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Tagging saccharides for signal enhancement in mass spectrometric analysis

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MALDI-MS provides a rapid and sensitive analysis of large biomolecules such as proteins and nucleic acids. However, oligo- and polysaccharides are less sensitive in MS analysis partly due to their neutral and hydrophilic nature to cause low ionization efficiency. In this study, four types of oligosaccharides including aldoses, aminoaldoses, alduronic acids and α-keto acids were modified by appropriate tags at the reducing termini to improve their ionization efficiency. Bradykinin (BK), a vasoactive nonapeptide (RPPGFSPFR), containing two arginine and two phenylalanine residues turned out to be an excellent MS signal enhancer for maltoheptaose, GlcNAc oligomers and oligogalacturonic acids. In the MALDI-TOF-MS analysis using 2,5-dihydroxybenzoic acid (2,5-DHB) as the matrix, the GalA4–BK and GalA5–BK conjugates prepared by reductive amination showed the detection limit at 0.1 fmol, i.e. ∼800-fold enhancement over the unmodified pentagalacturonic acids. The remarkable MS enhancement was attributable to the synergistic effect of the basic arginine residues for high proton affinity and the hydrophobic property phenylalanine residues for facile ionization. A tetrapeptide GFGR(OMe) and an arginine linked phenylenediamine $(H_2N)_2$Ph-R(OMe) were thus designed to act as potent tags of oligosaccharides in MS analysis. Interestingly, concurrent condensation and lactonization of α2,8-linked tetrasialic acid (SA4) was carried out with $(H_2N)_2$Ph-R(OMe) to obtain a quinoxalinone derivative, which showed >200-fold enhancement over unmodified SA4 in the MALDI-TOF-MS analysis.

Supporting information may be found in the online version of this article.

Keywords: MALDI-TOF-MS; signal-enhancing tags; oligosaccharides; bradykinin; phenylenediamine

Introduction

Complex carbohydrates play essential roles in living organisms. Glycosylation is one of the major post-translational modifications of proteins, which can greatly affect their biological properties. Glycoproteins and glycolipids (glycoconjugates) are abundant on the cell surfaces. Glycan parts of glycoconjugates and glycosaminoglycans play important roles in regulating cell–cell recognition, cellular developments and disease status of the individuals. A number of pathogens use host cell surface saccharides for foothold of their invasion.

To understand the exact mechanisms of these important biological reactions, the fundamental information needed is the structures of saccharide involved in these reactions. For such structural analyses, mass spectrometry (MS) is a tremendously valuable tool. MS has been widely used for the structural determination of macromolecules including proteins, oligonucleotides and oligosaccharides.[1,2] MS provides a rapid, sensitive and reliable analysis of mass and structural information for most biological compounds. There are many different ionization techniques, among which electrospray ionization (ESI)[3,4] and matrix-assisted laser desorption/ionization (MALDI)[5–7] are currently most frequently used. ESI-MS provides a mild ionization process to produce charged droplets by nanospray under electric field. MALDI-MS is gaining preference because of its relative ease of operation and feasibility of measurement of large molecules such as proteins. As matrices for MALDI-TOFMS (time-of-flight MS), 3-amino-4-hydroxybenzoic acid (AHB), 2,5-dihydroxybenzoic acid (2,5-DHB), 2,5-DHB/aniline, 2,5-DHB/*N*,*N*-dimethylaniline and 2′,4′,6′-trihydroxyacetophenone (THAP) are frequently used to induce the dominant quasi-molecular ions of saccharides.[8–10] Carbohydrate materials are notoriously poor MS signal generator compared with proteins and peptides, because of their numerous hydrophilic groups. Although stronger signals can be generated if the hydrophilic groups are modified by peracetylation or permethylation, these operations are not suitable in dealing with samples of minute quantities. On the other hand, chemical modification of the reducing group of oligosaccharides with UV-absorbing or fluorescent tags is highly adaptable to minute quantities of samples and has been used for liquid chromatography (HPLC)[11,12] or capillary electrophoresis (CE).[13] It was fortuitously found that

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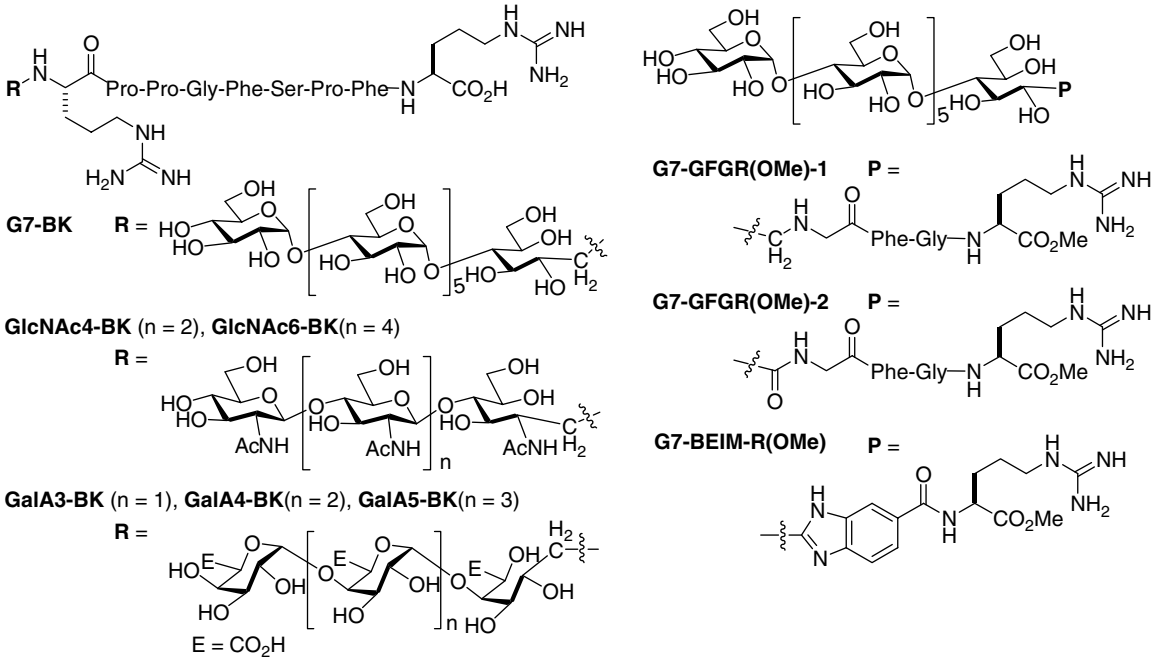

Figure 1. Structures of representative tagged derivatives of oligosaccharides.

the oligosaccharides tagged for HPLC or CE can also enhance MS signals.[11–14] For example, oligosaccharides derivatized with 2-(diethylamino)ethyl 4-aminobenzoate (ABDEAE, also known as procain) show much better sensitivity than the native oligosaccharides in ESI- and MALDI-MS.[15,16] Broberg and coworkers have modified oligosaccharides by reductive amination with benzylamine, followed by *N*,*N*-dimethylation with methyl iodide, to prepare positively charged derivatives, which could be detected by MALDI-TOF-MS with higher sensitivity than the unmodified oligosaccharides.[17] A recent report compares a number of such reagents for improvement of MALDI-MS signal generation.[18]

Derivatization of saccharides with hydrazide[19,20] was reported to have some advantage over the reductive amination because hydrazone formation without reduction of the C═N bond can be easily attained and no extensive clean-up of the hydrazone products was needed before MS analysis. For instance, derivatization of oligosaccharides with $[Me_3NCH_2CONHNH_2]^+Cl^-$ (Girard's T reagent) introduces a cationic moiety to increase the MS ionization efficiency.[21] Oligosaccharides have been elaborated with the hydrazides that carry hydrophobic and charged residues, e.g. quaternary ammonium, pyridinium and guanidino groups, to promote their ionization efficiency in MALDI-TOF-MS.[22,23]

Oxime formation is another popular method for saccharide derivatization. An aminooxyacetyl peptide, NH_2-OCH_2CO-KLEEQRPERVKG, containing four basic amino acid residues has been utilized to modify oligosaccharides to increase the MS response.[24] Nishimura and coworkers have developed a specific chemical ligation between reducing sugars and amino-oxy functionalized polymer-based particles to enable rapid and highly selective isolation of oligosaccharides,[25,26] which are subsequently released from polymer supports by treatment with alkoxyamine (e.g. benzyloxyamine) to achieve sample concentration of minute amounts of glycan samples and to obtain better overall detection sensitivity in MALDI-TOF-MS.

Our recent study[27–29] reveals that many saccharides, including maltoheptaose, pullulan and the glucan from *Ganoderma lucidum*, are easily converted to their corresponding naphthimidazole (NAIM) derivatives by an iodine-promoted oxidative condensation. These NAIM-labeled saccharides show high ionization efficiency in MALDI-TOF-MS.

It is known that the behaviors of peptides in MALDI-MS are highly dependent on the composition of amino acid residues.[30,31] In particular, the presence of arginine would facilitate protonation to enhance the signals of such arginine-containing peptide in MALDI-MS. This 'arginine effect' may be accounted for the outstanding gas-phase basicity of the guanidino side chain.[30,32,33] In addition to basic arginine, the hydrophobic amino acid residues (e.g. phenylalanine, tyrosine and leucine) may also improve the MALDI-MS signal intensity of peptides. For example, Rice and coworkers have converted oligosaccharides to the corresponding glycosylamines with NH_4HCO_3, which was then treated with *N*-*tert*-butoxycarbonyl-L-tyrosine to give the tyrosine-tagged glycans.[34] The presence of phenol chromophore in tyrosinamide facilitates the HPLC purification of oligosaccharides, and the aromatic hydrophobic property of tyrosinamide benefits the MALDI-MS analysis.[34,35] Siuzdak and coworkers have devised an on-chip enzymatic activity assay by nanostructure-initiator MS.[36] For example, lactose (an enzyme substrate) was attached to a fluorous tag for noncovalent immobilization on the fluorous liquid-coated chip. The enzymatic reactions of galactosidase or sialyltransferase were then performed on chip, followed by surface-washing, and direct MS measurements were pursued to determine the enzymatic activities. It has been shown that incorporation of an arginine residue into the fluorous tag can considerably increase the MS detectability.[36]

Though modification of saccharides with appropriate tags has proved to enhance ionization efficiency in MS, the detailed mechanism and key factors are not fully investigated. Towards such studies, we herein report systematic MALDI-TOF-MS analyses of tagged saccharide derivatives using different ligation methods (Fig. 1). In one approach, bradykinin (BK) and the related peptides were attached to saccharides for MS detection because peptides

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are known to have better ionization efficiency. Under the best conditions, the enhancement factor can reach near a thousandfold. In addition, other tags carrying charged or aromatic moieties were also investigated. Interestingly, modifications of oligosaccharides with aromatic sulfonic acids rendered excellent MS signals in negative mode.

Experimental

General

All chemicals and solvents were of analytical grade. Matrices of 2,5-DHB and THAP were purchased from Sigma (USA). Amine regents, iodine, acetic acid and *o*-phenylenediamine were purchased from Merck (USA) and were used without further purification. Sodium cyanoborohydride ($NaBH_3CN$), borane–pyridine reagent and maltoheptaose (G7) were purchased from Sigma–Aldrich (USA). The *tetra*-acetyl chitotetraose (GlcNAc4) and hexa-acetyl chitohexaose (GlcNAc6) were purchased from Megazyme (Ireland). The α1–4-trimer, tetramer and pentamer of D-galacturonic acid (GalA3, GalA4 and GalA5) were isolated from pectin.[37] *N*-Acetylneuraminic acid (α2–8) tetramer (SA4) was purchased from Nacalai Tesque (Japan). BK (nonapeptide RPPGFSPFR) was purchased from American Peptide Company. Partial peptides of BK were prepared according to the conventional methods either by solution-phase or solid-phase procedures.

Procedures for reductive amination of maltoheptaose and other saccharides

A mixture of saccharide (1–10 µmol), amine reagent (1.1 equiv) and $NaBH_3CN$ (1.1 equiv) in HOAc/DMSO solution (1 : 4, 1.0 ml) was stirred at 30–60 °C for 12–16 h to furnish a complete conversion of saccharide. The mixture was quenched by EtOH (0.5 ml) and then concentrated under reduced pressure. The residue was purified by size-exclusion chromatography on a LH-20 column with 0.1 M AcOH aqueous solution (sulfuric acid–phenol and visualized at 490-nm absorption) to give the reductive amination product for MS analysis.

Alternatively, borane–pyridine was used as the reducing agent in reductive amination of saccharides. Briefly, a mixture of saccharide (10 µmol), BK (or the peptide fragments, 1 µmol) and borane–pyridine (40 µmol) in EtOH/H_2O solution (v/v = 1 : 2, 1.5 ml) was stirred at room temperature for 24 h. The resulting solution was concentrated under reduced pressure and purified by G-25 column to give the saccharide–BK conjugates for MS analysis.

Procedures for oxidative amidation of maltoheptaose (G7)[38]

Iodine (1.2 equiv) was added to a stirring solution of maltoheptaose (10 µmol) and K_2CO_3 (1.5 equiv) in anhydrous MeOH (5 ml) at 40 °C under an atmosphere of nitrogen. The mixture was stirred for 0.5 h until the aldose was completely consumed as indicated by TLC (Acetone/EtOAc/H_2O/AcOH = 60 : 30 : 35 : 1, UV visualization) analysis. A trifluoroacetate salt of peptide methyl ester (1.0 equiv) and K_2CO_3 (1.0 equiv) were added, and the mixture was stirred at 40 °C for 12–16 h until the intermediate product of the first step was completely consumed as indicated by TLC analysis. The mixture was concentrated under reduced pressure to give a crude product, which was subsequently purified by reversed-phase chromatography (C18, 0–100% MeOH in water, sulfuric acid–phenol, visualized at 490-nm absorption) to produce the desired compound of aldonamide.

Oxidative condensation of maltoheptaose with *o*-phenylenediamine tag

A solution of maltoheptaose (10.0 mg, 9 µmol) in AcOH/H_2O (v/v = 10 : 1, 1.5 ml) was treated with iodine (2.0 mg, 8 µmol) and an *o*-phenylenediamine tag ($(H_2N)_2Ph$–R(OMe), 3.8 mg, 10 µmol). The mixture was stirred at room temperature for 18 h, concentrated under reduced pressure, diluted with water and triturated with EtOAc. The organic layer was removed, and the residue was concentrated *in vacuo* to give the crude product of aldo-benzimidazole, which was subsequently purified by reversed-phase chromatography (C18, 0–5% MeOH in water, sulfuric acid–phenol, visualized at 490-nm absorption) to give the desired bemzimidazole derivative (5.0 mg, 40% yield).

Concurrent condensation of lactonization of tetrasialic acid with *o*-phenylenediamine tag

A solution of α2,8-linked tetrasialic acid (5.0 mg, 4 µmol) in AcOH/H_2O (v/v = 4 : 1, 300 µl) was treated with an *o*-phenylenediamine tag [$(H_2N)_2Ph$–R(OMe), 2.0 mg, 5 µmol]. The mixture was stirred at room temperature for 20 h, and the residue was concentrated *in vacuo* to give a practically pure product of aldo-quinoxalinone with concurrent lactonization, which was directly subject to MALDI-MS analysis. Further purification could be done by dialysis with 100–500 D CE membrane to remove the residual reagents.

MALDI-TOF-MS analysis

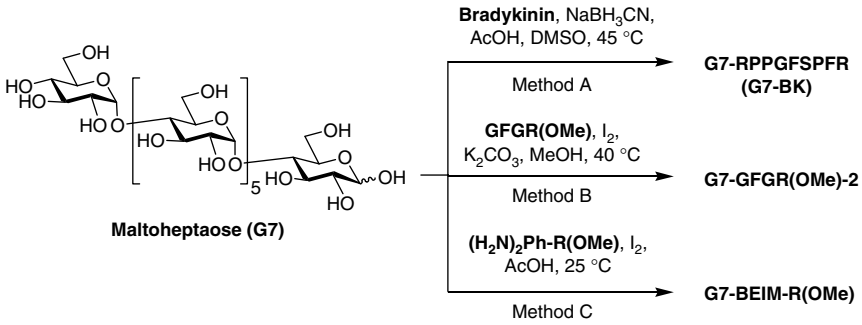
The sample of tagged saccharide with a fixed amount (100 nmol) of matrix 2,5-DHB was loaded by dried-droplet method or by vacuum drying process. For MALDI-TOF-MS analysis, a Voyager Elite (Applied Biosystems, Foster City, CA, USA) was used. The accelerating voltage was set at 20 kV in either positive or negative ion mode. Typically, for each (tagged) oligosaccharide, 80 spots are randomly measured by MALDI-TOF-MS, without discrimination of good or bad shots, and an average intensity of MS signal is taken for the calculation of the detection limit. Laser energy (80%) per pulse was calibrated with a laser power meter (PEM 101, Laser Technik, Berlin, Germany) so that laser fluence could be precisely measured. The delayed extraction time was adjusted from 100 to 500 ns. The grid voltage was set up as 95% of the accelerating voltage; the guide wire 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/cm^2. The pressure inside of the flight tube was always kept between 10^{-7} and 10^{-6} Torr.

A reflectron TOF is also tested in our preliminary study. The corresponding signals in the reflectron and linear modes show no significant difference. Since high resolution is not a focus of this work, only linear mode is operated and reported for the MALDI-TOF-MS measurements.

Results and Discussion

Derivatization of saccharides with BK and other peptides

BK is a vasoactive nonapeptide (RPPGFSPFR) with a molecular weight of 1060 Da. The BK derivatives of saccharides are expected to improve the MS ionization efficiency due to the effect of two basic (arginine, R) and two aromatic (phenylalanine, F) residues. For comparison, the BK fragments RPP (BK_{1-3}), RPPGF (BK_{1-5}),



Scheme 1. Derivatization of maltoheptaose (G7) by reductive amination (method A), oxidative amidation (method B) and oxidative condensation (method C).

Table 1. MALDI-TOF-MS analyses of maltoheptaose (G7) and the peptide tagged derivatives using 2,5-DHB as the matrix[a]

Entry	Peptide tags	Tagging methods[b]	Saccharide derivatives	Molecular ion (*m/z*)	Detection limits (fmol)[c]	Enhancement (fold)[d]
1			G7	$[M + Na]^+$ (1175)	54.6 ± 14.9	1
2	BK[e]	A	G7-RPPGFSPFR	$[M + H]^+$ (2197)	1.2 ± 0.6	45
3	KKKKKK	A	G7-K6	$[M + H]^+$ (1923)	83.2 ± 68.4	0.7
4	RRRRRR	A	G7-R6	$[M + H]^+$ (2091)	4.1 ± 3.2	13
5	HHHHHH	A	G7-H6	$[M + H]^+$ (1977)	21.6 ± 3.6	2.5
6	HHH	A	G7-H3	$[M + H]^+$ (1566)	96.5 ± 13.8	0.6
7	GF(OMe)	B	G7-GF(OMe)	$[M + H]^+$ (1387)	31.0 ± 16.0	1.8
8	GR(OMe)	B	G7-GR(OMe)	$[M + H]^+$ (1396)	2.8 ± 2.0	20
9	GFGR(OMe)	A	G7-GFGR(OMe)-1	$[M + H]^+$ (1586)	0.2 ± 0.1	273
10	GFGR(OMe)	B	G7-GFGR(OMe)-2	$[M + H]^+$ (1600)	1.1 ± 0.2	50
11	$(H_2N)_2$Ph–R(OMe)	C	G7-BEIM-R(OMe)	$[M + H]^+$ (1455)	0.4 ± 0.2	137

[a] The saccharide derivative was loaded with 100 nmol of 2,5-DHB matrix.
[b] Method A: reductive amination in the presence of $NaBH_3CN$. Method B: oxidative amidation in the presence of I_2. Method C: oxidative condensation with diamine in the presence of I_2.
[c] The detection limit is calculated from equation: sample (mole) ÷ observed *S/N* ratio × 3 (minimum *S/N*).
[d] Ratio of the detection limits of maltoheptaose to the tagged derivative.
[e] The MS detection limit of the free peptide BK (as the $[M + H]^+$ ion) is ~400-fold lower than the unmodified maltoheptaose (as the $[M + Na]^+$ ion).

RPPGFSP (BK_{1-7}) and PPGFSPFR (BK_{2-9}) as well as hexalysine (K6), hexaarginine (R6), hexahistidine (H6) and trihistidine (H3) were prepared and used to tag the saccharides by reductive amination (method A, Scheme 1).

Table 1 shows the MS enhancement of maltoheptaose (G7) derivatives using the peptide tags. While the unmodified G7 (MW 1152 Da) gave a major peak at *m/z* 1175 Da corresponding to the sodiated ion $[M + Na]^+$, the BK-tagged G7 showed the major peak of protonated ion $[M + H]^+$ at *m/z* 2197 Da, presumably due to the high proton affinity of the basic guanidino groups in BK. The detection limits for the MALDI-TOF-MS analyses of G7 and G7-BK were determined to be 54.6 and 1.2 fmol, respectively, according to the following equation: Sample (mole) ÷ observed *S/N* ratio × 3 (minimum *S/N*).

Although the molecular weight of the G7-BK derivative became nearly twice the original molecular weight of G7, it still improved the sensitivity of G7 detection by 45-fold. Attachment of BK and truncated BK fragments to G7 appeared to increase the proton affinity, and thus the ionization efficiency in MALDI-MS. As seen in Table 1, the arginine residue consistently showed better enhancing effect, so that hexaarginine tag was superior to hexalysine, hexahistidine and trihistidine (entries 3–6, Table 1). It should be noted that none of the K6, H6 and H3 tags was as good as R6, and none of these tags was as effective as BK itself. Presumably, a proper mix of hydrophobic residue and strongly cationic residue, such as that found in BK, can yield the best result. The dramatic signal enhancement by attaching GFGR(OMe) may be a proof of this notion (entry 9, Table 1).

Maltoheptaose was also labeled with the methyl esters of synthetic peptides GF(OMe), GR(OMe) and GFGR(OMe) by oxidative amidation (method B, Scheme 1).[38] Comparison of entries 7 and 8 (Table 1) shows that the arginine residue appeared to have greater enhancement effect than the phenylalanine residue. The oxidative amidation tagging with glycine–arginine dipeptide showed 20-fold increase of MS intensity over the unmodified G7 (entry 8, Table 1). Modification of G7 by oxidative amidation with a tetrapeptide tag GFGR(OMe) further improved the MS desorption/ionization efficiency, showing 50-fold enhancement (entry 10, Table 1). The oxidative amidation establishes a neutral amide bond with saccharides, whereas reductive amination introduces a basic amino group to saccharides. It is not surprising that the reductive amination derivative (entry 9, Table 1) exhibited even better improvement (273-fold) than that obtained by oxidative amidation (50-fold).

In view of our previous success in using 2,3-naphthalenediamine for conversion of saccharides to the naphthylimizole (NAIM) derivatives that exhibit high ionization efficiency,[29] we also prepared a phenylenediamine–arginine tag, $(H_2N)_2$Ph-R(OMe),

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Table 2. Positive mode MALDI-TOF-MS analyses of maltoheptaose derivatives using 2,5-DHB as the matrix[a]

Entry	Amine tags[b]	Tagging method[c]	Saccharide derivatives	Molecular ion (*m/z*)	Detection limits (fmol)[d]	Enhancement (fold)[e]
1			G7	$[M + Na]^+$ (1175)	54.6 ± 14.9	1
2	2AB	A	G7-2AB	$[M + Na]^+$ (1295)	7.5 ± 1.8	7.3
3	AMC	A	G7-AMC	$[M + Na]^+$ (1334)	5.0 ± 1.3	11
4	2AA	A	G7-2AA	$[M + Na]^+$ (1296)	4.6 ± 0.6	12
5	3AA	A	G7-3AA	$[M + Na]^+$ (1296)	3.8 ± 1.2	14
6	4AA	A	G7-4AA	$[M + Na]^+$ (1296)	3.8 ± 0.7	14
7	DAP	A	G7-DAP	$[M + H]^+$ (1246)	12.9 ± 4.7	4.2
8	PRC	A	G7-PRC	$[M + H]^+$ (1373)	4.6 ± 1.7	12
9	AMT	A	G7-AMT	$[M + H]^+$ (1240)	13.3 ± 4.8	4.1
10	PMA	B	G7-PMA	$[M + Na]^+$ (1404)	5.7 ± 1.3	9.6

[a] MALDI-TOF-MS was measured by loading the saccharide derivative with 100 nmol of 2,5-DHB matrix.
[b] 2AB, 2-aminobenzamide; AMC, 7-amino-4-methylcoumarin; 2AA, 2-aminobenzoic acid; 3AA, 3-aminobenzoic acid; 4AA, 4-aminobenzoic acid; DAP, 2,6-diaminopyridine; PRC, procain-HCl [2-(diethylamino)ethyl 4-aminobenzoate hydrochloride)]; AMT, 2-amino-*N*,*N*,*N*-trimethylethanaminium, PMA, 1-pyrenemethylamine.
[c] Method A: reductive amination in the presence of $NaBH_3CN$ or borane–pyridine complex. Method B: oxidative amidation in the presence of I_2.
[d] The detection limit is calculated from equation: sample (mole) ÷ observed *S*/*N* ratio × 3 (minimum *S*/*N*).
[e] Ratio of the detection limits of maltoheptaose to the tagged derivative.

having an arginine residue linked to the core structure of *o*-phenylenediamine. The phenylenediamine moiety acted as the main reaction site to react with G7 at the reducing end to give a derivative G7-BEIM-R(OMe) (method C, Scheme 1), in which the arginine moiety and the newly formed hydrophobic benzimidazole moiety were expected to improve the MS detection in a synergistic manner. Indeed, the protonated signal of G7-BEIM-R(OMe) was 137-fold greater than the unmodified G7 in the positive mode of MALDI-MS (entry 11, Table 1).

To compare the sensitivity of maltoheptaose with its tagged derivatives, the MALDI-TOF-MS measurements for mixed oligosaccharide components in different ratios were also conducted. The tagged saccharides consistently showed higher signals than unmodified G7 in these experiments (Figs S3–S5, Supporting Information). When a tagged derivative exhibits high MS enhancement over G7, the MS signal of G7 may not be observed in the sample containing low quantity (<1 pmol) of each component, whereas the tagged derivative still shows distinct signal. Though the detection limits may be not always linear in the range of measurements, these values are still useful to reflect the extent of MS signal enhancement (Fig. S6, Supporting Information).

In our previous study,[10] THAP was found to be a good matrix for the MALDI-MS analysis of polysaccharides, demonstrating the detection limit of G7 as 6.0 ± 2.0 fmol. The detection limit was slightly improved to 3.2 fmol in the G7-GF(OMe) derivative, whereas the sensitivity decreased for G7-BK, G7-R6, G7-GR(OMe) and G7-GFGR(OMe) derivatives, despite bearing arginine residues. These results indicate that ionization efficiency could change when different matrices were used.

Derivatization of saccharides with aromatic and aliphatic amines

Increasing the hydrophobicity of glycans typically improves the ionization efficiency, though the reason is unclear.[14] Most aromatic compounds are good candidates for tagging saccharides because such derivatives not only increase hydrophobicity but also are easily detected by their optical properties (e.g. UV absorptions and fluorescence emission) during chromatography or electrophoresis. Thus, G7 was modified with a variety of aromatic and aliphatic amines (see Fig. S1, Supporting Information for their structures). Table 2 shows that these G7 derivatives generally have better ionization efficiency than the unmodified G7 by 4- to 14-folds in the MALDI-MS using 2,5-DHB as the matrix.

Neutral saccharides usually give low intensity in the negative mode of MS even by using 3-aminoquinoline (3-AQ) as the matrix of choice.[39] The ionization efficiency was greatly improved by derivatization of saccharides via reductive amination, especially using the aromatic amines containing carboxyl or sulfoxyl groups (Table 3). For example, reductive amination of G7 with 2-amino-1-naphthalenesulfonic acid (ANS), yielding G7-ANS, showed 175-fold enhancement in the negative mode MALDI-MS using 3-AQ as the matrix (entry 2, Table 3). Under similar conditions, G7-AA showed 88-fold enhancement of the negative ion (entry 7, Table 3).

Mass spectrometric analysis of *N*-acetylglucosamine oligomers by derivatization with BK

Chitin is a long-chain polymer consisted of *N*-acetylglucosamine units in β1,4-linkage. The detection limits of *N*-acetylglucosamine tetramer (GlcNAc4) and hexamer (GlcNAc6) in positive mode MALDI-TOF-MS were found to be 2.5 and 17.9 fmol, respectively (Table 4). The sensitivity was somewhat improved by conjugation with BK, giving fourfold enhancement in GlcNAc4-BK and threefold enhancement in GlcNAc6-BK. On the contrary, GlcNAc4 and GlcNAc6 are much less sensitive in the negative mode MALDI-TOF-MS using 2,5-DHB as the matrix. They showed the predominant ions $[M + DHB - H]^-$ with the detection limits in the range of 1 pmol. On conjugation with BK by reductive amination, GlcNAc4-BK and GlcNAc6-BK produced the deprotonated ions $[M - H]^-$ with detection limits of 8.3 and 78.1 fmol, corresponding to 134- and 18-fold enhancements compared with the unmodified *N*-acetylglucosamine oligomers.

Mass spectrometric analysis of oligogalacturonic acid by derivatization with BK

The partial hydrolysis of polygalacturonic acid regions in pectin releases a series of oligogalacturonic acids, D-Gal*p*A-[(α1–4)-

Table 3. Negative mode MALDI-TOF-MS analyses of maltoheptaose derivatives using 3-aminoquinoline as the matrix[a]

Entry	Amine tags[b]	Saccharide derivatives	Molecular ion (*m/z*)	Detection limits (fmol)[c]	Enhancement (fold)[d]
1		G7	$[M-H]^-$ (1151)	526 ± 149	1
2	ANS	G7-ANS	$[M-H]^-$ (1358)	3 ± 1.8	175
3	A13S	G7-A13S	$[M-H]^-$ (1438)	7 ± 1.8	75
4	A27S	G7-A27S	$[M-H]^-$ (1438)	7 ± 5.0	75
5	2AB	G7-2AB	$[M-H]^-$ (1271)	44 ± 17.1	12
6	AMC	G7-AMC	$[M-H]^-$ (1310)	41 ± 11.7	13
7	2AA	G7-2AA	$[M-H]^-$ (1272)	6 ± 1.3	88
8	3AA	G7-3AA	$[M-H]^-$ (1272)	7 ± 3.6	75
9	4AA	G7-4AA	$[M-H]^-$ (1272)	9 ± 1.2	58
10	PRC	G7-PRC	$[M-H]^-$ (1371)	40 ± 5.9	13

[a] Reductive amination was conducted in the presence of $NaBH_3CN$ or borane–pyridine (method A). MALDI-TOF-MS was measured by loading the saccharide derivative with 100 nmol of 3-aminoquinoline matrix.
[b] ANS, 2-amino-1-naphthalenesulfonic acid; A13S, 7-amino-1,3-naphthalenedisulfonic acid; A27S, 7-amino-2,7-naphthalenedisulfonic acid; and see the footnote in Table 2 for abbreviations of other amine reagents.
[c] The detection limit is calculated from equation: sample (mole) ÷ observed *S*/*N* ratio × 3 (minimum *S*/*N*).
[d] Ratio of the detection limits of maltoheptaose to the tagged derivative.

Table 4. MALDI-TOF-MS analyses of *N*-acetylglucosamine oligomers and their bradykinin conjugates using 2,5-DHB as the matrix[a]

	Positive mode			Negative mode		
Compound	Molecular ion (*m/z*)	Detection limits (fmol)[b]	Enhance (fold)[c]	Molecular ion (*m/z*)	Detection limits (fmol)[b]	Enhance (fold)[c]
GlcNAc4	$[M+Na]^+$ (853)	2.5 ± 0.6	1	$[M+DHB-H]^-$ (903)	1112.1 ± 156.7	1
GlcNAc4-BK	$[M+H]^+$ (1875)	0.7 ± 0.2	4	$[M-H]^-$ (1873)	8.3 ± 2.8	134
GlcNAc6	$[M+Na]^+$ (1259)	17.9 ± 5.9	1	$[M+DHB-H]^-$ (1389)	1412.6 ± 285.8	1
GlcNAc6-BK	$[M+H]^+$ (2281)	6.3 ± 1.9	3	$[M-H]^-$ (2279)	78.1 ± 32.1	181

[a] Bradykinin conjugates of *N*-acetylglucosamine oligomers were prepared by reductive amination in the presence of $NaBH_3CN$ (method A). MALDI-TOF-MS was measured by loading the saccharide derivative with 100 nmol of 2,5-DHB matrix using dried-droplet method.
[b] The detection limit is calculated from the equation: sample (mole) ÷ observed *S*/*N* ratio × 3 (minimum *S*/*N*).
[c] Ratio of the detection limits of maltoheptaose to the tagged derivative.

D-Gal*p*A]$_n$-(α1–4)-D-Gal*p*A, and their partially methyl-esterified derivatives. Identification of their structures would give better understanding of their biological roles such as the regulation of physiological responses in plants. Chromatographic techniques,[40] electrophoresis[13] and MS[41,42] have been applied to analyze galacturonic acid oligomers. The MS method has the advantage of rapid analysis with only small amount of sample. The negative-ion mode ESI-MS of oligogalacturonic acids gives predominantly the deprotonated molecules $[M-H]^-$ along with the sodiated adducts and some satellite ions.[43] A direct injection ESI-MS technique has been successful in analysis of a mixture of mono-, di- and trigalacturonic acids under optimized conditions.[44] However, large molecules of oligogalacturonic acids usually exhibit a broad charge-state distribution and cation adduction, which lower signal intensity complicate the analysis in ESI-MS. It is suggested that the MALDI-TOF-MS analysis of partially methyl-esterified oligogalacturonic acid is best performed in the negative mode by using THAP as the matrix.[42]

In this study, the sensitivity of unmodified oligogalacturonic acids was low in the MALDI-MS by loading the samples using dry-droplet method. Using 2,5-DHB as the matrix, the major ions of sodiated tri-, tetra- and pentagalacturonic acids could be detected only with large quantities (>1.9 pmol in the positive mode and >3.3 pmol in the negative mode, respectively). The ionization efficiency could be improved by loading the samples using vacuum drying process or using THAP as the matrix. However, the MS detection limit could only reach ~75 fmol even under the optimized condition. In a sharp contrast, the BK conjugates of oligogalacturonic acids derived by reductive amination showed remarkably high sensitivity in the MALDI-TOF-MS (Table 5). In the positive mode, the conjugates yielded prominently the protonated dehydrative molecular ions $[M-H_2O+H]^+$ along with substantial signal of $[M+H]^+$ ions. The detection limits for the $[M-H_2O+H]^+$ ions of GalA3–BK, GalA4–BK and GalA5–BK were determined to be 0.2, 0.1 and 0.1 fmol, respectively. In comparison with the MS enhancement of G7-BK conjugate (45-fold) derived from the neutral maltoheptaose, the conjugation of BK to GalA oligomers exhibited much higher enhancement (365- to 810-folds) in the positive mode of MALDI-MS. A possible reason was attributed to the neutralization of the galacturonic acids by the basic arginine residues to render better ionization efficiency.

In the negative mode, the major ions of GalA3–BK, GalA4–BK and GalA5–BK conjugates were derived from deprotonation of the dehydration molecules $[M-H_2O-H]^-$, which were detected, respectively, at the limits of 1.8, 1.5 and 0.4 fmol. Thus, modification of tri-, tetra- and pentagalacturonic acids by reductive amination with BK rendered effective ionization to enhance the MALDI-MS measurements in both positive and negative modes.

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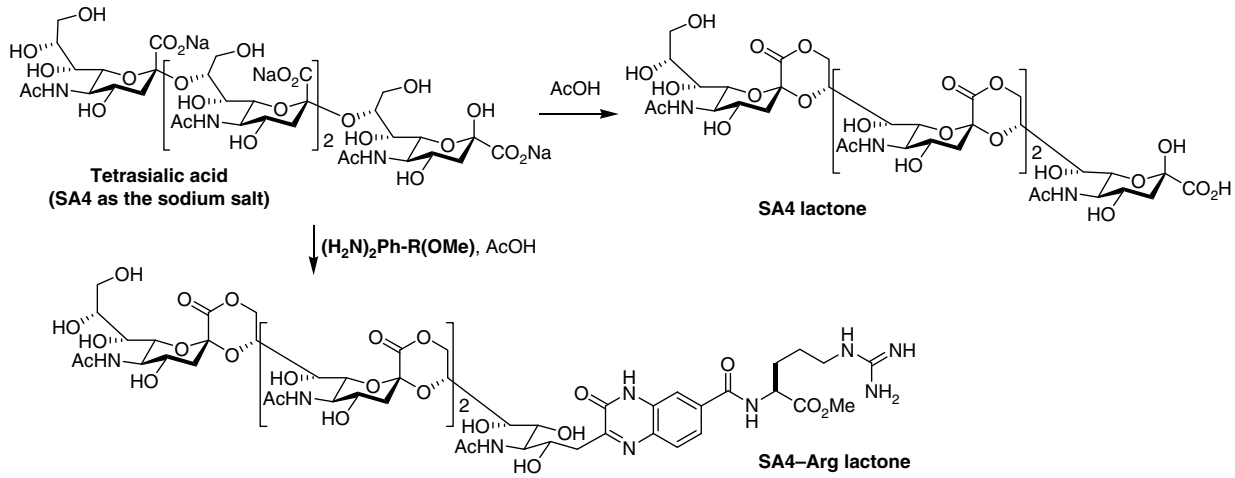
Table 5. MALDI-TOF-MS analyses of oligogalacturonic acids and their bradykinin conjugates using 2,5-DHB as the matrix[a]

Compound	Positive mode			Negative mode		
	Molecular ion (*m/z*)	Detection limits (fmol)[b]	Enhance (fold)[c]	Molecular ion (*m/z*)	Detection limits (fmol)[b]	Enhance (fold)[c]
GalA3[d]	$[M + Na]^+$ (569)	73 ± 10	1	$[M - H]^-$ (545)	442 ± 29	1
GalA3-BK[e]	$[M - H_2O + H]^+$ (1573)	0.2 ± 0.03	365	$[M - H_2O - H]^-$ (1571)	1.8 ± 0.4	246
GalA4[d]	$[M + Na]^+$ (745)	81 ± 8	1	$[M - H]^-$ (721)	306 ± 30	1
GalA4-BK[e]	$[M - H_2O + H]^+$ (1749)	0.1 ± 0.02	810	$[M - H_2O - H]^-$ (1747)	1.5 ± 0.4	204
GalA5[d]	$[M + Na]^+$ (921)	74 ± 10	1	$[M - H]^-$ (897)	121 ± 20	1
GalA5-BK[e]	$[M - H_2O + H]^+$ (1925)	0.1 ± 0.05	740	$[M - H_2O - H]^-$ (1923)	0.4 ± 0.1	302

[a] Bradykinin conjugates of oligogalacturonic acids were prepared by reductive amination in the presence of $NaBH_3CN$ (method A). MALDI-MS was measured by loading the saccharide derivative with 100 nmol of 2,5-DHB matrix using vacuum drying process.
[b] The detection limit is calculated from the equation: sample (mole) ÷ observed *S/N* ratio × 3 (minimum *S/N*).
[c] Ratio of the detection limits of maltoheptaose to the tagged derivative.
[d] Unmodified saccharides were loaded by vacuum drying process. When the sample was loaded by dry-droplet method, the sodiated ions of GalA3, GalA4 and GalA5 were detected at the limits of 1905 ± 1470, 2296 ± 830 and 2283 ± 1087 fmol in the positive mode, whereas the deprotonated ions were detected at the limits of 3781 ± 1565, 3733 ± 1455 and 3353 ± 672 fmol in the negative mode.
[e] The conjugates were loaded by vacuum drying process. When the sample was loaded by dry-droplet method, the protonated ions of GalA3–BK, GalA4–BK and GalA5–BK were detected at the limits of 0.3 ± 0.1, 0.1 ± 0.01 and 0.03 ± 0.01 fmol in the positive mode, whereas the deprotonated ions were detected at the limits of 1.1 ± 0.2, 1.7 ± 0.6 and 0.1 ± 0.03 fmol in the negative mode.



Scheme 2. Derivatization of 2,8-linked tetrasialic acid (SA4) with a phenylenediamine–arginine tag to the quinoxalinone derivative with concurrent lactonization.

Mass spectrometric analysis of oligosialic acid by derivatization with a phenylenediamine–arginine tag

MS detection of oligo- and polysialic acid (oligo/polySia) is a longstanding problem because they contain a high quantity of carboxylic acid groups. The reaction of sialic acid (*N*-acetylneuraminic acid, Neu5Ac) with 1,2-diamino-4,5-methylenedioxybenzene (DMB) gave a highly fluorescent quinoxalinone derivative for HPLC and MS analysis.[45–49] We recently found that the condensation reaction occurred smoothly in acetic acid to give high yields of quinoxalinone derivatives even with the phenylenediamine–arginine tag, $(H_2N)_2$Ph-R(OMe), which was less reactive than DMB. In this study, 2,8-linked tetrasialic acid (SA4) in AcOH solution was treated with $(H_2N)_2$Ph-R(OMe) at room temperature to give SA4–Arg lactone, a quinoxalinone derivative accompanied by concurrent lactonization (Scheme 2).[50–52] Thus, this reaction not only introduced an enhancing tag but also masked the multiple carboxy groups in SA4, and hence dramatically increased the MS sensitivity. In the absence of $(H_2N)_2$Ph-R(OMe), SA4 underwent lactonization in AcOH solution to give the corresponding lactone (SA4 lactone).

The unmodified tetrasialic acid showed low sensitivity in MALDI-MS, and the detection limit of $[M + H]^+$ signal (*m/z* 1183) was 1857 ± 603 fmol in the positive mode using 2,5-DHB as the matrix. Under the similar MALDI-MS conditions, the detection limit of SA lactone (as the sodiated ion $[M + Na]^+$ at *m/z* 1152) was down to 49.6 ± 11.4 fmol. The sensitivity was further improved in SA4–Arg lactone (as $[M + H]^+$ at *m/z* 1415) with a detection limit of 8.2 ± 1.4 fmol, i.e. 226-fold enhancement compared with unmodified tetrasialic acid. Thus, derivatization of oligoSia with the phenylenediamine–arginine tag, without tedious chromatographic purification, demonstrated a remarkable enhancement of the detection sensitivity in the positive mode of MALDI-TOF-MS.

Our results are consistent with an earlier report by Geyer *et al*.[52] They found that oligo/polySia could be treated with phosphoric acid to induce lactonization for direct MALDI-MS measurement. By masking the carboxyl residues, polySia containing 100 sialic

moieties is detected as low as 1 ng by MALDI-MS using 2,5-DHB or 6-aza-2-thiothymine (ATT) as the matrix. They also mentioned that the preformed DMB derivatives of oligo/polySia could be subjected to on-target lactonization and MALDI-MS analysis with comparable sensitivity (data not shown), though the efficacy of lactonization was reduced presumably due to the open-chained configuration of the reducing end Neu5Ac.

Conclusions

The hydrophilic nature of unmodified saccharides causes low ionization efficiency in MS analysis. Using maltoheptaose (G7) as an example, we have modified it by reductive amination and oxidative amidation with a variety of amine compounds, or by oxidative condensation with phenylenediamine derivatives. Such simple chemical modification rendered better desorption/ionization efficiency of maltoheptaose in MS. Among these examined signal-enhancing tags, a tetrapeptide GFGR(OMe), a nonapeptide RPPGFSPFR (BK) and an arginine-linked phenylenediamine $(H_2N)_2$Ph-R(OMe) were best for the modification of maltoheptaose to show 45- to 273-fold increased $[M + H]^+$ ions in MALDI-TOF-MS (entries 2 and 9–11, Table 1). Such high MS signal enhancement was attributable to the high proton affinity of the basic guanidino group in arginine moiety along with the increased ionization efficiency caused by the hydrophobic property of the aromatic moiety. On the other hand, tagging of G7 with aromatic carboxylic and sulfonic acids, such as 2-aminobenzoic acid (2AA) and 2-amino-1-naphthalenesulfonic acid (ANS), greatly improved the sensitivity of the deprotonated $[M - H]^-$ ions in the negative mode MALDI-MS (entries 2–4 and 7–9, Table 3). By conjugation with BK via reductive amination, the *N*-acetylglucosamine tetra- and hexamers showed great signal enhancement (134 and 181 folds) in the negative mode of MALDI-TOF-MS, albeit only slight enhancement (three- to fourfolds) in the positive mode.

MS detection of oligo- and polysaccharide acids (e.g. oligoGalA and oligo/polySia) is a longstanding problem because they contain a high quantity of carboxylic acid groups. We found that modification of tri-, tetra- and pentagalacturonic acids by reductive amination with BK rendered effective ionization for enhanced MALDI-MS measurements to reach the detection limit as low as 0.1 fmol. Treatment of 2,8-linked tetrasialic acid (SA4) in AcOH solution with a phenylenediamine–arginine tag $(H_2N)_2$Ph-R(OMe) gave the quinoxalinone derivative accompanied by concurrent lactonization, differing the previously reported posterior lactonization of the DMB derivatives of oligo/polysialic acids.[52] The SA4–Arg lactone showed a very high MS enhancement (226-fold) than the unmodified SA4.

Our present study amply demonstrates that MALDI-TOF-MS signals of neutral and acidic oligosaccharides can be dramatically enhanced by tagging with cationic and hydrophobic moieties (e.g. arginine and aromatics) at their reducing groups.

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Supporting information

Supporting information may be found in the online version of this article.

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