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Matrix-assisted laser desorption ionization mass spectrometry for the analysis of monosulfated oligosaccharides

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Abstract

Sulfated oligosaccharides are an important class of compounds in the field of glycobiology. Mass spectrometric analysis of these molecules is challenging due to their readiness to dissociate in sample preparation and their tendency to fragment during ionization. Moreover, their presence in small quantity in biological systems poses additional problems. We report the development of a mass spectrometric method based on matrix-assisted laser desorption ionization (MALDI) in a time-lag focusing time-of-flight mass spectrometer for the analysis of monosulfated oligosaccharides. It is found that coumarin 120 is an excellent matrix for the analysis of monosulfated disaccharides, whereas the use of a mixture of coumarin 120 and 6-aza-2-thiothymine is very effective for the ionization of sulfated trisaccharides and tetrasaccharides including those containing *N*-acetylneuraminic acid. Molecular ions for a series of synthetic sulfo/sialo βGal(1 → 3)GlcNAc and βGal(1 → 4)GlcNAc structures can thus be observed with subpicomole detection sensitivity using a uniform microcrystal matrix/sample preparation procedure. It is demonstrated that, with this matrix formulation, the presence of a high amount of sodium chloride or sodium phosphate buffer, which is often the case for the HPLC fractionated samples, does not deteriorate the MALDI performance. The analysis of mixtures containing different types of oligosaccharides is also examined. It is found that different classes of oligosaccharides require different matrix preparation methods. © 1997 Elsevier Science Ltd.

Keywords: Sulfated oligosaccharides; MALDI; Mass spectrometry

1. Introduction

Sulfated carbohydrates are an important class of compounds found in many biological systems [1,2]. For example, polysulfated carbohydrate polymers

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termed glycosaminoglycans, including hyaluronic acid, chondroitin sulfate, and heparin, have long been known as major constituents of mammalian tissues [1]. More recently, specifically sulfated sugar residues have been discovered on the carbohydrate chains of glycoproteins and glycolipids [2]. Such sulfated oligosaccharides, usually sulfated on galactose (Gal), mannose, *N*-acetylgalactosamine (GalNAc), *N*-acetylglucosamine (GlcNAc), or glucuronic acid residues, have been shown to directly control important biological activities such as those of glycoprotein hormones. Sulfated glycoprotein oligosaccharides have also been found to be active in cell–cell adhesion, including the control of leukocyte migration [2]. Because of the structural diversity and complexity of these oligosaccharides, the understanding of their biological functions at the molecular level requires initial identification and detailed structural characterization.

Mass spectrometry (MS), such as fast-atom bombardment mass spectrometry (FABMS), has traditionally played an important role in the analysis of oligosaccharides including sulfated oligosaccharides [3–5]. Recent advances of two relatively new biomolecule-compatible ionization methods, namely, matrix-assisted laser desorption ionization (MALDI) and electrospray ionization, are poised to provide further enhancement in performance [6]. A major advantage of the new ionization techniques is the improvement in detection sensitivity. The possibility of doing routine analysis of oligosaccharides in amounts ranging from subpicomoles to low picomoles should dramatically decrease the heroic effort that is often required to isolate nanomole samples for FABMS.

The MS analysis of sulfated oligosaccharides is particularly challenging [7–15]. Sulfated oligosaccharides can decompose during sample workup prior to MS analysis. The analysis is further complicated due to their readiness to form fragment ions upon ionization. Thus, obtaining the intact molecular ion with high detection sensitivity is an important first step for mass analysis. This is particularly true for mixture analysis where the presence of fragment ions may pose some ambiguity in chemical identification. Although the ionization of sulfated oligosaccharides by FAB has been extensively reported [7–12], microgram quantities are often required in FABMS. Several reports using electrospray MS for the analysis of these molecules are also present in the literature [13–16]. The detection sensitivity for electrospray MS has not been extensively reported. MALDI has the advantage of directly analyzing mixtures without extensive separation. It also has high tolerance to salt and buffer, as demonstrated for the analysis of non-sulfated oligosaccharides [17,18]. However, for MALDI, very few studies are reported for the detection of sulfated oligosaccharides, because matrices commonly used for non-sulfated oligosaccharides provide poor performance or fail for these compounds [19]. An indirect MALDI measurement, in which sulfated saccharides were mixed with a basic peptide or protein to form a non-covalent complex that was then detected by MALDI, was reported [19,20]. This method was shown to be particularly useful for the analysis of polysulfated polysaccharides [20].

In this report, we present a study of MALDI analysis for monosulfated oligosaccharides. A number of compounds ranging from sulfated disaccharides to tetrasaccharides, including those containing sialic acid are examined. We demonstrate that, by using a proper matrix/sample preparation protocol, it is possible to obtain soft ionization of monosulfated oligosaccharides with subpicomole detection sensitivity.

2. Results and discussion

Table 1 lists the sulfated oligosaccharides examined in this study. These range from disaccharides to tetrasaccharides, including those containing *N*-acetylneuraminic acid. As in the analysis of other classes of biomolecules by MALDI, the use of a proper matrix for the detection of sulfated oligosaccharides is very important. An analytically useful matrix formulation should provide molecular ions of the analyte of interest with high detection sensitivity. The interference from matrix-ion peaks should be kept to a minimum. In addition, an ideal matrix system should provide universal detection. For mixture analysis, no ion suppression of the analyte due to the presence of other components in a mixture should be observed.

For the sulfated disaccharides, it was found that intact molecular ions can be obtained from the use of 2-(4-hydroxyphenylazo)benzoic acid (HABA), sinapinic acid, and 7-amino-4-methylcoumarin (coumarin 120) as the matrix. However, for HABA and sinapinic acid, very intense, low-mass ion peaks from the matrix can provide interference in the detection of these molecules. This is shown in Fig. 1A and B for the mass spectra of **1** obtained with the use of HABA

Table 1
List of oligosaccharides examined in this study

Compound	Structure [a]	MW [b]	Best matrix [c]
1	βGal(1 → 4)βGlcNAc3SO_3-OR	633.23	A
2	βGal(1 → 4)βGlcNAc6SO_3-OR	633.23	A
3	βGal2SO_3(1 → 4)βGlcNAc-OR	633.23	A
4	βGal3SO_3(1 → 4)βGlcNAc-OR	633.23	A
5	βGal4SO_3(1 → 4)βGlcNAc-OR	633.23	A
6	βGal6SO_3(1 → 4)βGlcNAc-OR	633.23	A
7	βGal3SO_3(1 → 4)[αFuc(1 → 3)]βGlcNAc-OR	779.29	A
8	βGalSO_3Na(1 → 3)[αFuc(1 → 4)]GlcNAc ~ OH	631.14	B
9	αNeu5Ac(2 → 3)βGal(1 → 4)βGlcNAc6SO_3-OR	924.33	B
10	αNeu5Ac(2 → 3)βGal6SO_3(1 → 4)[αFuc(1 → 3)]βGlcNAc-OR	1070.39	B
11	αNeu5Ac(2 → 3)βGal6SO_3(1 → 3)[αFuc(1 → 4)]βGlcNAc-OR	1070.39	B
12	αNeu5Ac(2 → 3)βGal(1 → 4)[αFuc(1 → 3)]βGlcNAc6SO_3-OR	1070.39	B
13	αFuc(1 → 2)βGal(1 → 3)[αFuc(1 → 4)]βGlcNAc-OR	845.39	C

[a] R: -$(CH_2)_8COOCH_3$.
[b] Exact mass.
[c] A, coumarin 120; B, mixture of coumarin 120 and 6-aza-2-thiothymine; C, mixture of 2,5-dihydroxybenzoic acid and 5-methoxysalicylic acid.

and sinapinic acid as the matrix, respectively. The total amount of sample loaded is 2.5 pmol for Fig. 1A and 5 pmol for Fig. 1B. In comparison, the mass spectrum of the same compound obtained with the use of coumarin 120 as the matrix is shown in Fig. 1C. This spectrum is obtained with a sample loading of 0.5 pmol. Fig. 1C clearly shows that the coumarin 120 matrix provides not only better detection sensitivity but also less interference from the matrix ions. Note that coumarin 120 has been used in the past as a matrix for peptides [21]. For all sulfated disaccharides listed in Table 1, their mass spectra display a strong molecular-ion peak with no fragment-ion peaks when coumarin 120 is used.

One of the important observations from this study is that changes in oligosaccharide structure can significantly affect the MALDI performance. Table 1 also lists the optimal matrix found for the detection of the corresponding sulfated oligosaccharide. For example, coumarin 120 produces very weak signals for the sulfated trisaccharide (compound **8** in Table 1) and for the sulfated oligosaccharides containing *N*-acetylneuraminic acid such as **9–12** in Table 1. Considering compounds **7** and **8**, the major structural difference between these two compounds is the presence of methoxycarbonyloctyl aglycon in compound **7**. Previous studies of several non-sulfated oligosaccharides without the aglycon group and those with various types of aglycon groups have shown a matrix dependence in detection sensitivity [22]. It is likely that the hydrophobic matrix coumarin 120 cocrystallizes well with compound **7** containing the hydrophobic methoxycarbonyloctyl aglycon, resulting in sensitive detection. For compound **8**, HABA can generate a molecular-ion signal as shown in Fig. 2A. However, strong matrix-ion signals are also observed. For **9–12**, HABA generates relatively poor signals. On the other hand, 6-aza-2-thiothymine, a matrix introduced by Juhasz and Costello [23] for testing underivatized gangliosides, is found to generate good signals for these compounds. One drawback of this matrix is that it also gives strong matrix-ion signals that cover a broader low-mass range than does coumarin 120. If the analyte to be tested is in the range close to the matrix-ion signal region, interference can become a serious problem, a situation similar to that shown in Fig. 1A, B.

In analyzing **8**, it was found that a mixture of 6-aza-2-thiothymine and coumarin 120 provides the best results in terms of signal strength and background level. This is illustrated in Fig. 2B where an intense molecular-ion peak without fragmentation is obtained. This is also true for analyzing **9–12**. In each case, 6-aza-2-thiothymine forms the first layer, and to this layer coumarin 120 mixed with the analyte is then deposited. When the coumarin 120 and analyte solution is deposited, followed by immediate vacuum drying, the first layer appears to be partially dissolved. Cocrystals from the matrices and the analyte are likely formed in the interface region. If no vacuum drying is used after the second solution is applied, the first layer is completely dissolved. This is similar to the sample preparation where matrices and analyte are premixed and then deposited onto the

probe. However, the two-layer method without the total dissolution of the first layer was found to provide the best results. An additional observation is that the desorption/ionization laser threshold for coumarin 120 is about 3 times lower than that of 6-aza-2-thiothymine. By using the two-layer method, the laser threshold is about the same as that observed with the use of coumarin 120 as the matrix. It is likely that a lower laser flux is needed to desorb the cocrystals of 6-aza-2-thiothymine, coumarin 120, and the analyte, compared with the desorption of 6-aza-2-thiothymine and the analyte cocrystals. The top layer of microcrystals prepared by the two-layer method contains mainly coumarin 120. The reduction

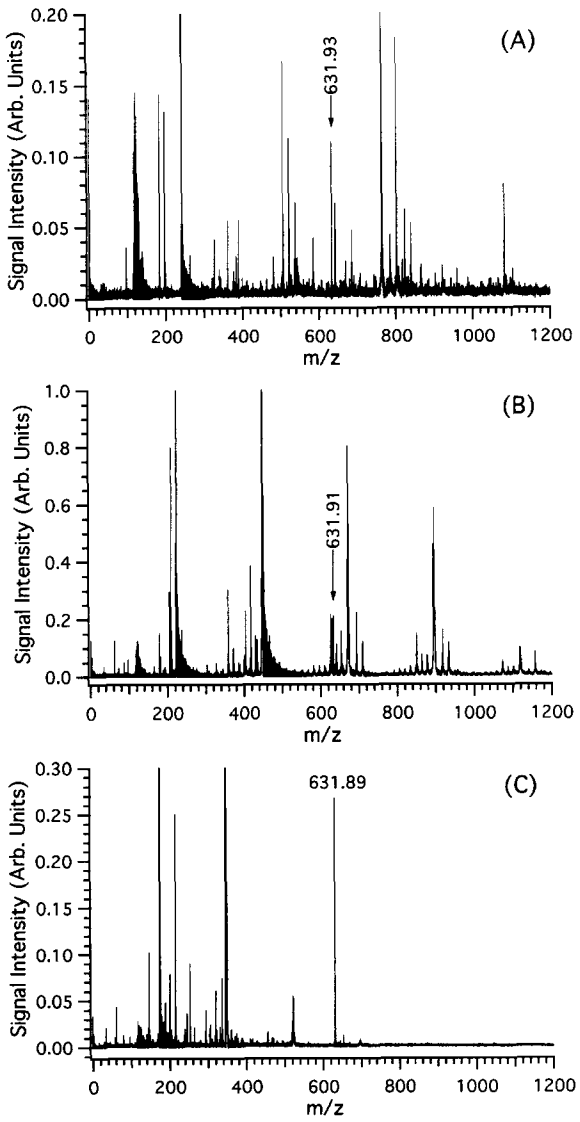

Fig. 1. MALDI mass spectra of **1** obtained with the use of (A) HABA, (B) sinapinic acid, and (C) coumarin 120 as the matrix. The calculated exact mass for $(M-H)^-$ of **1** is 632.23 and the measured masses are shown in the spectra.

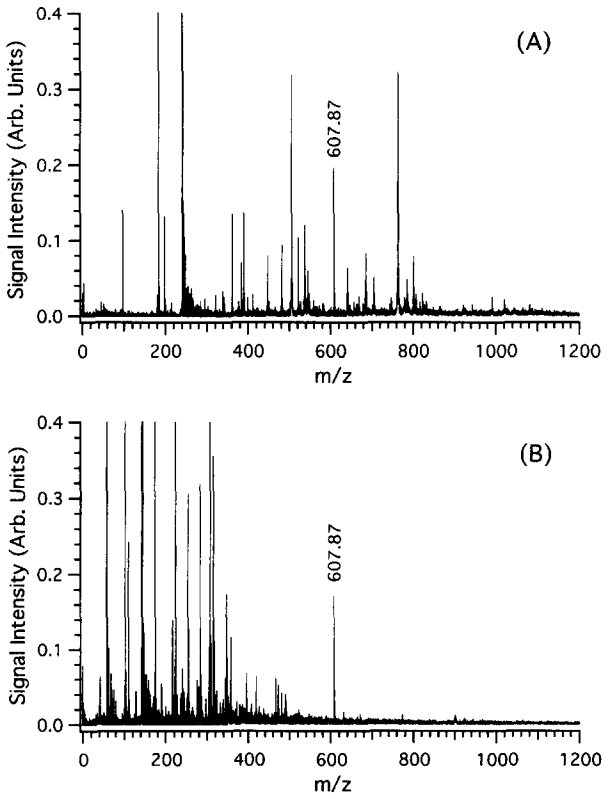

Fig. 2. MALDI mass spectra of **8** obtained with the use of (A) HABA and (B) a mixture of 6-aza-2-thiothymine and coumarin 120 as the matrix. The total amount of **8** loaded is 2.5 pmol for (A) and 1.0 pmol for (B). The calculated exact mass for $(M-Na)^-$ of **8** is 608.15 and the measured masses are shown in the spectra.

of both the laser threshold and the amount of 6-aza-2-thiothymine contributes to the reduction of the matrix background signals. Another example is illustrated in Fig. 3A for the mass spectrum of **10** obtained by using the two-layer sample preparation method. The intact molecular ion is observed with no apparent fragment-ion peaks. The total amount of sample loaded is about 0.5 pmol. Judging from the signal-to-background ratio observed, it is clear that subpicomole detection can be readily achieved.

One complication in analyzing oligosaccharides containing *N*-acetylneuraminic acid is that the molecular-ion region of the MALDI spectrum usually displays several peaks corresponding to different ionic forms of the acid group [24]. This is illustrated, as an example, in Fig. 3B for the MALDI analysis of **10**. The peaks at m/z 1069.45, 1091.44, and 1107.47 are from the molecular ions of the acid $(M-H)^-$, the analyte sodium salt $(M+Na-2H)^-$, and the analyte potassium salt $(M+K-2H)^-$, respectively. The formation of these multiple ions makes the peak assign-

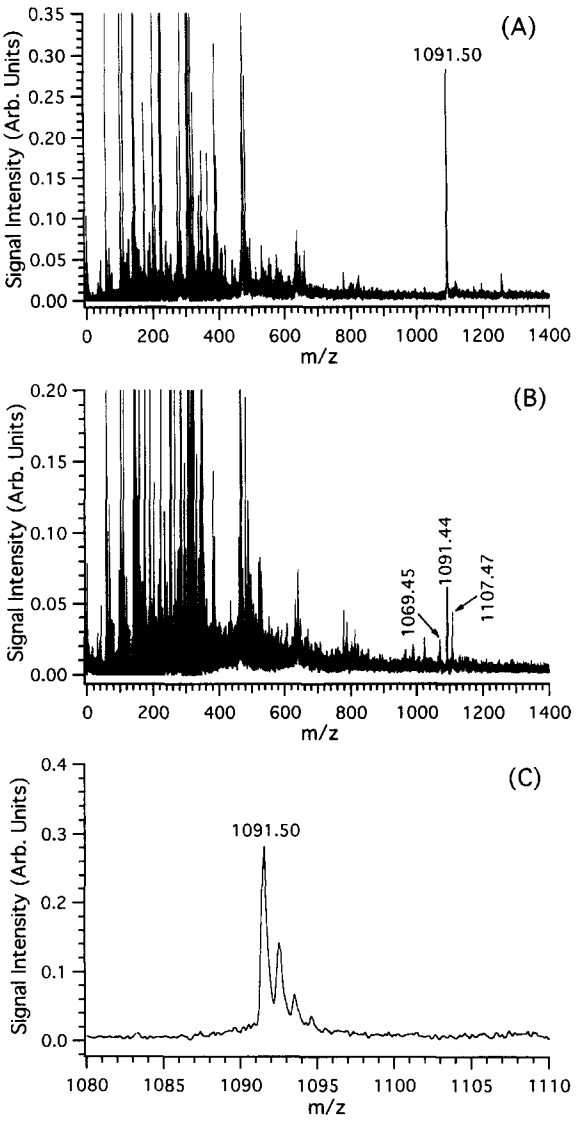

Fig. 3. MALDI mass spectra of **10** obtained by using a mixture of the matrices of 6-aza-2-thiothymine and coumarin 120: (A) with the addition of NaCl, (B) without the addition of NaCl, and (C) the expanded molecular-ion region of (A). The calculated exact masses for $(M-H)^-$, $(M+Na-2H)^-$, and $(M+K-2H)^-$ ions of **10** are 1069.38, 1091.36, and 1107.33, respectively. The measured masses are shown in the spectra.

ment more difficult, particularly in mixture analysis where signal overlap can occur. It also reduces the signal intensity because of the distribution of molecular-ion peaks. This problem can be averted by adding a small amount of NaCl to the sample, resulting in the domination of the sodium-cationized ions in the molecular-ion region (see Fig. 3A). In Fig. 3A, a clean spectrum and a much stronger $(M + Na - 2H)^-$ peak are obtained with the loading of half the amount of that used for Fig. 3B.

Note that accurate measurement of the molecular-ion mass can be readily made with the time-lag focusing MALDI instrument. However, the accuracy is dependent on a number of factors, including the sample/matrix preparation method, the type of calibrant and matrix used, and the method of calibration. The use of internal standards for mass calibration provides the best results. In general, an error of less than 70 ppm can be obtained [22]. With external calibration using the same sample preparation and the same type of molecules as the standards, an error of less than 100 ppm can be obtained. In this study, the instrument was externally calibrated with the peptide standards. The averaged mass measurement accuracy is within 500 ppm for all compounds tested, even with different sample preparation methods as described. For all sulfated oligosaccharides examined in this work, the instrumental resolution is sufficient to resolve the isotope peaks. Fig. 3C shows the expanded mass spectrum of the molecular-ion region for compound **10**. The mass resolution in this case is about 2440 FWHM (full width at half maximum). The measured mass for the molecular ion is 1091.50 and the calculated mass is 1091.36, representing a mass measurement error of 128 ppm.

In applying the MALDI method for real sample analysis, chemical composition of the sample can play an important role in detection performance. The presence of impurities as well as other chemical components can potentially have an adverse effect on the utility of the matrix preparation protocols. For example, in separation of oligosaccharides by HPLC, a high concentration of sodium phosphate or ammonium acetate is often used [15,16,25]. It is desirable to analyze the fractionated oligosaccharides with no or minimum sample cleanup to avoid possible structural degradation. We have examined the tolerance of the matrices 6-aza-2-thiothymine and coumarin 120 toward a large amount of salt in the sulfated oligosaccharide samples. We found that the matrix system is remarkably tolerant to a high concentration of salts. For example, almost the same mass spectrum as that shown in Fig. 3A can be obtained for compound **10** containing up to 0.1 M NaCl. Note that coumarin 120 is not particularly soluble in water, although 6-aza-2-thiothymine and the sulfated oligosaccharides dissolve well in water. On-probe washing with water can still be done with the sample prepared by the two-layer method. We speculate that both 6-aza-2-thiothymine and the analyte are entrained into the coumarin 120 crystals, and the insoluble crystals prevent further dissolution of the comatrix and the analyte. In the case of the samples containing 0.1 M

NaCl, spectra of equal quality can be obtained with or without on-probe cleaning.

Direct detection of sulfated oligosaccharides in a high concentration of ammonium acetate and sodium phosphate is also possible with the matrix system. However, it is found that the presence of ammonium acetate reduces the molecular-ion detection sensitivity. On the other hand, the use of the sodium phosphate buffer does not have a significant effect on the MALDI detection of the monosulfated oligosaccharides. As an example, Fig. 4 shows the mass spectra of compound **10** in 0.1 M sodium phosphate buffer obtained by MALDI without on-probe washing and with washing. The total amount of sample loaded is 1 pmol in both cases. The main spectral difference is the level of background signals. To obtain the spectrum from a sample without on-probe washing, about a 2-fold increase in laser power is required, compared with the sample with washing. The higher laser power produces stronger background signals as shown in the mass range from 600 to 1000 in Fig. 4A. The molecular ion is obtained in the form of the analyte sodium salt. No fragment ions are detected. These results indicate that the presence of sodium phosphate buffer does not alter the performance of the matrix formulation; thus, this buffer is highly recommended in

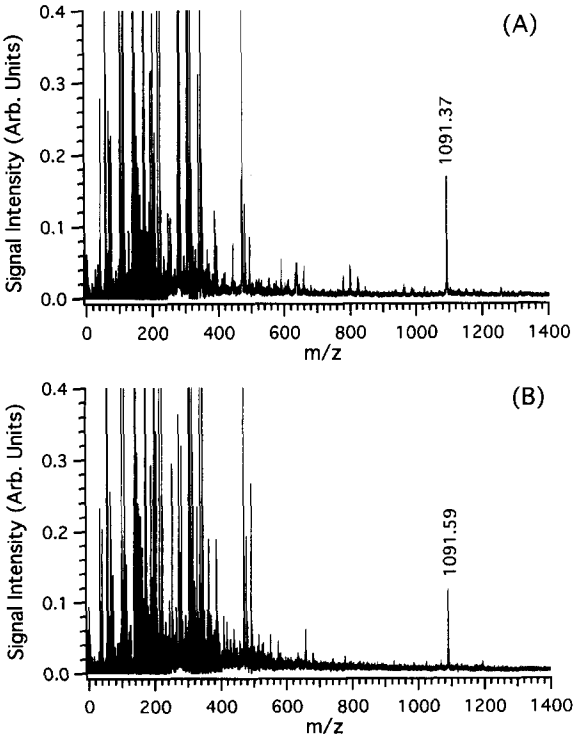

Fig. 4. Mass spectra of **10** in the presence of 0.1 M sodium phosphate buffer obtained (A) without rinsing the probe with water and (B) rinsing the probe with water.

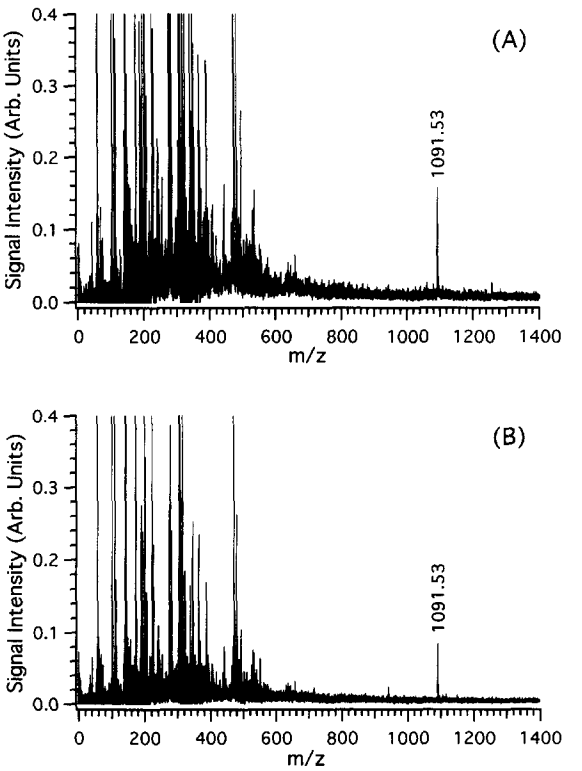

Fig. 5. Negative-ion MALDI mass spectra of a mixture of **10** and **13** in a molar ratio of (A) 1:1 and (B) 1:20. The total amount of **10** loaded is 1 pmol in both cases. The matrix preparation condition is the same as that used in Fig. 3A.

separation or sample preparation for monosulfated oligosaccharides from the MALDI analysis point of view.

In developing sample/matrix preparation protocols for MALDI, another major concern is related to the potential suppression of signals in direct analysis of mixtures. We find this not to be a problem at least for the analysis of mixtures containing both sulfated oligosaccharides and non-sulfated oligosaccharides. This is illustrated in Fig. 5 for the analysis of a mixture of a tetrasaccharide (**13**) and a sulfated tetrasaccharide (**10**) with different molar ratios. The matrix formulation used for obtaining spectra shown in Fig. 5 is the same as that used for the analysis of **10** alone. The peak observed at *m/z* 1091.53 corresponds to the molecular ion of **10**, $(M + Na - 2H)^-$. No signals from the tetrasaccharide (**13**) are detected. Fig. 5B also shows that a 20-fold excess of compound **13** reduces the signal level of compound **10** by about 40%. To detect the non-sulfated tetrasaccharide, a different matrix formulation is required. Fig. 6 shows the MALDI mass spectra of the same mixture with different molar ratios obtained by using 2,5-di-

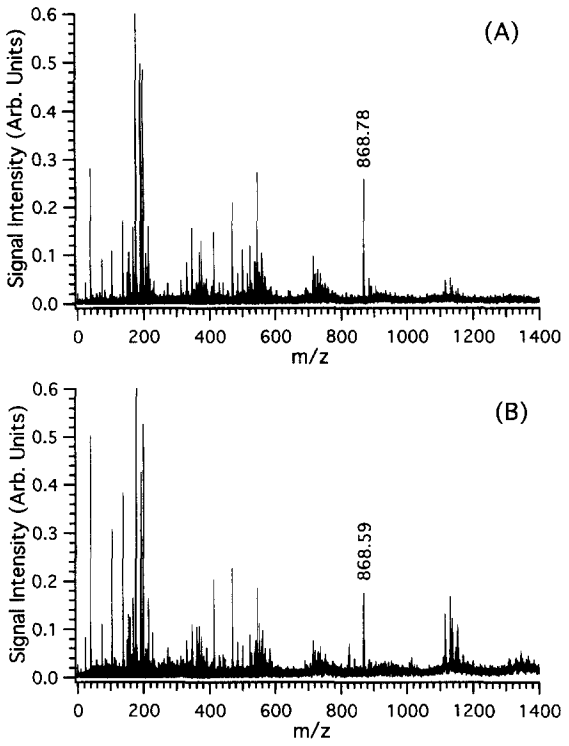

Fig. 6. Positive-ion MALDI mass spectra of a mixture of **10** and **13** in a molar ratio of (A) 1:1 and (B) 10:1. The total amount of **13** loaded is 5 pmol in both cases. The calculated exact mass for $(M+Na)^+$ of **13** is 868.38 and the measured masses are shown in the spectra.

hydroxybenzoic acid mixed with 5-methoxysalicylic acid as the matrix and operating the instrument in the positive-ion detection mode. In this case, only compound **13** is detected as the sodium-cationized molecular ion at *m/z* 868.78. No signals from the sulfated oligosaccharide are detected. Fig. 6B illustrates that the presence of a 10-fold excess of **10** in the mixture reduces the signal level by about 60%. Note that several peaks displayed at *m/z* ranging from 1100 to 1200 are from the matrix cluster ions.

The requirement to use different matrices for detecting oligosaccharides with different functional groups implies that fine tuning of the sample/matrix formulation is necessary for real-world sample analysis such as profiling the products of enzymatic reactions involving oligosaccharides. The real concern here is related to the total amount of sample required for multiple analysis in optimizing the sample/matrix preparation. This underscores the need to develop methods for improving the efficient use of sample in MALDI. The conventional sample loading method of placing 1 μL of sample over a large area on the sample probe, such as the one used in this work, is not efficient. To this end, we have been involved in developing a microspot MALDI method [26] aimed at reducing the amount of sample consumed in each preparation by loading the sample in a volume of 1 nL or less, thus allowing multiple analyses from a limited amount of analyte. Attomole detection of peptides has been demonstrated [26]. We expect improved detection sensitivity can be attained by using the microspot MALDI system for the detection of oligosaccharides. Work along this direction is planned.

In conclusion, matrix and sample preparation protocols have been developed for the analysis of monosulfated oligosaccharides, including those containing *N*-acetylneuraminic acid by MALDI MS. Coumarin 120 or a mixture of 6-aza-2-thiothymine and coumarin 120 prepared by using a two-layer method is found to be the optimal matrix for different monosulfated oligosaccharides. Molecular ions are detected with subpicomole detection sensitivity. It is also found that different matrix formulations are needed for the detection of different types of sulfated oligosaccharides. The presence of 0.1 M NaCl or 0.1 M sodium phosphate buffer does not alter the MALDI performance. In addition, for simple mixtures of a nonsulfated oligosaccharide and a sulfated oligosaccharide, the presence of other oligosaccharide in a mixture appears not to affect the detection of the oligosaccharide of interest. We anticipate that these matrix systems or modified versions of these are applicable to larger sulfated oligosaccharides. We have demonstrated that it is feasible to generate molecular ions of monosulfated oligosaccharides with subpicomole sensitivity by MALDI. We are in the process of extending this work to characterize sulfated oligosaccharides by tandem MS with both low- and high-energy collisional-induced dissociation in a sector/orthogonal-TOF instrument. The combination of MALDI MS and MALDI MS/MS should provide a new opportunity for detecting and identifying sulfated oligosaccharides in biological samples.

3. Experimental

Instrumentation.—Mass spectral data were collected on a linear time-lag focusing time-of-flight mass spectrometer. Details of the instrument have been described elsewhere [27]. Briefly, it features a four-plate source design, pulsed-ion extraction for time-lag focusing, and a one-meter linear flight tube.

Sulfated oligosaccharides are detected by operating the instrument in the negative-ion detection mode. The ions are generated by using a 3-ns-pulse-width laser beam from a nitrogen laser at 337 nm (Laser Science Inc., VSL 337ND, Newton, MA). A dual microchannel plate detector is used for ion detection, and a Hewlett–Packard MALDI data system combined with a LeCroy 9350M digital oscilloscope with a sampling speed of 1 Gigasample/s is used for mass spectral recording and data processing. In general, mass spectra from 50 to 100 laser shots are summed to produce the final spectrum.

Samples and reagents.—Compounds **1–7** [28] and **13** [29] (see Table 1) were prepared as previously described. Compounds **9–12** were prepared by total chemical synthesis using similarly established procedures of oligosaccharide synthesis [30]. Details of the synthesis will be reported separately (Y. Isogai and O. Hindsgaul, unpublished results). Compound **8** was obtained from V-LABS (Covington, LA). All the matrices examined in this work were purchased from Aldrich (Milwaukee, WI). Mass calibrants used in this work are N-t-Boc-Met-Asp-Phe-amide (Sigma, St. Louis, MO) and two synthetic peptides (acetyl-KLEALEA-amide and acetyl-KLEALEAKLEA-LEA-amide).

Sample preparation.—A uniform microcrystal formation method was used for sample/matrix deposition onto the MALDI probe [31]. In this method, the matrix solution is placed on a polished stainless steel probe and allowed to dry to form a microcrystalline layer. A solution containing both the analyte and the matrix is then added to the top of the matrix layer. The analyte solutions with known concentrations were made by dissolving appropriate amounts of sample in 1:4 H_2O–MeOH (v/v).

For the sulfated disaccharides and trisaccharide (**1–7** in Table 1), a 1-μL aliquot of a 0.05 M solution of 7-amino-4-methylcoumarin (coumarin 120) in 1:1 MeCN–MeOH (v/v) was used to form the first layer. The second layer solution was a 1:1 (v/v) mixture of analyte in about 10:1 H_2O–MeOH (v/v) and the supernant of coumarin 120 saturated in 1:2 MeCN–H_2O (v/v).

For the sulfated trisaccharide (**8**) without the methoxycarbonyloctyl aglycon, 2-(4-hydroxyphenylazo)benzoic acid (HABA) or coumarin 120 and 6-aza-2-thiothymine were used. With HABA as the matrix, the first layer was 1 μL of 10 g/L HABA in 1,4-dioxane, and the second layer was 0.5–1 μL of a solution of compound **8**. When coumarin 120 and 6-aza-2-thiothymine were used, the first layer was formed by using 1 μL of the matrix solution prepared from 16 mg/mL 6-aza-2-thiothymine in 6:4 MeOH–Me_2CO (v/v), and the second layer was the same as that used for the sulfated disaccharides.

For the sulfated trisaccharides or tetrasaccharides containing *N*-acetylneuraminic acid (NANA) (**9–12**), the first layer was the same as that described above for **8**. The second layer was formed by vacuum drying [32] from a 0.5-μL deposition of a solution containing 2 μL of analyte, 2 μL of supernant solution of coumarin 120 saturated in 1:2 MeCN–H_2O (v/v), and 1 μL of 2 mM NaCl.

With 2,5-dihydroxybenzoic acid (DHB) and 2-hydroxy-5-methoxybenzoic acid (HMB) as the matrices [17], 1 μL of a 1:1 (v/v) mixture containing the matrix solution and the analyte was applied. The matrix solution was prepared by mixing 10 g/L DHB in 1:2 MeCN–H_2O (v/v) with 10 g/L HMB in 1:2 MeCN–H_2O (v/v).

With sinapinic acid (SA) as the matrix, 1 μL of 0.12 M SA in 99:1 Me_2CO–H_2O (v/v) was deposited on the probe and allowed to dry in the air. A 1-μL aliquot of the analyte solution was then deposited on the top of the first layer [33].

For mass calibration in the *m/z* region between 500 and 800, N-t-Boc-Met-Asp-Phe-amide and acetyl-KLEALEA-amide were used as the external calibrants. With SA as the matrix, 1 μL of 0.10 M SA in 60:36:4 MeCN–MeOH–H_2O (v/v/v) was deposited on the probe and allowed to dry in the air. A 1-μL aliquot of 1:1 (v/v) mixture of the standard solution and the SA matrix solution was added to the top of the first layer and dried in vacuum [32]. For mass calibration in the *m/z* region between 800 and 1500, acetyl-KLEALEA-amide and acetyl-KLEALEAKLEALEA-amide were used as the external calibrants. 4-Hydroxy-α-cyanocinnamic acid (HCCA) was used as the matrix. A 1-μL aliquot of 0.12 M HCCA in 99:1 Me_2CO–H_2O (v/v) was applied to the probe and allowed to dry in the air. A 1-μL aliquot of the standard solution was added to the top of the first layer.

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