International Journal of Mass Spectrometry 305 (2011) 164–169

Contents lists available at ScienceDirect

International Journal of Mass Spectrometry

journal homepage: www.elsevier.com/locate/ijms

Infrared matrix-assisted laser desorption/ionization mass spectrometry for quantification of glycosaminoglycans and gangliosides

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ARTICLE INFO

Article history:
Received 26 July 2010
Received in revised form 21 October 2010
Accepted 25 October 2010
Available online 4 November 2010

Keywords:
Difference frequency generation
Urea
Sulfate
Heparin
Quantification

ABSTRACT

Infrared matrix-assisted laser desorption/ionization (IR-MALDI) is a "softer" ionization technique than UV-MALDI. In the present study, IR-MALDI using a 6 μm laser produced clean negative ion mass spectra of sulfated disaccharides, from heparin and chondroitin sulfate, without significant loss of sulfate groups. The sulfur content (wt%) of chondroitin sulfate could be calculated from the *m*/*z* and the signal intensity of di- and tetra-saccharides derived from chondroitinase digestion. Similarly, no significant loss of sialic acid occurred in poly-sialylated gangliosides including GD1b, GT1b and GQ1b. Interestingly, multiply deprotonated $[M-nH]^{n-}$ ions were observed in their mass spectra, and were useful for relative quantification of gangliosides in complex mixtures. These features of IR-MALDI were anticipated to be useful for robust discrimination and quantification of different gangliosides by a mass imaging technique. The multiply deprotonated ions observed in the 6 μm laser IR-MALDI mass spectrum suggested that the mechanism underlying deprotonated ion generation might differ between the 2.94 μm and 6 μm laser wavelengths.

1. Introduction

Glycans constitute the carbohydrate portion of glycoconjugates such as glycoproteins and glycolipids, or exist primarily as a free sugar chain. The biological functions of these sugars are quite diverse [1,2]. For example, N-linked glycans of glycoproteins are involved in the proper folding of newly synthesized polypeptides in the endoplasmic reticulum and in the subsequent maintenance of protein solubility and conformation. Various types of glycans of glycolipids and glycoproteins are present on the cell surface and play key roles in cell–cell interactions. All of these structural and modulatory functions are related to specific glycan structures [3,4]. Therefore, structural characterization of each glycan is essential to establish the structure–function relationship, but detection and identification of glycans in complex mixtures is also a crucial step preceding further analyses [5].

Mass spectrometry (MS) is the core technology in glycomics due to its high sensitivity and the capacity to analyze complex mixtures. The current advantage of MS is largely dependent on soft ionization methods, because raising the internal energy and thereby increasing fragmentation is often a major concern with ionization of labile biomolecules. In this regard, electrospray ionization (ESI) is generally considered to be softer than matrix-assisted laser/desorption ionization (MALDI). Especially, the glycans containing sialic acid residues or *O*- or *N*-sulfate groups are typically fragile, and often give signals derived from loss of these groups in the MALDI mass spectrum. However, MALDI has some advantages over ESI. For example, the ionization response of MALDI for neutral oligosaccharides is more constant as mass increases than that of ESI [6]. MALDI produces simple mass spectra of single or smaller charge state ions than ESI. MALDI allows rapid data acquisition from a small surface area. All of these MALDI features allow profiling of complex mixtures and high-throughput analysis as typically exploited by tissue imaging MS. These advantages of MALDI have been confirmed by a series of multi-institutional studies on *N*- and *O*-glycans [7,8].

There are three classes of internal energy build-up resulting in prompt fragmentation and post-source decay of UV-MALDI ions [9]. Thermal activation and chemical activation in the MALDI plume cause prompt fragmentation, and the latter is due to reactions with hydrogen radicals. The third class of activation is caused by extraction-induced collision with neutral components of the plume. One of the recent attempts to overcome the fragmentation of acidic glycoconjugates was the use of ionic liquid matrices. For example, 1-methylimidazolium (Im) salt of α-cyano-4-hydroxicinnamic acid (CHCA), suppresses the fragmentation of (poly)sialylated gangliosides and sulfated oligosaccharides, probably due to limited thermal activation [10–12]. However, some degree of sulfate group loss was observed for the latter compounds even with the next generation of ionic liquid matrices [13,14]. Costello and her colleagues extended the idea of collisional (vibrational) cooling of hot MALDI ions during the desorption/ionization

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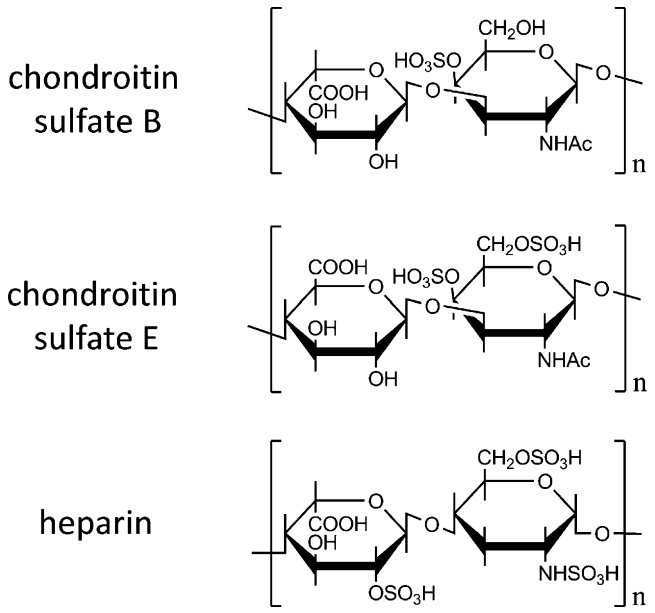
doi:10.1016/j.ijms.2010.10.029



Scheme 1. Chemical structures of sulfated sugars.

event in high-pressure MALDI to their Fourier transform MS and were able to reduce the cleavage of the sialic acid linkages in gangliosides [15,16]. Zhang et al. reported atmospheric pressure MALDI MS of gangliosides and sulfated sugars, and showed less fragmentation than the conventional vacuum MALDI MS [17]. However, loss of sialic acid from multiply sialylated gangliosides or sulfated sugars was observed in all of these UV MALDI experiments. Another approach is the use of an infrared (IR) laser for MALDI, or IR-MALDI. IR-MALDI is a softer ionization method than UV-MALDI, as demonstrated by a striking application to large nucleic acids [18], and is, therefore, anticipated to be useful for labile glycoconjugates. Indeed, the combination of an IR laser (ER:YAG, 2.94 μm) with urea or glycerol matrices for MALDI was reported for the detection of ganglioside species separated on a thin-layer plate [15,19].

In our previous study, we scrutinized the features of IR-MALDI at a 6 μm wavelength and found the thermal and chemical classes of activation to be quite low compared with UV [20]. Especially, hydrogen radical formation was minimal at this wavelength, but probably not at 2.94 μm. These merits as well as low photon energy (0.21 eV for λ = 6 μm vs. 3.67 eV for λ = 337 μm) allowed detection of labile molecules such as S-nitrosylated peptides and clean mass spectra of sialylated or sulfated carbohydrates without fragmentation. To our knowledge, this was the first application of IR-MALDI to sulfated glycans. Herein, we extend that study and explore the capability of IR-MALDI in the quantitative profiling of labile glycans or glycoconjugates including glycosaminoglycans (GAGs) and gangliosides (Schemes 1 and 2).

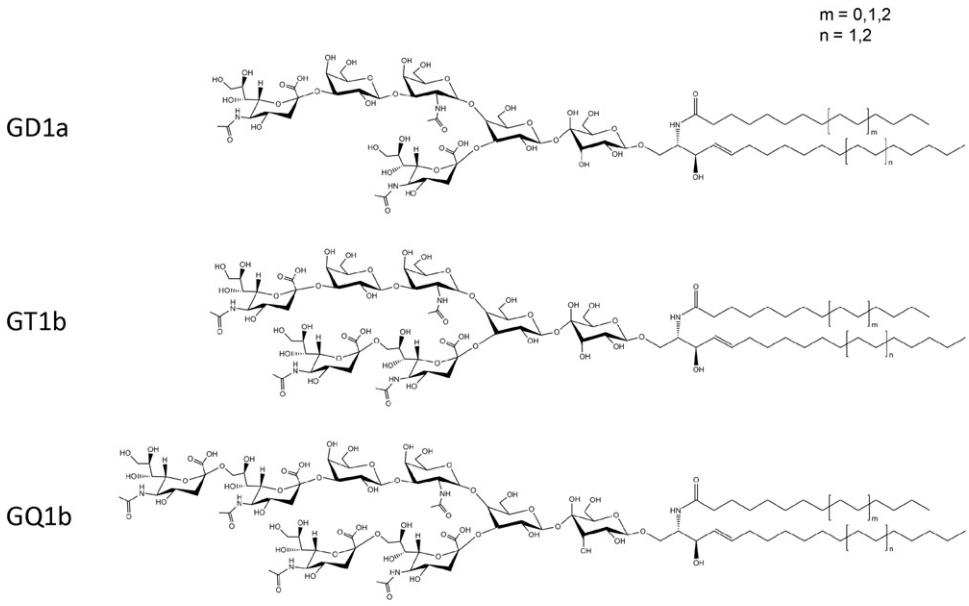
2. Experimental methods

2.1. Materials and sample preparation

Urea and GD1a (from bovine brain) were purchased from Wako Pure Chemical Industries (Osaka, Japan). GT1b (bovine brain), 1,1,3,3-tetramethylguanidium (TMG) and *p*-coumaric acid were obtained from Sigma–Aldrich (St. Louis, MO). GQ1b (bovine brain) was purchased from Calbiochem (Darmstadt, Germany). Gangliosides dissolved at 1 mM in 10% methanol were subjected to ZipTip C18 (Millipore, MA) to remove salts, and an aliquot of the eluent in 100% acetonitrile was dried and dissolved in 50% methanol.

Heparin disaccharide (1-S, sodium salt) was purchased from Sigma–Aldrich and was dissolved in distilled water at 50 μM. Heparin-derived tetrasaccharide (0.27 mM) was a gift from Dr. Ryoichi Sakiyama [21]. It was derived from a heparinase digest of heparin and was isolated using a size-exclusion chromatography column equilibrated with ammonium hydrogencarbonate.

Chondroitin sulfate B (CS-B) (pig skin dermatan sulfate) and E (CS-E) (squid cartilage) were purchased from Seikagaku Kogyo (Tokyo, Japan). Each 0.2 mg chondroitin sulfate was digested with 10 mU chondroitinase ABC (Seikagaku Kogyo) in a 0.1 mL solution of 50 mM Tris–HCl, pH 7.0, at 37 °C for 3 h. Subsequently, the solution was added to Ultrafree-MC (10,000 NMWL) (Millipore, Bedford, MA) and centrifuged at 8000 × *g* for 15 min, and the filtrated solution was recovered.



Scheme 2. Chemical structures of gangliosides.

2.2. Mass spectrometry

Both IR- and UV-MALDI mass spectra were acquired using a Voyager DE Pro time-of-flight (TOF) mass spectrometer (Applied Biosystems, Foster City, CA) which was equipped with a 337 nm nitrogen laser (3 ns pulse width and 20 Hz repetition). The acceleration voltage was 20 kV. The mass spectra were acquired by negative ion extraction and reflector TOF mode operation. The signals from 500 laser shots were accumulated to form a spectrum.

For IR MALDI, a tunable wavelength pulsed laser (5 ns pulse width and 10 Hz repetition) in a range of 5.5–10.0 μm generated by difference frequency generation (DFG) (Kiss-laser, Kawasaki Heavy Industries, Tokyo, Japan) was introduced through a Zn:Se window of the ion-source of the mass spectrometer [20]. A fixed 5.9 μm wavelength corresponding to the carbonyl stretching absorption was employed in the present study. The maximum energy outside the ion source chamber was approximately 700 μJ. The IR laser beam diameter was approximately 0.5 mm. The sample matrix was urea. An aliquot of 1 M urea in 50% acetonitrile was mixed with analyte solution on the sample target.

For UV-MALDI MS, an ionic liquid matrix of G_3CA, *i.e.*, the TMG salt of *p*-coumaric acid, was prepared according to the method of Fukuyama et al. [14]. Briefly, *p*-coumaric acid was mixed with TMG at a 1:3 molar ratio in methanol. After evaporation of the methanol in a SpeedVac for 2–3 h, the solution was left in a vacuum overnight and then dissolved in methanol again at a concentration of 9 mg/0.1 mL. The solution was diluted 5% with methanol before use, and an aliquot was mixed with analyte solution on the MALDI sample target.

ESI (nanospray) MS was carried out using an LTQ XL ion trap mass spectrometer (Thermo Fisher, San Jose, CA). Samples were dissolved in 50% methanol solution and directly infused into the mass spectrometer using a PicoTip emitter (New Objective, Woburn, MA). Typical nanospray source conditions were 2.2 kV source voltage, −42.8 V capillary voltage, and 200 °C capillary temperature.

3. Results and discussion

3.1. Characteristics of 6 μm IR-MALDI MS

In the present study, a TOF mass spectrometer with IR (DFG, λ = 5.5–10 μm) and UV (nitrogen, λ = 337 nm) lasers was used, and DFG was set at the 6 μm wavelength region corresponding to the C=O stretching vibrations. In our previous study using this wavelength, the sensitivity of IR-MALDI for the protonated ions of peptides was two or three orders of magnitude lower than that of UV. On the other hand, the sensitivity for detecting alkali adducted ions of neutral sugars was equal to or slightly better than that of UV MALDI. However, the "softness" feature of IR MALDI was typically encountered in the detection of deprotonated molecules for acidic glycans or glycoconjugates, giving clean negative ion mass spectra with one or two orders of magnitude better sensitivity for sialylated or sulfated sugars [20]. Therefore, we focused on negative ion extraction throughout this study, and used urea, the softest matrix among a variety of compounds so far examined. Dissolving urea in 50% acetonitrile is important to avoid chunk formation of the dried urea/analyte mixture.

3.2. Heparin-derived oligosaccharides

The sulfate groups of GAGs and heparin are quite fragile in UV-MALDI MS, resulting in a loss of SO_3 (−80 u) or $NaSO_3$ (−$NaSO_3$ + H: −102 u). Recently, ionic liquid matrices such as Im or TMG salt of CHCA (ImCHCA or G_2CHCA, respectively) and G_3CA have been used to suppress the loss of these groups [10,11,13,14]. However, sulfate group loss still occurs to various degrees even with these matrices, while the fragments observed in the mass spectrum are lower in the positive than in the negative ion mode [13,14]. We examined all three ionic liquid matrices and found that G_3CA gave the best results with respect to loss of sulfate groups (data not shown). In Fig. 1a showing the negative ion UV-MALDI mass spectrum of heparin disaccharide trisulfate measured with G_3CA, deprotonated ions of di- and tri-sodium adducts (or salts) were observed, and a signal at *m/z* 518.0 indicated a loss of $NaSO_3$ (−$NaSO_3$ + H, see above) from $[M-3H+2Na]^-$ (*m/z* 619.9) or SO_3 from $[M-2H+Na]^-$ (*m/z* 598.0). Similarly, a signal at *m/z* 496.0 corresponded to a fragmentation product from either or both $[M-2H+Na]^-$ and $[M-H]^-$, but there were no signals corresponding to a loss from $[M-4H+3Na]^-$ (*m/z* 641.9), being consistent with the notion that pairing sulfate groups with sodium ions stabilizes O–SO_3 bonds [22]. This is the reason that cationization of sulfated glycans is necessary. However, optimization of the ratio of analyte molecules and sodium atoms and the corresponding sample preparation are obviously burdensome. In addition, strong matrix interference, as shown in Fig. 1a, is a distinct drawback to the use of an ionic liquid matrix for small molecules.

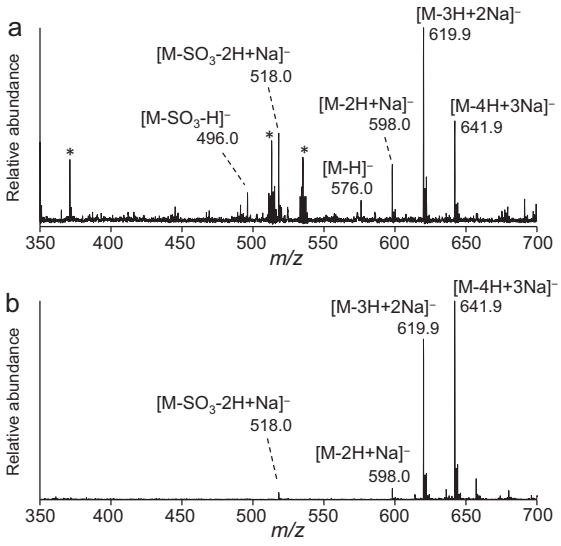


Fig. 1. Negative ion MALDI reflector TOF mass spectra of heparin-derived disaccharides containing three sulfates. (a) UV-MALDI mass spectrum with G_3CA ionic liquid matrix. The peaks indicated by an asterisk are matrix-derived ions. (b) IR-MALDI mass spectrum.

In contrast, the IR-MALDI mass spectrum of the same sample was associated with minimal sulfate group loss despite the presence of insufficiently cationized $[M-3H+2Na]^-$ ions (*m/z* 619.9), giving a clean mass spectrum with a high signal-to-noise ratio (Fig. 1b). Fig. 2a shows an IR-MALDI mass spectrum of heparin-derived tetrasaccharides after addition of an approximately 100-fold molar excess of sodium acetate. The ions at *m/z* 1306.8 carried six sulfate groups. The ions observed at *m/z* 1204.9 were the tetrasaccharide containing five sulfate groups but not the product of $NaSO_3$ loss, because the ratio of the five and six sulfated species in this mass spectrum was comparable to that in the ESI mass spectrum shown in Fig. 2b.

3.3. Chondroitin sulfate

Vertebrate GAGs consist of repeating sulfate-substituted disaccharide units polymerized into long chains. The biological functions of proteoglycans are mediated by the interactions of these GAGs with different protein ligands such as growth factors, and the interaction capacity depends on the specific pattern of GAG sulfation.

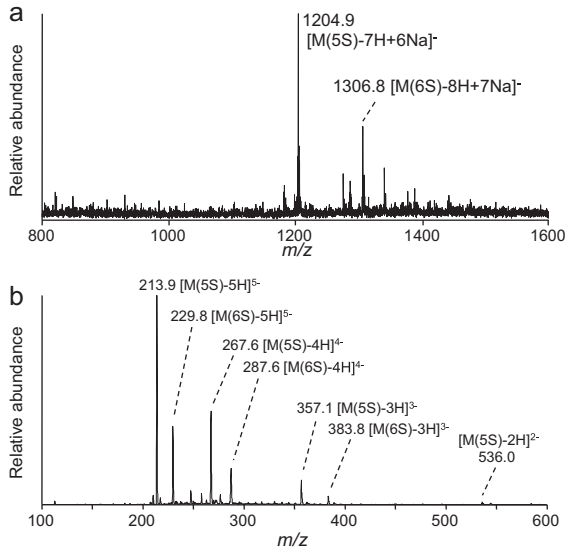


Fig. 2. Negative ion IR-MALDI and ESI mass spectra of heparin-derived tetrasaccharide. (a) IR-MALDI reflector TOF mass spectrum. The molecule containing five sulfate groups corresponding to the ion at *m/z* 1204.9 was present in the sample, but not the fragmentation product. (b) ESI (nanospray) mass spectrum.

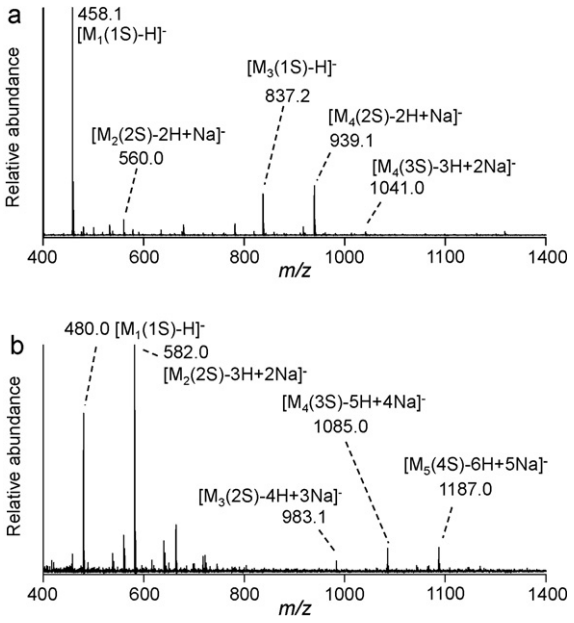


Fig. 3. Negative ion IR-MALDI reflector TOF mass spectra of CS oligosaccharides from chondroitinase ABC digestion. (a) Chondroitin (dermatan) sulfate B from pig skin. (b) Chondroitin sulfate E from squid cartilage.

Therefore, the sulfation level is among the basic pieces of information to be obtained from GAG specimens, while the locations of sulfate groups on disaccharide units are a major structural characterization issue. This is readily known from the adverse clinical event caused by oversulfated chondroitin sulfate as a contaminant of heparin [23,24]. Considering the simple and clean IR-MALDI mass spectra of sulfated saccharides in Figs. 1b and 2a, it was anticipated that the overall sulfation levels, or quantification of the sulfur content, of GAGs or heparin could be calculated from the mass spectrum of disaccharides derived from glycosidase digestion. This method is a type of label-free quantification, which has been applied to glycopeptides for *N*- and *O*-glycan profiling [25,26].

To address this strategy, chondroitin sulfate samples from different sources, CS-B and CS-E, were digested with chondroitinase ABC, and the resulting disaccharides were analyzed by IR-MALDI MS. To simplify the mass spectral profile and to stabilize the ions, an approximately 200-fold molar excess of sodium acetate was added to the CS-E disaccharide sample. In the mass spectra shown in Fig. 3, the signal intensity of the disulfated disaccharide ions is much higher for the CS-E than for the CS-B disaccharide sample. The difference is substantiated by calculations using the following equations, among which Eq. (1), or the percent content of each molecular species, is the same as that used for glycopeptide profiling [25,26]:

$$\text{peak}_i\,\% = \frac{\text{peak}_i\ \text{intensity}}{\sum \text{peak}_i\ \text{intensity}} \times 100 \quad (1)$$

where peak_i is the intensity of the ion for molecule-*i* in the mass spectrum.

The average content (wt%) of sulfur in the original GAGs is then calculated using Eq. (2):

$$S\,(\text{wt}\%) = \sum \left[\frac{32 \cdot n_i}{M_i} \times \text{peak}_i\,\% \right] \quad (2)$$

where M_i, mass of molecule-*i*, is determined based on the *m/z*, and n_i is the number of sulfates in molecule-*i*. The number 32 is the atomic weight of sulfur.

Taking all of the signals of differently sulfated species as well as their sodium adducts into account, the values 6.9% and 9.9% for CS-B and CS-E, respectively, were obtained from these mass spectra. These calculated values were consistent with the respective sulfur contents, 6.4% and 8.75%, based on elementary analysis and labeled for these specimens. A major concern with our calculation is the possible presence of disaccharides without sulfates not being taken into account, because negative ion generation from neutral glycans would be inefficient as compared with sulfated glycans.

3.4. Gangliosides

Use of a 2.94 μm IR laser for MALDI MS of gangliosides has been studied in considerable detail [15,19]. From a quantification point of view, a good correlation between IR-MALDI ion signal intensity and the sample amount of a singly sialylated ganglioside, GM3, has been reported [19]. In our previous study, 6 μm IR-MALDI of GD1a and negative ion extraction produced a clean mass spectrum composed of $[M-H]^-$ species with minimal sialic acid loss signals [20], similar to the results obtained with a 2.94 μm laser. In the IR-MALDI mass spectrum of GQ1b shown in Fig. 4, cation-adduct ions such as $[M(Na)-H]^-$ and $[M(K)-H]^-$ are abundant and

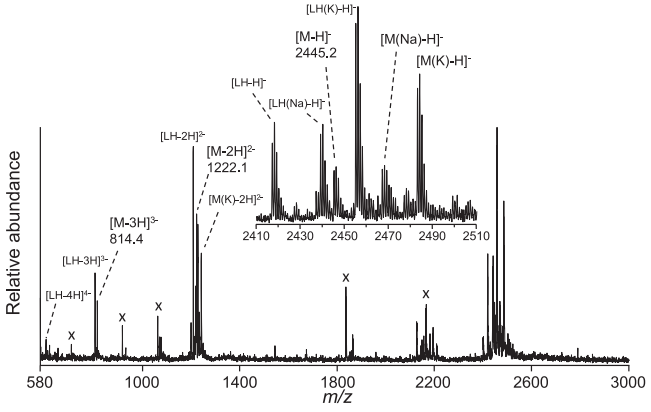


Fig. 4. Negative ion IR-MALDI reflector TOF mass spectrum of ganglioside GQ1b Ceramide structures are C20:1 and C18:1 for M and LH, respectively. The singly charged ion region is depicted in the inset. The peaks indicated by "x" are derived from poorly or desialylated species, which were detected by ESI MS as well (data not shown), and thus were present in the sample before MALDI measurements.

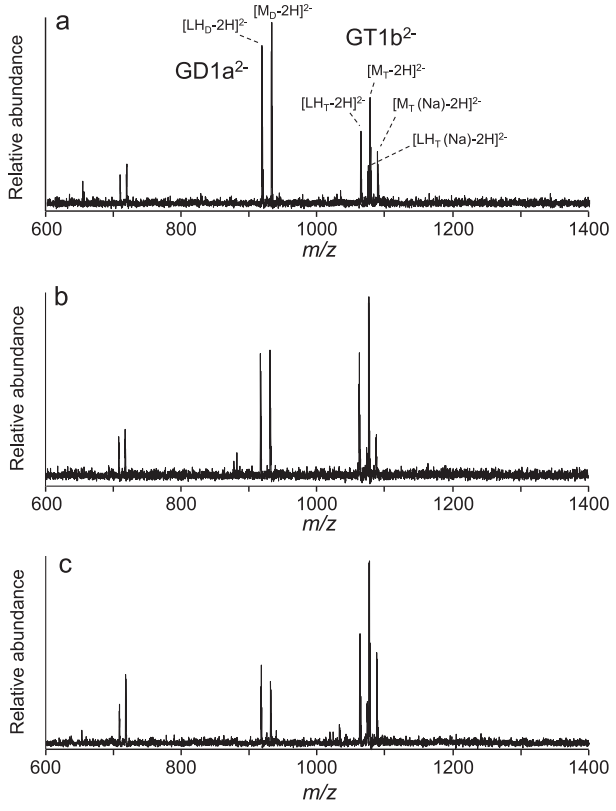


Fig. 5. Negative ion IR-MALDI reflector TOF mass spectra of mixtures of GD1a and GT1b gangliosides. The GD1a/GT1b ratios were 2.0 (a), 1.0 (b) and 0.5 (c).

the mass spectrum is complicated in the singly charged ion region. Interestingly, however, multiply charged ions were observed in the 6 μm IR-MALDI mass spectrum while no cation-adducted ions were observed among the triply charged species. In addition, the presence of multiply charged ions, which have higher internal energy than the singly charged species, demonstrated the "softness" feature of IR-MALDI.

Fig. 5 shows the mass spectra of GD1a and GT1b mixture samples, in which the GD1a/GT1b ratios were 2.0, 1.0 and 0.5. Differences in the GD1a/GT1b ratio among these samples are readily recognized by viewing $[M-2H]^{2-}$ ions, indicating that this method allows robust discrimination and determination of the ratios of different gangliosides. This result also suggested that use of IR-MALDI for mass imaging allows reliable localization of each differently sialylated ganglioside, a key issue in brain science and pathology.

Both 2.94 μm and 6 μm IR-MALDI techniques generate multiply protonated ions from proteins [20,27]. However, in contrast to the 6 μm wavelength, there were no $[M-nH]^{n-}$ multiply charged ions in the 2.94 μm IR-MALDI mass spectra of GT1b, as described by Ivleva et al. [15], despite the same urea matrix being used. The mechanism underlying deprotonated ion generation may differ between the 2.94 μm and 6 μm laser wavelengths, which use stretch vibrations of O–H and N–H (2.94 μm) and C=O (6 μm) bonds for the MALDI process.

4. Conclusions

IR MALDI using a 6 μm laser causes little or no loss of acidic groups from polysulfated oligosaccharides or polysialylated gangliosides. The softness of IR MALDI for these labile molecules appears to exceed those of all other UV MALDI methods including atmospheric pressure MALDI or the use of an ionic liquid matrix. The multiply charged ions observed in the mass spectra are free of cation adducts, and are useful for relative quantification or profiling of the biomolecules modified with different numbers of acidic groups. The softness feature of IR MALDI observed here is probably unique to a 6 μm wavelength range, providing a new perspective on IR MALDI applications.

Acknowledgement

This study was supported in part by a grant-in-aid for Scientific Research (B) #19390093 from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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