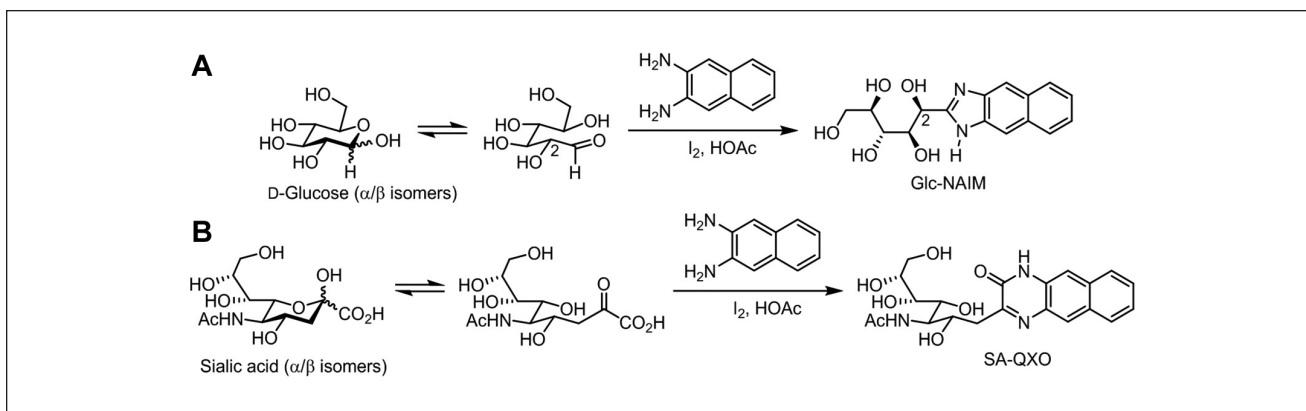Email: jmfang@ntu.edu.tw

Figure 1. Derivatizing glucose and sialic acid with NADA.

prepared to assist the structural assignment of parent sugars in the chromatographic and spectrometric analyses.[22–26]

A reducing sugar may exist in the cyclic form as the α- and β-anomers, which sometimes obscure ^{1}H-NMR signals. The sugar-NAIM derivative eliminates this obstacle in NMR analysis.[25] We have previously shown that NAIM derivatization provides a simple method for quantitative NMR analysis of monosaccharides and disaccharides, including arabinose (Ara), xylose (Xyl), rhamnose (Rha), glucose (Glc), mannose (Man), galactose (Gal), *N*-acetylgalactosamine (GalNAc), glucuronic acid (GlcUA), maltose (Mal), and lactose (Lac).[25] The NAIM derivative of each saccharide shows a single characteristic vinyl H-2 proton at a distinct position to facilitate the quantitative analysis. This NAIM method is especially useful for the identification and quantification of multiple kinds of glycans for their compositional analysis. In addition, the sugar-NAIM carries a hydrophobic NAIM group that can enhance ionization in MS detection.[26] The UV- and fluorescence-active NAIM modifier can also assist in the LC analysis. The limit of detection for sugar-NAIM compounds can possibly reach the submicromolar range upon using a fluorescence detector. Furthermore, the D-/L-enantiomeric pairs of sugar-NAIM compounds derived from common monosaccharides, including ribose (Rib), Ara, Xyl, Rha, fucose (Fuc), Glc, Man, Gal, GalNAc, GlcUA, and galacturonic acid (GalUA), are resolved on an uncoated fused-silica capillary using sulfated-α-cyclodextrin as the chiral selector.[27]

The use of a microreactor greatly facilitates the NAIM derivatization, resulting in a shorter reaction time and improved yield.[28] Flow chemistry systems for the multiple-step synthesis of many other bioactive compounds and natural products[29–32] optimize the yield as well as contribute to safety. In this report, we show that combining glycan degradation and saccharide derivatization in a flow system, along with the use of chromatography, MS, and NMR techniques, leads to rapid carbohydrate compositional analysis.

Materials and Methods

Materials

Iodine, glacial acetic acid, NADA, HCl, and D_2O were purchased from Merck & Co., Inc. (Darmstadt, Germany). 2,5-Dihydroxybenzoic acid (2,5-DHB), glucose, maltose, maltotriose, lactose, and other monosaccharides were purchased from Sigma-Aldrich (St. Louis, MO). GM3-sugar was purchased from Dextra Laboratories Ltd. (Reading, UK). Maltotetraose was purchased from Supelco Analytical (Mainz, Germany). All chemicals and solvents were of analytical grade and used without further purification. The NAIM labeling kit used in this study was a gift from Sugarlighter Co., Inc. (New Taipei City, Taiwan).[25]

Batch Preparation of Sugar-NAIM Derivatives

The procedure follows the published method.[22] A mixture of monosaccharide (2.0 mg, 11 µmol), NADA (2.0 mg, 13 µmol), and iodine (2.0 mg, 8 µmol) in glacial acetic acid (1.0 mL) was stirred at room temperature. The labeling reaction was completed in 3 h as indicated by thin-layer chromatography (TLC). The mixture was concentrated by rotary evaporation under reduced pressure to give the sugar-NAIM derivative. Other sugars were also derivatized in this fashion. Alternatively, sugar-NAIM derivatives were prepared by using a NAIM labeling kit (Sugarlighter Co.).[25]

Vapourtec E-Series Flow Chemistry System

A Vapourtec flow reactor E-series with V-3 peristaltic pumps (Vapourtec Ltd., Bury St. Edmunds, Suffolk, UK) was used for flow chemistry. Our setup is shown in **Supplemental Figure S1**. The reactor comprises a 10.0 mL 1/16-inch polytetrafluoroethylene (PTFE) tube (0.81 mm i.d. × 200 cm). The E-series comes with a touchscreen interface, mounted at an ergonomically optimal height with
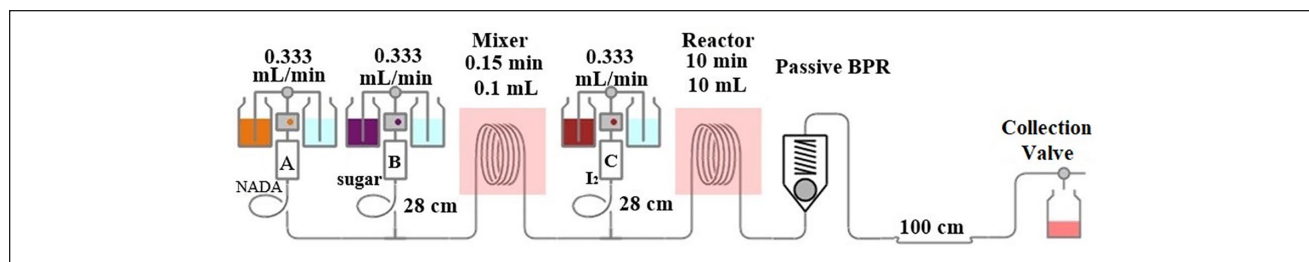
Figure 2. Diagram for preparation of sugar-NAIM derivatives in a flow chemistry system. Glacial acetic acid is shown in aqua blue. BPR, back-pressure regulator.
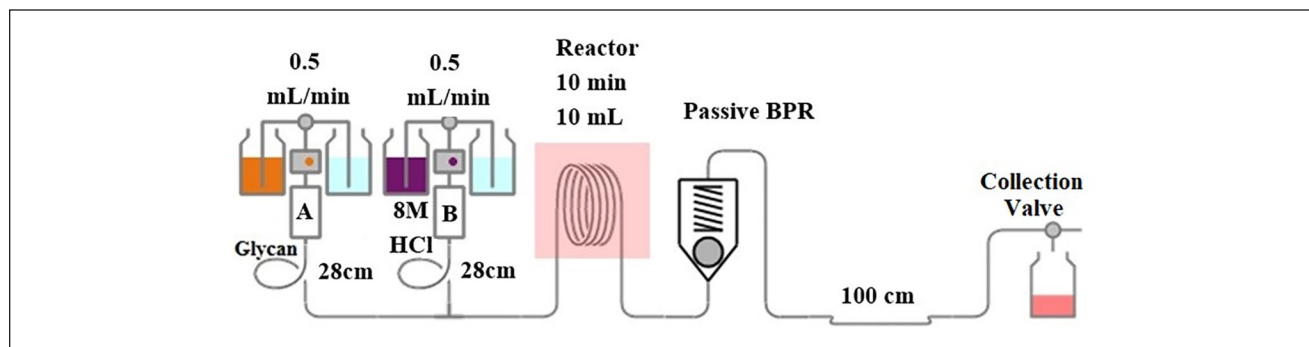

Figure 3. Diagram for glycan hydrolysis in a flow chemistry system. The gauge pressure of the back-pressure regulator was set at 3 and 4 bar for the reactions at 120 and 150 °C, respectively.

full tilt adjustment. It allows setting the key flow rates and temperature (± 1 °C) through a feedback system.

Method for Preparation of Sugar-NAIM Derivatives in Flow Chemistry System

The procedure for preparing sugar-NAIM in a flow chemistry system was modified from the batch preparation method.[22,25] The flow diagram of the NAIM tagging process in a Vapourtec easy-MedChem flow chemistry system is shown in **Figure 2**. All solutions were in glacial HOAc: vial A, NADA (1000 mg/100 mL); vial B, sugar samples (500 mg/100 mL); and vial C, iodine (127 mg/100 mL). The reaction was performed by pumping solutions A, B, and C at the same rate (0.33 mL/min). The final amount of monosaccharide (15.0 mg, 0.08 mmol), NADA (30.0 mg, 0.18 mmol), and iodine (3.8 mg, 0.03 mmol) was conducted at 60 °C (the reading of the instrument setting) over a period of 10 min. After the reaction was completed, the mixture was concentrated by rotary evaporation under reduced pressure to give the desired sugar-NAIM derivative, which was directly subjected to ^{1}H-NMR and LC-MS analyses without further purification. This reaction protocol is applicable to prepare other sugar-NAIM derivatives, including those of mixed sugars, oligosaccharides, and glycans.

Procedure for Glycan Hydrolysis in Flow Chemistry System

The diagram of the glycan hydrolysis setup in a flow chemistry system is shown in **Figure 3**. Vial A containing a solution of glycan (100 mg) in doubly distilled water (dd-H_2O; 100 mL) and vial B containing a solution of 8 M HCl (100 mL) were prepared for glycan hydrolysis. The reaction was performed by pumping solutions A and B at the same rate (0.5 mL/min) at various temperatures over a period of 10 min. This generated a hydrolysis volume of 10.0 mL with a concentration of 5.0 mg of glycan in 4 M HCl. After the reaction was complete, the solution was concentrated by rotary evaporation under reduced pressure to give the glycan hydrolysate, which was directly subjected to ^{1}H-NMR and LC-MS measurements without further purification. This reaction protocol is applicable to the hydrolysis of other glycans.

MALDI-TOF-MS

The stock solutions of saccharides (1.2×10^{-3} to 5×10^{-3} M) were prepared in dd-H_2O containing 0.1% formic acid and 50% CH_3CN. The stock solutions of matrix 2,5-DHB (10 mg/mL, 6.5×10^{-2} M) and NaCl (1.7×10^{-2} M) were

prepared in dd-H_2O containing 0.1% formic acid/CH_3CN (1:1 v/v). The sample for MALDI-MS measurement was typically prepared by combining 10 μL of saccharide stock solution with 10 μL of matrix stock solution and 5 μL of NaCl solution to give a final volume of 25 μL in an Eppendorf tube. Then, 2 μL of this sample solution was applied to the sample plate by a dried-droplet method (i.e., placing a droplet of the sample solution on a mass spectrometer's sample stage and drying the droplet at room temperature),[24] instead of a vacuum drying process. Samples of saccharide-NAIM derivatives were similarly prepared for MALDI-MS determination. The mass spectrometer used to acquire the spectra was a Voyager Elite Applied Biosystem (Foster City, CA). The accelerating voltage was set at 20 kV in either positive or negative ion mode. Typically, spectra were obtained by accumulating 800–1000 laser shots for quantification. Laser energy per pulse was calibrated with a laser power meter (PEM 101; Laser Technik, Berlin, Germany) so that laser fluence could be precisely measured. The delay extraction time was adjusted from 10 to 500 ns. The grid voltage was set at 95% of the accelerating voltage; the guidewire voltage was 0.2% of the accelerating voltage. The laser beam diameter was measured as ~100 μm on the sample target. The laser fluence was in the range of 50–300 mJ/cm². The flight tube pressure inside the vacuum was always kept between 10^{-7} and 10^{-6} torr.

LC-MS

The Velos Pro dual-pressure linear ion trap MS from Thermo Fisher Scientific (San Jose, CA) was used for linear trap quadrupole Fourier transform mass spectrometry (LTQ-FTMS). The saccharide sample was similarly prepared as described above and subjected to LC-MS analysis. In brief, a sample solution was prepared by dissolving the saccharide (or sugar-NAIM derivative) in dd-H_2O (0.5 mL) containing 0.1% formic acid. The sample solution (5 μL) was then injected into an Xbridge C18 column (1.0 mm i.d. × 15.0 cm, 3.5 μm particle size, 130 Å pore size). The flow rate was set at 0.05 mL/min, the gradient elution was applied (0–20 min, 2%–98% ACN/H_2O), and a UV detector was used for the LTQ-FTMS analysis.

NMR

^{1}H-NMR spectra were recorded on a Bruker AV600 MHz NMR spectrometer (Rheinstetten, Germany). This is a two-channel system equipped with a 5 mm DCI dual cryoprobe for high-sensitivity ^{1}H/^{13}C observation. The sugar-NAIM sample was dissolved in D_2O solution containing $(CH_3)_2SO$ (0.03%–0.1%) as an internal standard. Quantification of sugars was based on the integral areas of the characteristic proton signals. For example, the area

of H-2 in an individual hexose-NAIM derivative was compared with that of $(CH_3)_2SO$ (integral region from δ 2.792 to 2.727 ppm for six protons of the two methyl groups). The acquisition parameters were equipped with a high-performance actively shielded standard-bore 14.09-Tesla superconducting magnet. The following parameters were used: ^{1}H-NMR acquisition: 90° pulse, P1 = 9.95 μs, PL1 = –0.8 dB; relaxation delay D1 = 2 s; number of acquisition (aq) = 1.9530824 (s); type of baseline correction: quad; window function: EM; LB = 0.5 Hz; software for spectral processing and regression analysis: TopSpin 3.0.

Results and Discussion

Preparation of Sugar-NAIM Derivatives in a Flow Chemistry System

We have previously prepared a series of sugar-NAIM derivatives in a batch-wise manner by treating aldoses with NADA and iodine in a flask with magnetic stirring.[22] This reaction was usually complete in 3–6 h at room temperature. The reaction time was reduced to 1–2 h upon using a NAIM labeling kit through the enhanced concentration of NADA and iodine.[25] Using a flow chemistry system further improved the labeling reaction (**Fig. 2**). In a typical procedure, a solution of NADA (30.0 mg, 0.18 mmol) in HOAc (glacial, 3.0 mL), a solution of monosaccharide (15.0 mg, 0.08 mmol) in HOAc (3.0 mL), and a solution of iodine (3.8 mg, 0.03 mmol) in HOAc (3.0 mL) were mixed and reacted in a flow system over a period of 10 min at 60 °C (the reading of the instrument setting) at a flow rate of 1 mL/min. The desired sugar-NAIM products were obtained and concentrated under reduced pressure to remove HOAc. The product was analyzed by ^{1}H-NMR, MALDI-TOF-MS, and LC-MS without further purification.

Taking D-glucose as an example, the formation of Glc-NAIM derivative was ~ 20% for 5 min at 25 °C in a flow system, and essentially completed at 20 min (**Fig. 4**). The reaction time was reduced at 60 °C for 10 min to give an essentially completed reaction. The reaction was monitored by ^{1}H-NMR spectra (600 MHz, D_2O). Glucose initially showed the C-1 proton signals at δ 5.22 and 4.64 for the α- and β-anomers, respectively. Both anomers were converted to a single NAIM compound, which displayed the characteristic C-2 and C-3 protons at δ 5.38 and 4.39, respectively.

Figure 5 shows that various monosaccharides including D-Glc, D-Gal, D-GlcUA, L-Fuc, D-Man, and D-Xyl were effectively transformed into their corresponding NAIM derivatives by mixing with NADA and iodine at 60 °C for 10 min in a flow system. This protocol of flow chemistry was applicable to prepare the NAIM derivatives of
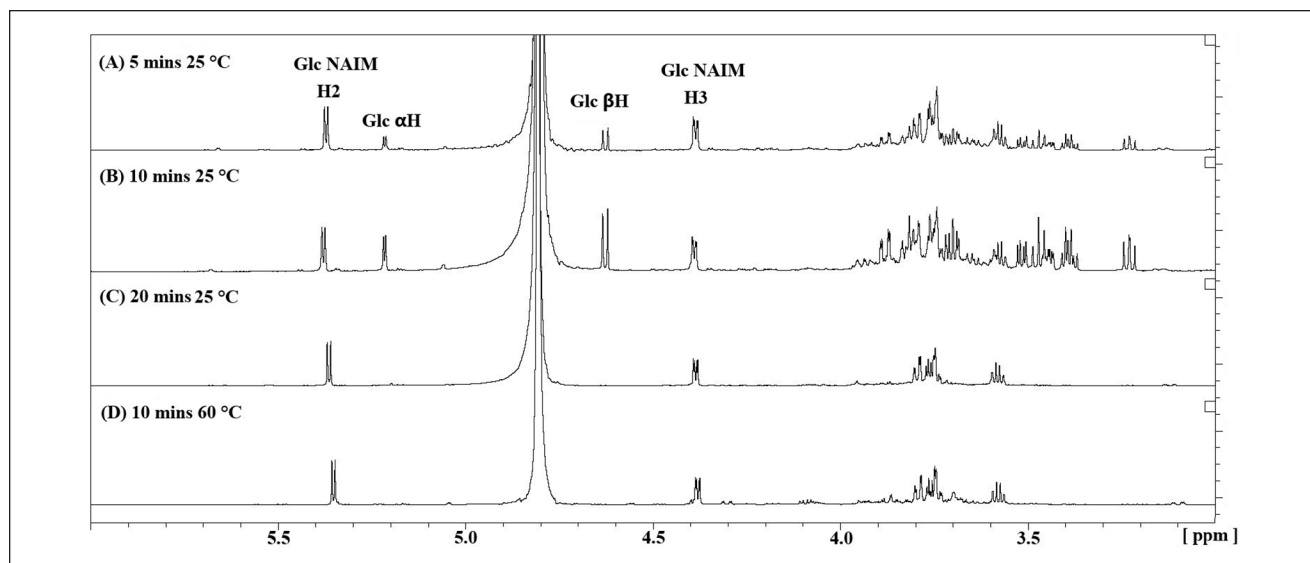
Figure 4. Glc-NAIM formation at different times and temperatures in a flow chemistry system: (**A**) 5 min at 25 °C, (**B**) 10 min at 25 °C, (**C**) 20 min at 25 °C, and (**D**) 10 min at 60 °C. The reaction was monitored by ^{1}H-NMR spectra (600 MHz, D_2O). The α- and β-anomers of glucose showed anomeric H signals at δ 5.22 and 4.64, respectively. The Glc-NAIM derivative showed the characteristic C-2 and C-3 protons at δ 5.38 and 4.39.
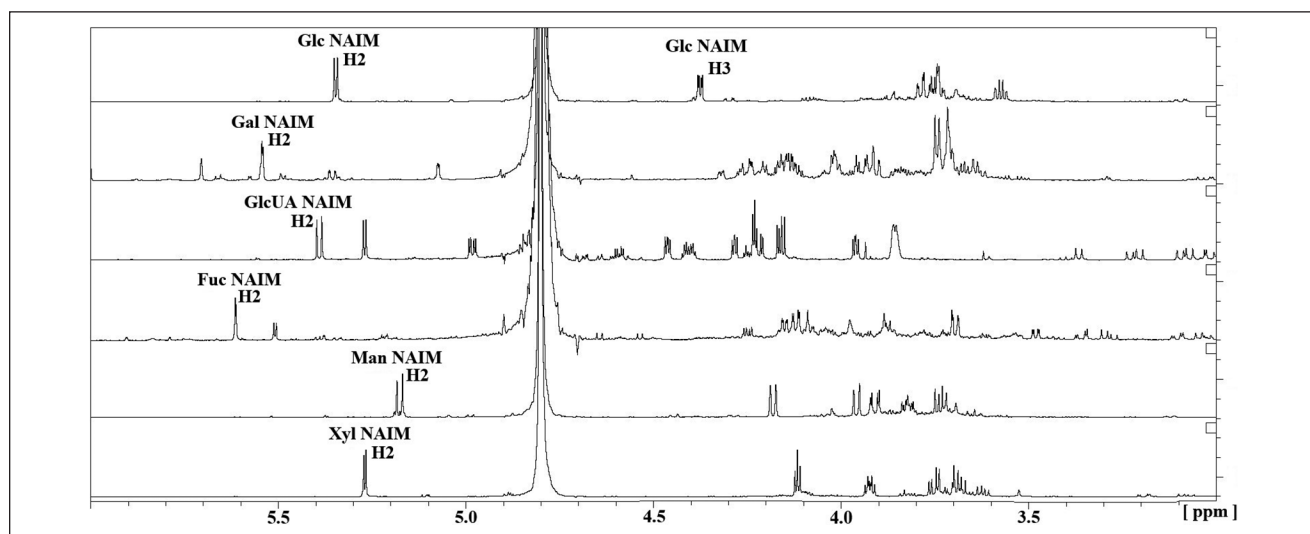

Figure 5. Formation of various sugar-NAIM derivatives at 60 °C for 10 min in a flow chemistry system. The final amounts of monosaccharide (15.0 mg, 0.08 mmol), NADA (30.0 mg, 0.18 mmol), and iodine (3.8 mg, 0.03 mmol) were used in each run of NAIM tagging reaction. ^{1}H-NMR spectra (600 MHz, D_2O) were measured to characterize the sugar-NAIM derivatives by their C-2 protons: Glc-NAIM at δ 5.38, Gal-NAIM at δ 5.54, GlcUA-NAIM at δ 5.39, Fuc-NAIM at δ 5.61, Man-NAIM at δ 5.18, and Xyl-NAIM at δ 5.27.

oligosaccharides and higher glycans, albeit requiring a somewhat longer reaction time (~20 min).

Glycan Hydrolysis in a Flow Chemistry System

We first investigated the acidic hydrolysis of di-, tri- and tetrasaccharides in a flow chemistry system. Maltose

(1.0 mg/mL) was treated with 4 M HCl at 80 °C for 10 min in a flow system to cause partial hydrolysis (~65%) according to the MALDI-TOF-MS analysis of the product mixture (**Suppl. Fig. S2**). The hydrolysis was accelerated at higher temperatures (120 and 150 °C) and completed in 10 min. The temperature effect was further supported by the acidic hydrolysis of maltotriose (**Suppl. Fig. S3**). Upon treatment
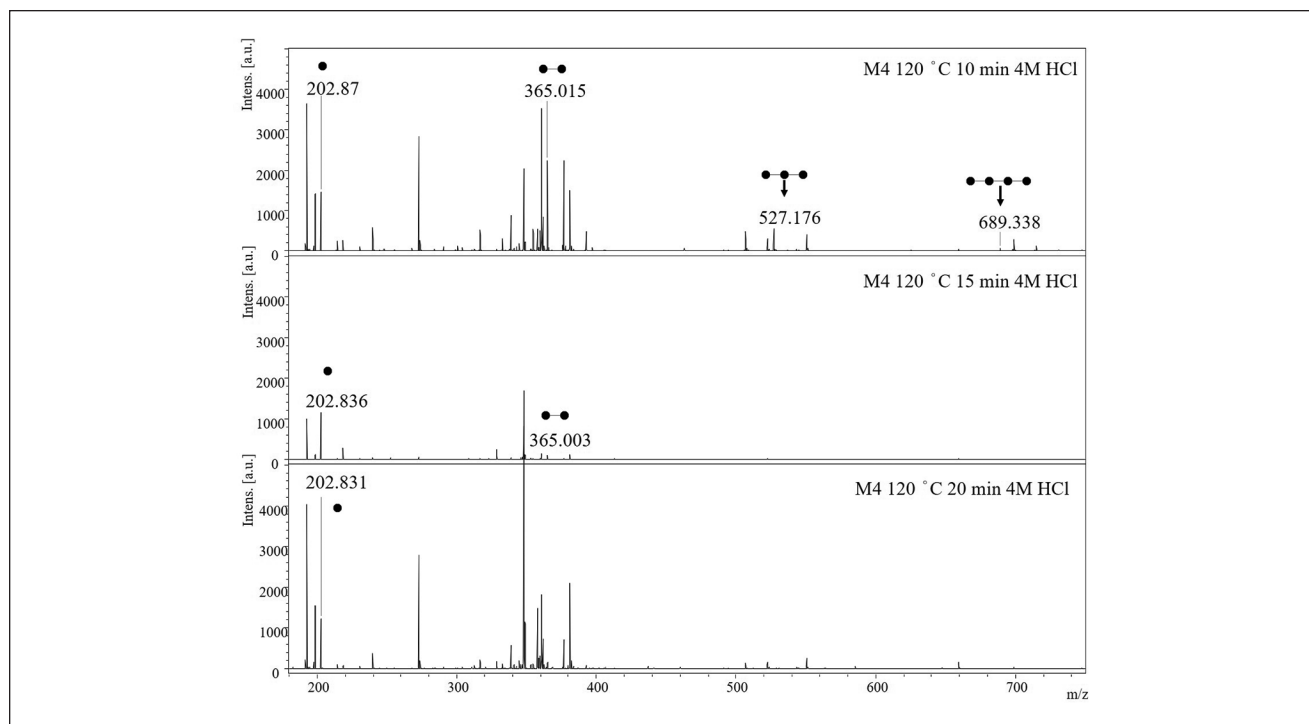
Figure 6. Hydrolysis of maltotetraose (1.0 mg/mL) upon treatment with 4 M HCl at 120 °C for different periods of time (10, 15, and 20 min) in a flow chemistry system. The reaction was monitored by MALDI-TOF-MS measurement.

of maltotriose with 4 M HCl at 25 °C for 10 min in a flow system, 15% of glucose and 30% of maltose were obtained, while 55% of maltotriose remained. The rate of hydrolysis increased as the reaction temperature increased. After the acid treatment at 120 °C for 10 min, 95% of maltotriose was hydrolyzed to give 65% glucose and 30% maltose. Compared with **Supplemental Figure S2**, it seemed that the saccharide of higher size would slow down the hydrolysis rate. **Supplemental Figure S4** compared the hydrolysis efficiency of maltotriose upon treatment with 4 or 2 M HCl at 120 °C for 10 min in a flow system. The hydrolysis of maltotriose apparently decreased in lower concentrations of HCl. Thus, the hydrolysis of higher oligosaccharides, such as maltotetraose, was best conducted with 4 M HCl at 120 °C (**Fig. 6**). After 10 min of reaction time, a mixture of maltotetraose (5%), maltotriose (15%), maltose (50%), and glucose (30%) was obtained according to the MALDI-TOF-MS analysis. Further degradation occurred after longer hydrolysis time (15 and 20 min); only glucose and maltose were observed as the sodiated ions at *m/z* 202 and 365, respectively.

We then investigated the degradation of a disaccharide that contained two different monosaccharide components in the flow chemistry system. Common saccharides (e.g., Glc, Man, and Gal) are hardly distinguished by MS when they have the same molecular weight. High-performance

anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) is often used for direct separation and detection of the saccharide components by elution with a strong base (NaOH).[33–36] In comparison, conventional reverse-phase high-pressure liquid chromatography (HPLC) is more easily accessed to separate the proper derivatives of sugar components, such as sugar-NAIM compounds.[22,37] In addition, HPLC can be linked with MS for the analysis of oligosaccharides with prior derivatization.[37,38] For glycan compositional analysis, those monosaccharides obtained from glycan hydrolysis were recycled into the flow system to generate the sugar-NAIM derivatives, even at low sample loads. The prepared sugar-NAIM derivatives were concentrated by rotary evaporation under reduced pressure and analyzed by LC-MS without further purification.

Taking lactose as an example, the glucose and galactose components were obtained by hydrolysis in a flow chemistry system. After NADA labeling, the Glc-NAIM and Gal-NAIM derivatives were analyzed by LC-MS. The residue was separable on a C18 capillary column and identified by LTQ-FTMS (**Suppl. Fig. S5**). By tagging the NAIM chromophore, Glc-NAIM and Gal-NAIM occurring at the retention times of 13.3 and 13.9 min were easily detected using a UV detector at a wavelength of 330 nm. The NAIM derivatives exhibited higher hydrophobicity than their parent

saccharides to show enhanced MS signals.[24,26] The isobaric isomers Glc-NAIM and Gal-NAIM both showed the protonated ions at m/z 319.

GM3 is a common glycosphingolipid in tissues. The carbohydrate portion (GM3-sugar) is a trisaccharide SA(2α,3)Gal(1β,4)Glc comprising sialic acid, galactose, and glucose. In this study, GM3-sugar (5.0 mg, 8.0 μmol) was hydrolyzed with 4 M HCl at 120 °C for 10 min in a flow system, and the hydrolysate was analyzed by MALDI-TOF-MS (**Fig. 7**). Glc and Gal showed the sodiated molecular ion at m/z 203, whereas the signal at m/z 291 was attributable to SA with elimination of one water molecule. In comparison, the signal at m/z 365 ascribed to the Gal-Glc disaccharide (as the sodiated ion) was much stronger than the signal at m/z 453 attributable to the SA-Gal disaccharide (as the dehydrated ion). This result demonstrated that the sialyl glycoside bond was more susceptible to acid treatment, as expected.

In addition to composition analysis, the lysate of GM3-sugar was concentrated and labeled with NADA in a flow chemistry system to obtain the corresponding NAIM and QXO derivatives (**Fig. 1**). The LC-MS analysis revealed four species of Glc-NAIM, Gal-NAIM, Lac-NAIM, and SA-QXO occurring at 13.54, 13.96, 13.18, and 14.01 min, respectively (**Fig. 8**). Glc-NAIM, Gal-NAIM, and Lac-NAIM displayed the [M + H]$^+$ ions at m/z 319, 319, and 481, respectively, whereas SA-QXO exhibited the [M–H]$^-$ ion at m/z 430. Of the most importance, Lac-NAIM (i.e., Gal-Glc-NAIM), but not Glc-Gal-NAIM, was identified by comparison with the retention time of the authentic sample in the LC diagram (**Fig. 8A**), and the structure was confirmed by ^{1}H-NMR spectroscopic determination (**Suppl. Fig. S6**). Taken together, the results shown in **Figures 7 and 8** led to the conclusion that the Glc moiety is at the reducing end, the SA moiety is at the nonreducing end, and the two moieties are linked with Gal to form the SA-Gal-Glc trisaccharide. This study thus provides an example of carbohydrate sequencing.

Developing a Continuous Protocol for Glycan Hydrolysis and Tandem NADA Tagging in a Flow Chemistry System

We further combined glycan hydrolysis and NADA tagging in a continuous flow system to simplify the procedure for preparation of sugar-NAIM (or QXO) derivatives. An additional peristaltic pump reactor was installed to the Vapourtec E-series Flow Chemistry System (**Fig. 9**). For example, lactose (2.0 mg, 5.8 μmol) was suspended in HOAc (glacial, 2.0 mL) containing a small amount (2 μL) of 12 M HCl and pumped into reactor 1 for hydrolysis. The reaction was performed at 120 °C for 15 min, and the glycan hydrolysate was pumped into reactor 2 for NADA tagging in HOAc
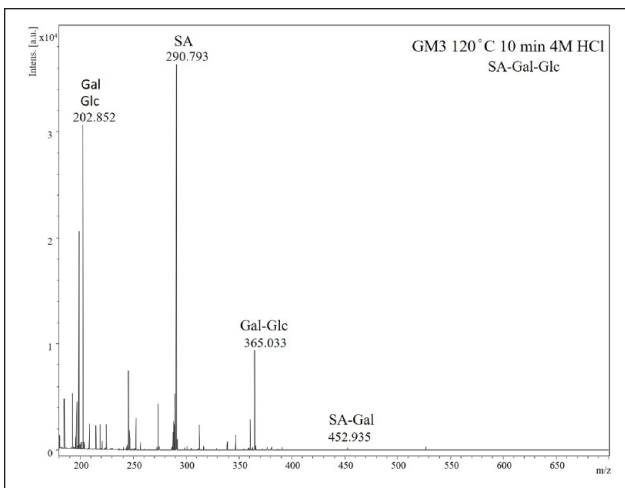

Figure 7. Hydrolysis of SA-Gal-Glc trisaccharide (GM3-sugar, 1.0 mg/mL) with 4 M HCl at 120 °C for 10 min in a flow chemistry system. The reaction was monitored by MALDI-TOF-MS measurement.

solution at 60 °C for 10 min. The final mixture was concentrated by rotary evaporation under reduced pressure to give the sugar-NAIM derivatives, which were directly analyzed by MS and NMR to determine the composition of the glycan precursor.

Prospect for Glycan Structural Analysis

Automated polymer-supported synthesis of oligosaccharides is rapidly progressing.[39–41] Immobilization of complex glycan onto a polymer or solid surfaces can be advanced to structural analysis with the assistance of the flow chemistry system. We have previously demonstrated that an arginine-tagged phenylenediamine can successfully catch tetrasialic acid.[24] We thus propose to modify the surface of a polymer (or solid) with *ortho*-phenylenediamine moieties as depicted in **Figure 10**. Many polymeric and solid materials carrying linkers with terminal amine groups are either commercially available or readily prepared.[42] For example, porous silica beads are treated with 3-aminopropyltriethoxysilane to graft amino functional groups onto their surfaces. The solid support carrying linkers with terminal amine groups will be modified with the *tert*-butoxycarbonyl (Boc)-protected 3,4-diaminobenzoic acid (DAB) via amide bond formation.[24] Then, the DAB-encapsulated solid support can be used to catch the target glycan via the condensation reaction with its terminal aldehyde (or ketoacid) group at the reducing end.

We have previously demonstrated the use of α-amylase, endo-β-1,3-glucanase, and cellulose for specific digestion of maltohexaose, laminarihexaose, and cellohexaose, respectively (**Suppl. Fig. S7**).[43] Northen and coworkers
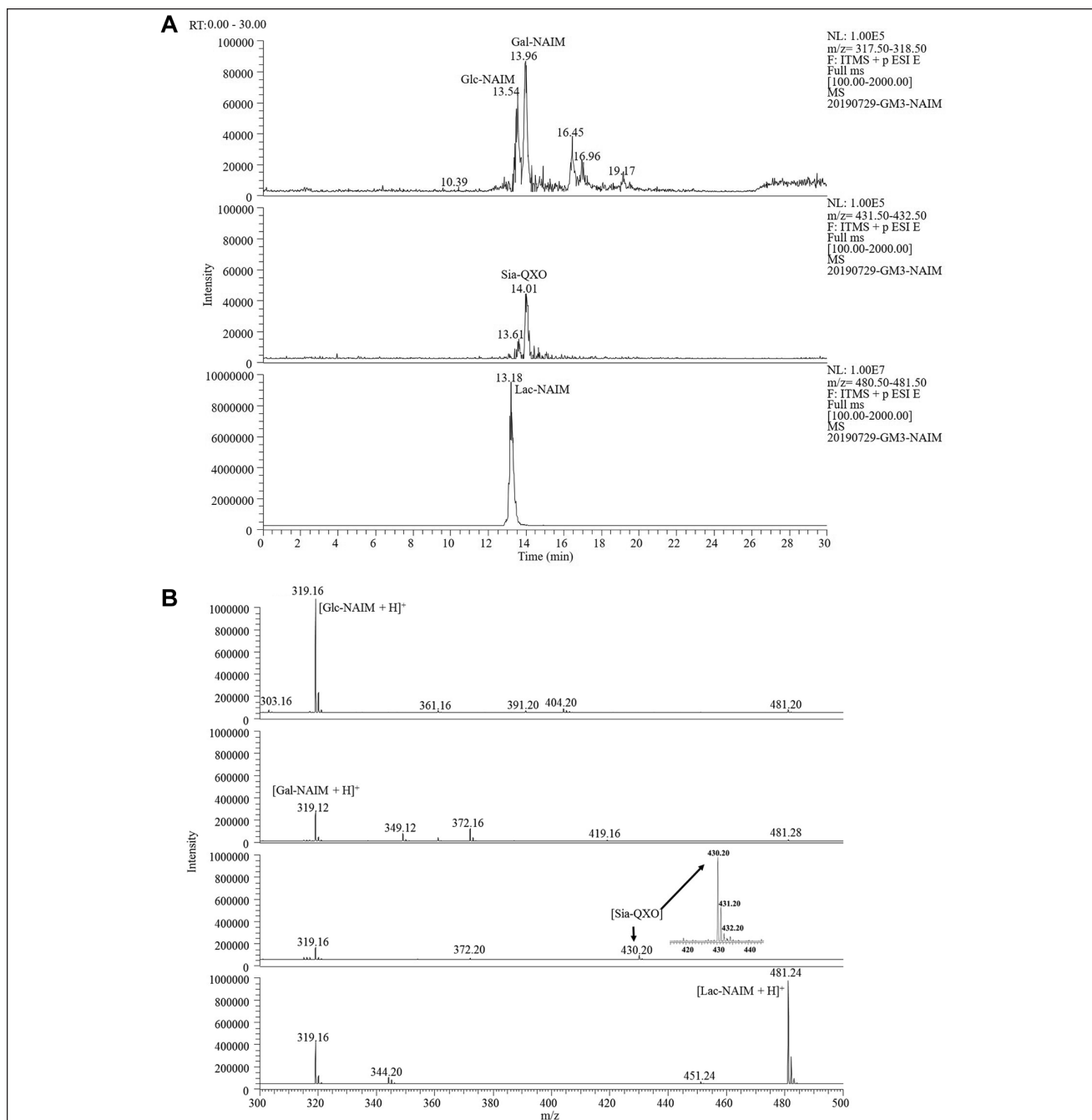
Figure 8. LC-MS analysis of the GM3-sugar hydrolysate after tagging with NADA to the corresponding NAIM and QXO derivatives. The reaction was monitored by LC-MS analysis: (**A**) LC diagram on a C18 capillary column and (**B**) LTQ-FTMS spectra.

have also devised an on-chip enzymatic reaction of galactosidase and sialyltransferase.[44] Therefore, enzymatic digestion (or acidic hydrolysis) of glycan on beads will be feasible to release saccharide components. Different glycosidases can be used to digest a specific type of glycans,[9,42] and the degree of glycoside bond cleavage can be controlled by the reaction conditions. The glycan hydrolysate can be subjected to NADA labeling in a flow system to obtain the corresponding sugar-NAIM (or sugar-QXO) derivatives for compositional analysis. This procedure is also possibly applicable to sequencing the glycan that contains heterosugars.
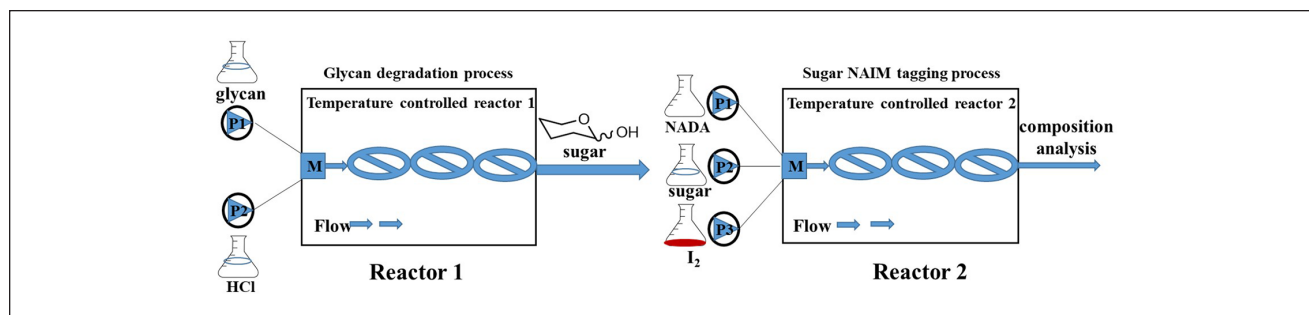
Figure 9. Protocol of a continuous tandem strategy for glycan hydrolysis toward sugar-NAIM (or sugar-QXO) derivatives in a flow chemistry system.
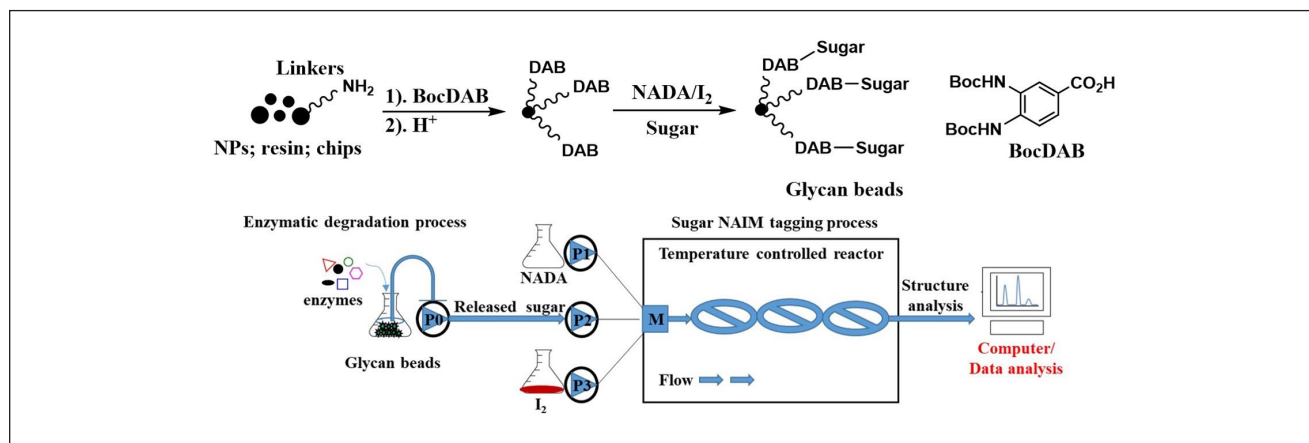

Figure 10. Protocol for glycan structural analysis by preparation of glycan beads for enzymatic cleavage and labeling the released saccharides in a flow chemistry system.

Conclusion

In this study, we demonstrated that glycan hydrolysis and saccharide tagging were accelerated in a flow chemistry system. Aldoses and α-ketoacid-type saccharide components were mixed with NADA and iodine at 60 °C for 10 min to form the light-absorbing sugar-NAIM and sugar-QXO derivatives. This new method improved the structural determination, compositional analysis, and possibly sequencing of the parent glycan by using a combination of LC, MS, and NMR techniques. For example, the heterotrisaccharide GM3-sugar was hydrolyzed in 4 M HCl at 120 °C for 10 min and NADA-labeled in a flow system. Since the product mixture was found to contain Glc-NAIM, Gal-NAIM, Lac-NAIM, and SA-QXO by MALDI-TOF-MS, LC-MS, and ^{1}H-NMR analyses, the results concluded that GM3-sugar has a sequence of SA-Gal-Glc out of six possible permutations. As demonstrated in this study, application of the flow chemistry system for continuous glycan hydrolysis and NADA labeling can assist the existing methods in glycan sequencing. At this moment, we still use micromolar amounts of glycan sample; however, one should be able to conduct this experimental protocol with smaller amounts of glycans when advanced instruments are available. For complete glycan sequencing, one must elucidate the linkage position and anomeric configuration in each monosaccharide component. This is still a challenging task, even though many hurdles have been overcome by using chemical, biological, and instrumental methods in concert.[2,3,9,17]

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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