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Analysis of acidic carbohydrates as their quaternary ammonium or phosphonium salts by matrix-assisted laser desorption/ionization mass spectrometry

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Abstract—New two-component systems using quaternary ammonium or phosphonium salts as a co-matrix have been developed for the analysis of acidic carbohydrates by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS). In the analysis of the sodium salt of heparin disaccharide I-S, the combination of 2-amino-5-nitropyridine with tetraphenylphosphonium bromide gave the best result. In the analysis of gangliosides containing the sialic acid moiety, the combination of 2,4,6-trihydroxyacetophenone with dimethyldipalmitylammonium bromide was determined to be the system of choice. Under optimum conditions all acidic carbohydrates gave molecular ions in the form of $[M(Q_n)-Q]^-$, where $M(Q_n)$ is the molecular mass of a molecule containing n molecules of quaternary ions as salt.

Keywords: Heparin disaccharide; Gangliosides; MALDI-TOFMS

1. Introduction

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) is now one of the most important tools in structural studies of carbohydrates.[1] However, many problems still remain in the analysis of acidic carbohydrates by MALDI-TOFMS; difficulty in ionization and desulfation of sulfated carbohydrates,[2] loss of sialic acid from sialylated glycans and gangliosides,[3] reduction of signal intensity by formation of alkali-metal adducts,[2,3] and others.

Recently, we reported that N^{α}- and side-chain *O*-fully sulfated homooligomers of tyrosine up to 19-mer in length, isolated as their tetrabutylammonium (TBA) salts, could be easily desorbed and ionized using α-cyano-4-hydroxycinnamic acid (CHCA) as the matrix in the negative-ion mode of MALDI.[4,5] This method was then modified to a more general method consisting of in situ conversion of acidic analytes to their TBA salts using the TBA salt of CHCA (CHCA·TBA) during preparation of an analyte–matrix mixture, and this modified method was then successfully applied to multiply sulfated and phosphorylated peptides.[6] In these measurements, peptides with acidic functions gave molecular ions in the form of $[M(TBA_n)-TBA]^-$, where $M(TBA_n)$ corresponded to the molecular mass of a molecule containing n molecules of TBA as salt, rather than $[M(\text{free acid})-H]^-$ or $[M(TBA_n)-H]^-$. This suggested superior ionization potential of the quaternary ammonium salt in MALDI. A similar approach for the ionization of sulfated glycans involving non-covalent complex formation with basic peptides has been reported.[2,7] Strong interaction between the basic and acidic components completely displaced alkali cations from the anionic groups of the acidic components to give sufficient structural information. However, the method requires fairly large peptides, such as synthetic ones with the composition of $(\text{arginyl-glycine})_n$, where $n = 10$ or 15. In addition, the small number of sulfate groups was shown not to be sufficient to provide strong binding to the basic peptides.[7]

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On the other hand, ammonium acetate,[8] citrate,[9,10] halides,[11] and tartrate[9,10] have been used as good ionization components in two-component matrix systems in MALDI-TOFMS of oligonucleotides. The two-component system using ammonium citrate has also been applied to acidic oligosaccharides.[12] The presence of the ammonium salts as a co-matrix effectively suppressed the formation of alkali-ion adducts and gave a clear $[M-H]^-$ signal in the negative-ion mode. More recently, spermine (*N*,*N*′-bis[3-aminopropyl]-1,4-butanediamine tetrahydrochloride), in conjunction with 2,5-dihydroxybenzoic acid (DHB),[13] was shown to be effective in minimizing alkali-ion adduct formation in MALDI of sialylated glycoconjugates.[14] These results prompted us to study a new two-component system consisting of a matrix and a quaternary ammonium salt ($Q^N{\cdot}X$). As expected, this combination also gave a similar pattern containing the molecular ion in the form of $[M(Q^N{}_n)-Q^N]^-$, where *n* corresponded to the number of acidic groups, as observed when CHCA·TBA was used for sample preparation.[15] Among the quaternary ammonium salts examined, dimethyldipalmitylammonium (DMDPA) and dimethyldistearylammonium (DMDSA) bromides gave the best results, both in terms of signal intensity and suppression of desulfation.[15] A triply phosphorylated peptide gave a similar result.[15] Then, in this study the new two-component system was applied to acidic carbohydrates, making it possible to obtain the intact molecular ion with high sensitivity. Structures of the samples used in this study are shown in Figure 1.

2. Results and discussion

In MALDI-TOFMS, selection of a proper matrix is very important.[1] CHCA, the most popular matrix for measurements of peptides, gave excellent results in our previous studies on sulfated and phosphorylated peptides.[15] However, no signal appeared when a combination of CHCA and TBA hydroxide (TBA·OH) was applied to the sodium salt of heparin disaccharide I-S (α-ΔUA-2S-[1–4]-GlcNS-6S, $C_{12}H_{15}NO_{19}S_3Na_4$) containing one carboxyl and three sulfate groups. Then, DHB,[13] 2,4,6-trihydroxyacetophenone (THAP),[9,12] 3-hydroxypicolinic acid (HPA),[16] and 2-amino-5-nitropyridine (ANP)[17] were tried. Among them, only measurement with ANP and TBA·OH gave a similar pattern of signals to that observed in polysulfated peptides (Fig. 2a). ANP has been used most conveniently in oligonucleotide analysis as the matrix with a strong inhibitory effect on the formation of alkali-metal adducts.[11] However, the spectrum was not simple because the desired molecular ion, $[M(TBA_3)-TBA]^-$ (1059.3), accompanied two additional signals, $[M(TBA_3)-2TBA+Na]^-$ (840.2) and $[M(TBA_3)-H]^-$ (1300.8). This problem was partly improved by use of dimethyldipalmitylammonium (DMDPA) and dimethyldistearylammonium (DMDSA) bromides as the co-matrix. These phase-transfer catalyst-type quaternary ammonium salts had been used as preferred components in the analysis of sulfo- and phosphopeptides.[15] As expected, formation of the Na adduct ion could be suppressed, but selectivity of the molecular ion

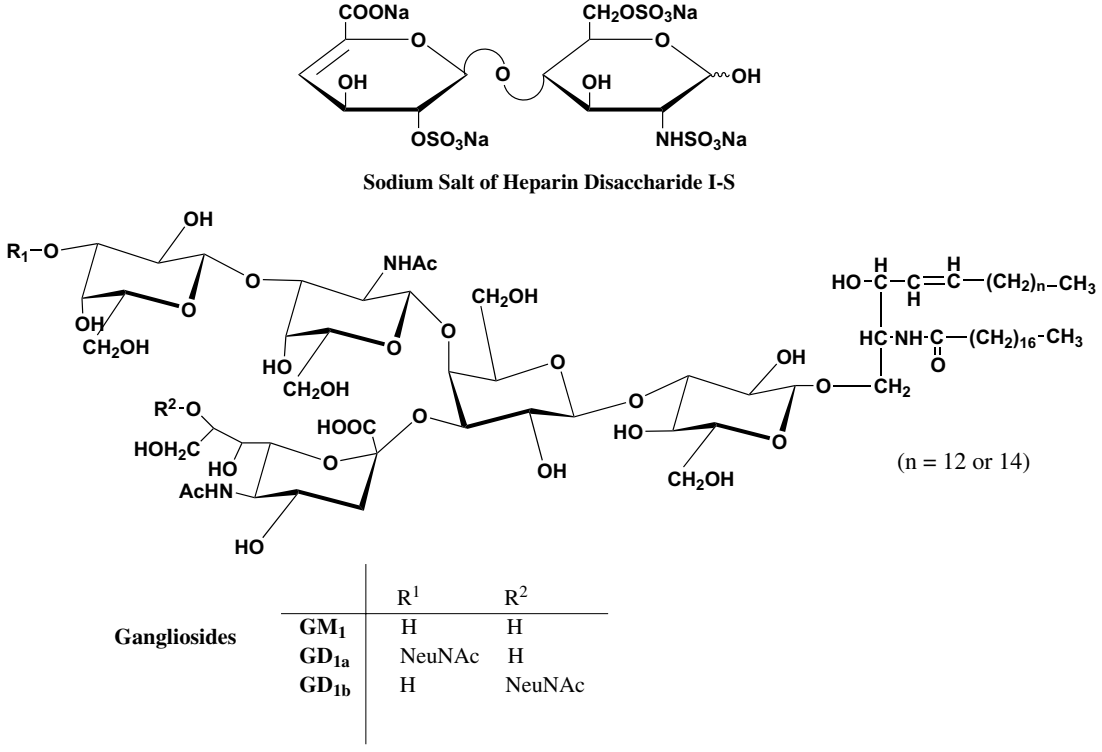

Figure 1. Structures of sodium salt of heparin disaccharide and gangliosides investigated.

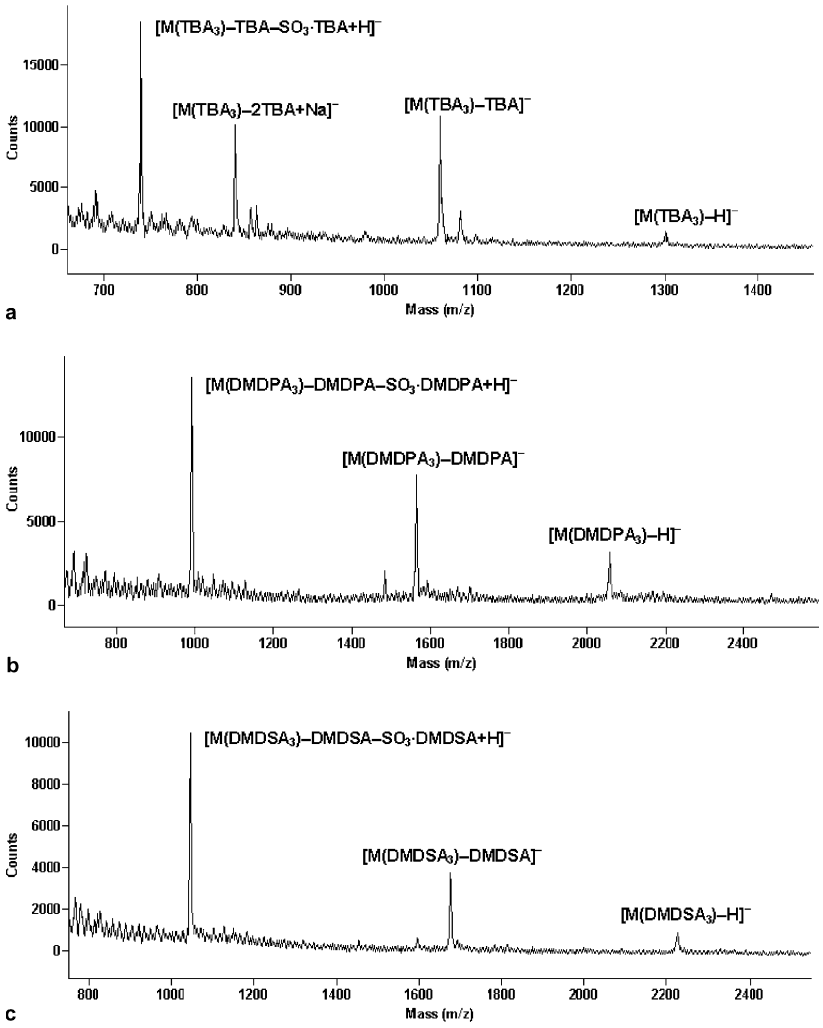

Figure 2. MALDI-TOFMS of the sodium salt of heparin disaccharide I-S measured using various quaternary ammonium salts (10 nmol/μL) as co-matrix of ANP in the negative mode: (a) TBA·OH; (b) DMDPA·Br; (c) DMDSA·Br.

$[M(Q^N{}_3)-Q^N]^-$/$[M(Q^N{}_3)-H]^-$ was not sufficient (Fig. 2b and c). As a more substantial problem, degradation by loss of the sulfate group should be minimized. For this purpose a new type of compound was necessary.

Various kinds of quaternary phosphonium salts are now commercially available. The tested ones were tetrabutylphosphonium (TBP) bromide (Fig. 3a), butyltriphenylphosphonium (BTPP) bromide (Fig. 3b), tetraoctylphosphonium (TOP) bromide (Fig. 3c), and tetraphenylphosphonium (TPP) bromide (Fig. 3d). In TBP·Br the sodium adduct ion (978.8) was still observed; however, the $[M(Q^P{}_3)-H]^-$ signal disappeared, except in one case (TOP·Br). While the desulfation could not be suppressed, both BTPP·Br and TPP·Br gave very simple spectra showing the molecular ions in the form of $[M(Q^P{}_3)-Q^P]^-$ (Q^P = BTPP or TPP) with high intensity. From these results, preference for the quaternary phosphonium salts over the quaternary ammonium salts in the analysis of polysulfated carbohydrates was demonstrated.

The more important difference between the quaternary ammonium and phosphonium salts was seen in measurements in the positive-ion mode. Generally, polysulfated compounds do not ionize in the positive-ion mode. However, when the heparin disaccharide was analyzed in the two-component matrix consisting of ANP and the quaternary phosphonium salt, measurements in the positive-ion mode were made possible for the first time (Fig. 4a–d). As we have reported before in the fast-atom bombardment mass spectrometry of the TBA salt of 9-fluorenylmethyloxycarbonylated tyrosine sulfate,[4] molecular ions were observed as adducts with the quaternary phosphonium groups. The heparin disaccharide used as the sample contains one carboxyl and three sulfate groups. In the measurement with ANP/TPP·Br, a molecular ion of the tetrakis-TPP salt in the form of $[M(TPP_4)+TPP]^+$ appeared as the most abundant signal, excepting a strong signal corresponding to $[2TPP+Br]^+$ (759.7) (Fig. 4d). In the other three cases, signals of the same type of molecular ions for both

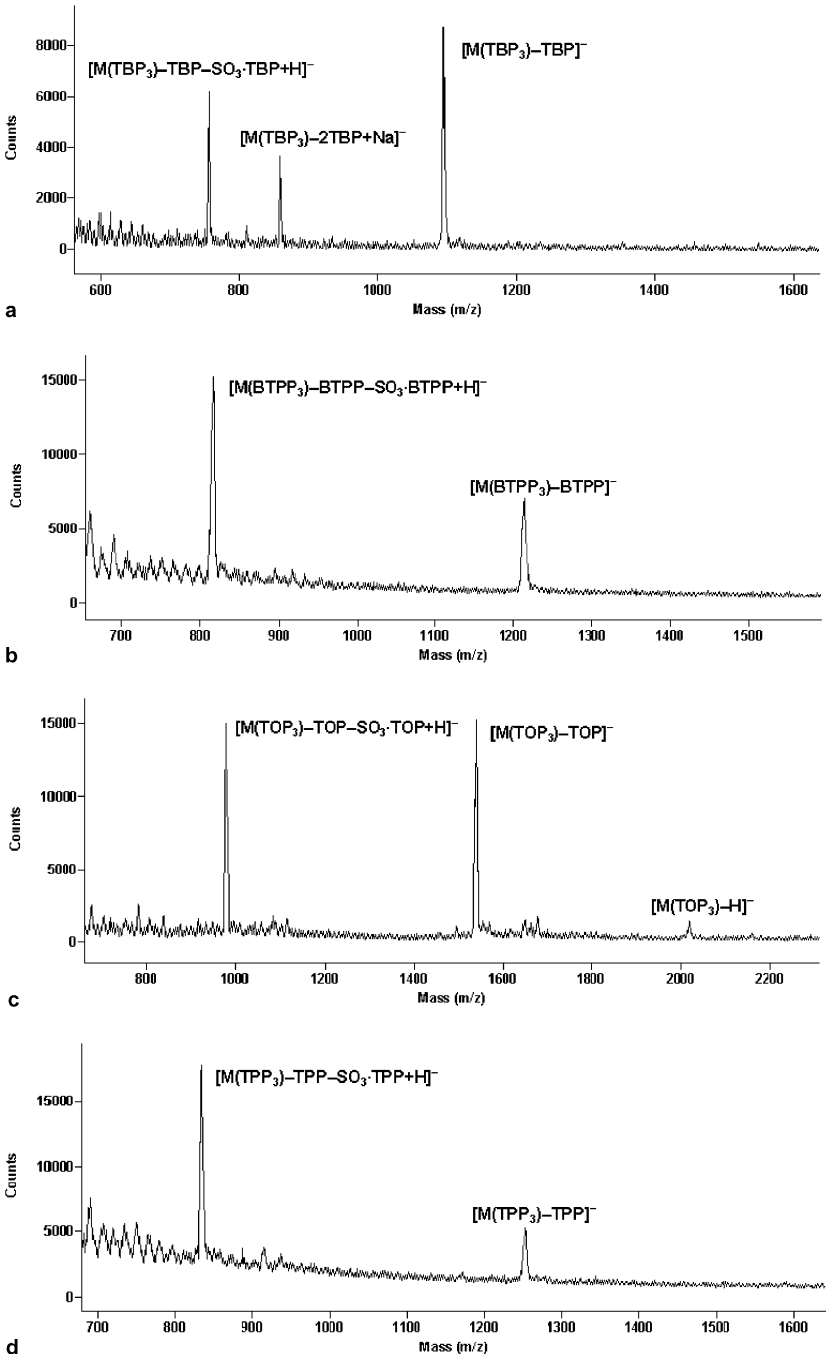

Figure 3. MALDI-TOFMS of the sodium salt of heparin disaccharide I-S measured using various quaternary phosphonium salts (10 nmol/μL) as co-matrix of ANP in the negative mode: (a) TBP·Br; (b) BTPP·Br; (c) TOP·Br; (d) TPP·Br.

tris- and tetrakis-phosphonium salts and desulfated ions made their spectra complicated (Fig. 4a–c). In conclusion, ANP and TPP·Br would be the two-component system of first choice in the analysis of polysulfated saccharides in both the negative- and positive-ion modes. However, all other phosphonium salts would also be good options in measurements of polysulfated carbohydrates.

Sialic acid is another troublesome sugar moiety in MALDI-TOFMS. Sialylated oligosaccharides and glycopeptides lose their sialic acid easily under MALDI conditions.[18] The THAP–ammonium citrate system solved the problem for simple sialylated oligosaccharides.[12] However, gangliosides seem to be more difficult to analyze,[14] requiring derivatization such as permethylation[3] or methyl ester formation.[19] To find a better solution to this problem, application of the aforementioned two-component system was tried. Three commercially available gangliosides, monosialylated GM_1 and disialylated GD_{1a} and GD_{1b} with structures shown in

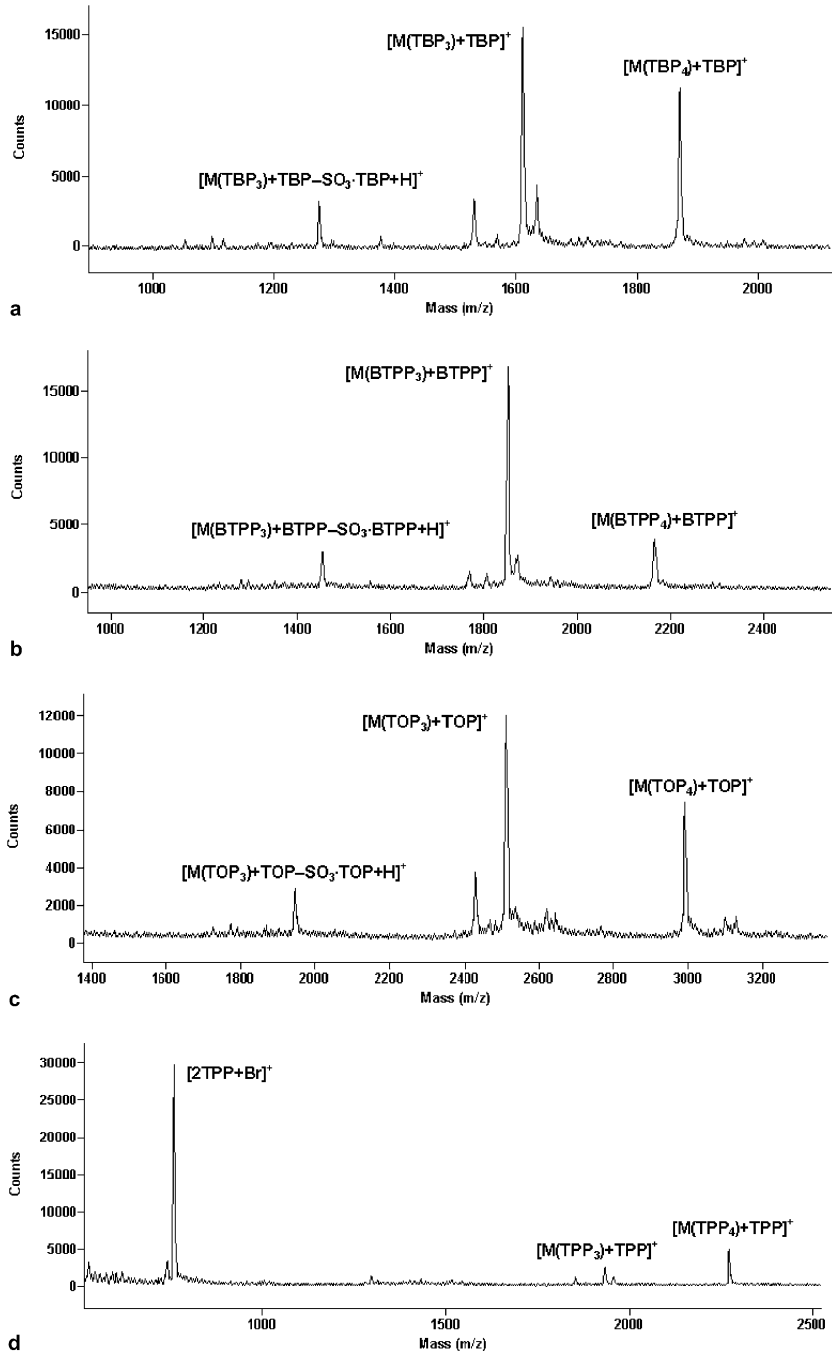

Figure 4. MALDI-TOFMS of the sodium salt of heparin disaccharide I-S measured using various quaternary phosphonium salts (10 nmol/μL) as co-matrix of ANP in the positive-ion mode: (a) TBP·Br; (b) BTPP·Br; (c) TOP·Br; (d) TPP·Br.

Figure 1, were used as samples. Considering the change of the acidic group from the sulfate to carboxylate in the gangliosides, the best combination of matrix and co-matrix was determined again. Signal intensities also being strongly dependent on the concentration of the quaternary ammonium co-matrix, three concentrations were tested. Table 1 shows the results of the analysis of GM_1 obtained with various combinations of matrix and quaternary ammonium co-matrix in the optimum concentration. Since this ganglioside contains two components differing in their hydrocarbon chain lengths in the ceramide part, signal intensities for two components (SI_1 and SI_2) and their ratio (SI_1/SI_2) are listed for evaluation of the matrix system. A substantial deviation of the SI ratio from the range of 1.10–1.25 would imply a difference in sensitivity to each component. By consideration of both strength in intensity and SI ratio, combinations of THAP with TBA·OH or DMDPA·Br and ANP with TBA·Br or TBA·F were selected as best.

Table 1. Effects of matrices and quaternary ammonium salts in the linear negative-ion analysis of GM_1

Entry	Matrix	Co-matrix (concentration/nmol/μL)[a]	Signal intensity (SI)[b]		Ratio
			SI_1	SI_2	SI_1/SI_2
1	THAP	None	20,320	17,258	1.18
2	THAP	TBA·OH (1)	41,057	34,371	1.19
3[c]	THAP	TBA·OH (1)	905	660	1.37
4	THAP	TBA·Br (1)	12,399	7055	1.76
5	THAP	TBA·F (1)	17,053	12,397	1.38
6	ANP	TBA·OH (1)	20,703	13,686	1.51
7	ANP	TBA·Br (1)	35,958	28,234	1.27
8	ANP	TBA·F (1)	27,988	25,434	1.10
9	HPA	TBA·OH (1)	2976	1916	1.55
10	DHB	TBA·OH (1)	2758	2260	1.22
11	THAP	DMDPA·Br (1)	16,275	14,086	1.16
12	THAP	DMDSA·Br (1)	7690	4235	1.81
13	ANP	DMDPA·Br (1)	20,239	13,958	1.45
14	ANP	DMDSA·Br (1)	4557	1398	3.26

[a] The optimum concentration among those tested (1.0, 10, and 20 nmol/μL except for DHB) is shown.
[b] SI_1 and SI_2 are counts for lower and higher molecular weight components, respectively.
[c] The analyte concentration was 50 fmol/μL.

Next, the same comparison was made using quaternary phosphonium salts as co-matrix. Results are summarized in Table 2. In contrast to the case of ammonium salts, higher intensities were obtained using a higher concentration (10 nmol/μL) of the phosphonium salts; however, differences among matrices and co-matrices were slight. Among all the combinations, that of THAP and TPP·Br would be the first choice, showing a high intensity for both components, followed by THAP and ANP with TOP·Br.

When the analyte concentration was reduced to 50 fmol/μL, signals (S/N = 5) could also be observed using the standard stainless steel platform (Table 1, entry 3 and Table 2, entries 3 and 6). Recent development of more effective platforms such as silicon nanowires[20] would be helpful to lower the detection limit further.

When the separately disialylated ganglioside GD_{1a} was used as the sample, the effectiveness of the quaternary co-matrices was remarkable. Without addition of quaternary salts as co-matrix, only weak signals of $[M-H]^-$ and the alkali-metal adducts were observed (Fig. 5a). With use of the co-matrices, two sets of signals differing in extent of salt formation of the two carboxyl groups appeared. Among them, the clearest spectrum was obtained with THAP–DMDPA·Br (Fig. 5b). This combination could suppress both alkali-metal adduct formation and desialylation almost completely to give $[M(DMDPA_2)-DMDPA]^-$ as the most abundant signal. When THAP–TPP·Br was used, in contrast, $[M(TPP)-TPP]^-$ appeared more strongly than $[M(TPP_2)-TPP]^-$ and desialylation was serious (Fig. 5c). This means that the phosphonium ion interacts less effectively with the carboxyl group of the sialyl moiety. We have already reported such partial salt formation in the analysis of multiply phosphorylated peptides.[6]

The sequentially disialylated ganglioside GD_{1b} also gave signals with *m/z* values that depended on the mass of co-matrix. When TBA·OH (20 nmol/μL) was used with THAP, loss of the sialic acid was significant (Fig. 6a). This was suppressed with THAP–DMDPA·Br (1 nmol/μL), and the signals for both double and single

Table 2. Effects of matrices and quaternary phosphonium salts in the linear negative-ion analysis of GM_1

Entry	Matrix	Co-matrix (concentration/nmol/μL)[a]	Signal intensity (SI)[b]		Ratio
			SI_1	SI_2	SI_1/SI_2
1	THAP	TBP·Br (10)	9436	7837	1.20
2	THAP	TOP·Br (10)	27,673	22,369	1.24
3[c]	THAP	TOP·Br (10)	2216	2067	1.07
4	THAP	BTPP·Br (10)	16,040	13,384	1.20
5	THAP	TPP·Br (10)	33,186	27,276	1.22
6[c]	THAP	TPP·Br (10)	1833	1489	1.23
7	ANP	TBP·Br (10)	11,187	10,350	1.08
8	ANP	TOP·Br (10)	27,897	24,088	1.16
9	ANP	BTPP·Br (10)	26,413	18,289	1.44
10	ANP	TPP·Br (10)	20,876	13,412	1.57

[a] The optimum concentration among those tested (1.0, 10, and 20 nmol/μL).
[b] SI_1 and SI_2 are counts for lower and higher molecular weight components, respectively.
[c] The analyte concentration was 50 fmol/μL.

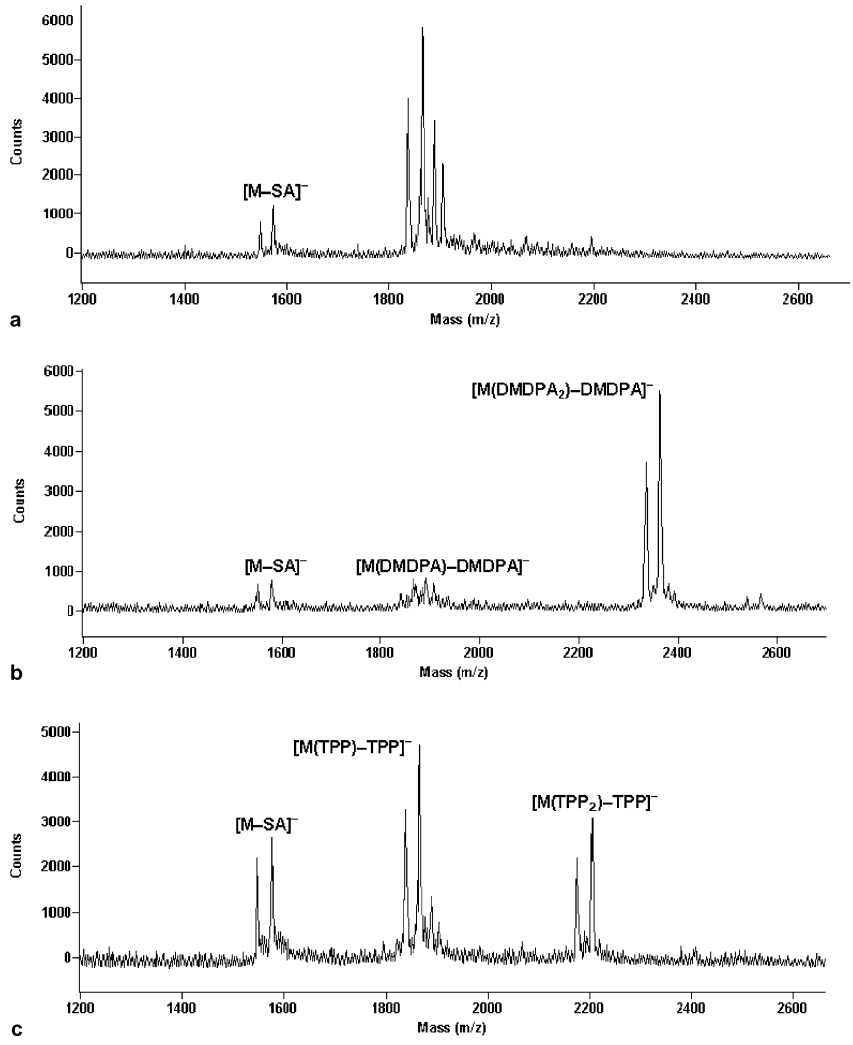

Figure 5. MALDI-TOFMS of disialoganglioside GD_{1a} measured using various quaternary salts as co-matrix of THAP: (a) no addition; (b) DMDPA·Br (1 nmol/μL); (c) TPP·Br (10 nmol/μL).

salts were observed with similar intensities (Fig. 6b). This difference in signal appearance between GD_{1a} and GD_{1b} in the same matrix–co-matrix system would be the result of the difficulty of salt formation of the inner sialyl moiety in GD_{1b}. When the phosphonium salts, BTPP·Br (10 nmol/μL, Fig. 6c) and TPP·Br (10 nmol/μL, Fig. 6d), were used with THAP, more intense signals were observed. However, the intensities for the double salts decreased, and signals for the single salts were observed as the most abundant signal. The latter signals may possibly be due to the $[M-H]^-$ ion for the free acid, having the same elemental composition as the $[M(Q)-Q]^-$ ion from the single salt. Contrary to the case of GD_{1a} (Fig. 5c), the extent of desialylation was suppressed, being very low (Fig. 6d). Comparing BTPP·Br and TPP·Br, the latter gave a somewhat clearer spectrum, completely removing Na^+ and K^+ adducts formation. In all cases, ANP was less effective than THAP (10 nmol/μL, Fig. 6e).

Summarizing all the data for the three gangliosides, the combination of THAP–DMDPA·Br gave the most reliable results. Use of this new two-component system allowed the appearance of the $[M(DMDPA_n)-DMDPA]^-$ ion to give the clearest information of the molecular mass of gangliosides.

In conclusion, in this study we were able to establish a new two-component matrix system using quaternary ammonium and phosphonium salts as co-matrix for MALDI-TOFMS measurements of sulfated and sialylated carbohydrates.

3. Experimental

3.1. MALDI-TOFMS Matrix chemicals

α-Cyano-4-hydroxycinnamic acid (CHCA), 2,5-dihydroxybenzoic acid (DHB), 2-amino-5-nitropyridine

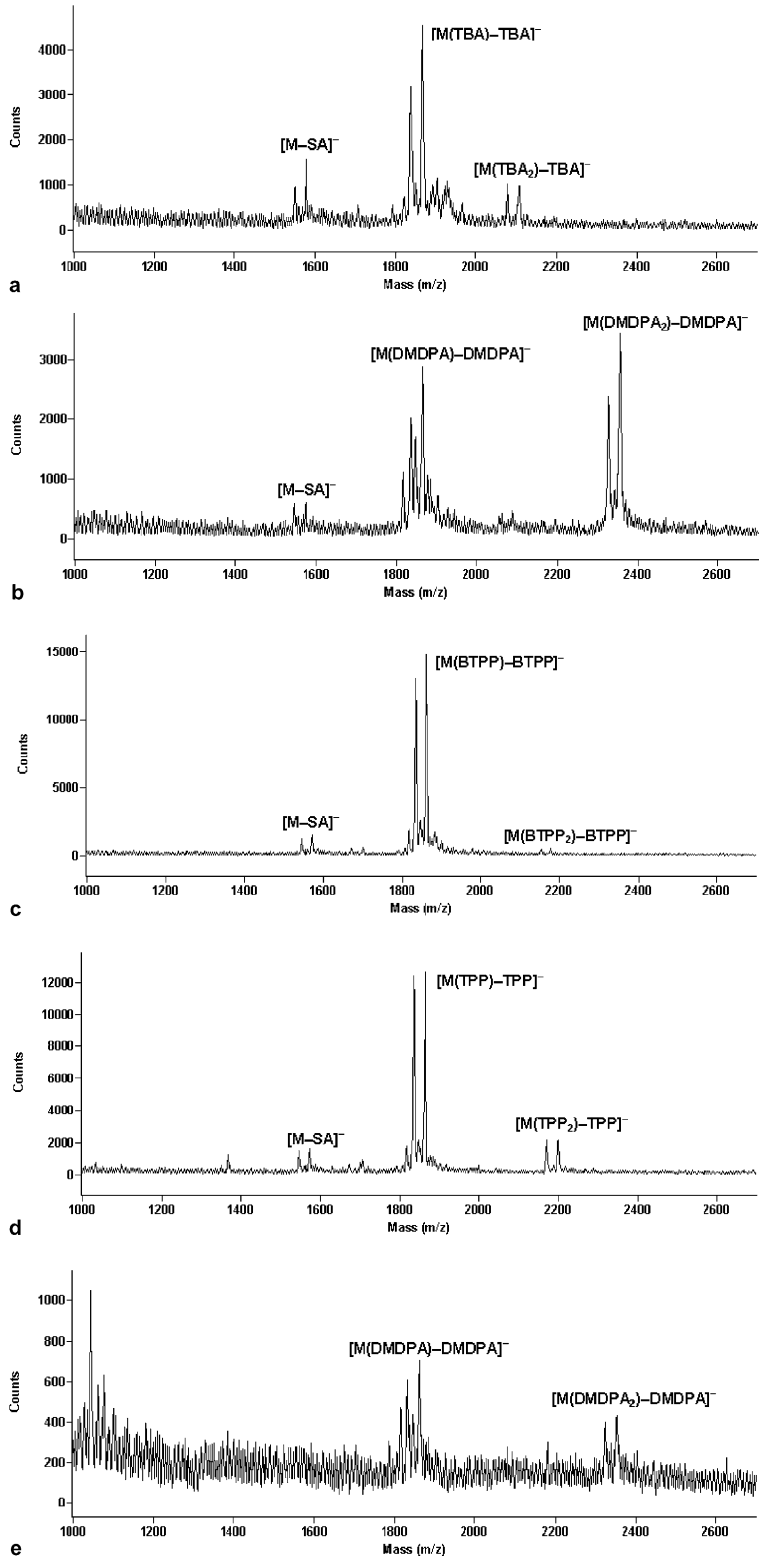

Figure 6. MALDI-TOFMS of disialoganglioside GD_{1b} measured using various quaternary salts as co-matrix: (a) TBA·OH–THAP; (b) DMDPA·Br–THAP; (c) BTPP·Br–THAP; (d) TPP·Br–THAP; (e) DMDPA·Br–ANP.

(ANP), 2,4,6-trihydroxyacetophenone (THAP), and 3-hydroxypicolinic acid (HPA) were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA).

Tetrabutylammonium hydroxide (TBA·OH, 10% in water), dimethyldipalmitylammonium bromide (DMDPA·Br), tetrabutylphosphonium bromide (TBP·Br),

tetraphenylphosphonium bromide (TPP·Br), and tetraoctylphosphonium bromide (TOP·Br) were obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Dimethyldistearylammonium bromide (DMDSA·Br) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Butyltriphenylphosphonium bromide (BTPP·Br) was obtained from Aldrich Chemical Co.

3.2. Analytes

Sodium salt of heparin disaccharide I-S, monosialoganglioside GM_1, disialoganglioside GD_{1a}, and disialoganglioside GD_{1b} were obtained from Sigma Chemical Co.

3.3. Calibrating chemicals

Bradykinin fragment 1–7 (MW 756.9), angiotensin II (human, MW 1046.2), ACTH fragment 18–39 (human, MW 2465.7), and insulin oxidized B chain (bovine, MW 3495) were obtained from Sigma Chemical Co.

3.4. Solvents

MeOH, ethanol, and acetonitrile were obtained from Kanto Chemical Co. Inc. (Tokyo, Japan) and used without further purification.

3.5. Instruments

Measurements were performed using a Voyager DE time-of-flight mass spectrometer (PerSeptive Biosystems, Framingham, MA, USA), equipped with a pulsed nitrogen laser ($\lambda = 337$ nm; pulse width = 3 ns). The accelerating voltage in the ion source was 20 kV. The sample was irradiated just above the threshold laser power for obtaining ions. Thus, the irradiance used for producing mass spectra was analyte dependent. Each mass spectrum was produced by accumulating data from 64 laser shots. All samples were measured in the linear mode, and in both the positive- and the negative-ion modes. External calibration was performed by using peaks of the standard chemicals with CHCA as matrix [10 mg in 1 mL of MeCN/0.3% aqueous trifluoroacetic acid (1:1, v/v)].

3.6. Matrix preparation

CHCA matrix solution was prepared by dissolving 5 mg of CHCA in 0.5 mL of MeCN/EtOH/H_2O (2:2:1, v/v). DHB matrix solution was prepared by dissolving 5 mg of DHB in 0.5 mL of EtOH/H_2O (1:9, v/v). ANP matrix solution was prepared by dissolving 5 mg of ANP in 0.5 mL of EtOH/H_2O (1:1, v/v). THAP matrix solution was prepared by dissolving 5 mg of THAP in 0.5 mL of MeCN/H_2O (1:1, v/v). HPA matrix solution was prepared by dissolving 12.5 mg of HPA in 0.5 mL of MeCN/H_2O (1:1, v/v).

Quaternary ammonium or phosphonium salt solutions were prepared by dissolving the selected compound in solvent [H_2O for TBA·OH, MeOH/H_2O (7:3, v/v) for DMDPA·Br and DMDSA·Br, MeCN/H_2O (1:1, v/v) for quaternary phosphonium salts]. Concentrations of each quaternary ammonium or phosphonium salt solutions were described elsewhere.

3.7. Sample preparation

Solution of the sodium salt of heparin disaccharide I-S was prepared by dissolving 0.26 mg of the analyte in 0.39 mL of water. Solutions of gangliosides were prepared by dissolving 0.3 mg of the analytes in 0.2 mL of EtOH.

Equal portions of the analyte and quaternary ammonium or phosphonium salt solutions were mixed, then 2 μL of the resultant solution was mixed with 18 μL of the matrix solution. A 0.5–1 μL of aliquot of the analyte–matrix solution was applied to the sample plate and dried under ambient conditions before loading into the mass spectrometer.

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