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Hydrazinonicotinic acid as a novel matrix for highly sensitive and selective MALDI-MS analysis of oligosaccharides

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Jing Jiao,[ab] Ying Zhang,[ab] Pengyuan Yang[b] and Haojie Lu*[ab]

Analysis of oligosaccharides with matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) remains challenging due to their low ionization efficiency. The sensitivity achieved by MS for oligosaccharides lags far behind that for proteins/peptides. Here, the hydrazinonicotinic acid (HYNIC) is proposed as a new matrix to realize highly sensitive and selective analysis of oligosaccharides in MALDI-MS. The detection limit of maltoheptaose provided by HYNIC is as low as 1 amol, which is five orders of magnitude lower than that provided by traditional matrix 2,5-dihydroxybenzoic acid (DHB). Interestingly, HYNIC displayed remarkable selectivity for ionization of oligosaccharides, making glycans from glycoprotein become more accessible to be detected even without pre-purification, as demonstrated by the direct detection of the oligosaccharides from human serum without pre-separation of the proteins/peptides. The HYNIC matrix also possessed the virtue of higher homogeneity of crystallization and better salt tolerance (up to 200 mM NaCl, 140 mM urea and 40 mM sulfocarbamide et al.) compared with traditional matrix DHB. Furthermore, HYNIC matrix afforded adequate fragmentation, thus providing rich information for the structure elucidation of oligosaccharide. Therefore, HYNIC as the matrix to directly analyze oligosaccharides is inherently simple and straightforward.

Introduction

Glycosylation is one of the most common and complicated form of post-translational modifications (PTMs) in eukaryotic proteins.[1] Oligosaccharides, which are structurally diverse, participate in key biological progresses including protein folding, cell-cell recognition, signaling and cancer metastasis in living systems.[2-6] Due to its rapidity and accuracy, matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) has been the most important technique for the analysis of oligosaccharides.[7-9] However, the lack of basic group to be protonated and the hydrophilic nature of oligosaccharides make them more difficult to be ionized than peptides and proteins. In an attempt to redress the problem, chemical derivatization has frequently been used to improve the limit of detection (LOD) of oligosaccharides in MS.[10,11] However, the gain in sensitivity through chemical derivatization is often somewhat counterbalanced by sample loss during the time-consuming sample preparation procedure.[12] What's more, problem resulting from the incomplete derivatization complicates the mass spectrum due to the coexistence of signals from native and derived glycans. Comparing with the tedious derivatization methods, analysis of oligosaccharides using proper matrix is simple and straightforward for giving signals in their native state and reducing the complexity of the spectrum. 2,5-Dihydroxybenzoic acid (DHB) is the most conventional matrix whose weak points are also obvious. First, DHB affords just passable sensitivity with LOD merely at the fmol level as far as we know.[13] Second, inhomogeneous needle-shaped crystals of DHB are often formed around the rim of the MALDI spot after drying, as a result, the search for "sweet spot" where the analytes are ionized is required.[14] Therefore, development of novel matrices with high sensitivity is always a hot research topic.[13-16]

Suppression effect caused by analytes with higher proton affinity (i.e. peptides) leads to another difficulty in detecting glycans.[7] Traditional solution to circumvent this problem is to selectively enrich the glycans from the mixtures.[15,16] However, necessary manipulation of incubation and elution is a time-consuming and labor-intensive task. Selective improvement of ionization efficiency of oligosaccharides with a proper matrix

would undoubtedly facilitate direct and effective mass spectrometry measurements of the trace amount of native carbohydrates from peptide mixtures. Recently, diamond nanoparticles (DNP) were incorporated as the MALDI matrix to selectively enhance the sensitivity of the mass spectrometer to carbohydrates for the first time.[17] However, like other inorganic matrices, DNP with the size about 500 nm may lead to possible contamination of MALDI ion source. Matrix with both high sensitivity and selectivity would specially help to discerning of glycans in complex samples. Herein, a novel matrix 6-hydrazinonicotinic acid (HYNIC) was investigated for highly sensitive and selective analysis of oligosaccharides. We systematically evaluated the advantages of HYNIC as a novel matrix for different kinds of oligosaccharides analysis including oligosaccharides, permethylated oligosaccharides, fucosylated oligosaccharides and sialylated oligosaccharides. Several aspects including limit of detection for oligosaccharides, ability to selectively ionize oligosaccharides, shot-to-shot reproducibility, salt tolerance and the impact on the oligosaccharides fragmentation pattern were performed for investigating the superiority of HYNIC matrix. Results showed that HYNIC could be an outstanding matrix for the analysis of oligosaccharides, eg. enabling highly sensitive detection of maltoheptaose (DP7) as low as 1 amol accompanied by selective enhancement of oligosaccharides ions, high homogeneity of crystalline and high tolerance to several kinds of buffers. At last, more than 40 glycans released from human serum were successfully detected using HYNIC as matrix.

Experimental section

Materials and reagents

Hydrazinonicotinic acid (HYNIC) and standard peptide (LSITGTYDLK) were purchased from ChinaPeptides Co., Ltd and the purity of the peptide was more than 99%. Maltoheptaose (DP7, 95%) was purchased from Hayashibara Biochemical Laboratories (Okayama, Japan). NA2F glycan and A1 glycan were purchased from Ludger. 2,5-Dihydroxybenzoic acid (DHB), trifluoroacetic acid (TFA), dithiothreitol (DTT), urea, sulfocarbamide, ammonium bicarbonate (ABC), Dextran 1000 analytical standard (Dextran 1000, 98%), nicotinic acid, trypsin (Proteomics Grade), ribonuclease B (RNase B) and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Peptide N-glycosidase (PNGase F) was obtained from New England Biolabs (Ipswich, MA, USA). Sodium chloride (NaCl) was purchased from Shanghai Chemical Reagent Company (Shanghai, China). HPLC-grade methanol and acetonitrile (ACN) were purchased from Merck (Darmstadt, Germany). Acetic acid (HAc) of analytical grade was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Distilled water was purified by a Milli-Q system (Milford, MA, USA).

Sample preparation

HYNIC was dissolved in methanol containing 2.0% HAc. The DHB matrix solution was prepared at the concentration of 10 mg/mL in ACN/water (50:50, v/v) containing 0.1% TFA. All oligosaccharides were dissolved in water in a series of concentration and stored at 4 ^{o}C for further use. In order to generate peptide mixtures, BSA was dissolved in 50 mM ABC and denatured by boiling for 10 min, trypsin was then added into protein solution at an enzyme/substrate ratio of 1:50 (w/w) for 12 h at 37 ^{o}C to produce peptides. The tryptic peptide mixtures were stored at -20 ^{o}C until further use. RNase B (0.5 mg) was dissolved in 50 mM ABC buffer (pH 8.0) and denatured by the same method. After incubating with trypsin for 12 h, PNGase F solution (0.5 μL) was added and the enzymatic deglycosylation reaction was carried out at 37°C for 24 h. The sample were directly stored at -20 ^{o}C without separation.

Human serum (10 μL) from a healthy volunteer was denatured by heating for 5 minutes in 8M urea/0.2M Tris-HCl buffer. Then the sample was treated with 25 mM dithiothreitol (DTT) at 57 °C for 30 minutes and alkylated with 30 mM iodoacetamide (IAA) at room temperature for 45 minutes in the dark. The denaturant and other low-molecular-weight molecules were removed by ultrafiltration. One μL PNGase F solution was added and the enzymatic deglycosylation reaction was carried out at 37°C for 24 h. The released N-glycans were obtained by repeated ultrafiltration with pure water and lyophilized before MALDI analysis.

Mass Spectrometric Measurements

One μL amount of sample and one μL of matrix solution were spotted on the MALDI plate for MS analysis. The MALDI-TOF measurements were performed on 5800 Proteomics Analyzer (Applied Biosystems, Framingham, MA, USA). Mass spectra were acquired in reflection mode with a Nd:YAG laser at 355 nm, a repetition rate of 400 Hz, and an acceleration voltage of 20 kV. MS/MS spectra were interpreted manually with the assistance of the GlycoWorkbench software. External mass calibration was performed by using standard peptides from myoglobin digests.

MSI acquisition and data processing

Mass spectrometry imaging was conducted in 5800 Proteomics Analyzer (Applied Biosystems, Framingham, MA, USA) using 4800 imaging software. Mass conditions were set as described above. Mass spectrometric analysis was performed in the positive reflection mode and MALDI-MS spectra were recorded in the m/z range of 1000-2000. Two-dimensional ion density maps were created using the image reconstruction software (BioMap, Novartis, Basel, Switzerland).

Results and discussions

Evaluation of HYNIC matrix for highly sensitive detection of standard oligosaccharides

The concentration of HYNIC was optimized on the basis of the minimum amount needed to produce uniform dried crystals throughout a target spot as well as a clear mass spectrum. Our preliminary optimized work contributed to confirm that HYNIC matrix dissolved in methanol at the concentration of 2.0 mg/mL

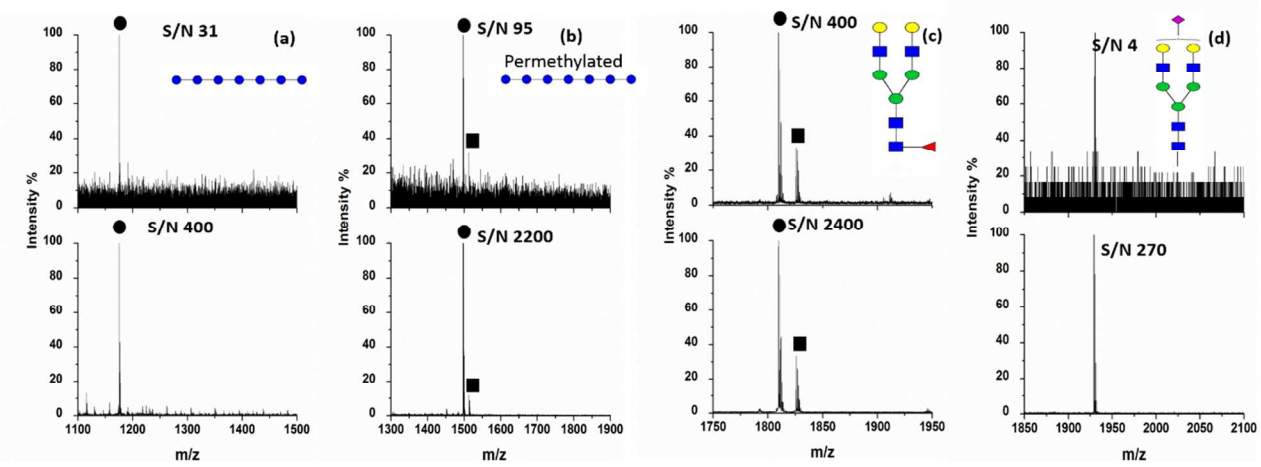

Figure 1. MALDI mass spectra of (a) 1 pmol DP7, (b) 1 pmol permethylated DP7, (c) 5pmol NA2F and (d) 5 pmol A1 using matrix DHB (upper panel) and HYNIC (lower panel). ● denotes $[M+Na]^+$ signals. ■ denotes $[M+K]^+$ signals.

containing 2% HAc can generate high quality spectrum. Higher concentration of HYNIC generated more cluster peaks of matrix, while HYNIC of lower concentration did not crystallize well with the analytes. We also tried other solvents including system of acetonitrile and water and the system of methanol and water, in which HYNIC didn't crystallize on the target well after solvent evaporation. After optimization the concentration and matrix buffer, HYNIC was used as the matrix to help ionizing different kinds of oligosaccharides. Figure 1 displays the MALDI mass spectra of four kinds of representative oligosaccharides including DP7, permethylated DP7, fucosylated oligosaccharide NA2F and sialylated oligosaccharide A1 using DHB and HYNIC as matrix respectively. The signals of neutral oligosaccharides are often observed as the form of alkali metal adducts like $[M+Na]^+$ or $[M+K]^+$ because oligosaccharides have a high affinity for alkali cations.[18] Therefore, the $[M+Na]^+$ is chosen for the comparison of the signal to noise (S/N) ratio between the DHB and HYNIC. The signals of sialylated oligosaccharides are obtained in negative modes. Obvious higher S/N and superior spectrum quality are obtained using HYNIC matrix (Figure 1, lower panel) than DHB matrix (Figure 1, upper panel) for all of the four types of oligosaccharides. From the spectra (Figure 1) we believed that HYNIC didn't react with the reducing end of oligosaccharides because the corresponding product signals were not detected. Therefore, HYNIC acted as matrix but not derivatization reagent to help improve the ionization efficiency of oligosaccharides. In addition, the molecular weight of HYNIC is 153.1 Da and the matrix peaks of HYNIC can be observed in the low mass range of the mass spectrum (data not shown), which won't interfere with the analysis of oligosaccharides. DP7 was used as a model glycan to further compare the ionization ability of HYNIC and DHB. Table S1 made a comparison of S/N for DP7 with different loading amounts using DHB and HYNIC. With the decrease of loading amount, DHB lost the ability to help ionize DP7. By contrast, HYNIC was demonstrated as a more effective matrix. DP7 was also used as a model sample to determine the LOD, the loading amount of DP7 was decreased to the amount on a single well at which the S/N just exceeded 3. When HYNIC matrix was used, LOD for DP7 is 1 amol (Figure S1b). For comparison, LOD of provided by the traditional matrix DHB is 100 fmol (Figure S1a). LOD provided by HYNIC increases five orders of magnitude than that of DHB, and it is almost one order of magnitudes lower than the LOD that have been reported in a very recent report.[13] Dextran 1000, a more complex, branched polysaccharide composing of many glucose molecules joined into chains of different lengths[19] was used to further evaluate the performance of HYNIC matrix. Several reports developed proper matrix and derivatization approach to enhance the ionization efficiency of dextran.[12,20] In our study, a degree of polymerization (DP) from 3 to 15 could be detected with fine intensity and higher S/N with HYNIC (Figure S2b) compared to DHB (Figure S2a). Table S2 lists the exact m/z values of the signals detected by HYNIC and DHB as form of sodium adducts $[M+Na]^+$ and potassium adducts $[M+K]^+$. Due to the structural similarity between HYNIC and nicotinic acid, we also compared the ionization effect between them. Figure S2c shows the MALDI mass spectrum of the same amount of Dextran 1000 obtained by nicotinic acid. Only five oligosaccharides (DP from 3 to 7) were successfully detected. Therefore HYNIC exhibits advantage over nicotinic acid by comparing the ionization effect for oligosaccharides. To make a summary, HYNIC matrix demonstrated outstanding ionization efficiency covering from simple oligosaccharide to complex oligosaccharides.

Evaluation of HYNIC matrix for highly selective detection of oligosaccharides from peptide mixtures

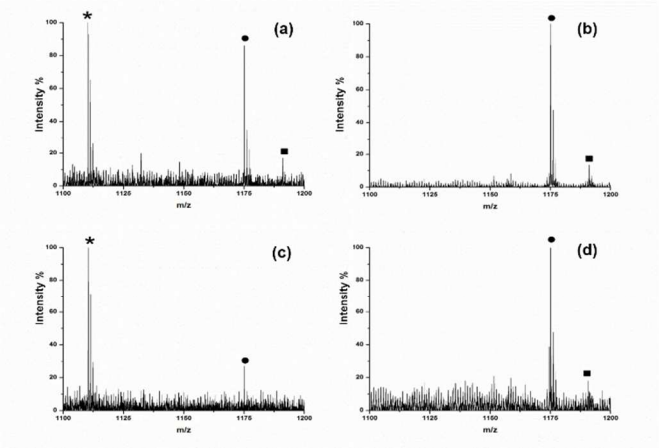

Figure 2. MALDI mass spectra of the mixture of standard DP7 and peptide with DP7: peptide at a molar ratio of (a, b) 1:1 and (c, d) 1:10 using DHB (left panel) and HYNIC (right panel). * denotes standard peptide, ● denotes $[DP7+Na]^+$ signals. ■ denoted $[DP7+K]^+$ signals.

Another striking advantage of HYNIC matrix is that it can be readily extended to selectively enhance the ionization efficiency of oligosaccharides and suppress ionization of other components (e.g. peptides). A mixture of standard peptide (LSITGTYDLK) and DP7 was used as a model sample to evaluate its ionization selectivity toward oligosaccharides. We blend DP7 and standard peptide in different molar ratios (with 1:1 and 1:10) to examine whether highly abundant peptide would affect the oligosaccharide ionization efficiency. DHB was adopted as the matrix for comparison. As shown in Figure 2, when the molar ratio of DP7 and peptide was 1:1, both DP7 and peptide could be detected with DHB (Figure 2a). By contrast, HYNIC reveals the excellent ionization selectivity toward oligosaccharides (Figure 2b). When the molar ratio of DP7: peptide decreased to 1:10, DP7 could still be selectively detected with HYNIC matrix (Figure 2d), but DP7 almost could no longer be detected using DHB (Figure 2c). To further verify the selectivity effect of HYNIC, DP7 was mixed with tryptic peptides of BSA in different mass ratios (with 1: 1 and 1:10) and analyzed by MALDI-MS. As shown in Figure S3a and Figure S3c, lots of peptides signals coming from BSA were detected using DHB. Although the signal of DP7 was also observed, it was suppressed by the presence of large amounts of peptides. However, when HYNIC was used, DP7 could be easily detected with a clean background in the mass spectra resisting interference of the peptides, which made it easy for later identification. The ability to selectively ionize toward glycans facilitated the direct identification of glycans from the deglycosylated glycopeptides mixture without the need to purify the glycans before analysis. Ribonuclease B (RNase B) is a glycoprotein containing five different glycosylated variants with 5-9 mannoseresidues attached to the chitobiose core at the site Asn34.[21] Without purification of the glycans, a series of five ion peaks with an interval of 162 Da was observed after detaching the glycans and using HYNIC as the matrix for analysis (Figure 3b), corresponding to $[M+Na]^+$ peaks from $GlcNAc_2Man_5$, $GlcNAc_2Man_6$, $GlcNAc_2Man_7$, $GlcNAc_2Man_8$, $GlcNAc_2Man_9$. Whereas only tryptic peptides were observed when DHB was used (Figure 3a). Therefore, we concluded that HYNIC was advantageous for the efficient oligosaccharide-specific ion embossment on the MALDI-TOF analysis.

Evaluation of reproducibility of HYNIC matrix

As previously mentioned, DHB suffered from the disadvantage of forming hot spots due to inhomogeneous crystallization. In most cases, the center of the target spot is devoid of crystals.[22] Therefore, it is essential to upgrade crystal formation within the analyte-matrix system, aiming towards a more homogenous embedding of analytes on the target spot and finally improving the mass resolution as well as reproducibility.[23-25] DP7 was chosen as a standard oligosaccharide to evaluate the performance of HYNIC matrix. All samples were spotted on one target plate with thin layer spotting method and analyzed in the same round of experiments to ensure reliability of the comparison. Higher signal intensity and better shot-to-shot reproducibility were obtained by comparison of peak intensity, shown in Figure 4 (left panel). The MALDI imaging analysis was utilized to further verify that HYNIC matrix formed more homogeneous crystals with analyte on the target well. As shown in Figure 4b (right panel), the signals of DP7 using DHB were detected around the rim of the MALDI spot, which is accordance with previous study.[22] The signals of DP7 from HYNIC matrix located uniformly throughout the spot shown in Figure 4a (right panel), which is thought to be quite useful for automated data acquisition and quantitative analysis using

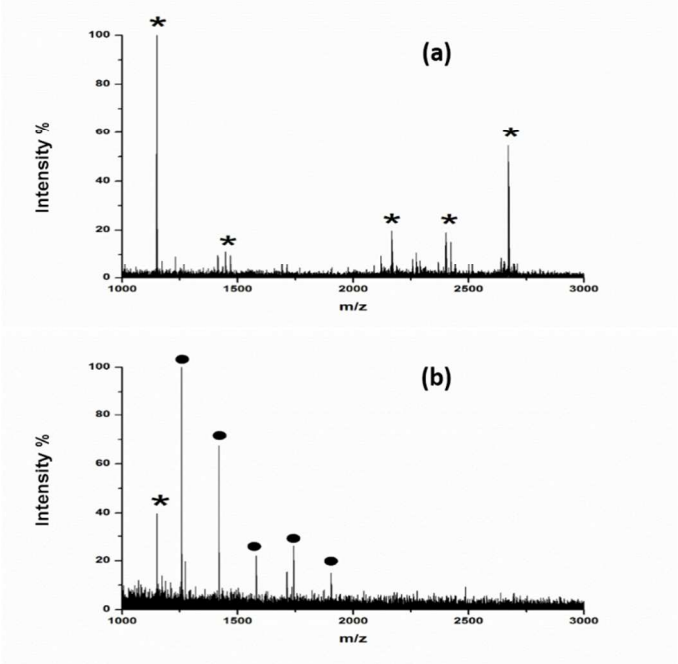

Figure 3.MALDI mass spectra of tryptic and PNGase F digests of RNase B (equivalent to 10 pmol amount of glycoprotein) using (a) DHB and (b) HYNIC. * denotes peptides, ● denotes glycans.

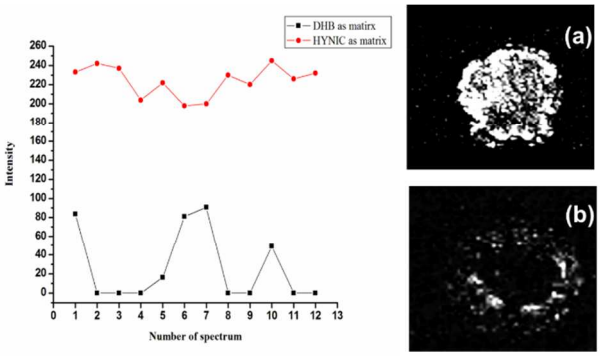

Figure 4. Comparison of peak intensity (left panel) and signal distribution (right panel) with HYNIC matrix and DHB matrix. The signal distribution was measured by method of MALDI imaging with (a) HYNIC and (b) DHB. Loading amount of DP7 is 1 pmol with thin layer spotting method. The peak intensity (left panel) comes from spectra accumulated by 20 sub-spectrum, and each sub-spectrum was collected from 50 randomized positions within the spot.

MALDI-MS. In fact, the spotting technique can make a large difference in the crystallization form. We adopted three other spotting techniques including acetonitrile recrystallization of DHB, dried droplet method and double-layer method. As shown in Figure S4, acetonitrile recrystallization and dried droplet method helped to form more homogeneous crystals but no obvious improvement of signal intensity was observed (Figure S5). Whereas, more uniform crystals and better shot-to-shot reproducibility were obtained by HYNIC without deposition method optimization.

Evaluation of the buffer tolerance ability of HYNIC matrix

Although MALDI-TOF MS is considered to have a tolerance for a low concentration of salts, analyte signals are usually severely suppressed or undetectable when the salt concentration reached a certain amount.[26] Several desalting methods including HPLC, C18Ziptip, microextraction chip have been extensively applied to sample pretreatment prior to MALDI MS analysis.[27] Nevertheless, sample loss and poor recovery of analyte have been observed. Therefore, matrix with high salt tolerance to enable direct MALDI MS analysis of analytes is favorable. Sodium chloride (NaCl) buffer was chosen as a representative non-volatile salt to test the salt tolerance of HYNIC matrix, with the concentration of NaCl ranged from 25 mM to 200 mM. HYNIC matrix demonstrated excellent buffer tolerance as it enabled the detection of the DP7 in all buffer concentrations, despite a little decrease of S/N (Figure S6 e-h). However, for DHB matrix, the signals of DP7 of the same amount could rarely be detected with poor S/N (Figure S6 a-d). High buffer tolerance ability brought HYNIC matrix more extensive application scope, because trace concentrations of analyte dissolved in buffer can be directly analyzed without desalting. We also evaluated the salt tolerance of HYNIC by mixture the sample (DP7) with diluted cell lysis solution (140 mM urea and 40 mM sulfocarbamide) and common used digestion buffers (50 mM ammonium bicarbonate). As shown

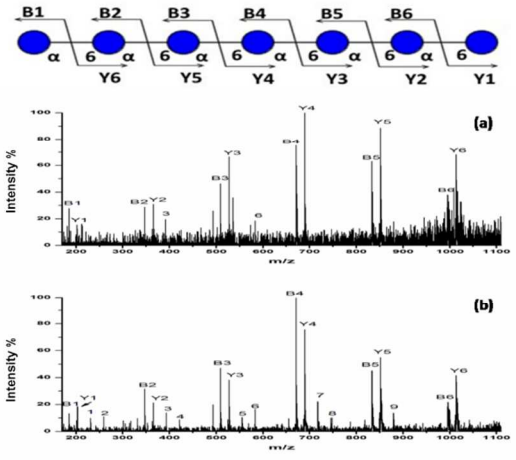

Figure 5. MALDI-TOF/TOF mass spectra of (a) 10 pmol of DP7 with DHB matrix and (b) 1 pmol of DP7 with HYNIC matrix.

in Figure S7, more than an order of magnitude higher S/N was obtained by HYNIC matrix than DHB. The dominant peaks are observed as form of sodium adducts $[M+Na]^+$, although there are some contaminated peaks which may come from the lysis solution.

Evaluation of the impact of HYNIC matrix on fragmentation pattern in tandem mass spectrometry

To further investigate the performance of HYNIC matrix, we performed the collision-induced dissociation (CID) for the MS/MS analysis of sodium adducts $[DP7+Na]^+$ from DP7 as precursor in positive ion mode. Fragmentation of the $[DP7+Na]^+$ generated with DHB was used as a comparison. 10 pmol DP7 was loaded when DHB was used and 1 pmol amounted for HYNIC to ensure the similar intensity. Suitable laser energy was adjusted in order to get a relatively high quality spectrum. The legend for fragment ions is provided on the top of Figure 5. The two spectra were very similar for the most part in the spectra between the DHB and HYNIC. Fragments representing a glycosidic bond dissociation (B and Y ions) are the most abundant features both in Figure 5a and Figure 5b. Differences are also observed, the most notable being the high signal intensity of cross-ring cleavage of HYNIC (Figure 5b) relative to DHB (Figure 5a). Corresponding m/z values and fragmentation types are summarized in Table S3. What's more, the use of HYNIC resulted in improved quality MS/MS spectra due to a much higher signal-to-noise ratio compared to the spectra obtained using DHB. Therefore, HYNIC allowed more of the sample ionized per amount deposited on a target and provided a more informative tandem MS analysis, which is quite effective for the structural characterization of oligosaccharides.

Real Sample Analysis

In order to further test the feasibility of HYNIC, we adopted glycans released and purified from human serum as a more complex sample. Human serum is a kind of significant body

liquid and serum glycan profiles can provide information which have potential in early disease diagnosis. Alterations of glycans are correlated with many kinds of disease, such as cancer, diabetes and immune disorders. It is crucial for the analysis of limited quantities of glycans in this complex sample. Herein we used glycans coming from healthy human serum to test whether HYNIC is still applicable to real sample. After treatment of PNGase F digestion and ultrafiltration, the sample was lyophilized and sent to be analyzed by MALDI with HYNIC directly. More than 40 glycan signals were successfully detected (Figure S8) and their detailed information of structures were summarized in Table S4. Tandem mass analysis were performed to further verify the identification results. Displayed in Figure S9, the fragments of precursor m/z 1663.6 were assigned to the precursor whose structure has been reported earlier.[28] Therefore, HYNIC is turned out to be applicable to many kinds of oligosaccharides.

Conclusions

A new matrix HYNIC was found to be advantageous for oligosaccharides as it enabled high ionization efficiency in MALDI analysis. Direct profiling the oligosaccharides without derivatization and desalt procedures streamlined the analysis process by minimizing the number of handling steps in sample preparation. HYNIC boasts not only forming more uniform crystals with analytes on MALDI target but also dramatically increasing the detection sensitivity as low as 1 amol loading amount per target well. Besides, selectively enhancing ionization efficiency of oligosaccharides and significantly suppressing ions of other contaminants, such as peptides, minimized the sample preparation steps. To some extent, it provides a new way to solve the inherent limitation of mass spectrometry in the direct analysis of oligosaccharides from complex mixtures. Furthermore, higher quality spectrum of tandem mass spectrometry was achieved. Dominantly by B and Y ions, MS/MS spectra of oligosaccharides obtained from HYNIC matrix also contained necessary signals from cross-ring fragments, facilitating the elucidation of the oligosaccharides. Compatibility with high concentration of buffer made it possible to omit process of desalting and avoid loss of trace analytes. In a word, the adoption of HYNIC matrix delivered outstanding results by quick, simple, straight and sensitive analysis for oligosaccharides in MALDI mass spectrometry measurements.

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[a] Shanghai Cancer Center and Key Laboratory of Glycoconjugates Research Ministry of Public Health, Fudan University, Shanghai 200032, P. R. China. E-mail luhaojie@fudan.edu.cn. Fax: (+86)21-5423-7961

[b] Department of Chemistry and Institutes of Biomedical Sciences, Fudan University, Shanghai, 200032, P. R. China.

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