APPLICATION NOTE

Enhanced In-Source Fragmentation in MALDI-TOF-MS of Oligonucleotides Using 1,5-Diaminonaphthalene

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Abstract

The capability to rapidly and confidently determine or confirm the sequences of short oligonucleotides, including native and chemically-modified DNA and RNA, is important for a number of fields. While matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) has been used previously to sequence short oligonucleotides, the typically low fragmentation efficiency of in-source or post-source decay processes necessitates the accumulation of a large number of spectra, thus limiting the throughput of these methods. Here we introduce a novel matrix, 1,5-diaminonaphthalene (DAN), for facile in-source decay (ISD) of DNA and RNA molecular anions, which allows for rapid sequence confirmation. *d*-, *w*-, and *y*-series ions are prominent in the spectra, complementary to the (*a*-B)- and *w*- ions that are typically produced by MALDI post-source decay (PSD). Results are shown for several model DNA and RNA oligonucleotides, including combinations of DAN-induced fragmentation with true tandem TOF MS (MS/MS) for pseudo-MS³ and “activated-ion PSD.”

Keywords: Matrix-assisted laser desorption/ionization, In-Source decay, DNA, RNA, 1,5-Diaminonaphthalene

Introduction

The capability to accurately determine or confirm the sequences of short oligonucleotides is important for a variety of applications (e.g., for quality control in the development of novel DNA- or RNA-based therapies, in human and microbial forensics, structural elucidation of ribonucleoprotein complexes, and in proof-reading and error correction of data obtained by orthogonal sequencing methods, such as NextGen DNA sequencing-by-synthesis technologies). This is particularly true for oligonucleotides shorter than 50 bases as well as those containing modified bases that are

difficult to sequence by traditional methods [1]. The accurate mass measurements and resolution afforded by mass spectrometry (MS) make this an ideal technique to characterize oligonucleotides [2]. Both matrix-assisted laser desorption/ionization (MALDI) and electrospray (ESI) MS have been previously applied to determine the sequence of DNA [3–5], RNA [6–9] and oligonucleotides containing base or backbone modifications [1, 8, 10–12].

Laser-induced dissociation (LID) in MALDI has also been used for oligonucleotide sequence analysis [4]. LID encompasses a number of physical and chemical processes that occur as a result of photon-induced interactions between the analyte and matrix molecules both on the surface and during the subsequent expansion of the plume. In-source decay (ISD) fragments are produced as a result of prompt (rapid on the time scale of ion extraction) dissociation in the source region. ISD fragments will be separated and detected in both a linear and reflectron TOF analyzers [13].

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Concurrently, post-source decay (PSD) fragment ions are produced in the field-free drift region of a TOF instrument by metastable decay following ion extraction. Having attained the same velocity as the precursor (and thus different kinetic energies), PSD ions must be separated in a tandem TOF instrument or by using stepped or curved-field reflectron voltages. ISD of DNA has been shown to produce mainly *d*-, *w*-, and (*a*-*B*)-ions [4]. In contrast to ISD, PSD of DNA results in mainly neutral base loss from the precursor and (*a*-*B*)- and *w*-sequence ions [14–17] while PSD of RNA produces primarily *c*- and *y*-ions [18]. Detailed reviews of oligonucleotide fragmentation mechanisms have been published [19–21]. However, the typically poor ISD and PSD fragmentation efficiency results in relatively low fragment ion abundances, which necessitate accumulation of many spectra and substantially decreases the throughput of MALDI-TOF for analysis of oligonucleotides. The relative efficiencies of ISD and PSD are a function of a number of parameters, including type of analyte and matrix, as well as instrument configuration (laser fluence per pulse, spot size, accelerating voltages, etc.).

Here we demonstrate a MALDI-based in-source fragmentation method that allows rapid and unambiguous sequence analysis of short oligonucleotides to be performed. The

method utilizes 1,5-diaminonaphthalene (DAN, Supplementary Information Scheme 1). DAN has been previously used as a MALDI MS matrix for on-target protein disulfide reduction [22] and has been reported to increase ISD of proteins by producing mainly *c*- and *z*-positive fragment ions [23]. To our knowledge, DAN has not been used previously as a MALDI matrix for studying DNA and RNA. We demonstrate that DAN promotes facile ISD in short (≤ 20 base) DNA and RNA oligonucleotides, and thus provides complementary sequence information to that obtained by TOF/TOF or PSD alone. Combining DAN-induced ISD with true tandem TOF MS (MS/MS) allows us to perform pseudo-MS3 and “activated-ion PSD” for complete sequence coverage of the DNA or RNA molecule.

Methods

Single-stranded DNA and RNA oligonucleotides (from 5 to 20 bases) were commercially synthesized (IDT, Coralville, IA, USA) and dissolved in plasma-grade water (Thermo Fisher Scientific, Waltham, MA, USA) to a stock concentration of 100 μ M. Ten mg/mL DAN and 50 mg/mL 3-hydroxypicolinic acid (HPA) (both from Sigma Aldrich) were freshly prepared in 1:1 acetonitrile:water and used as

Figure 1. Negative ion MALDI TOF spectra of the DNA oligonucleotide CATCCCCATTAG: **(a)** reflectron mode with HPA matrix; **(b)** TOF/TOF LIFT mode with HPA matrix; **(c)** reflectron mode ISD spectrum using DAN matrix; fragment ion notations provided in the respective insets

matrices. All solvents were purchased from Thermo Fisher Scientific at LC-MS grade or higher. 10 mM diammonium citrate (Sigma Aldrich, St. Louis, MO, USA) was prepared in water as a co-matrix. Prior to MALDI sample preparation, both oligonucleotide and matrix solutions were desalted over Dowex 50 W-X8 cation-exchange resin (Sigma Aldrich) using standard procedures [24]. A mixture of 1:4:0.5 oligonucleotide:matrix:co-matrix was prepared and spotted on a polished stainless steel target using the dried droplet method. Positive and negative ion MALDI TOF spectra were obtained in linear and reflectron modes on an Autoflex TOF/TOF instrument (Bruker Daltonics, Billerica, MA, USA) equipped with a 337 nm N₂ laser. A standard peptide mixture was used for external mass/charge calibration in both polarities. For comparison with ISD fragments, negative ion LID TOF/TOF spectra (i.e., LIFT mode [25]) of selected oligonucleotides were also acquired using standard instrument parameters [26]. Typically, from 250 to 1000 individual laser shots were averaged for each MS or MS/MS spectrum. Assignment of observed fragment ions was performed using Web-accessible DNA/RNA MS bioinformatics software: Mongo Oligo Calculator (<http://library.med.utah.edu/masspec/mongo.htm>) and MS2Links [27]. (Additional methodological considerations may be found in the online supplementary materials.)

Results and Discussion

Several short DNAs were chosen as model systems to investigate the effect of DAN on the ISD of oligonucleotides compared to typical DNA MALDI matrices. The fragmentation behavior of a mixed-base 11-mer DNA (CATCCATTAG) is shown in Figure 1. When the commonly used HPA matrix was used, no fragmentation was observed and only the singly- and doubly-deprotonated species were seen in the negative ion mode (Figure 1a). In contrast, when DAN was used as the MALDI matrix at the same laser fluence as for HPA, very intense ISD fragments were observed in the negative ion mode (Figure 1c). No precursor or fragment ions were observed in the positive ion mode when DAN was used as a matrix, again a stark difference in MALDI behavior compared to HPA and other typical DNA matrices. While increased laser fluence and several hundred to thousands of individual laser shots were required to produce good quality PSD spectra with HPA (Figure 1b), intense DAN-induced fragments were clearly observed after as few as 50 shots. In contrast with the (*a*-B)- and *w*-ion series produced by PSD with HPA matrix, DAN-induced fragments mapped primarily to *d*-, *w*-, and *y*-ion types providing rich fragmentation and nearly complete sequence coverage. In addition, neutral base-loss, demonstrated to be the first step in the production of the typical (*a*-B)- and *w*-ion series, was greatly reduced or absent in the DAN-induced fragmentation. These observations are in contrast to the typical *d*-, (*a*-B)-, and *w*-fragments that have been noted for ISD of DNA when using other matrices [4, 28, 29].

Four short (≤ 6 base) homopolymeric DNAs were analyzed using DAN matrix to compare relative fragmentation

efficiency as a function of nucleobase identity. As shown in Figure 2, ISD fragments were observed in all cases, although they varied with regard to intensity. Similar to the longer heteropolymeric DNA, fragment ions mainly corresponded to *b*/*y* and *d*/*w* series (which could not be resolved into individual ion series due to isobaric forward and reverse homopolymeric fragments). Additionally, we observed ions consistent in mass with adducts of the matrix with the precursor oligonucleotide and its fragments (either 158 Da or 286 Da higher). These assignments were confirmed by tandem MS using LIFT (data not shown). Matrix adducts of 158 Da have been previously reported in studies of peptide ISD induced by DAN [22, 30], although the structure of the 286 Da putative matrix adduct is not known. The efficiency

Figure 2. Negative ion MALDI TOF ISD spectra (reflectron mode) using DAN matrix: (a) dT5, (b) dC6, (c) dA6, and (d) dG5 homopolymeric oligonucleotides. (Note that “AAAAA” in panel (c) is a result of internal fragmentation of the precursor.)

of DAN-induced backbone fragmentation of homopolymeric 5- and 6-mers exhibited base composition preference: G, A, C>T. This was qualitatively determined based on fragment ion intensity relative to the precursor averaged over a large number (>1000) of laser shots. The specificity observed with DAN is similar to the base preference observed by others (G~C>A >>T) for ISD fragmentation of model DNA oligonucleotide ions, attributed to the stabilities of the nucleobases correlated to their relative proton affinities [4, 28]. However, little to no neutral nucleobase-loss was observed in DAN-induced fragmentation.

A short RNA oligonucleotide was also subjected to DAN-induced ISD (Supplementary Figure 3). DAN matrix induced similar *d*- and *w*-fragment ions in this RNA sequence as was previously observed for DNA. In our experiment, no fragmentation was observed from the RNA oligonucleotide when HPA matrix was used at the same laser fluence (data not shown). Although full sequence coverage was obtained for this short RNA oligonucleotide, the fragment ion intensity was notably lower than was observed for comparable DNA sequences. Other short RNA sequences also produced similarly limited fragmentation with DAN, indicating that this effect was not due to the particular RNA sequence chosen. Such reduced fragmentation efficiency may be caused by the increased stability of RNA compared to DNA due to the additional hydroxyl group, noted previously in MALDI studies with different matrices [31].

Conclusions

We have demonstrated that DAN is a useful fragmentation-inducing matrix in MALDI TOF MS of DNA and RNA. The DAN-oligonucleotide interactions during the desorption/ionization event in negative ion mode result in significant fragmentation of the intact molecular ions. The observed fragment ions are primarily *d*-, *w*-, and *y*-types with little to no base loss, more typical of non-ergodic excitation methods such as EDD. Using DAN as a matrix for DNA and RNA in combination with other “cooler” (non-ISD-inducing) matrices can provide both molecular ion and sequence information complementary to that traditionally derived by TOF/TOF and PSD methods. Online supplementary data demonstrate that better sequence coverage was achieved through combined use of ISD with true MS/MS (“activated-ion PSD” and pseudo-MS³). DAN can also be applied to characterization of more labile modifications, due to the reduced tendency for base loss. Additional work is planned to characterize the mechanisms of DAN-induced fragmentation using longer oligonucleotides.

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