RESEARCH ARTICLE

N-(1-Naphthyl) Ethylenediamine Dinitrate: A New Matrix for Negative Ion MALDI-TOF MS Analysis of Small Molecules

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Abstract

An organic salt, N-(1-naphthyl) ethylenediamine dinitrate (NEDN), with rationally designed properties of a strong UV absorbing chromophore, hydrogen binding and nitrate anion donors, has been employed as a matrix to analyze small molecules ($m/z < 1000$) such as oligosaccharides, peptides, metabolites and explosives using negative ion matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Compared with conventional matrixes such as α -cyano-4-hydroxycinnamic acid (CCA) and 2,5-dihydroxybenzoic acid (DHB), NEDN provides a significant improvement in detection sensitivity and yields very few matrix-associated fragment and cluster ions interfering with MS analysis. For low-molecular-weight saccharides, the lowest detection limit achieved ranges from 500 amol to 5 pmol, depending on the molecular weight and the structure of the analytes. Additionally, the mass spectra in the lower mass range ($m/z < 200$) consist of only nitrate and nitric acid cluster ions, making the matrix particularly useful for structural identification of oligosaccharides by post-source decay (PSD) MALDI-MS. Such a characteristic is illustrated by using maltoheptaose as a model system. This work demonstrates that NEDN is a novel negative ion-mode matrix for MALDI-MS analysis of small molecules with nitrate anion attachment.

Key words: N-(1-naphthyl) ethylenediamine dinitrate, Negative ion-mode matrix, MALDI MS, Small molecule analysis

Introduction

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has the characteristics of easy sample preparation, rapid analysis, high sensitivity, and, most notably, tolerance of contaminants in analysis. Since 1980s [1, 2], it has been widely used to analyze

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large molecular weight compounds such as proteins [3], polysaccharides [4], DNA/RNA [5], polymers [6], and even bacteria [7]. Although the high-throughput nature of MALDI-MS makes it a powerful tool for the analysis of those molecules, its application in the field of small molecules has been rather limited because commonly used organic matrixes [8, 9] always produce a large number of matrix-associated interfering ions that obscure the detection of analytes in the low m/z region.

A number of inorganic materials have been developed as alternative matrixes to avoid the drawbacks of using conventional organic matrixes [10–17]. Although low fragmentation of matrix ions can be attained with these techniques, several experimental factors, including surface morphology and laser fluence, need to be optimized simultaneously to achieve a reliable analysis. Recently, conjugated amino aromatic systems have been explored as matrixes for negative ion MALDI [18–24]. For example, 9-aminoacridine was used as an efficient matrix to analyze low molecular compounds [18, 20, 21]. Owing to the presence of matrix-related ions in the mass spectra, it makes spectral interpretation obscure. Based on Brønsted-Lowry acid-base theory, Shroff et al. reported the use of proton sponge as a superbasic matrix for MALDI analysis of acidic analytes from biological samples [22, 23]. Hence, there is a continued need to develop new matrix interference-free methods for the analysis of small molecules by MALDI MS.

In MALDI MS analysis of small molecules, a suitable MALDI matrix should meet a number of requirements, including (1) strong absorption at the applied laser wavelength, (2) efficient ionization/desorption of the analytes, (3) little or no fragmentation of analyte, and (4) devoid of matrix interfering ions in the low m/z region [25, 26]. Here, we report a rationally designed matrix, *N*-(1-naphthyl) ethylenediamine dinitrate (NEDN), for small molecule analysis by negative ion MALDI MS. Interestingly, NEDN preferentially forms intact molecular radical cation in the gas phase, producing no or little interfering fragment ions in the low mass region. Additionally, the generations of nitrate ions during laser desorption/ionization (LDI) allow for detection of small molecules by anion attachment. These unique physical and chemical properties meet the above four requirements of being an effective matrix. The applicability of NEDN in performing small molecule analysis is demonstrated.

Experimental

N-(1-naphthyl) ethylenediamine dihydrochloride (NEDC) was purchased from Tianjin Xinbote Chemical Engineering (Tianjin, China). Silver nitrate, ethanol, methanol, and acetonitrile were obtained from Beijing Chemical Engineering (Beijing, China). Stachyose and pentafluorobenzoic acid were from Acros Organics (Morris Plains, NJ, USA). All chemicals were used in the highest purity and without further purification. The deionized water used was purified with a Mill-Q water system from Millipore (Milford, MA, USA).

Preparation of Matrix and Sample Solutions

To prepare NEDN, NEDC, and silver nitrate (at a molar ratio of 1:2) were dissolved together in ethanol/water (vol/vol, 30:70). After 10 min, silver chloride gradually precipitated, according to the reaction:

The $\text{AgCl}_{(s)}$ precipitates were then removed by centrifugation at 5000 rpm for 15 min and filtration with filter paper. The newly formed NEDN solution was purified once by passing through cation ion exchange resins (part no. 732; Jiangsu Linhai Resin Technology, China) and the effluent was used as the matrix solution without further treatment.

Matrix solutions were prepared at a concentration of 100 mM in ethanol/water (vol/vol, 30:70) and were diluted to desired concentrations as required. Except hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), which was prepared in tetrahydrofuran, and pentafluorobenzoic acid, uric acid, ascorbic acid, which were prepared in pure ethanol, the other analytes were all dissolved in methanol/water (vol/vol, 50:50) to a final concentration of 10 mM and diluted to desired concentrations prior to MS analysis.

Optical Characterization

Ultraviolet-visible (UV-Vis) absorption spectra of NEDN and DHB were analyzed using a TU-1900 double beam UV-Vis spectrophotometer (Purkinje General Instrument, Beijing, China) in a 1-cm path length cuvette at room temperature. Micrographs of NEDN crystallites were acquired with an S-4800 scanning electron microscope (Hitachi, Tokyo, Japan).

Mass Spectrometry

All MS measurements were performed with either a Microflex or a Biflex III MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a nitrogen laser ($\lambda = 337\text{nm}$). Mass spectra (20 summed shots) were acquired in the reflector mode with a 19 kV accelerating voltage and a 20 kV reflector voltage. The laser pulse energy was set marginally above the threshold of ionization to avoid fragmentation. Prior to MS analysis, matrix solution and analyte solution were mixed at an equal volume, and 1 μL of the mixed solution was spotted on the MALDI target plate. After being dried in air, the samples were subjected to analysis.

Results and Discussion

NEDN as a Negative Ion-Mode Matrix for Small Molecule Analysis

In analysis of small molecules by MALDI MS, the matrix plays a key role since it serves not only as a laser light

energy absorbent and a thermal energy transporter, but also as a potential charge donor. For the typical MALDI matrixes, such as DHB, there is a significantly large absorption in the UV region because of its unique benzoic acid core structure [9]. *N*-(1-naphthyl) ethylenediamine dinitrate contains a naphthalene core, which acts as a strong UV absorbing chromophore and shows a strong and broad absorption band centered at 324 nm. The molar extinction coefficient ($\epsilon=7.10\times10^3\text{ M}^{-1}\text{ cm}^{-1}$) of NEDN is about 3-fold larger than that of DHB ($\epsilon=2.17\times10^3\text{ M}^{-1}\text{ cm}^{-1}$) at the applied laser wavelength of 337 nm (Figure 1S in Supplementary Material). This important feature makes NEDN a good MALDI matrix in the first place.

It is of interest to note that the positive ion LDI spectrum of the NEDN shows no signals associated with protonated *N*-(1-naphthyl) ethylenediamine (NED) at m/z 187.12 [22, 23] (Figure 2S in Supplementary Material). Instead, only one intense peak corresponding to the NED radical cation appears at m/z 186.12 [27]. This unique property allows effective desorption upon laser irradiation to proceed but with little background interference due to matrix fragmentation. The negative ion mass spectrum of NEDN is remarkably simple and essentially free of background signals except two characteristic peaks at m/z 61.98 and 124.98, corresponding to $[\text{NO}_3]^-$ and $[\text{HNO}_3+\text{NO}_3]^-$, respectively. Interestingly, neither molecular ions corresponding to NED nor their fragments could be detected in its mass spectrum. It can be envisioned that the NED group in the matrix absorbs the laser photon energy and transfers the energy to the analytes, and the nitrate acid serves as an anion donor in the MALDI process.

Good cocrystallization of matrix and analyte is one of the most important factors in producing effective laser desorption/ionization [28]. In this process, solvent plays a crucial role and profoundly modulates the analyte-matrix interactions, resulting in the increase or decrease of the MALDI ion signals [29–31]. For example, when using NEDN as a matrix, the mass spectrum of sucrose shows only a very weak signal if the matrix is dissolved in solvent such as pure water, methanol, or ethanol. Titration experiments at different ratios of ethanol/water led to an optimal condition of ethanol:water=30:70 (vol/vol) (Figure 3S in Supplementary Material).

In addition to the matrix solvent, we also found that the composition of the analyte solvent strongly influences the peak intensity of the nitrate adduct ions in MALDI mass spectra. Using sucrose as the test compound, when the sample was dissolved in 50:50 methanol/water, the highest signal intensity can be gained (Figure 4S in Supplementary Material). We therefore selected 30:70 ethanol/water as the matrix solvent and 50:50 methanol/water as the analyte solvent in the subsequent experiments.

We studied the effect of the molar ratio of matrix to analyte on the signal intensity of the adduct ions [32]. In conventional MALDI-TOF MS analysis, the typical matrix-to-analyte ratio varies from 1500:1 to 3000:1, depending on the

species and molecular weights of analytes [33]. Surprisingly, we found that the optimal molar ratio used for this benchmark system was 10:1 (Figure 5S in Supplementary Material). This is very different from the widely accepted idea that a very high matrix/analyte ratio is required for effective laser desorption/ionization. The observation of a relatively low matrix/analyte ratio suggests that the NEDN matrix behaves very differently from the conventional DHB matrix. The nitrate anion donating capability of the NEDN to the analyte is very effective, which minimizes the required amount of matrix for charge transfer ionization. When the ratio is greater than 100:1, the signals of $[\text{NO}_3]^-$ and $[\text{HNO}_3+\text{NO}_3]^-$ ions are too strong, making it very difficult to detect the nitrate adduct ions in the mass spectra, presumably due to the ion competition effect. However, when the ratio is less than 1:5, there is not enough matrix to assist laser desorption/ionization of analyte and the cocrystallization of matrix with analyte is poor. It is considered that because of the free NH hydrogen bonds derived from primary and secondary amines, NEDN can provide additional hydrophilic bonds for analyte to be embedded and isolated. Hence, the NEDN can easily form molecular complexes with analytes in the matrix, which facilitates the process of laser desorption/ionization of analyte.

Figure 1 shows the MALDI mass spectra of 5 nmol of oligosaccharides and 5 nmol of di- and tetra-peptides using NEDN as the matrix, respectively. The peaks at m/z 242.05, 404.10, and 728.21 are assigned to the nitrate adducts of D-galactose, sucrose, and stachyose, respectively. In comparison to the mass spectrum of pure NEDN, only the nitrate-attached molecular anion was newly found in the spectra. There is no fragmentation of the analyte and the

Figure 1. MALDI-TOF mass spectra of (a) D-galactose, (b) sucrose, (c) stachyose obtained with NEDN as matrix. The spectra were acquired in the negative ion mode

NEDN matrix, making the low-mass range (m/z 150–1000) a clear window for observation of small oligosaccharides. From a series of experiments with NEDN as matrix, we conclude that small neutral saccharides such as sucrose can form anionic adducts with nitrates. These results also show that there is little or no fragmentation of the analytes and matrix molecules, illustrating the excellent performance of NEDN in MALDI-MS analysis especially in the low-mass region.

The matrixes, CCA and DHB, have long been regarded as the preferred matrixes for MALDI-MS analysis of carbohydrates [8, 9, 31, 34]. To illustrate the advantage of using NEDN over CCA and DHB for MALDI-MS analysis of small molecules, we conducted a comparative study using sucrose as test compounds (Figure 6S in Supplementary Material). The signals of sucrose were not detected in the negative ion mass spectra when CCA or DHB were used as matrixes. With the use of NEDN as a matrix, the analyte is readily detected as the nitrate adducts with high peak intensities, sensitivity, and, more importantly, the interference from matrix ions is completely eliminated. No fragment ions of the analytes are observed, suggesting that NEDN is an effective negative ion-mode matrix for MALDI-TOF MS analysis of small molecules.

Very recently, we have reported that NEDC can be used as an effective matrix to analyze small molecules with chloride-attached product [27]. It is instructive to compare the efficacy of adduct ion formation between Cl^- and NO_3^- with the small molecules investigated herein. In this experiment, an equal molar ratio of NEDN and NEDC was mixed and used as a binary anion donor matrix for the analysis of sucrose and Gly-Leu-Tyr. The mass spectrum of pure binary matrix (NEDN/NEDC; 1:1 molar ratio) shows strong signals of $[\text{NO}_3]^-$ and $[\text{HNO}_3 + \text{NO}_3]^-$, but neither $[\text{Cl}]^-$ nor $[\text{HCl} + \text{Cl}]^-$ appears in the spectrum (Figure 7S in Supplementary Material). Similar results were found in the mass spectra of both analytes, indicating that the nitrate adducts of both oligosaccharides and peptides can be generated more readily than their corresponding anionic chloride adducts. The finding could be explained by the fact that the gas-phase basicity of chloride ions is higher than that of nitrate ions, and this places a tighter restriction on the ability to observe nitrate adducts in MALDI MS [19, 35, 36].

The exceptionally few interfering low-mass ions in the mass window below m/z 150 can enable NEDN as an effective matrix for MALDI-MS analysis of small molecules. Hexahydro-1,3,5-trinitro-1,3,5-triazine is a reduced symmetrical triazine ring compound, which is explosively unstable because of the presence of three N-nitro substituents. The spectrum shows that RDX can form a nitrate anionic adduct at m/z 284.02 in the process of laser desorption/ionization of NEDN (Figure 2). When NEDN was used as the matrix to analyze pentafluorobenzoic acid, nitrate adducts, deprotonated and decarboxyl ions can all be

Figure 2. MALDI-TOF mass spectra of (a) RDX, (b) pentafluorobenzoic acid, (c) uric acid, and (d) ascorbic acid obtained with NEDN as matrix. RDX was prepared in tetrahydrofuran at a concentration of 10 mM, the other three acids in pure ethanol at a concentration of 10 mM, and the matrix in 30:70 ethanol/water. The spectra were acquired in the negative ion mode

found at m/z 273.98, 210.98 and 166.99 in its mass spectrum. The reason that the signal of the decarboxylated ion is stronger than that of the deprotonated ion is because the bond length (1.54 Å) between the benzene ring and the carbon atom on the carboxyl group is longer than that of $-\text{C}-\text{O}$ (1.41 Å) in the carboxyl group and, hence, the $-\text{COOH}$ loss can readily occur. When NEDN was used as a matrix to analyze metabolites such as uric acid and ascorbic acid, both deprotonated ions and nitrate adducts could be observed in their mass spectra. They reside at m/z 167.02 and 230.02 for uric acid, and at m/z 175.02 and 238.02 for ascorbic acid, respectively.

We further extensively studied the feasibility of generating anionic nitrate adducts with NEDN as the new matrix to facilitate the analysis of small molecules in the negative ion mode of MALDI-MS using a variety of low molecular weight compounds including oligosaccharides, peptides, explosives, and metabolites. The laser power was adjusted to a value slightly above the desorption/ionization threshold, and each mass spectrum was obtained by averaging 20 laser shots scanned across the sample spot. The samples were extensively diluted with 50:50 methanol/water solution to desired concentrations prior to analysis. Taking the analysis of glucose as an example, we defined a positive detection of nitrate-attached glucose ions based on the peak detection criteria of above a signal-to-noise (S/N) to 3 (Figure 3) [29, 37–39]. Experimentally determined limit of detections (LODs) for the above mentioned analytes were listed in the Supplementary Table 1. The detection limit of glucose was 500 amol, significantly lower than that obtained using conventional DHB matrix [40]. The NEDN-assisted LDI process is shown to achieve highly sensitive detection for

Figure 3. MALDI-TOF mass spectra of glucose obtained with NEDN as matrix. The amounts of glucose deposited on the MALDI plate are (a) 500 pmol, (b) 500 fmol, and (c) 500 amol, respectively. Glucose was only detected as nitrate attached anionic adduct

numerous small molecules with molecule weight below 1000 Da, and is expected to find potential applications in the biological and biomedical research.

Structural Characterization of Oligosaccharide

Sensitive detection of small oligosaccharides with NEDN matrix can be achieved in the negative-ion mode MALDI MS analysis. In this mode, the background interference derived from sample and matrix fragmentation is minimized, which is well-suited for post-source decay (PSD) fragment ion mass analysis by MALDI mass spectrometry. *N*-(1-naphthyl) ethylenediamine dinitrate was thus used as a negative-ion mode matrix in the structural characterization of maltoheptaose using PSD-MALDI mass spectrometry. The signals of analyte at m/z 1214.4 were attributed to the nitrate adduct of oligosaccharide in the negative mode MALDI mass spectrum. To further characterize the structure of the maltoheptaose, the adduct ions [M+NO₃]⁻ were selected as precursors for PSD fragmentation. The negative ion mode PSD spectrum of maltoheptaose with NEDN matrix was shown in Figure 4. Domon and Costello nomenclature [41] is used to describe the MALDI-TOF/TOF fragmentation patterns of maltoheptaose. Subscripts refer to the number of rings retained, and superscripts indicate cleavages within carbohydrate rings. A nonreducing

Figure 4. Negative ion mode MALDI-PSD mass spectrum of maltoheptaose with NEDN as matrix. Nitrate attached maltoheptaose ions are selected as MS/MS precursors

end without the glycosidic oxygen is referred to as an A_n ion and a nonreducing ion that retains the glycosidic oxygen is referred to as a C_n ion. If the anions are retained by the reducing end of a glycosidic cleavage ion, Y_n and Z_n ions are used to designate the ions. The use of two superscripts k and l (i.e., $^{k,l}A_i$ and $^{k,l}X_j$) to indicate the sugar ring bonds that have been broken. For A_i , the situation is more complex, and the loss of water needs to be specified (e.g., $[^{0,2}A_4 - H_2O]$, $[^{0,2}A_5 - H_2O]$, $[^{0,2}A_6 - H_2O]$ and $[^{0,2}A_7 - H_2O]$ observed at m/z 588, 751, 912 and 1074, respectively), while for X_i and Y_i , nitrate adducts are readily produced (e.g., $[Y_7 + NO_3]^-$, $[^{1,5}X_7 + NO_3]^-$, $[^{2,5}X_7 + NO_3]^-$, and $[^{3,5}X_7 + NO_3]^-$ at m/z 1053, 1082, 1112, and 1141, respectively). The PSD fragmentation spectrum of nitrate attached maltoheptaose ions revealed abundant of cross-ring fragmentation. This observation may be explained by the nitrate attachment of maltoheptaose via hydrogen bond formation increased the activation energy required for glycosidic bond cleavages and resulted in the increase of cross-ring fragmentation ions. The obtained mass spectrum was essentially matrix interference-free, which greatly facilitated the fragmentation pattern assignment in the low mass region. The NEDN is demonstrated to be a suitable matrix for sensitive analysis of structurally informative fragment ions derived by negative ion PSD spectra of nitrate-attached adduct of oligosaccharides.

Conclusion

With the combined properties of strong UV absorption, hydrogen bonding, and nitrate anion donation capabilities, NEDN has been demonstrated to be useful as a negative ion-mode matrix to analyze small molecules by MALDI MS. Compared with commonly used matrixes, such as CCA and DHB, NEDN is advantageous in that it allows straightforward mass spectrometric identification of analytes without resorting to high m/z resolution and accuracy. We envision further applications of this new matrix, particularly in imaging of small molecules in biological tissues with MALDI-TOF MS [42].

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