Analysis of Oligodeoxynucleotides by Negative-Ion Matrix-Assisted Laser Desorption Mass Spectrometry

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Thirty compounds were tested in combination with ammonium acetate for the ability to desorb and ionize oligodeoxynucleotides by ultraviolet matrix-assisted laser desorption mass spectrometry. Negative ion yields using matrices such as 2,5-dihydroxybenzoic acid and 3-hydroxy-4-methoxybenzaldehyde are enhanced by the addition of ammonium salts at a molar ratio of 1:1, pH 7. 3-Hydroxy-4-methoxybenzaldehyde was tested with 12 different ammonium, alkylammonium, and pyridinium salts for the ability to cocrystallize with oligodeoxynucleotides and to improve desorption and ionization. Ions of oligodeoxynucleotides 9, 10, and 11 nucleotides in length were observed with a matrix of 3-hydroxy-4-methoxybenzaldehyde and ammonium acetate, pH 7, at a mass resolution of 100–150 (fwhm). A small oligodeoxynucleotide (11-mer) was observed at the femtomole level with a combination of 2,5-dihydroxybenzoic acid and ammonium acetate as the matrix. Ions from single stranded DNA (60 nucleotides in length) were also observed using this same matrix combination. The results of these studies have shown that both sensitivity and desorption conditions need to be further improved before complex mixtures of large pieces of DNA can be effectively analyzed. (*J Am Soc Mass Spectrom* 1993, 4, 955–963)

Completion of the human genome project in a reasonable time frame requires the development of new technologies for rapid large scale DNA sequencing [1]. Mass spectrometry has enormous potential as an approach for DNA sequencing because of its inherent sensitivity and high sample throughput. However until recently, the most useful ionization methods for analysis of oligodeoxynucleotides have been fast-atom bombardment [2] and ^{252}Cf -plasma desorption [3]. Analysis by these techniques is still fairly limited by the size of the oligodeoxynucleotides. In addition, ions resulting from fragmentation are often observed in the spectra and complicate the analysis of unknown mixtures. Recently, matrix-assisted laser desorption ionization (MALDI) has enabled the analysis of proteins as large as 300 kDa with time-of-flight (TOF) mass spectrometers [4–9]. TOF mass spectrometers with their high mass range and high sensitivity are attractive instruments for large molecule analysis. However, proteins and DNA are very different molecules and matrix-assisted laser desorption TOF mass spectrometry has been less successful in the

analysis of DNA. To be competitive with current automated DNA sequencing approaches, mass spectrometry must be capable of analyzing DNA fragments as large as 500 bases [1].

Several laser desorption approaches have been applied to the analysis of DNA. Spengler et al. [12] analyzed small oligodeoxynucleotides as protonated molecules using sinapinic acid as the matrix and laser irradiation at 266 nm. A 5S-ribosomal RNA (38,000 Da) has been desorbed by MALDI as well as a $[[\text{d}(\text{pT})]12\text{--}[\text{d}(\text{pT})]18]$ ladder [11]. Nelson et al. [12] laser ablated DNA across a distance of 2.0 cm to a microscope slide and used gel electrophoresis to determine the size of the ablated DNA. Fragments as large as 410,000 Da were observed to traverse the gap between the copper substrate and the microscope slide. To determine if the ablated DNA was intrinsically charged, this same experiment was attempted on a TOF mass spectrometer and a double-stranded DNA fragment of mass 17,900 Da was observed [13]. A similar experiment was attempted by Levis and Romano [14] using a rhodamine matrix, demonstrating the desorption of a DNA fragment 1000 bases long, across a gap to a nitrocellulose membrane. In addition, they demonstrated the stability of the phosphodiester bond during the desorption process. Hettich and Buchanan [15] successfully desorbed small oligodeoxynucleotides using MALDI on a Fourier transform mass spectrometer (FTMS). Al-

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though they were able to obtain resolution in excess of 3500 (fwhm), they were unable to detect ions larger than 6-mers. In addition, substantial fragmentation of the DNA was observed in spectra obtained by these methods.

In all of these approaches, the matrix serves to carry the analyte into the gas phase during the thermal expansion and may promote "cooling" of the analyte. The matrix in the MALDI process is critical for the efficient ejection of analyte ions. Several factors appear to be important for the desorption/ionization of sample molecules: the matrix must have a high extinction coefficient at the wavelength of the laser, the matrix must cocrystallize with the sample (although some liquids matrices have been used), and laser energy must be transferred into the matrix in a short time frame [4]. Initial theories of the mechanism of desorption and ionization proposed ion formation prior to desorption [5]. Continued investigations suggest that photochemistry of the excited matrix molecules and matrix-analyte clusters may contribute to ion formation. A recent theory by Russell et al. [16] proposes ion formation by proton transfer from an electronically excited matrix molecule to the sample molecule.

To further improve the ability to desorb/ionize DNA, an understanding of the necessary parameters for efficient laser desorption of oligodeoxynucleotides must be developed. We have been investigating the analysis of oligodeoxynucleotides by UV MALDI to develop a qualitative understanding of the process of the desorption and ionization of DNA. We have identified several matrices that are useful for desorption and ionization of oligodeoxynucleotides to at least 60 bases in length.

Experimental

MALDI TOF spectra were acquired on a Finnigan MAT (San Jose, CA) LaserMATTM laser desorption TOF instrument. This instrument has been described in detail elsewhere [17]. Briefly, the instrument has a 50 cm linear flight tube operating with a 20 kV accelerating voltage and a 5 kV discrete dynode multiplier. Ions were desorbed using a nitrogen laser at a wavelength of 337 nm and a pulse width of 5 ns. The output of the laser is 120 μ J and this is attenuated using two polarizing filters, one fixed and one rotating. Output from the laser is focused through a lens onto a 1 mm² spot. This corresponds to approximately 1×10^7 W/cm² at maximum laser power, which can be attenuated down to 0 W/cm². Samples were prepared by depositing ~ 0.5 μ L of matrix solution and ~ 0.5 μ L of sample on the center of a gold-plated metal target. In general, the ratio of analyte to matrix was about 1:10,000. The target was allowed to air dry, viewed under a microscope to verify crystallization within the target area, and placed in the instrument. The minimum laser power density needed to observe analyte ions from a particular spot on the target was determined. If no ions

were observed from a particular position on the sample target, the aim of the laser was changed to one of four different positions. Multiple laser shot spectra were acquired by firing the laser at a rate of 1 Hz on a fixed target spot. Typically more than 40 mass spectra could be acquired from a single spot. Calibration was performed by addition of an internal standard of known molecular weight or by using the molecular weight of one of the analytes and generating a two-point calibration curve within the data system using the mass-to-charge ratio of the standard and the zero time point. Spectra were acquired in either the positive or negative ion mode, although negative ion spectra generally showed better resolution and sensitivity.

Organic compounds for the laser desorption experiments were purchased from Aldrich Chemical Co. (Milwaukee, WI). Laser desorption matrices were prepared by dissolving the compound and ammonium acetate (molar ratio of 1:1) in ~ 2 mL of acetonitrile/water (40-60%) to produce a solution ~ 0.1 M in matrix. The pH of this solution was then adjusted to 7.5 by the dropwise addition of 30% ammonium hydroxide. The pH was measured using a Corning pH electrode and Beckmann Instruments pH meter which had been calibrated with a standard buffer/acetonitrile (50%) mixture (pH 4.0 and 7.0). The pH of the matrix solutions was verified by adding a drop of matrix solution to pH paper which had been soaked in distilled and deionized water and comparing the color change to the color chart provided with the pH paper. Oligodeoxynucleotides are most soluble at a pH of ~ 7 so this was the pH at which the analyses were performed. To test the ability of these matrices to desorb and ionize oligonucleotides, two separate sample preparations were made with each matrix, and at least four analyses from different parts of the sample target were attempted. All matrices were prepared fresh on the day of use. The 3-hydroxy-4-methoxybenzaldehyde/ammonium acetate has to be prepared a maximum of 1 or 2 hours before use, as it decomposes in approximately 3 hours. The 2,5-dihydroxybenzoic acid/ammonium acetate matrix is still useful at least 3 days after preparation if stored at 4 $^{\circ}$ C.

The 5'-dT¹¹TTTTTTT-3' oligodeoxynucleotide was purchased from Pharmacia-LKB Biotechnology (Piscataway, NJ) and desalted on reverse phase C₁₈ packing with 100% 50 mM aqueous triethylammonium acetate and eluted off the column with 20% 50 mM aqueous triethylammonium acetate/80% acetonitrile. The following oligodeoxynucleotides were synthesized using an Applied Biosystems 394 DNA/RNA synthesizer with standard phosphoramidite chemistry: 5'-dGC(s)ATGCATG-3' (MW 2800 Da), 5'-dGC(s)ATGCATGG-3' (MW 3129 Da), 5'-dGC(s)ATGCATGGG-3' (MW 3458 Da), 5'-dGCATGCATGC-3' (MW 3005 Da), 5'-dGCATGCAT₁₁GC-3' (MW 15456 Da) and 5'-dGCATGCAT₁₃GCAT-3' (MW 18543 Da). The oligodeoxynucleotides were purified with the 5' dimethoxytrityl protecting groups left in

place by reverse-phase high-performance liquid chromatography on a C_{18} (Axxiom, 4.6 mm $\times$ 250 mm) column. A linear gradient of 100% 50 mM aqueous triethylammonium acetate to 30% 50 mM aqueous triethylammonium acetate in acetonitrile was used to elute the oligodeoxynucleotides. The purified oligodeoxynucleotide was collected, dried in vacuo, and the dimethoxytrityl protecting group was removed by dissolving the oligodeoxynucleotide in 80% acetic acid for 10 minutes at room temperature. The acetic acid was removed in vacuo, water added, and the dimethoxytrityl alcohol was removed by extracting with ethyl acetate. The oligodeoxynucleotides were then dissolved in ultrapure water to a concentration of 10–50 pmol/mL.

To determine the limit of detection for the oligodeoxynucleotide dGC(s)ATGCATGGG, 103 μ g of the synthetic material was weighed and dissolved in 100 μ L of ultrapure water to give a final concentration of 300 pmol/ μ L. This solution was serially diluted to produce solutions with a final concentration of oligodeoxynucleotide of 3 pmol/ μ L, 630 fmol/ μ L and 200 fmol/ μ L. Aliquots consisting of 1 μ L of these solutions were deposited on sample slides and mixed with 1 μ L of a 2,5-dihydroxybenzoic acid/ammonium acetate, pH 7 solution.

Results and Discussion

Initial studies of the desorption and ionization of DNA focused on the use of matrices effective for the desorption of proteins [4]. Most of these compounds proved to be poor matrices for oligodeoxynucleotides and failed to produce mass spectra or produced poor mass spectra in the negative or positive ion mode. Table 1 lists 31 compounds tested with ammonium acetate and the thiophosphate oligodeoxynucleotides 5'-dGC(s)ATGCATGG-3', 5'-dGC(s)ATGCATGG-3' or 5'-dGC(s)ATGCATGGG-3'. The matrices were examined for their ability to desorb and ionize the test oligodeoxynucleotides in the presence of equimolar ammonium acetate at pH 7. The strongest ion signals (signal-to-noise > 100/1) were observed with 2,5-dihydroxybenzoic acid and 3-hydroxy-4-methoxybenzaldehyde.

It is not readily apparent why a matrix should work well for proteins and not at all for oligodeoxynucleotides, although a possible explanation may be the solid state interactions between matrix and analyte. To influence the interactions of matrix and oligodeoxynucleotides in the crystalline state and to promote cluster formation, compounds such as boric acid, ammonium acetate, ammonium hydroxide, and ammonium bicarbonate, were added in various molar ratios. Table 2 lists a variety of salts tested with 3-hydroxy-4-methoxybenzaldehyde as the matrix, the effects on the crystallization of the matrix, and an indication of whether ions were observed with the thiophosphate oligonucleotides 5'-dGC(s)ATGCATGG-3' and 5'-

Table 1. Compounds tested for generation of ions of thiophosphate oligodeoxynucleotides using a 1:1 mixture of ammonium acetate at pH ~ 7*

S/N	Matrix
220/1	2,5-dihydroxybenzoic acid
30/1	Sinapinic acid
35/1	Sinapinic acid, methyl ester
—	Sinapinic acid, C_{12} ester
83/1	3-aminopyrazine-3-carboxylic acid
107/1	3-hydroxy-4-methoxybenzaldehyde
18/1	3-amino-4-hydroxybenzoic acid
23/1	4-hydroxy-3-methoxybenzaldehyde
12/1	4-hydroxy-3,5-dimethoxybenzaldehyde
> 3/1	Hydroxyazobenzene
> 3/1	8-anilino-1-naphthalenesulphonic acid
> 3/1	5-hydroxyisophthalic acid
> 3/1	4-aminoisophthalic acid
> 3/1	2,4,6-trinitroaniline
> 3/1	2,4,6-trinitrophenol
> 3/1	3,5-dinitrobenzoic acid
> 3/1	2,4-dinitrobenzoic acid
> 3/1	4-hydroxy-3-methoxybenzonitrile
—	Tetrafluoroisophthalic acid
—	3-hydroxy-4-methoxybenzaldehyde, semicarbazone derivative
—	2,3-pyrazinedicarboxamide
—	N,N,N-trimethyl-5-amino-isophthalic acid
—	5-chlorosalicylaldehyde
—	3 cyanobenzaldehyde
—	Silver fulminate
—	3-nitrobenzyl alcohol
—	Benzophenone
—	9-hydroxy-4-methoxyacridine
—	2-methoxy-4-nitroaniline
—	5-nitroisophthalic acid
—	Tetrafluoroisophthalic nitrile

*The oligodeoxynucleotides used in this study were 5'-dGC(s)ATGCATGG-3', 5'-dGC(s)ATGCATGG-3', or 5'-dGC(s)ATGCATGGG-3'; each contains one phosphorothionate ester at the 5' cytidine. Twenty noise measurements were taken on either side of the oligodeoxynucleotide ion and averaged.

dGC(s)ATGCATGGG-3'. We cocrystallized the oligonucleotides with compounds of increasing gas-phase basicity ($NH_3 < CH_3NH_2 < (CH_3)_2NH < (CH_3)_3N$) to determine if ionization efficiency could be influenced by desorption of these mixtures. However,

Table 2. MALD results for the analysis of the thiophosphate oligodeoxynucleotides (5'-dGC(s)ATGCATGG-3' and 5'-dGC(s)ATGCATGGG-3') using various salts added to 3-hydroxy-4-methoxybenzaldehyde

Cation	Counterion	Signal/Noise
NH_4^+	Acetate	100/1
NH_4^+	Butyrate	DNC
NH_4^+	Chloride	—
NH_4^+	Formate	—
NH_4^+	Benzoate	—
$MeNH_3^+$	Acetate	DNC
$Me_2NH_2^+$	Acetate	DNC
Me_3NH^+	Acetate	DNC
$(PhCH_2)_3NH^+$	Acetate	—
Pyridinium	Acetate	DNC

— = Did not observe oligodeoxynucleotide ions
DNC = did not crystallize

many of the alkylammonium salts prevented macroscopic crystallization and we did not observe ions from any of the test thiophosphate oligodeoxynucleotides in experiments without crystallization. The most dramatic improvements were observed with ammonium salts at a pH of 7. Figure 1a shows a negative ion MALDI TOF mass spectrum of a mixture of three thiophosphate oligodeoxynucleotides ranging from a 9-mer to an 11-mer (5'-dGC(s)ATGCATG-3', 5'-dGC(s)ATGCATGG-3', 5'-dGC(s)ATGCATGGG-3') using a 3-hydroxy-4-methoxybenzaldehyde/ammonium acetate matrix in a 1:1 molar ratio adjusted to pH 7. The major components in the mass spectrum are the matrix ions. In all the mass spectra acquired the matrix ions were the most intense ions in the spectrum, but are always restricted to the low mass region. Figure 1b shows an expanded view of the oligodeoxynucleotide region of the mass spectrum. The ions are assigned to be $[M-H]^-$ although the mass accuracy is insufficient to unequivocally assign this ion; the mass accuracy was determined to be 0.03% using the mass of the 9-mer as an internal calibrant. Although the oligodeoxynucleotides used for the analysis shown in Figure 1 contain one phosphorothionate ester linkage, no appreciable difference in sensitivity or resolution was observed between the negative ion spectra of these oligodeoxynucleotides and analogous oligodeoxynucleotides (such as 5'-dGCATGCATGCAT-3') which contain all phosphate esters.

Some general observations on the effect of adding ammonium salts to 3-hydroxy-4-methoxybenzaldehyde can be made. Desorption and ionization of oligodeoxynucleotides was observed with compounds that readily decompose in a vacuum such as the ammo-

nium salts of acetate, carbonate, or bicarbonate. No ions were observed from the thiophosphate oligodeoxynucleotides with the addition of 1:1 mixtures of ammonium formate, ammonium benzoate, and ammonium chloride salts with the matrix 3-hydroxy-4-methoxybenzaldehyde.

The desorption and ionization of small oligodeoxynucleotides with the matrix 2,5-dihydroxybenzoic acid was observed without the presence of the ammonium salts. Shown in Figure 2a and b are spectra of the oligodeoxynucleotide 5'-dTTTTTTTTTT-3' desorbed and ionized with and without ammonium bicarbonate added to the matrix 2,5-dihydroxybenzoic acid, respectively. The size of oligodeoxynucleotide which could be observed increased with the addition of ammonium salts. The mass spectrum of 5'-dGCAT(GCAT)₁₃GCAT-3' (MW 18543 Da) in Figure 3 and the mass spectrum

Figure 1. MALDI mass spectrum of a mixture of three oligodeoxynucleotides (approximately 15 pmol of each loaded onto sample platform) using a matrix of 3-hydroxy-4-methoxybenzaldehyde/ammonium acetate. The oligodeoxynucleotides are 5'-dGC(s)ATGCATG-3' (MW 2800 Da), 5'-dGC(s)ATGCATGG-3' (MW 3129 Da), and 5'-dGC(s)ATGCATGGG-3' (MW 3458 Da); each contains one phosphorothionate ester at the 5' cytidine. The spectrum is an accumulation of 11 laser shots. Laser power was attenuated to 65%.

Figure 2. (a) MALDI mass spectrum of approximately 10 pmol of 5'-dTTTTTTTTTT-3' using a matrix of 2,5 dihydroxybenzoic acid. This spectrum results from one laser shot. Laser power was attenuated to 42% and an external calibration was used to assign masses. (b) MALDI mass spectrum of approximately 10 pmol of 5'-dTTTTTTTTTT-3' using a matrix of 1:1 2,5 dihydroxybenzoic acid and ammonium bicarbonate. This spectrum results from one laser shot. Laser power was attenuated to 60% and an external calibration was used to assign masses.

Figure 3. MALDI mass spectrum of approximately 50 pmol of a 60-mer oligodeoxynucleotide (5'-dGCAT(GCAT)₁₃GCAT-3') (MW 18543 Da). The matrix was 2,5-dihydroxybenzoic acid/ammonium acetate and the spectrum is an accumulation of 17 shots. Laser power was attenuated to 70%. An external calibration was used.

of the mixture of 5'-dGCAT(GCAT)₁₃GCAT-3') and 5'-dGCAT(GCAT)₁₁GC-3' in Figure 4 were obtained using a 1:1 mixture of ammonium acetate/2,5-dihydroxybenzoic acid. Ions for these larger oligodeoxynucleotides were not observed with 2,5-dihydroxybenzoic acid in the absence of ammonium acetate.

The amount of buffer added to the matrix was an important parameter. In studies with the matrix 3-hydroxy-4-methoxybenzaldehyde, ions for the three test thiophosphate oligodeoxynucleotides were not observed unless the ratio of salt to matrix was between 1:1 and 2:1. At a ratio 3:1 ammonium acetate to 3-hydroxy-4-methoxybenzaldehyde, the oligodeoxynucleotide, 5'-dGC(s)ATGCATGG-3' shown in Figure 5, is barely observed in the mass spectrum. When ammo-

Figure 4. MALDI mass spectrum of approximately 50 pmol of two oligodeoxynucleotides (5'-dGCAT(GCAT)₁₁GC-3' (MW 15456 Da) and 5'-dGCAT(GCAT)₁₃GCAT-3') (MW 18543 Da). The smaller oligodeoxynucleotide was used as an internal mass calibrant. The matrix was 2,5-dihydroxybenzoic acid/ammonium acetate and the spectrum is an accumulation of 16 shots. Laser power was attenuated to 96%.

Figure 5. A single laser shot mass spectrum of the oligodeoxynucleotide 5'-dGC(s)ATGCATGG-3' with a 3:1 ratio of 3-hydroxy-4-methoxybenzaldehyde/ammonium acetate. The laser power was attenuated to 98% and the masses were assigned with an external calibration.

nium acetate is present in a ratio larger than 2:1, the mixture does not dry completely without heating since ammonium acetate is hygroscopic. A 3:1 mixture of ammonium acetate and matrix was forced to dry by application of a heat source and immediately placed in the mass spectrometer, but as seen in Figure 5 no improvement in the ionization of the oligodeoxynucleotide is observed.

The role of the solid and gaseous state chemical interactions between matrix and analyte in promoting desorption and ionization is not well understood. Consequently, it is difficult to assess the role of the salt in this process simply from the observation of ions. Clearly, critical parameters for successful matrix-assisted laser desorption are the formation of crystals incorporating the analyte and the nature of the chemical interactions of the matrix and analyte in the crystalline state. Presumably, the analyte is incorporated into a loosely packed structure of the crystal as an impurity, since high levels of analyte appear to significantly disrupt the crystallization process and prevent the formation of crystals or desorption. While it may be possible that cocrystallization of matrix and analyte involves segregation of the analyte within the crystalline structure, it is unlikely [18]. In fact, Strupat et al. [19] have found that crystals of 2,5-dihydroxybenzoic acid incorporating a protein, have a homogeneous distribution of analyte and show minimal disruption of the crystal packing. However, molecular crystals of matrices with polar or charged substituents can produce stronger interactions between matrix molecules than between matrix and analyte [18]. Since most of the effective matrices have acidic or basic functionality, strong interactions between matrix and analyte are expected. Thus, the ammonium salts may have a role in either disrupting or weakening these interactions, or alternatively may encourage the formation of loosely

bound clusters with matrix/analyte during the desorption process [20].

A recent theory by Russell et al. [16] suggest that ionization of analytes in UV MALDI is dependent upon gas-phase interactions between the analyte- and excited-state matrix molecules, and that clustering of matrix and analyte molecules may play a significant role in promoting protonation or deprotonation of the analyte. For example, compounds such as phenols become more acidic when excited, while aromatic carbonyls become more basic [21]. Droz et al. [22] and Jouvot et al. [23] have shown that excited state proton transfer occurs in clusters of ammonia with phenols and that the proton transfer is a function of the number of ammonia ligands. While it would be difficult to extend their findings to MALDI, clustering and the

concomitant interactions of the matrix, ammonium ions, and oligodeoxynucleotides may play a role in the formation of negative ions of oligodeoxynucleotides. Ammonium ions may also promote enhanced gas-phase cooling of the desorbed oligodeoxynucleotides by evaporation of matrix and ammonia from the analyte, thereby stabilizing an ion that might otherwise decompose. These possibilities bear further investigation.

An important consideration for the MALDI of oligodeoxynucleotides is the gas-phase stability of the ions created in this process. For example, at laser power densities significantly above the threshold for ionization, small oligodeoxynucleotide decomposition products are observed. Figure 6a shows a spectrum of a 10-mer (5'-dGCATGCATGC-3') oligodeoxynucleotide

Figure 6. (a) MALDI spectrum of a 10-mer oligodeoxynucleotide (5'-dGCATGCATGC-3') desorbed at full laser power, showing substantial decomposition. The spectrum is an accumulation of 10 laser shots. The mass scale is not accurately calibrated. The matrix is 2,5-dihydroxybenzoic acid/ammonium acetate (pH 7). (b) MALDI spectrum of a 10-mer oligodeoxynucleotide (5'-dGCATGCATGC-3') desorbed with laser power attenuated to 38% of full power or just above the threshold for ionization of this oligonucleotide. The spectrum is an accumulation of five laser shots. The mass scale is calibrated with an external calibration procedure. The matrix is 2,5-dihydroxybenzoic acid/ammonium acetate (pH 7). (c) MALDI spectrum of a 50-mer oligodeoxynucleotide (5'-dGCATGCATGC-3') desorbed at full laser power. The spectrum is an accumulation of six laser shots. The lower mass ions correspond to multiply charged analyte species. The matrix is 2,5-dihydroxybenzoic acid/ammonium acetate (pH 7). (d) MALDI spectrum of a 50-mer oligodeoxynucleotide (5'-dGCATGCATGC-3') desorbed with laser power attenuated to 67% of full power. The spectrum is an accumulation of six laser shots. The matrix is 2,5-dihydroxybenzoic acid/ammonium acetate (pH 7).

desorbed with a laser power density of $\sim 7 \times 10^6$ W/cm² with a matrix of 2,5-dihydroxybenzoic acid/ammonium acetate. The broad peaks below the parent ion are presumably ions resulting from thermally induced decomposition, which must occur in a very short time frame, since ions decomposing after acceleration out of the ion source will not appear as separate ions. The best laser power density for the analysis of a sample is generally just above threshold for onset of desorption and ionization (Figure 6b). Significantly above this level the peak shapes broaden distorting mass accuracy and resolution. Consequently, the exact mass of the decomposition ions cannot be determined. In desorbing an oligodeoxynucleotide 50 bases in length under the same laser power density conditions ($\sim 7 \times 10^6$ W/cm²), only a small amount of decomposition was noted and some multiply charged ions were formed (Figure 6c). Figure 6d shows a mass spectrum of the same oligodeoxynucleotide desorbed with a laser power slightly above threshold. Some care has to be taken in evaluating these results. Even though both spectra were obtained under identical conditions, these conditions may not be the optimum for both samples. Nevertheless, this result suggests that the negative ions of larger oligodeoxynucleotides created during the laser desorption process are reasonably stable and traverse the acceleration field without decomposition, even though higher laser energy is usually required for observation.

The sensitivity of analysis for oligodeoxynucleotides by UV MALDI is still poor compared to gel electrophoresis with radioactive or fluorescent detection (10^{-15} – 10^{-18} moles). In general, 1–10 pmol is required for smaller oligodeoxynucleotides (<20-mer) and this increases to approximately 50 pmol for the larger oligodeoxynucleotides. Figure 7a–c shows mass spectra of an 11-mer (5'-dGC(s)ATGCATGGG-3') obtained from 3 pmol, 630 fmol and 200 fmol, respectively, using a 2,5-dihydroxybenzoic acid/ammonium acetate (1:1, pH 7.5) mixture as the matrix. Improvements in sample preparation such as concentrating the analyte in a small spot may yield increases in the sensitivity of analysis.

The ability to differentiate between two oligodeoxynucleotides differing by one base is essential if MALDI TOF is to be used for sequencing DNA. Figure 1 shows a mixture of three oligodeoxynucleotides with a resolution of 150 (fwhm) using a matrix of 3-hydroxy-4-methoxybenzaldehyde/ammonium acetate. In practice, desorption/ionization of small oligodeoxynucleotides (up to 12-mers) with this matrix produces a mass resolution of between 100–150 (fwhm), but this resolution deteriorates rapidly as the size of the oligodeoxynucleotide increases (see below).

Mass accuracy is another common problem in TOF instruments and perhaps one of the greatest limitations in this area is obtaining a reference sample of similar structure and nearby mass. The advantage of internal calibrants of molecular structure similar to the

Figure 7. MALDI mass spectrum of an 11-mer (5'-dGC(s)ATGCATGGG-3') using (a) 3 pmol, (b) 630 fmol, and (c) 200 fmol. The matrix was 2,5-dihydroxybenzoic acid/ammonium acetate. The spectra are an accumulation of 10 laser shots each. The laser power was attenuated to approximately 90% for each analysis.

sample is that they ensure that chemical interactions of the sample and calibrant are similar. This results in effective cocrystallization, similar peak shapes and compatible desorption/ionization efficiencies. It can be seen from Figure 1 that sequences of oligodeoxynucleotides provide a natural ladder of molecular weight reference points, which helps with mass accuracy even

at high masses. The oligodeoxynucleotides analyzed in Figure 1 differ each by one guanine deoxynucleotide, which is equivalent to 329 mass units. The observed differences between the ions in Figure 1 is 329 and 328 mass units which corresponds to a mass accuracy of 0.03%. This mass accuracy and resolution easily allows oligodeoxynucleotides differing by one base in this mass range to be distinguished. Figure 3 shows the desorption of a synthetic 60-mer oligodeoxynucleotide (MW 18,543 Da) with 2,5-dihydroxybenzoic acid/ammonium acetate at a resolution of 18 (fwhm). The dimer of the 60-mer can also be observed in Figure 3 at a mass of approximately 37,062 Da. This mass spectrum was produced with a laser power density of $\sim 1 \times 10^7$ W/cm². The occurrence of dimers is sample, concentration, and laser power dependent, and they are generally less abundant than the monomer and subsequently produce little confusion in the analysis of the spectra.

All our experiments with large oligodeoxynucleotides (>40-mer) using various matrices and laser powers produced low resolution. The formation of adducts is one possible explanation for the increased peak width at high mass. Although matrix adducts were not observed in the negative ion spectra of small oligodeoxynucleotides, ammonia adducts were sometimes observed in the spectra (Figure 8). Figure 4 shows a spectrum of a mixture of two oligodeoxynucleotides, 50 and 60 bases long. The sample was run numerous times to check reproducibility and when the ion of one of the oligodeoxynucleotides was used as an internal calibrant the mass of the other peak always appeared within 0.03% of its calculated mass. It is possible that the broad peak shapes observed for the larger oligodeoxynucleotides are a result of metastable decomposition. In such a case skewing of peaks to higher masses (adduct formation) would not be observed.

Figure 8. A single laser shot mass spectrum of the oligodeoxynucleotide 5'-dGC(s)ATGCATGC-3' with a 1:1 ratio of 3-hydroxy-4-methoxybenzaldehyde/ammonium acetate showing ammonia adducts at m/z 3142 and 3159. The laser power was attenuated to 98% and the masses were assigned with an external calibration.

Conclusion

Addition of salts, such as ammonium acetate, to a UV absorbing matrix has been shown to have an effect on the sensitivity, reproducibility, and mass range in the analysis of oligodeoxynucleotides by negative ion UV MALDI. The desorption and ionization of a synthetic single stranded oligodeoxynucleotide of 60 bases in length was shown by this approach. This is the largest oligodeoxynucleotide we have attempted to desorb by this method and may not represent a limit in the mass range applicable to the method. Negative ion MALDI has been demonstrated as an effective method for the analysis of large single stranded oligodeoxynucleotides without significant decomposition.

Although mass resolution is adequate for small oligodeoxynucleotides, further improvements in resolution and sensitivity of analysis of larger oligodeoxynucleotides is necessary. Increases in desorption and ionization efficiencies may occur with laser irradiation at wavelengths more closely tuned to the absorbance maximum of the matrix. In addition, the use of picosecond lasers may also lead to increases in mass resolution [24]. Future experiments will focus on the desorption/ionization of large oligodeoxynucleotides and improving the sensitivity of analysis.

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