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Matrix-assisted laser desorption/ionization mass spectrometry of polysaccharides with 2′,4′,6′-trihydroxyacetophenone as matrix

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So far, there have been only a few matrices reported for detection of polysaccharides with molecular weight higher than 3000 Daltons by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). In this work, we found that 2′,4′,6′-trihydroxyacetophenone (THAP) is a good matrix for MALDI time-of-flight MS analysis of polysaccharides with broad mass range. Large polysaccharides, dextrans, glycoproteins and polysialic acids have been successfully detected by MALDI-MS with THAP as matrix. Copyright © 2007 John Wiley & Sons, Ltd.

Matrix-assisted laser desorption/ionization (MALDI)[1,2] was successfully developed as a soft ionization technique for biopolymers and macromolecules less than two decades ago.[1,3,4] This technique made direct mass measurement of high molecular weight compounds such as synthetic polymers,[5] proteins,[6] and oligonucleotides[7] feasible. The benefit of MALDI-mass spectrometry (MS) for analysis of polymers resulted predominantly from the formation of singly charged molecular ions that leads to a simple mass spectrum for polymer distribution analysis. In view of the increasing biological importance of saccharides associated with cell-cell recognition, protein targeting and metabolic diseases, polysaccharide and glycoconjugate research has been attracting ever more attention.[8,9] Up to now, gel permeation chromatography (GPC) has been the method of choice for measurement of molecular information of polysaccharides. However, it is tedious and resolution is generally poor. Moreover, molecular weights and hydrodynamic sizes do not always correlate linearly. Although some ionic polysaccharides, such as polysialic acids and polygalacturonic acids, have been analyzed by high-performance anion-exchange chromatography (HPAEC),[10–12] it is still time consuming and molecular size identification is not direct. In addition, upper limits of determination are rather limited. Thus direct mass spectrometric measurement of large polysaccharides is highly desirable to overcome these shortcomings. Electrospray ionization mass spectrometry (ESI-MS) and MALDI-MS are two major tools for biopolymer analysis. Since most polysaccharide samples are polydisperse with respect to molecular size, the use of ESI-MS is not suitable due to the fact that multiple peaks are produced from a single compound by ESI. Furthermore, the ionization efficiency of polysaccharides is low in ESI due to the low proton affinity of carbohydrates. Therefore, MALDI is preferred for polysaccharide mass analysis. Nevertheless, detection of non-derivatized polysaccharides by MALDI-MS is still a challenge due to the lack of adequate matrices. To the best of our knowledge, there have been no reports on MALDI-MS measurements of linear non-derivatized polysaccharides with molecular weights higher than 7000 Da. In order to detect carbohydrate compounds by MALDI-MS, derivatization processes such as permethylation,[13] peracetylation, and reductive amination[14] were often utilized. Unfortunately, derivatization processes can alter chemical properties from those of the intrinsically neutral sugars. In addition, the efficiencies for derivatization of different sizes of polysaccharides usually are different. It is quite difficult to achieve a complete derivatization of all hydroxyl or other groups.[15] Thus, a capability of direct detection of non-derivatized polysaccharides by MALDI becomes critically important. In this work, we measured both derivatized and non-derivatized polysaccharides as well as glycoproteins and neoglycoproteins with MALDI-MS.

The first investigation of non-derivatized oligosaccharides by MALDI with 3-amino-4-hydroxybenzoic acid as matrix was reported by Mock *et al*. in 1991.[16] Stahl *et al*. subsequently discovered that 2,5-dihydroxybenzoic acid (DHB) yielded better reproducibility and higher signal-to-noise ratio[17] than

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3-amino-4-hydroxybenzoic acid. Since then, DHB has become the primary choice of matrix for oligosaccharides. Improvements in sensitivity with a concomitant improvement in resolution were achieved with the addition of 10% 2-hydroxy-5-methoxybenzoic acids to DHB[18] and these co-matrices were referred as 'super-DHB'. 1-Hydroxyisoquinoline (HIQ)[19] was also found to be an effective additive to DHB to produce more homogeneous samples for MALDI detection. DHB with or without additives has been broadly used as matrix on MALDI for neutral saccharides.

Although MALDI allows the detection of very large proteins (>100 000 Da), it has not been very useful for detection of large polysaccharides. To alleviate this deficiency, it is highly desirable to explore more effective matrices for MALDI analysis on saccharides and their derivatives. Since MALDI mass detection so far has been limited to polysaccharides of ~3000 Da with DHB as matrix, more effective matrices for larger polysaccharides have been actively sought. Recently, 5-amino-2-mercapto-1,3,4-thiadiazole[20] was reported as a matrix for MALDI of neutral polysaccharides with molecular weight up to approximately 5000 Da. Several other matrices have also been reported.[14,21,22] However, the detection barrier of low mass-to-charge ratio (m/z) (less than 7000) still needs to be improved. In the past, 2′,4′,6′-trihydroxyacetophenone (THAP) was tested for the analysis of acidic carbohydrates[14] and oligonucleotides[23,24] with some success, but it has not been successfully applied to neutral polysaccharides. We now have found that THAP is a good matrix for MALDI of polysaccharides and various glycoconjugates. For example, a linear neutral polysaccharide with m/z higher than 47 000 was readily detected by MALDI using THAP. Use of THAP as matrix always yielded high quality spectra with good reproducibility. Spot-to-spot repeatability and dominant molecular ions were always obtained with THAP as matrix.[25] We found THAP is a better matrix than DHB, not only for linear neutral polysaccharides, but also for dextrans, polysialic acids, and glycoproteins.

In this work, we also report negative ion and positive ion mass spectra[26–28] of polysaccharides with THAP as matrix. This observation is noteworthy due to its departure from conventional expectations from the previous literature.[17,29–31] MALDI experiments of neutral carbohydrates are commonly performed in positive ion mode. Our observation of both positive and negative ions of polysaccharides can give some clues on the MALDI mechanism of polysaccharides. It has been speculated that the polysaccharide desorption/ionization efficiency by MALDI is less than that for proteins although few quantitative studies have been reported. In this paper, we also compared the efficiency of MALDI for polysaccharides to proteins.

EXPERIMENTAL

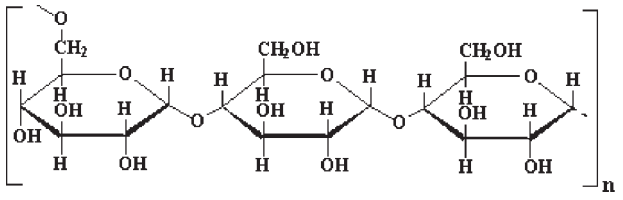
The approach employed in the sample preparation for MALDI targets was the 'dried-droplet' method instead of a vacuum drying process.[19] THAP itself, under ambient condition, can cause the analyte to be embedded in the matrix homogeneously because of its low degree of phase segregation. Therefore, 'sweet spots'[32] are less significant than for DHB as matrix.

Materials

The matrices 2,5-dihydroxybenzoic acid (DHB) and 2′,4′,6′-trihydroxyacetophenone (THAP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA), and used without further purification. Cesium iodide (CsI) and acetonitrile (ACN) were obtained from Merck & Co., Inc. (USA). All chemicals were of analytical grade and double distilled water was used throughout. The PL polysaccharide standards (pullulans), produced by the microorganism *Aureobasidium pullulans*, with molecular weights (MWs) of ~5900, ~11 800, ~22 800, and ~47 300 Da (abbreviated as PL-6k, PL-12k, PL-23k and PL-47k in this report), were acquired from Polymer Laboratories (Varian, Inc., Amherst Fields Research Park). They are characterized by ultracentrifugal sedimentation equilibrium, and have been broadly used for calibration in GPC. The pullulans consisting of maltotriose repeating units, forming a linear and polysaccharide chain, are known as α-1,4-α-1,6-glucans. Three glucose units in maltotriose are connected by an α-1,4-glycosidic bond, whereas consecutive maltotriose units are connected by an α-1,6-bond. Scheme 1 shows the chemical structures of PL samples. The modified sugar of mannose octamer $C_{53}H_{93}NO_{41}$, abbreviated as Man-8 (Scheme 2), with MW of 1400.3 Da, was synthesized as the mimic of the epitopes of HIV surface glycoprotein, gp120. Dextrans of high MW comprising mainly α-1,6-linked glucose units and some short α-1,3-linked glucose branch units were purchased from Fluka BioChemika (Switzerland). Man_{51}-BSA was prepared according to the published method.[33]

MALDI sample preparation

THAP or DHB was dissolved in 50% ACN by mixing 1:1 (v/v) ACN and double distilled water (ddH_2O) to the final concentrations of 50 nmol/μL with or without addition of CsI. When CsI was used as additive to the matrix solution, the concentration was typically fixed as 1/20 of matrix concentration. For polysaccharide samples, typical concentrations ranged from1 nmol/μL to 1 pmol/μL. Powders of PL-6k, Pl-12k, PL-23k and PL-47k were dissolved in ddH_2O to obtain solutions of 1 nmol/μL concentration used as stock solutions. These stock solutions were further diluted to 100, 50, 10, 2 pmol/μL for necessary experiments. Typical samples for MALDI analysis were prepared by combining 1 μL of matrix solution (50 nmol/μL, maybe with salts or electrolyte) with 1 μL of diluted saccharide solutions of interest in an Eppendorf tube. Then 1 μL of this mixture was applied onto the sample plate.



Scheme 1. Maltotriose repeating units comprise the pullulan polysaccharides.

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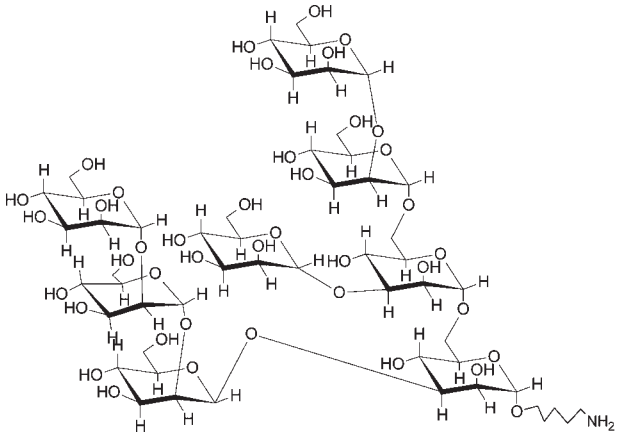

Scheme 2. Chemical structure of Man-8.

Instrumentation

The MALDI-TOF mass spectrometer used to acquire the spectra was a Voyager Elite (Applied Biosystems, Foster City, CA. USA), equipped with a nitrogen pulsed laser (337 nm). The acceleration voltage was set at 20 kV in either positive or negative ion mode. Typically, spectra from 80–100 laser shots were accumulated to obtain the final spectrum. All mass spectra obtained in this work are from a linear TOF. A reflectron TOF was also tested but the corresponding signals were much lower than those obtained in linear mode. For larger polysaccharide (MW >10 000 Da) no signals were observed when using the reflectron TOF mass spectrometer. Laser energy per pulse was calibrated with a laser power meter (PEM 101, Laser Technik, Berlin, Germany) so that laser fluence could be precisely measured. The delay extraction time[34] could be adjusted from 10 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 applied was in the range of 50–300 mJ/cm^2. The vacuum inside the flight tube was always kept between 10^{-7} and 10^{-6} Torr.

RESULTS AND DISCUSSION

Despite the wide use of MALDI analyses of proteins in proteomic research, its detailed mechanism is still not understood. MALDI of polysaccharides is even less clear. For example, most polysaccharide ions detected appear to contain alkali atoms, and, yet, the source of the alkali atoms and the mechanism of alkalization are not known. Due to the relatively poor mass accuracy and polydispersity of polysaccharides, it is often difficult to identify the types of polymer ions (protonated, Na^+ attached, K^+ attached, etc.) in a MALDI spectrum. Figure 1 shows a MALDI mass spectrum of a pure branched mannose octamer ($C_{53}H_{93}NO_{41}$) with THAP as matrix. Only the Na^+-attached ion peak was observed, indicating MALDI with THAP as matrix can give a simple mass spectrum, which is an advantage for analyzing samples containing mixtures of different size polysaccharides. Furthermore, the low ratio of matrix-to-analyte eliminates interference in the low-mass region due to matrix compound.

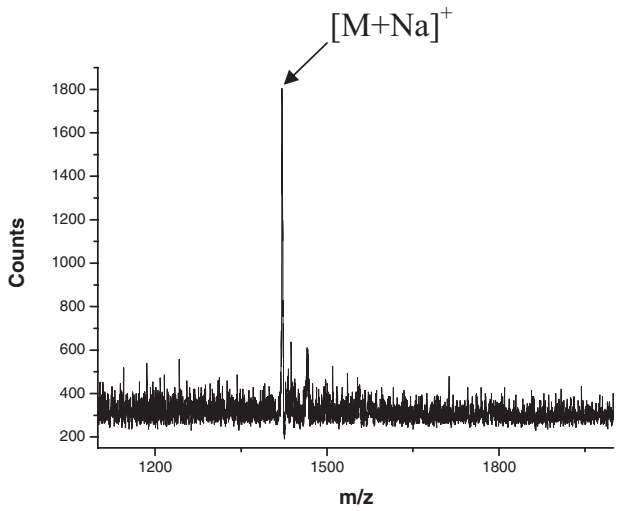

Figure 1. Mass spectrum of Man-8. Sodium-attached adduct of Man-8 is clearly observed. The concentrations of Man-8 and THAP are both 1 nmol/μL. Laser fluence is applied as 160 mJ/cm^2. The low matrix/sample ratio eliminates matrix interference in the mass spectrum on the analysis of oligosaccharide.

MALDI of linear polysaccharides

Up to now, MALDI has not been successfully used for analyzing large non-derivatized linear polysaccharides. In this work, THAP was used to probe the feasibility of measuring various large polysaccharide compounds. Positive ion MALDI spectra of polysaccharides with different MWs are shown in Figs. 2–5 with various matrices as listed in Table 1. For PL-6k polymers, different matrices do not make any significant difference in intensity (Fig. 2). Spectra from all four different matrices show the repeating unit of mass 486 Da which corresponds to the anhydromaltotriose moiety ($C_{18}H_{30}O_{15}$). In addition to the series with a 486 Da gap, there is a minor series with a 162 Da increment corresponding to the mass of anhydro-glucose. It may be due to the presence of a stunted maltotriosyl moiety that might result from incomplete synthesis or from degradation of the polysaccharide. Two categories of cation adducts with a mass difference of 16 Da are observed in Figs. 2(a), 2(b) and 2(d). One corresponds to the sodium attachment and the other is the potassium adduct $[M_n+K]^+$ where the suffix '11' indicates that n is equal to 11. Since sodium is more abundant than potassium in nature, the $[M_n+Na]^+$ peak series are expected to be higher than the corresponding peaks for $[M_n+K]^+$, as shown in Figs. 2(a) and 2(d). However, this ratio is reversed in Fig. 2(b) when super-DHB is used as matrix. (The height of the peaks of *m/z* at 5387 Da, namely $[M_{11}+Na]^+$, is smaller than that of 5403 Da, namely $[M_{11}+K]^+$.) Since the detailed mechanism for alkalization of polysaccharides is not known, it is difficult to give a good explanation at present. A broad peak at *m/z* 5400 Da presumably contains $[M_{11}+Na]^+$ and $[M_{11}+K]^+$ peaks, which are not resolved when DHB-HIQ is used as matrix (Fig. 2(c)).

Figure 3 shows mass spectra of the 'PL-12K polysaccharide' sample with the same four matrices. In these mass spectra, peaks for the series of maltotriose repeating units

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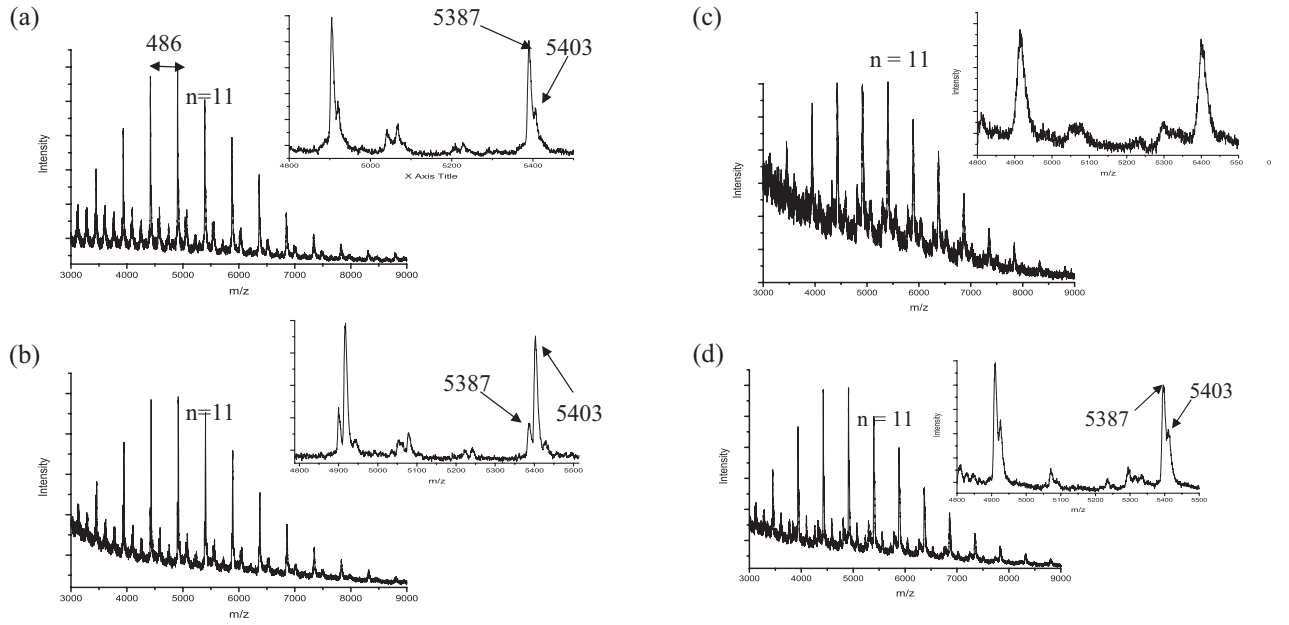

Figure 2. Mass spectra for PL-6k polysaccharide using matrix (a) DHB only, (b) super-DHB, (c) DHB-HIQ, and (d) THAP in positive ion mode. All matrices above are at the level of 50 nmol/μL and PL-6k compound at 10 pmol/μL. Laser fluence is estimated as 300 mJ/cm^2. Sodium and potassium ion attachments could be clearly identified in these denotations except in (c).

still dominate the spectrum. In the past, osmometry and light scattering have been used to determine weight-average MW, number-average MW and polydispersity index.[35,36] MALDI-MS provides a more direct way for mass distribution measurement. Nevertheless, the MALDI-measured MW was often found to be lower than that obtained by GPC and the peak distribution is prominently asymmetric (i.e. the top of the Gaussian-bell shifts towards the low-mass region). Only a small deviation from a Gaussian distribution for MALDI of PL-6K (Fig. 2) was observed. For MALDI of PL-12k shown in Fig. 3, results are similar to organic polymers. It is obvious that the average MW from mass distributions obtained in Fig. 3 does not completely agree with the values obtained by GPC. This discrepancy increases as the average MW of the polysaccharides increases. This discrepancy can be due to the following three reasons: (1) higher desorption and ionization efficiency for smaller polysaccharide than larger ones; (2) degradation of large polysaccharides into smaller polysaccharides during the MALDI process; and (3) low detection efficiency with a microchannel plate detector (MCP) for the larger ions compared to the small ones. This effect is more pronounced when DHB, super-DHB or DHB-HIQ is used as matrix (Figs. 3(a), 3(b) and 3(c)) than when THAP is used. It is quite clear that THAP as matrix gives a mass spectrum with a better mass resolution and less bias on mass distribution (Fig. 3(d)). Since ion detection efficiency is always lower for high-mass ions with the same kinetic energy, the relative signal amplitudes should be adjusted for different detection efficiencies for various masses. For large ions with velocity near the threshold of secondary electron ejection, detection efficiency for a MCP is approximately proportional to v^4 where v is the velocity of the ion.[37] If we adjust amplitude for this differential detection efficiency, the average MW shifts to the higher mass side (Fig. 3(e)). The average MW was obtained as 10618 Da that is closer to the result obtained by GPC. It indicates no significant degradation of large polysaccharides into smaller polysaccharides when THAP is used as matrix. It also indicates that THAP is a better matrix in terms of profiling size distribution of polysaccharides. Figure 4 shows mass spectra for the PL-23K polysaccharide sample with THAP. Clear signals with good resolution were only obtained with THAP as matrix. For all other matrices, no signals were observed (spectra not shown). The averaged MW including detection efficiency correlation is still lower than that obtained by GPC. When even higher mass polymers (PL-47K sample) are analyzed, signals can still be observed with THAP as matrix. Nevertheless, mass resolution was not good enough to resolve each individual polysaccharide (Fig. 5). No polysaccharide signals were observed when other matrices were used. Low mass resolution is mostly due to the broad energy distribution of large analyte molecules.[38] The performances of various matrices used for polysaccharides by MALDI-MS are listed in Table 1. THAP is clearly a better matrix than DHB. As of now, THAP is the only matrix suitable for MALDI detection of large linear polysaccharides with m/z higher than 20 000 Da.

Effect of molar ratio of matrix/polysaccharide

For MALDI of proteins and oligonucleotides, samples are typically prepared with a molar ratio of matrix-to-analyte between 10^3 and 10^6. In general, MALDI of proteins or nucleotides with a higher MW requires a high ratio of matrix-to-analyte to obtain a good mass spectrum. For MALDI of polysaccharides, the matrix-to-analyte ratio for signal optimization has not been established. Table 2 shows the results obtained from polysaccharides of various MWs analyzed using different amounts of THAP as matrix. The concentrations of polysaccharides were maintained as 0.01 nmol/μL. For the of PL-6k and PL-12k polysaccharide

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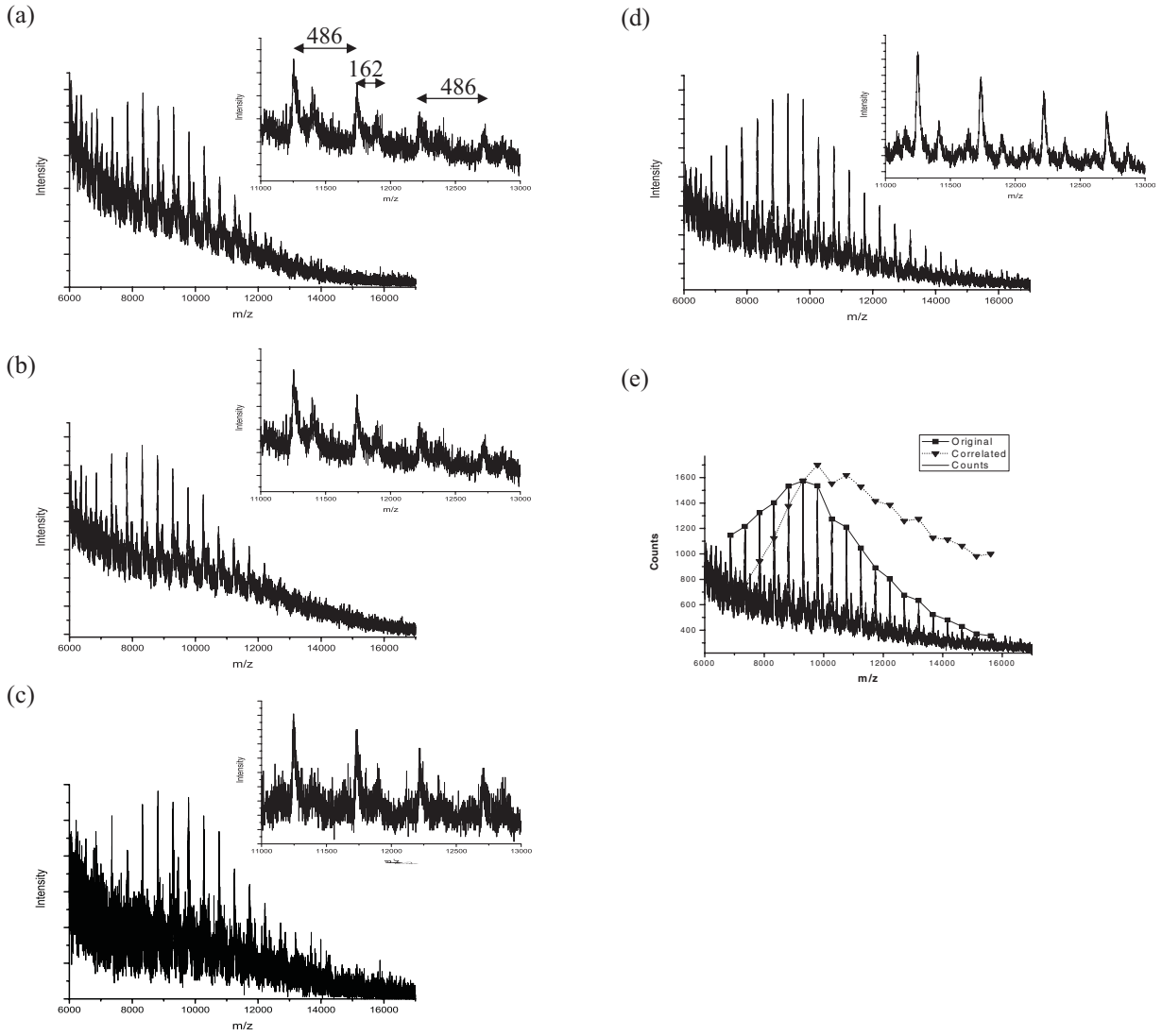

Figure 3. Mass spectra of PL-12k polysaccharide using matrix (a) DHB only, (b) super-DHB, (c) DHB-HIQ, and (d) THAP in positive ion mode. All the matrices above are at the level of 50 nmol/μL and PL-12k compound at 10 pmol/μL. Laser fluence is estimated as 300 mJ/cm^2. In addition to typical 486 Da peak-to peak increments, there are gaps of 162 Da adding to the dominant series. (e) Correlated mass distribution of (d). ▼: corrected; ■: original counts.

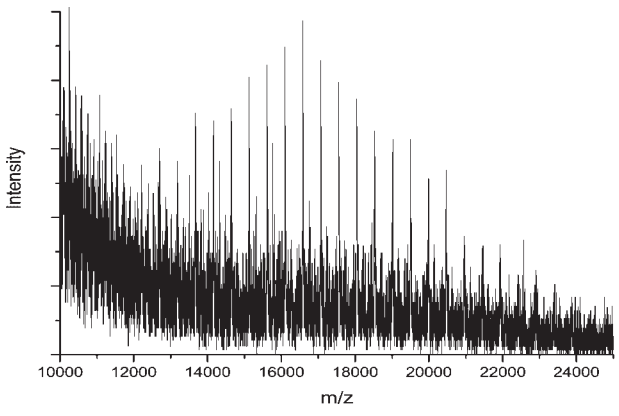

Figure 4. Mass spectrum of PL-23k polysaccharide using THAP as matrix in positive ion mode. The matrix is at the level of 50 nmol/μL and PL-23k compound at 10 pmol/μL. Laser fluence is estimated as 375 mJ/cm^2. Only when THAP used as matrix could signals be obtained.

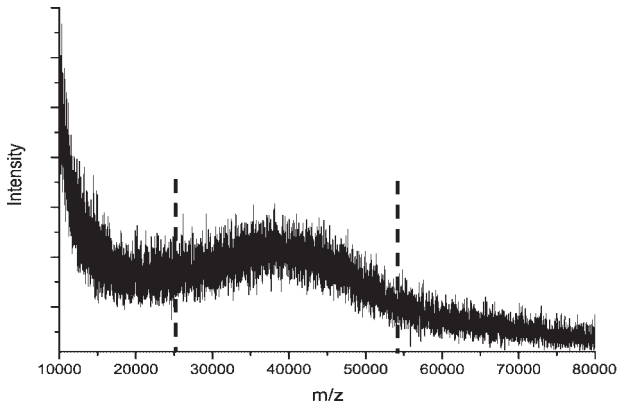

Figure 5. Mass spectrum of PL-47k polysaccharide using THAP as matrix in positive ion mode. A wide range of mass band instead of a series of peaks was obtained. The matrix concentration is 50 nmol/μL and PL-47k compound at 10 pmol/μL. Laser fluence is estimated as 400 mJ/cm^2. The region within the two dashed lines indicates the broad band. The mass resolution is not high enough to resolve different sizes of polysaccharides.

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Table 1. MALDI signals of polysaccharides with different matrices. The concentrations of polysaccharide and matrix are 50 nmol/μL and 0.01 nmol/μL, respectively

MW	6k	12k	23k	47k
DHB only	++[a]	+	−	−
Super-DHB[b]	++	+	*	−
DHB-HIQ[c]	++	+	−	−
THAP only	++	+	+	○

[a] ++: excellent; +: weak; −: no analyte signal observed; *: faint; ○: not resolved.
[b] Super-DHB.[15]
[c] DHB-HIQ.[16]

samples, sensitivity is not a strong function of the matrix-to-analyte ratio. For the PL-23k sample, no signals were observed even when the matrix-to-analyte ratio was above 5000. It is noteworthy that a smaller matrix-to-analyte ratio usually gave better MALDI signals for polysaccharides. A strong polysaccharide signal can still be obtained with a matrix-to-analyte ratio as low as 100. In contrast, for MALDI of large proteins or oligonucleotides, few signals can be obtained with the matrix-to-analyte ratio less than 500. This result may imply that the desorption/ionization process of polysaccharides is different from that of proteins. In order to compare the desorption/ionization efficiency of MALDI for polysaccharides and proteins, we mixed polysaccharides with a known quantity of protein in the same sample for MALDI mass spectra measurement. The results are shown in Fig. 6 where matrix concentration is 500 times higher than that of the polysaccharide; the polysaccharide concentration is 1000 times higher than that of the protein with polysaccharide concentration of 1 nmol/μL. Since the signal for the insulin ion is about 50% higher than the sum of all polysaccharide signals, it indicates that the MALDI efficiency for proteins is about 1500 times better than polysaccharides of a comparable mass when THAP is used as matrix. The obstacle of matrix selection for neutral polysaccharides awaits further exploration.

Cationization with alkali metal ions

Cationization mechanism in MALDI has been reported by different groups concerning polymers and peptides.[39–42] For

Table 2. MALDI signals of polysaccharides with different matrix-to-analyte ratios. The former concentration belongs to the matrix and the latter is of polysaccharide

matrix: THAP				
MW				
nmol/μL	6k	12k	23k	47k
1 vs. 0.01	++[a]	+	+	**
10 vs. 0.01	++	+	+	**
25 vs. 0.01	++	+	+	**
50 vs. 0.01	++	+	+	*
75 vs. 0.01	+	−	−	−
100 vs. 0.01	+	−	−	−

[a] ++: excellent; +: weak; −: no analyte signal observed; **: broad peak but clear; *: faint.

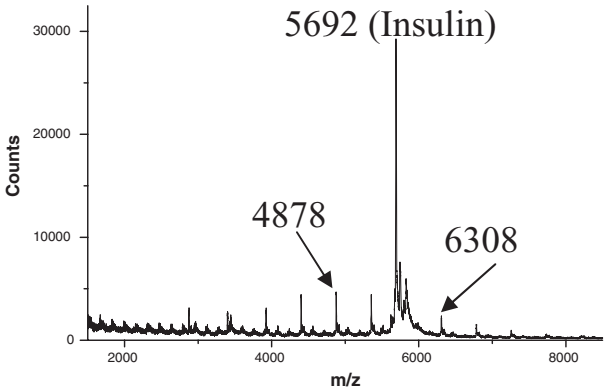

Figure 6. MALDI mass spectrum of polysaccharides and insulin mixtures. Laser fluence is estimated as 160 mJ/cm^2. Even though the concentration of insulin is 1/1000 that of polysaccharide, the sum of the signals arisen from polysaccharide is still lower than that from insulin.

polysaccharides, alkalization occurred much more frequently than protonation (Fig. 1). In order to locate the source of alkalization, CsI was intentionally added in the mixture during sample preparation. As a result, Cs^+ would compete

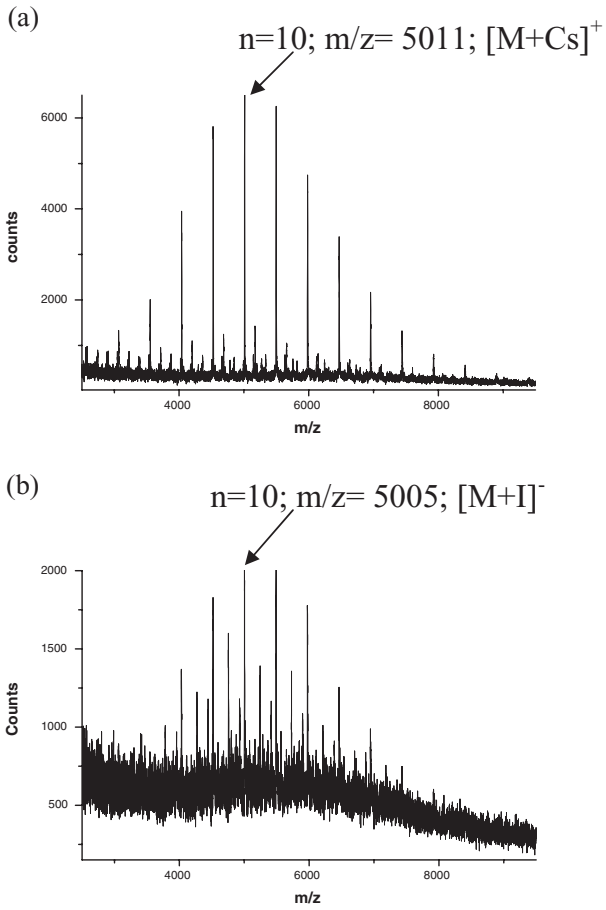

Figure 7. Mass spectra of PL-6k polysaccharide with an addition of CsI. The concentrations of polysaccharide mixtures and CsI are 10 pmol/μL and 5 pmol/μL, respectively. Laser fluence is estimated as 160 mJ/cm^2: (a) positive ion mode and (b) negative ion mode with THAP as matrix. A series of cesium-attached ions are clearly observed in (a) while iodine-attached negative ions are observed in (b).

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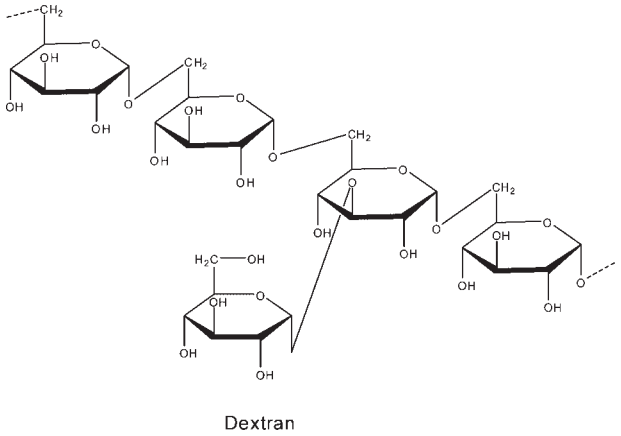

Scheme 3. Structure of dextran.

with naturally occurring K^+ and Na^+ for alkalization of polysaccharide. Experimental results are shown in Fig. 7(a). Positive polysaccharide ions with a Cs adduct can be clearly resolved. So far, few negative ion MALDI mass spectra of polysaccharides have been reported. With the addition of CsI, we also observed a negative ion spectrum of linear polysaccharides, as shown in Fig. 7(b). All ion peaks can be easily identified with a negative iodine ion attached.

MALDI of dextrans

In addition to large linear polysaccharides, THAP was also used for MALDI of polysaccharides with side chains such as dextran. Dextrans are somewhat complex, branched polysaccharides made of many glucose molecules joined into chains of different lengths. The straight chain consists of α-1,6-glycosidic linkages between glucose molecules, while branches often begin from an α-1,3-linkage. The structure of dextran is shown in Scheme 3. Dextrans with MWs ranging from 10 000 to 150 000 Da have been commonly used in microsurgery. The results shown in Fig. 8(a) indicate that either larger polymer ions can be fragmented into smaller polysaccharide ions during the MALDI process or smaller dextran polymer ions are produced much more easily than larger ones. Positive ion peaks in Fig. 8(a) only reach ~8000 Da while the average MW of ~19 000 Da was obtained by GPC. Since there is little reason to expect the overall desorption/ionization efficiency for smaller dextran compounds would be so much higher than the larger ones in this mass region, we speculate that fragmentation is a possibility to cause the stronger signals for smaller dextran compounds. On the other hand, GPC, relying on the hydrodynamic dimension of the analytes, is not reliable for very accurate mass determination of branched polysaccharides that tend to have larger hydrodynamic volumes than their linear counterparts. In the past, permethylation[43] was often used to increase the ion signals of some polysaccharides. We also pursued permethylation on dextrans to check the possibility to extend the detection to higher mass range. Experimental results are shown in Fig. 8(b). With permethylation, polymeric peaks exceeding 16 000 Da were observed. The mass spectrum also shows a gap of mass peaks of 204 Da that corresponds to the per-O-methylated inner Glc residue. The signal-to-noise ratio was also clearly improved after

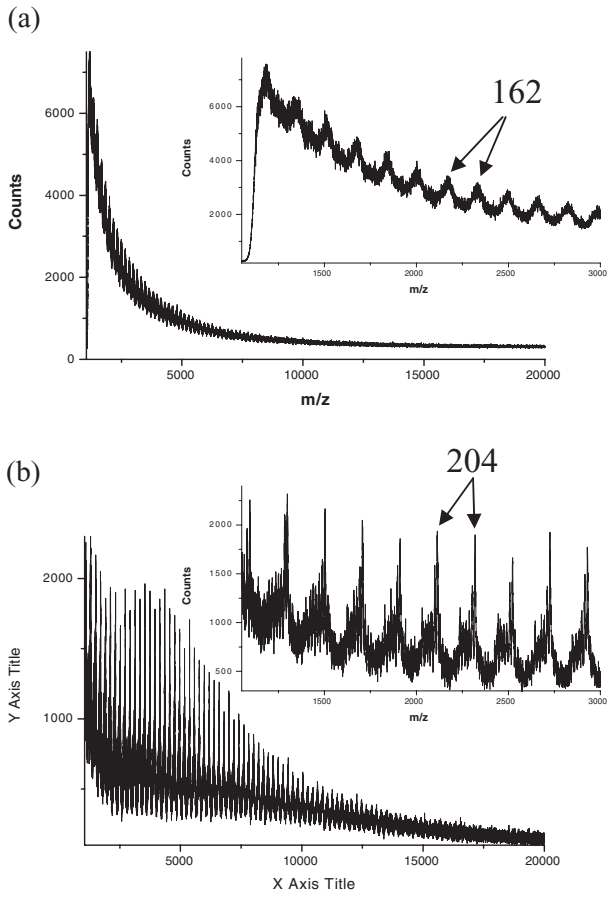

Figure 8. Mass spectra of (a) natural and (b) methylated dextrans in positive ion mode. Condensed natural dextrans have three free hydroxyl groups possible for methylation. Thus, 162 Da increments would switch to 204 Da. Matrix concentration is 50 nmol/μL and dextran concentration is 10 pmol/μL. Laser fluence is estimated as 200 mJ/cm^2.

permethylation. Even with permethylation and correction for differential efficiency, the average MW by MALDI was still less than that determined by GPC.

Detection of glycoproteins, neoglycoproteins and polysialic acids

THAP was also used as matrix for the detection of glycoproteins to probe structural information of glycans on proteins. Bovine fetuin type III, obtained from fetal calf serum, plays important roles in many physiological aspects. It can stimulate endocytosis, influence bone marrow maturation during the mineral phase, promote the action of lymphocytes and lipid binding activity and bind to thyroid hormones. The MALDI-TOF mass spectrum of bovine fetuin with THAP as matrix is shown in Fig. 9. Besides the precursor ion, a doubly charged ion and the dimer can also be clearly identified in this figure. Figure 10 displays the mass spectrum of Man_{51}-BSA (51 is the average value of Man/BSA, colorimetrically determined). Its structure is shown in Scheme 4. R denotes one of the lysyl side chains. The formula weight of the residue inside the bracket is 236 Da. Thus, all Man units will be summed up to be around

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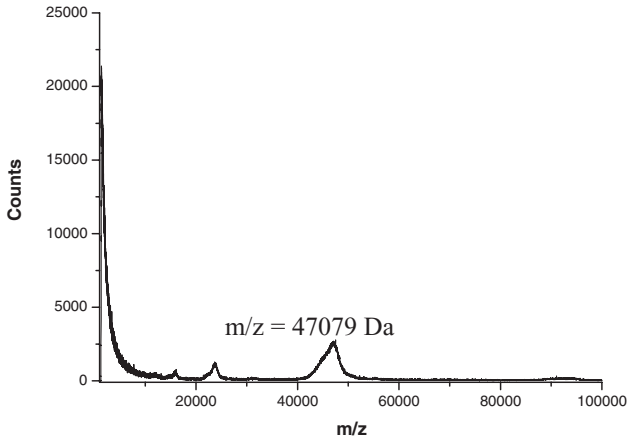


Figure 9. MALDI-MS of bovine fetuin within mass range 1000–100 000 Da in linear positive ion mode. The ratio matrix to fetuin is 10000/1. The concentration of THAP is 100 nmol/μL and that of bovine fetuin is 10 pmol/μL. Laser fluence is applied at 160 mJ/cm^2.

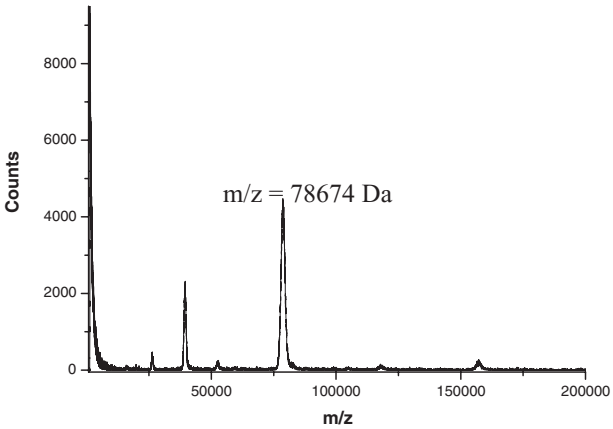
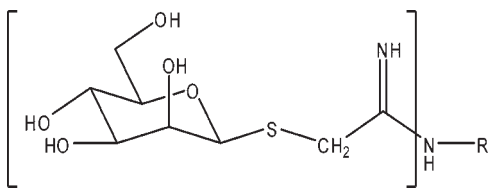


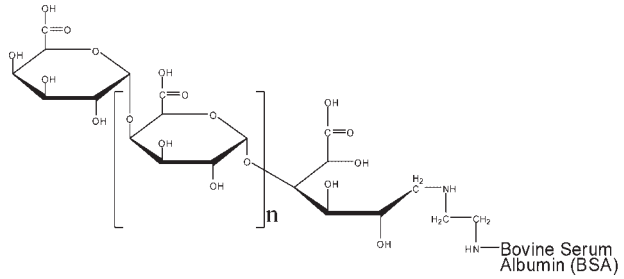
Figure 10. The MALDI-TOF spectrum of Man_{51}-BSA. The concentration of THAP is 100 nmol/μL and that of Man_{52}-BSA is 10 pmol/μL. Laser fluence is applied at 160 mJ/cm^2.

12 036 Da. Finally, with the MW of BSA added, the estimated MW of the total glycoprotein will be 78 466 Da; our result of a peak at 78 674 Da is in good agreement with the value determined colorimetrically.

MALDI-MS of oligogalacturonic acid (oligo-Gal A)-linked BSA with THAP as matrix was also tested (Scheme 5 and Fig. 11). The glycan portion which constitutes part of the glycoprotein contains seven units of partially O-acetylated



Scheme 4. Chemical structure of Man_{51}-BSA. R is the ε-amine of lysine on BSA.



Scheme 5. (Galacturonic acid)$_7$-BSA, abbreviated as (GalA)$_7$-BSA, contains total number of cyclic and acyclic GalA heptamers.

galacturonic acid with an estimated molecular weight 1780–1980 Da. A zoom-in illustration of mass range 1000–20 000 shows (Gal A) units of ~1870 Da increments. The mass spectrum in Fig. 11 shows BSA peaks with discrete mass separation of on average 1870 Da, correspondent to the (Gal A)$_7$ side chains. These results indicate that THAP as matrix allows MALDI to be used for measurement of large glycoproteins.

Recent studies[44] revealed the structural and functional diversity of polysialic acids that exist in organisms ranging from bacteria to humans. In this study, direct mass detection of natural and lactonized polysialic acids was pursued. Figure 12(a) shows the mass spectrum of a sample of a mixture of large polysialic acids with THAP as matrix. The mean MW obtained from Fig. 12(a) is almost equivalent to the results from HPAEC.[43] When DHB was used as matrix, fragmentation was observed since peaks were only observed in the low-mass region (see Fig. 12(b)). Nevertheless, a gap of 291 Da, which is equivalent to the mass of sialic acid, was clearly observed (See Fig. 12(b)). Figures 12(a) and 12(b) again show that THAP is a better matrix for detecting large polysialic acid compounds than DHB. It is known that polysialic acid can form lactones spontaneously even under mild acidic conditions.[45] (The chemical structure is shown in Scheme 6.) The MALDI mass spectrum of lactonized

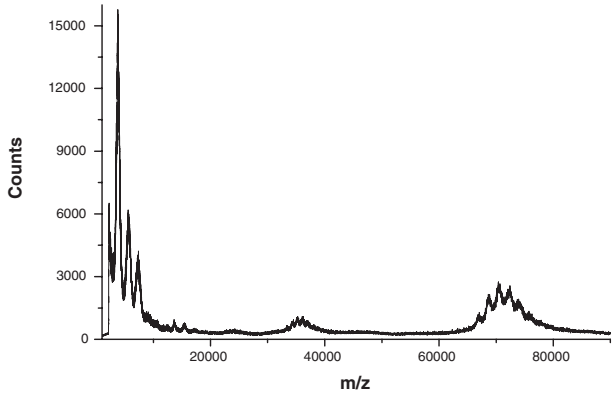


Figure 11. MALDI-MS of (GalA)$_7$-BSA within the mass range1000–90 000 Da in linear positive ion mode. The ratio of matrix to (GalA)$_7$-BSA is 10000/1. The concentration of THAP is 100 nmol/μL and that of (GalA)$_7$-BSA is 10 pmol/μL. Laser fluence is estimated as 160 mJ/cm^2.

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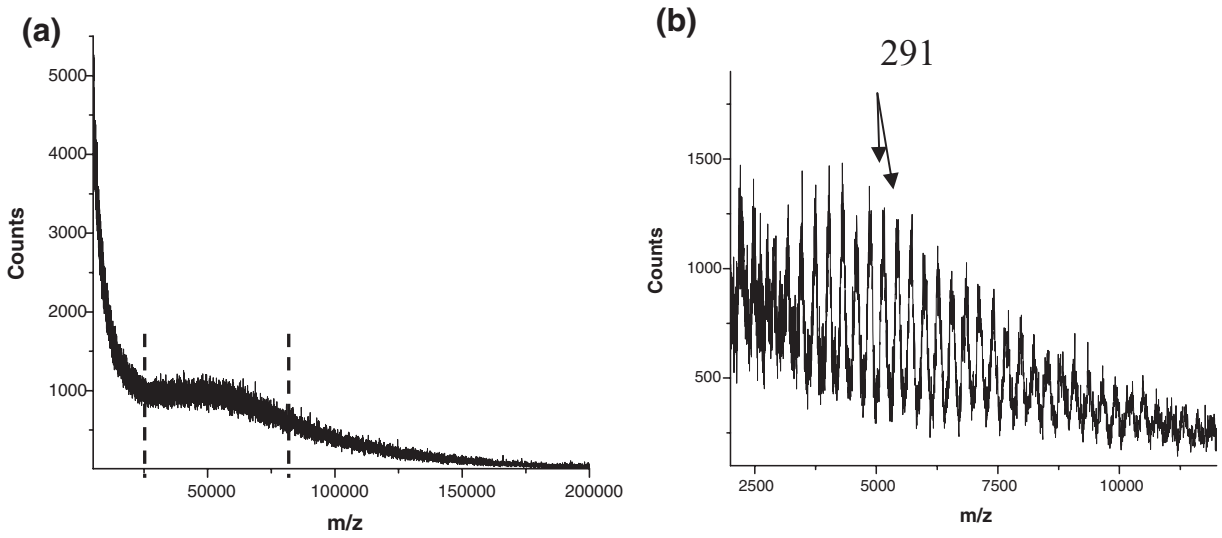
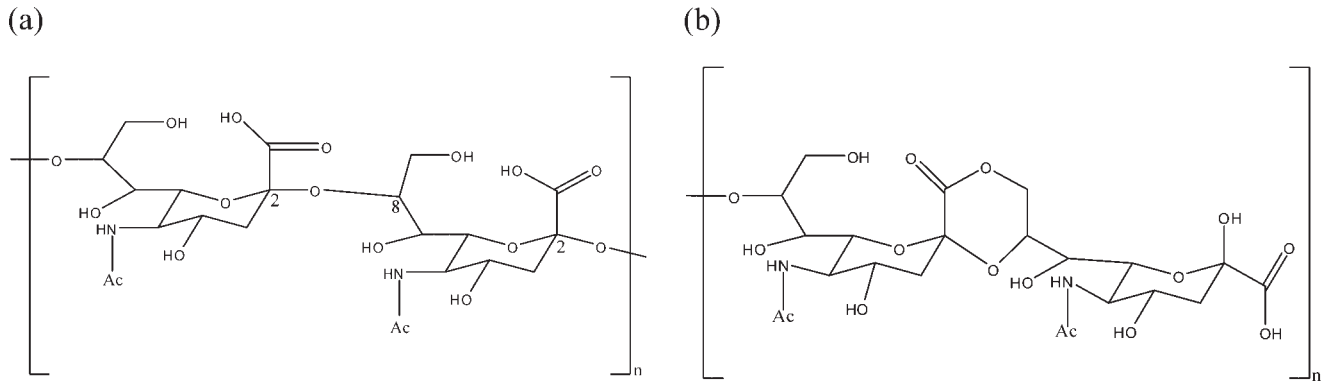


Figure 12. Underivatized polysialic acid with THAP as matrix in positive ion mode. Matrix is used as 100 nmol/μL and polysialic acid is used as 25 μg/μL. Laser fluence is applied as 160 mJ/cm^2. (a) Mass spectrum of the full range of 2000–200 000 Da and a broad band signal (within the region of two dashed lines) is obtained without fragments in low mass range only when THAP is used as matrix. (b) DHB is used as matrix, the expected band is not observed but only fragments in the low mass region were obtained. This comparison indicates that THAP works better than DHB.



Scheme 6. (a) Polysialic acid: α(2 → 8) linkage and (b) lactonized structure of polysialic acid with α(2 → 8) linkage.

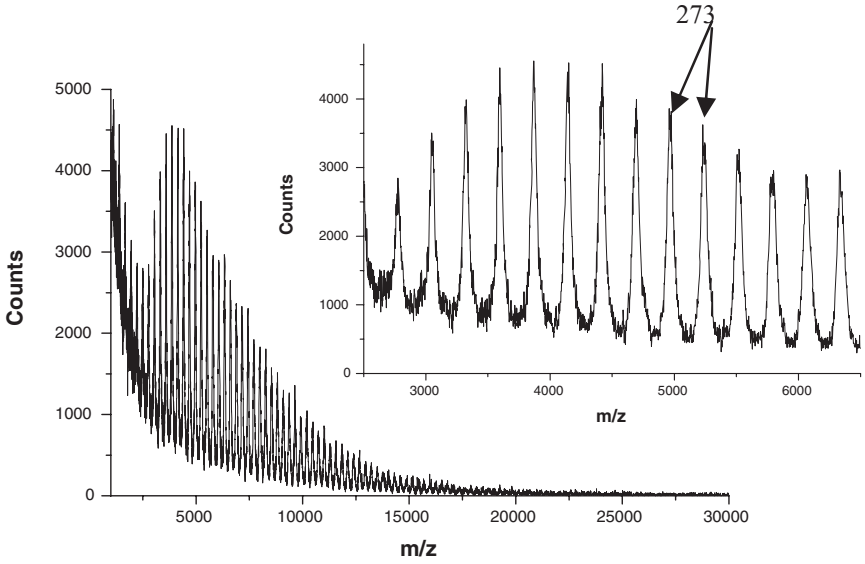


Figure 13. MALDI-TOF MS of lactonized polysialic acid (PSA-lactone) in positive ion mode. The concentrations of matrix and PSA-Lactone are 50 nmol/μL and 100 pmol/μL respectively. With lactonization, the mass spectra of polysialic acids could show well resolved peaks with an increment of 273 Da. Laser fluence is applied at 160 mj/cm^2.

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α-2,8-linked oligo/polysialic acid with THAP as matrix is shown in Fig. 13. The mass spectrum with the same peak increment of 273 Da is a reflection of lactonized polysialic acids with different degrees of polymerization. The average of this increment (273 Da) reflects a sialic acid (291 Da) with dehydration from condensation between carboxyl and hydroxyl groups. It clearly demonstrates that THAP is a good matrix for the efficient detection of lactonized polysialic acid compounds. Capillary electrophoresis also can provide a platform for separation or analysis of oligosialic/polysialic acids.[46] MALDI-MS apparently is an alternative for modified polysialic acid analysis with a faster speed and better resolution.

CONCLUSIONS

We have demonstrated that THAP as matrix yields excellent MALDI-MS results for successful analysis of large non-derivatized and derivatized polysaccharides and glycoproteins. Nevertheless, the overall detection efficiency for polysaccharides compared to proteins is still low. The search for better matrices and for understanding of the detailed desorption/ionization mechanism must continue vigorously for further improvement of large polysaccharide analysis by MALDI-MS.

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