5-Methoxysalicylic Acid and Spermine: A New Matrix for the Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry Analysis of Oligonucleotides

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5-Methoxysalicylic acid (MSA) is demonstrated to be a useful matrix for matrix-assisted laser desorption/ionization time-of-flight (TOF) mass spectrometry of oligonucleotides, when desorption/ionization without fragmentation is desired. When MSA is combined with the additive spermine, the need for desalting is reduced. The MSA/spermine matrix yields linear TOF mass spectra with improved resolution, less fragmentation, and less intense alkali ion adduct peaks than those spectra obtained using 3-hydroxypicolinic acid and 6-aza-2-thiothymine with spermine or diammonium hydrogen citrate as additives. Instrumental conditions are discussed to improve the spectral resolution, specifically the use of longer delay times in the delayed-extraction ion source. (J Am Soc Mass Spectrom 2001, 12, 456–462) © 2001 American Society for Mass Spectrometry

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS), developed by Karas et al. [1], has been extensively used to analyze biological compounds at the subpicomole level. To date, most of the published MALDI MS work has involved the analysis of peptides and proteins. MALDI MS has also been used for the analysis of oligonucleotides [2, 3]. The development of MALDI matrices optimized for the analysis of oligonucleotides has lagged behind that for peptides [4], but is currently receiving considerable attention. This report discusses the performance of 5-methoxysalicylic acid, with spermine as a matrix additive, for the detection of oligonucleotides in MALDI with the suppression of prompt fragmentation in the linear time-of-flight (TOF) MS experiment.

Oligonucleotides and DNA behave very differently than peptides and proteins in the MALDI experiment. Differences of note include the sensitivity, extent of prompt fragmentation, the need for cleanup prior to analysis, and resolution.

Certainly, for all classes of biomolecules studied by MALDI, as sample handling methods and TOF instruments have been developed, typical sample sizes used for analysis have decreased. Peptide analysis at the subpicomole level was rapidly achieved, although it has taken longer to realize similar MALDI detection limits for oligonucleotides. There have been significant advances from earlier reports of MALDI analyses of oligonucleotides, in which 10–100 pmol of material were required, to recent reports that show clear detectability at the femtomole level [5, 6].

When a pure peptide is analyzed by MALDI-TOF MS, it usually yields a single mass spectrometric peak representing the intact molecule (in protonated or deprotonated form). Matrices and sample conditions have been reported to increase the extent of prompt fragmentation of peptides in MALDI [7], but experimentalists have little predictable control over the process, generally. In contrast, oligonucleotides frequently fragment promptly and extensively in linear TOF MS [8].

The analysis of pure samples certainly makes mass spectral interpretation easier for all biomolecules. Depending on whether compounds are isolated from biological sources, from electrophoresis experiments (gels or membranes), or are synthesized, peptides and oligonucleotides may be presented for mass spectrometry analysis in the presence of compounds such as salts, buffers, and glycerol, frequently in higher relative amounts than the analytes. In all cases, high amounts of salts can sufficiently change the MALDI target material to lower analyte signals. Glycerol is usually a problem as a component when the first step of the MALDI experiment is to grow crystals. In terms of the spectra that result, salts have a very different impact when analyzing peptides compared to oligonucleotides. If NaCl is present, two peaks may appear representing a single peptide in positive ion mode, most frequently representing the $[M + H]^+$ and $[M + Na]^+$ ions, separated by 22 Da. As the salt contribution increases,

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1044-0305/01/$20.00
PII S1044-0305(00)00212-4

Received September 7, 2000
Revised November 30, 2000
Accepted November 30, 2000

sodium adducts become more intense. The presence of salts has a much more dramatic influence on the MALDI spectra of oligonucleotides. With an ionic phosphodiester backbone, oligonucleotides can contain a large number of anionic sites that must combine with an assortment of cationic species such as H^+, Na^+, and K^+ to desorb in a singly charged form. Combinations of cations to provide a charge balance can lead to a substantial number of peaks representing the intact molecule. Whenever ions for a single species are generated with a range of *m/z* values, detection limits are lowered. Also, it becomes much more difficult to detect multiple components of similar mass. Certainly, a simple situation is to generate one mass spectral peak per component, not several.

In the analysis of oligonucleotides with MALDI TOF MS, the resolution achieved is often less than what would be realized for peptides of similar size. Adduct formation and nucleotide base loss have frequently been cited as causes of the reduced resolution [9]. For larger oligonucleotides, this is clearly the case. However, even for smaller sequences with resolved sodium and potassium adducts, resolution for an oligonucleotide is less than that obtained for a peptide of similar mass on the same instrument. The use of an ion reflector does not dramatically improve the resolution of oligonucleotides in MALDI MS [10]. One of the few instances in which relatively small oligonucleotides were analyzed by MALDI and spectra containing isotopic resolution were obtained was recently published, using a MALDI TOF instrument with an extended flight tube [11]. We show that, for the TOF instrument used in this work utilizing gridded ion optical components and delayed extraction (DE), "nontraditional" ion source settings can improve the resolution.

Although peptides and oligonucleotides have many differences in terms of their MALDI MS analysis, they also have some notable similarities in terms of the development of the analytical utility of mass spectrometry for their analysis. Early in the history of MALDI, demonstrations of the ability to generate signals from large, intact, ionized proteins maintained excitement for this new method, allowing it to be further developed. Whereas positive results had been reported for proteins with molecular weights (MW) of several hundred thousand, the mass spectral peaks were very broad, and it was difficult to extract what would be considered in mass spectrometry an accurate mass. The early results showed that, in most cases, MW's determined were more useful than those derived from gel electrophoresis. Today, the real strength of MALDI in protein analysis does not lie in its ability to generate ions of intact proteins, but in the subpicomolar sensitivity obtainable for smaller peptides. The analysis of enzymatic and chemical digestion products is the application that is making the real contributions to protein analysis. Scientists have certainly worked to demonstrate that large oligonucleotides can be characterized by MALDI as well. Whereas a few reports have been published demonstrating the detection of 50-mers, the MALDI response is much more sharply a function of MW than is observed for peptides, making the detection of large oligonucleotides far from routine. Because a potential application was the use of mass spectrometry to replace gel electrophoresis when sequencing DNA using the Sanger method, the requirement of the mass spectrometry method was different than when sequencing peptides. Peaks with good resolution must be detectable not only at low *m/z* values, but over a very wide mass range, because the approach for DNA analysis is a ladder sequencing method. Although mass spectrometry may not in the near future replace gels for DNA sequencing, other powerful applications are emerging, notably the analysis of single nucleotide polymorphisms (SNPs) [12, 13]. In this area, mixtures of small oligonucleotides, usually with molecular weights less than 6000 Da, are generated which are indicative of errors in genetic code. This is an application to which MALDI MS is well suited. Certainly, the need continues to develop methods for improved analysis of oligonucleotides of all sizes. The SNP application is clearly driving method development for analyses of oligonucleotides of sizes for which the challenges are not the creation of new instruments, but improved matrix chemistry. The goals continue to be lower detection limits, the ability to perform analyses with less or no cleanup (recognizing the contaminants most commonly encountered, and that purification steps always lead to sample loss), and to identify experimental conditions in which fragmentation is decreased or eliminated. Certainly, in the analysis of mixtures, if one could rely on generating only one peak per component, as is common for peptides, then the analysis of mixtures of oligonucleotides would be much easier.

Many matrices have been developed for peptide analysis [14, 15] by MALDI such as nicotinic acid, ferulic acid, sinapinic acid, and 2,5-dihydroxybenzoic acid. Because, generally, these are not as useful for oligonucleotide analysis, alternate matrices have been identified [16, 17]. Several matrices were found to be compatible for the analysis of oligonucleotides using UV lasers, including 3-hydroxypicolinic acid (HPA) [18] and 6-aza-2-thiothymine (ATT) [19]. Since the development of these matrices, HPA has become the standard matrix for the analysis of larger nucleic acids while ATT is used for sequences containing less than 25 bases [2]. In addition to UV-MALDI, work has been done using infrared lasers [20, 21]. Successful matrices for IR-MALDI include succinic acid and urea [22]. Because matrix selection alone does not overcome some of the problems associated with the MALDI MS analysis of oligonucleotides, a number of matrix additives have been developed. The utility of ammonium salts has been demonstrated to reduce the formation of alkali ion adducts. Ammonium acetate was the first to be used in the MALDI experiment [8]. Since then, other ammonium salts have appeared in the literature with the most successful being diammonium hydrogen citrate [23, 24].

Thus, if an oligonucleotide exists in solution in polyanionic form as P^{n-}, it forms a singly charged anion by adding combinations of H^+, Na^+, and K^+ ions. If NH_4^+ ions are present, these compete effectively with alkali ions in complexing with negatively charged phosphates. During the desorption/ionization process, ammonia is lost, leaving protons behind. In this paper, the fully protonated form of an oligonucleotide, $[P^{n-} + nH^+]$, the "free acid form," will be referred to as M. If no alkali ions are involved and all of the phosphates are neutralized by protons, negative ion MALDI yields deprotonated molecules, designated as $[M - H]^-$. In our lab, the role of polyamines as matrix additives has been explored and spermine was found to improve MALDI spectra for single-stranded oligonucleotides, eliminating both the need for ammonium citrate as well as desalting [5, 25].

We demonstrate here that 5-methoxysalicylic acid (MSA) is useful in the MALDI analysis of oligonucleotides. In our experiences, it is unique in generating intact oligonucleotide ions with very little fragmentation. MSA is not a new matrix in the field of MALDI. It has been suggested that a mixture of MSA, 2,5-dihydroxybenzoic acid, and fucose is a useful matrix for the analysis of peptides [26], whereas MSA with 9-anthracenecarboxylic acid has been demonstrated as a matrix for the analysis of polymers [27]. MSA has also been used in a multicomponent matrix when quantitation was being attempted [28]. We show here that MSA is also an effective matrix for the analysis of oligonucleotides. The MALDI spectra obtained using MSA are compared here to those obtained using the traditional HPA and ATT matrices. Best results were obtained using spermine as an additive, rather than diammonium hydrogen citrate.

Experimental

The 12-mer oligodeoxyribonucleotide d(CGCGAATTCGCG) was purchased from the Michigan State University Macromolecular Structure, Sequencing, and Synthesis Facility (East Lansing, MI). The stock solution used had a concentration of 46.6 pm/μL and was used without desalting. The matrices 6-aza-2-thiothymine and 5-methoxysalicylic acid were purchased from Aldrich (Milwaukee, WI). Spermine and 3-hydroxypicolinic acid were purchased from Fluka (Milwaukee, WI). All matrices were used without further purification. When spermine was used as a matrix additive, it was prepared at a concentration of 25 mM in water. When diammonium hydrogen citrate (J.T. Baker, Phillipsburg, NJ) was used, it was prepared at a concentration of 50 mM in water. Saturated matrix solutions were made using a 1:1 acetonitrile/additive solution. Samples were prepared by mixing 1 μL of analyte solution with 1 μL of the matrix solution on a gold sample plate and allowing the mixture to air dry.

MALDI mass spectra were recorded using an Applied Biosystems (Framingham, MA) Voyager delayed extraction linear time-of-flight mass spectrometer equipped with a nitrogen laser (337 nm, 3 ns pulse). For the negative ion MALDI spectra reported here, the accelerating voltage was -15 kV, the delay time was 700 ns, the grid voltage was 94.5% of the accelerating voltage, and magnitude of the guide wire voltage was 0.20% of the accelerating voltage. Typically, 50 laser shots were averaged for each spectrum.

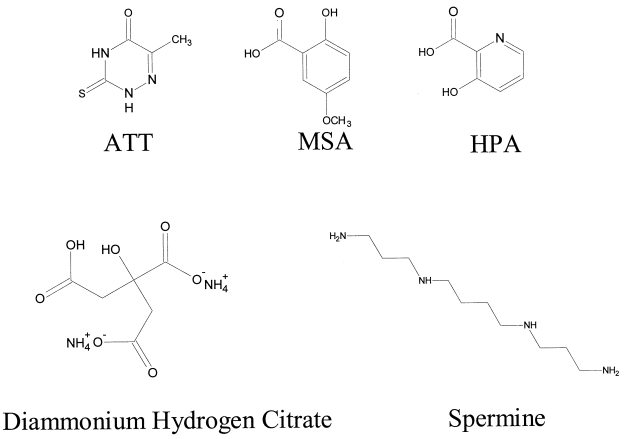

Figure 1. Structures of the matrices and additives used in this study.

UV/visible spectra of aqueous solutions of the matrices were recorded using an ATI Unicam (Cambridge, UK) UV2 spectrophotometer. All spectra were acquired with a scan speed of 120 nm/min, a data interval of 0.5 nm, and a 2.0 nm bandwidth. Spectra were acquired for three matrices, each at a concentration of 2.0×10^{-5} M. Quartz cuvettes were used with water in the reference cuvette.

Results and Discussion

To demonstrate the advantages of MSA as a MALDI matrix for oligonucleotide analysis, we selected the Dickerson dodecamer, d(CGCGAATTCGCG), referred to here as DD, as an example of a typical oligonucleotide. We have obtained similar results for a variety of other oligonucleotides, including d(ACCCACCCACCC), d(AAAAACCAAAAA), d(TTTTTGGTTTTT), and d(CCGGAATTGGCC). DD was chosen as an example, because it has been used extensively in the literature, and contains all four bases. Furthermore, while all of these oligonucleotides fragment in typically used matrices such as ATT, DD fragments most extensively. The nomenclature suggested by McLuckey [29] is used to identify fragment ions.

Data are shown for the analysis of DD using the three matrices HPA, ATT, and MSA. Their structures are shown in Figure 1. Using the traditional additive, diammonium hydrogen citrate (DHC), these three matrices yield the spectra shown in Figure 2. The ATT/DHC mixture yields the complex spectrum shown in Figure 2a. The resolution for the peak representing the deprotonated molecule at *m/z* 3645 is low, and extensive

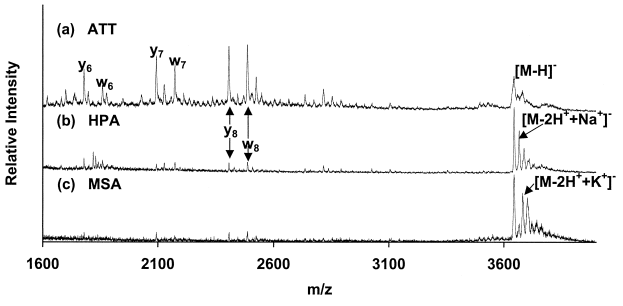

Figure 2. MALDI-MS negative-ion mass spectra of DD with DHC as an additive using (*a*) ATT, (*b*) HPA, and (*c*) MSA.

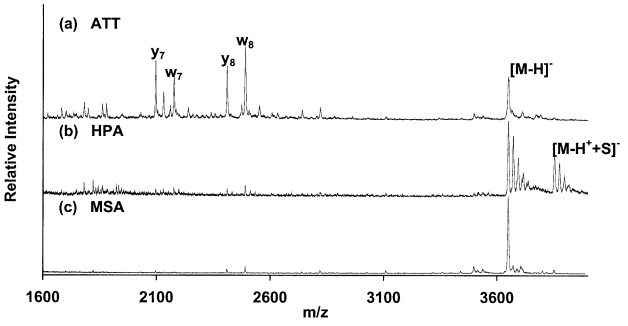

Figure 3. MALDI-MS negative-ion mass spectra of DD with spermine (S) as an additive using (*a*) ATT, (*b*) HPA, and (*c*) MSA.

fragmentation occurs. The y_8 and w_8 peaks, which are more intense than that for the deprotonated molecule, are formed following phosphodiester bond cleavage between the G and A nucleosides. In contrast, the HPA/DHC matrix produces a much different MALDI spectrum, Figure 2b. The deprotonated molecule is the dominant peak in the spectrum, with only small fragment ion peaks present. In comparing Figure 2a, b, the fragment ions do not change when the matrix is changed, but HPA produces fewer fragment ions. The resolution for the deprotonated molecule peak is clearly higher than in the ATT/DHC spectrum. At *m/z* values above *m/z* 3645, a series of sodium ion adducts are observed, in which an H^+ is replaced by a sodium ion in the mono-anion. The low mass shoulder on the *m/z* 3689 peak ($[M - 3H^+ + 2Na^+]^-$) is a potassium adduct, $[M - 2H^+ + K^+]^-$.

The spectrum obtained using MSA/DHC as the matrix, shown in Figure 2c, closely resembles that obtained using HPA/DHC. There are fewer detectable fragment ions in Figure 2c and the peaks are less intense, compared to those in Figure 2a, b. Again, the change in matrix does not yield a substantial change in the fragmentation pattern, but changes the extent of fragmentation. The deprotonated molecule peak is again the most intense in the spectrum. It is interesting to note that the potassium adduct has greater intensity than the monosodium adduct. In Figure 2c, alkali ion adducts contribute to the unresolved high *m/z* "tail." Although the addition of ammonium citrate reduces cation adduction, there is still a need for desalting. The ammonium citrate clearly works more efficiently when used with HPA or MSA, and does provide spectra that are superior to those obtained using the matrices alone. Clearly, the choice of matrix (as well as the choice of additive [30]) has a substantial impact on the extent of fragmentation, with MSA allowing for desorption of intact oligonucleotides with minimal formation of fragment ions. A reduction of fragmentation accompanied by formation of an intense peak representing the doubly charged molecular ion has also been seen using 3-HPA, at an excititation wavelength of 355 nm [31].

In our experience, spermine is found to be consistently superior to ammonium citrate as a matrix additive. As shown in Figure 3, the choice of matrix affects the performance of spermine as an additive. The negative ion MALDI spectrum obtained using ATT/spermine as the matrix. Figure 3a, yields a fragmentation pattern similar to that seen using DHC as an additive, Figure 2a. Regardless of the additive, fragment ions are more abundant than the deprotonated molecule. With spermine as the additive, the resolution of the $[M - H]^-$ peak increases, and only small sodium and potassium adduct peaks are present. In addition to the alkali ion adducts, there are two small higher-*m/z* peaks that represent a matrix and a spermine adduct. This is not the case in the HPA/spermine spectrum shown in Figure 3b. Although the fragmentation is minimal, the spermine adduct becomes a dominant peak in the spectrum. Spermine is ineffective in eliminating the formation of alkali ion adducts in HPA for this experiment. The five sodium adducts in the *m/z* range 3667–3755 increased in intensity from those observed when HPA/DHC is the matrix. Adduct ions are also formed in which both spermine and alkali ions are attached.

When combined with spermine, MSA is an ideal matrix for the desorption/ionization of intact oligonucleotides, as shown in Figure 3c. The spectrum obtained using the MSA/spermine matrix contains very small w_8–w_{10} and y_8 fragment ions. The peak representing the intact analyte is fully resolved with the sodium and potassium adducts greatly reduced. The spermine adduct is of low intensity compared to the intact, deprotonated molecule. Also, there is no peak representing the doubly deprotonated molecule as seen previously in other spectra of oligonucleotides containing negligible fragmentation [31]. Thus, if the goal is to obtain spectra in which one oligonucleotide yields a single peak, MSA will limit fragmentation and spermine will limit the formation of adducts, and this combination of MSA and spermine retains the favorable properties of each matrix component. The performance of spermine has been discussed previously and it is not our intention to reintroduce it here. We do note that the oligonucleotide samples were used as supplied from the Michigan State University Macromolecular Structure, Sequencing, and Synthesis Facility. As synthesized, using standard methods [32], these samples had a high, but poorly defined salt content, and contained other contaminants

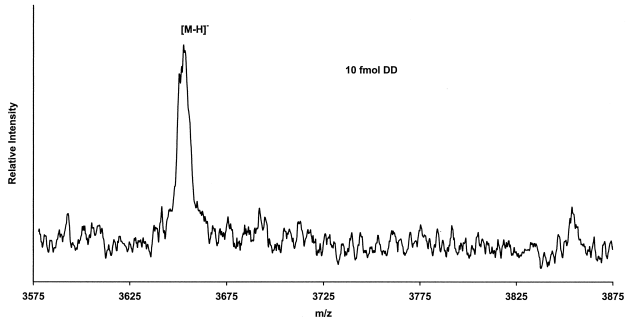

Figure 4. MALDI-MS negative-ion mass spectrum of 10 fmol of DD, using MSA/spermine as the matrix.

such as a buffer. For oligonucleotides made in this way, desalting is unnecessary if spermine is used.

The spectra in Figures 2 and 3 were obtained using 47 pmol of oligonucleotide as the analyte. With this amount, the spectra are strong and the differences can be clearly documented. The MSA/spermine combination allows for spectra to be obtained with sample amounts below 1 pmol. Figure 4 shows a portion of the spectrum obtained using the MSA/spermine combination with a total of 10 fmol of DD in the target. A signal-to-noise ratio of 12:1 is achieved (prior to smoothing).

The results presented here clearly show that matrix selection can have a dramatic influence on the extent of fragmentation of oligonucleotides. For the three matrices studied, MSA and HPA are both organic acids, and ATT is considered as a neutral matrix. If ATT were drawn in the enol form, all three would have aromatic hydroxy groups that could be the source of protons for analyte ionization in the positive ion MALDI experiment. Rather than considering structural features such as specific functional groups, there may be a correlation between matrix performance and molar absorptivities. The UV/visible spectra of equimolar aqueous solutions of the three matrices used in this work are shown in Figure 5. As suggested by their spectra, MSA has the greatest molar absorptivity at 337 nm with a value of 2240 M^{-1} cm^{-1}. ATT and HPA have molar absorptivities at 337 nm of 1135 and 911 M^{-1} cm^{-1}, respectively. Although the UV experiment was conducted in solution, we expect a similar result for the solid matrices. This is reflected in the fact that, when MSA is used as a matrix, significantly lower laser powers are required to generate intense signals. If a matrix is an efficient "light harvester," with a high molar absorptivity, absorbing crystals may quickly achieve higher temperatures than would crystals of molecules with lower extinction coefficients. Because the matrix molecules absorb the energy, time is required for energy to flow from matrix molecules into analyte molecules [33]. At higher temperatures, desorption rates increase, decreasing the time in which energy can flow into the analyte. The result would be less prompt fragmentation. This is one possible explanation for the reduced fragmentation observed when MSA is used. With the data provided here, our intention is not to develop predictive capabilities for fragmentation of oligonucleotide ions as a function of matrix choice, but rather to establish that fragmentation is greatly reduced by use of the MSA/spermine matrix combination.

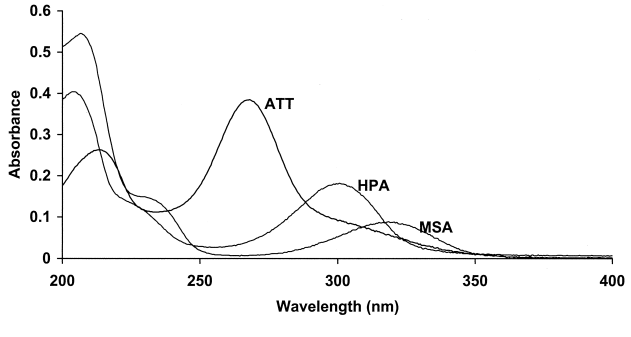

Figure 5. UV-visible spectra of the matrices. For each, the concentration (aqueous) was 2.0×10^{-5} M.

Whenever new matrices are proposed, the question of sample spot homogeneity should be addressed. Formation of MSA crystals is visibly different than for ATT and HPA. The MSA solution, applied to a MALDI plate, tends to spread more than other matrices, yielding a thinner, more uniform bed of crystals. Spectra such as those shown can be obtained at most points across the target, unlike the other matrices for which the crystal growth is more extensive around the outer edges of the target.

All of the spectra shown were obtained with what we consider to be "nontraditional" tuning of the MALDI DE-TOF instrument used for this work. The PE Biosystems TOF mass spectrometer uses a "gridded" ion source and a linear TOF mass analyzer; changes in voltages suggested here may not have the same effect on spectra obtained using instruments that do not employ grids. If analyzing a peptide with a molecular weight in the 3000–4000 range, an accelerating voltage of 20 kV, a grid voltage of 94%–95% the accelerating voltage, a guide wire voltage of approximately 0.05%, and a delay time of 100 ns are the typical conditions selected for optimal resolution. Using these conditions, the resolution obtained for an oligonucleotide is much less than that for a peptide of similar mass. The reduction in resolution for oligonucleotides could result from a number of causes including ion fragmentation in the ion source during acceleration, and slow desorption/ionization-kinetics following laser irradiation. Fragmentation which occurs on a time scale longer than the ion generation and shorter than the acceleration time will be manifested in a linear TOF experiment as tails on the fragment ion peaks towards higher *m/z* values [34]. However, the spectra shown here have negligible tailing on the fragments peaks and more prominent tailing on the peak correlating to the intact ion. Processes other than fragmentation during acceleration must occur which lead to the decreased resolution in an oligonucleotide spectrum. The slow desorption/ionization ki-

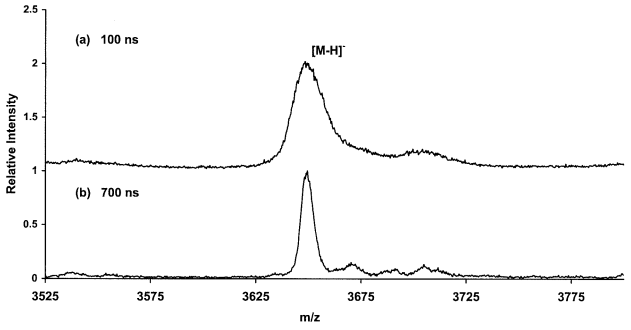

Figure 6. MALDI-MS negative-ion mass spectra of DD with MSA/spermine as the matrix with (*a*) 100 ns delay and (*b*) 700 ns delay.

netics for an oligonucleotide may be another cause of decreased resolution. Oligonucleotides are more polar than peptides or proteins and, thus, require more energy to generate intact, gas phase ions [10]. Increasing the delay time is one way to compensate for the processes that may lead to low resolution. Improvements in resolution as a function of tuning are not unique to the one instrument on which these experiments were performed. Improvements were also observed on a PerSeptive Biosystems Voyager STR DE instrument. A portion of the DD spectrum obtained using MSA/spermine and the standard tuning is shown in Figure 6a; the same region of the spectrum, obtained using a much longer delay time, is shown in Figure 6b. For longer delay times, slightly higher guide wire voltages are recommended. This combination results in much improved resolution. The optimized conditions, a guide wire voltage of 0.200% and a delay time of 700 ns, provide improved resolution for all peaks in the spectrum in both positive and negative ion modes. This result is not matrix specific. Although we make this observation, and routinely use long delay times when obtaining MALDI spectra of oligonucleotides, there is no one process that could clearly be linked to the delay time-resolution correlation. Low resolution for oligonucleotides under standard ion source conditions does not appear to be due to decomposition of the intact anions during acceleration, because spectra obtained using long delay times do not contain fragment ion peaks that are absent or less intense when short delay times are used. Desorption kinetics, linked to the unique variety of charge states of the oligonucleotides that may be trapped in the matrix, may be very different than those for peptides that are trapped in predominantly neutral form.

If the combination of a new matrix and modified tuning leads to the advantage of increased resolution, one may expect the improvement to be further realized by using a reflectron. Again, oligonucleotides behave very differently than do peptides in MALDI MS [10]. Resolution is much lower for oligonucleotides and the use of a reflectron does not significantly improve the resolution [10]. When analyzing DD with each of the three matrices on a Voyager STR in reflectron mode, there is no change in resolution. The $[M - H]^-$ peak is smaller and the spectra are worse than if a linear TOF MS is used. The intensity of the $[M - H]^-$ peak may decrease in reflectron mode because we have delayed all fragmentation from prompt (in source) to occurring in the flight tube. If this is the case, a post-source decay experiment should yield an intense spectrum. This is not observed. It is possible an electron may be ejected from the $[M - H]^-$ species leaving $[M - H]$ in the flight tube. Whatever the mechanism, best results are achieved for oligonucleotides in negative mode in a linear MALDI experiment.

Conclusion

Although HPA/DHC and ATT/DHC are matrix/additive combinations commonly used for oligonucleotide analyses, MSA proves to be a superior matrix for detection of the intact analyte in the 12–20 base pair range when spermine is used as an additive. The MSA/spermine matrix provides alkali ion adducts of low abundance, little fragmentation, and better resolution when compared to ATT and HPA with either DHC or spermine. Spermine, when used with MSA, reduces the need for desalting of oligonucleotide samples.

Although analyses of double-stranded oligonucleotides have been reported with several matrices [21, 22], such noncovalently bound complexes are still difficult to routinely detect intact. In most cases, if a double-stranded oligonucleotide is analyzed using MALDI MS, peaks representing only the single strands are observed in the spectrum. Although MSA/spermine is very effective as a matrix for single strands, it does not allow for double strands to be detected as intact ions. The search continues for a matrix that will routinely allow such noncovalent interactions to remain intact.

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1276–1277 Nucleic Acids Research, 1995, Vol. 23, No. 7

6-Aza-2-thiothymine: a matrix for MALDI spectra of oligonucleotides

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Received January 3, 1995; Revised and Accepted February 23, 1995

Matrix Assisted Laser Desorption Ionization (MALDI) is a powerful tool for the analysis of biomolecules (1). Thanks to its high sensitivity and tolerance towards chemicals commonly used in experimental protocols, this 'soft ionization' mass spectrometric technique is becoming the first choice for the analysis of biological compounds with high molecular weight (MW). Lately, easy-to-use benchtop instruments have became available on the open market and together with simple experimental protocols for sample preparation, they allow for routine analysis by non-expert users. Peptides and proteins are now routinely analyzed by MALDI; to the contrary, oligonucleotides are still problematic, due to cation (Na^+ and K^+) and matrix adducts that reduce resolution and the accuracy of the determination of the molecular weight (2). As a result, time-consuming procedures or sophisticated and expensive instruments are often necessary to achieve high resolution and sensitivity with oligonucleotide samples (3,4).

Considerable research has gone into the selection of an appropriate matrix able to transfer energy from the laser beam to the oligonucleotide without artefactual modifications (5–7). Several factors are crucial for the matrix (8), including: (i) high absorbance at the wavelength of the laser beam, (ii) solubility in the same solvent used for the analyte, (iii) co-crystallization with analyte, and (iv) a pH ~7 to avoid DNA depurination. In this latter respect, ammonium salts have been used as a co-matrix, proving beneficial also in the suppression of peak broadening due to multiple alkali ion adducts (2).

We demonstrate below that on a benchtop MALDI-TOF instrument (Kompact MALDI III, Shimadzu Scientific Instrument, Columbia, MD) 6-aza-2-thiothymine (ATT) (6i5-76-9) crystallized with ammonium citrate is a good matrix for the analysis of oligonucleotides, since it gives good resolution (500 FWHM), good accuracy (better than ±0.02% using an internal standard) and good shot-to-shot and sample to sample reproducibility. Furthermore, with ATT no fragmentation or depurination of the oligonucleotide has been observed during the analytical procedure.

All the matrices were purchased from Aldrich and used without further purification. Each matrix solution was prepared fresh every day at a concentration of 10 mg/ml in a solution of CH_3CN/ammonium citrate 20 mM 1:1 (v/v). Samples were prepared by mixing 3 µl of matrix and 1 µl oligonucleotide (1–10 µM solutions in H_2O); 1 µl of this mixture was used for the analysis and the solvent removed by leaving the sample slide in a vacuum desiccator for ~10 min. Samples were analyzed on a Kompact MALDI III using a 337 nm N_2 laser. The negative ions desorbed were accelerated by a static electric potential of 22 KeV and the instrument was run in linear mode. With ATT, 20–50 laser shots were usually enough to achieve the best signal-to-noise ratio.

Table 1 shows the MW values of a 16mer oligodeoxynucleotide (TTTAGAGTCTGCTCCC calculated mass 4822.2) obtained after 30 individual analysis (three experiments, 10 determinations each) with: 2′,4′,6′-trihydroxy acetophenone (THA), 3-hydroxy picolinic acid (3-HPA) and ATT. In each determination the laser intensity used was just above the threshold. Calibration was achieved by using bovine insulin β-chain (3493.5) and bovine insulin (5731.5) as internal standard. ATT consistently gave clearly higher sample to sample reliability and resolution over the other matrices. Furthermore it was quite easy to find an 'hot spot' on the sample target with the laser beam, even without the assistance of a video camera or computer analysis. This is likely due to the higher homogeneity of the surface of the matrix-analyte crystal as was observed when the probe was examined under a microscope. The matrix analyte formed a continuous 'crystalline' surface rather than individual crystals as observed with the other matrices.

Table 1. Comparison of reliability and reproducibility of 6-aza-2-thiothymine (ATT) compared to two common matrices used for the analysis of oligonucleotides by MALDI: 2′ 4′ 6′-trihydroxy acetophenone (THA) and 3-hydroxy picolinic acid (3-HPA)

	ATT	THA	3-HPA
Resolution (FWHM)	480	240	280
Mean (n = 30)	4822.35	4821.65	4822.54
Std deviation	1.04	4.38	5.01
Interassay var. %	±0.006	±0.057	±0.045

Figure 1 shows the spectrum of a crude deprotected oligodeoxynucleotide 24mer (5 µM) with the highest MW peak representing the intact 24mer $[M–H]^-$ 7331.2 (calc. 7331.9). The multiple peaks below *m/z* 3200 represent a mixture of by-products of the synthesis and deprotection steps (see Fig. 1). As shown in this spectrum, the matrix gives a low background even at relatively low MW and no evidence of matrix adduct peaks. It is important to emphasize that ATT gives clear molecular ion

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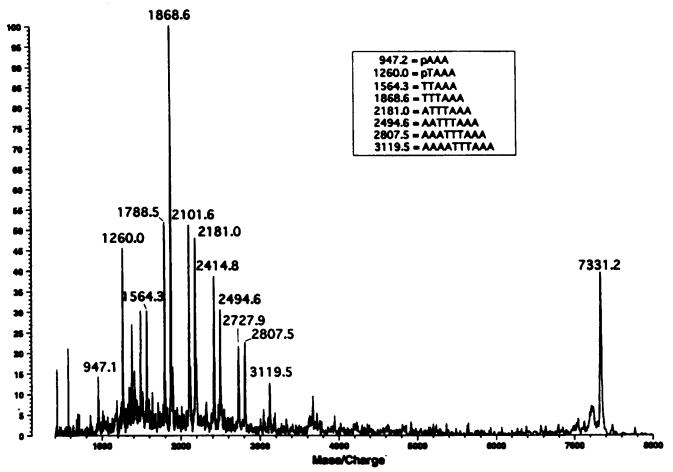

Figure 1. 24mer oligodeoxynucleotide TAAAGTTACTTTTCAAAATT-TAAA: a quasi-molecular ion $[M-H]^-$ can be seen at *m/z* 7331.2 (calc. 7331.9); below *m/z* 3200 by-products from incomplete synthesis and deprotection steps are observed. The table lists the assignments of their phosphorylated peaks; the peaks 80 daltons lower are the unphosphorylated equivalents.

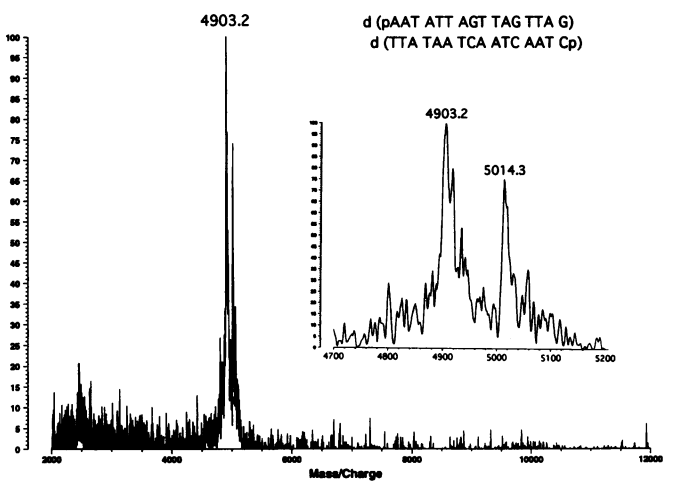

Figure 2. MALDI spectrum of a blunt ended double-stranded DNA fragment 16 bp (Sigma catalog # S 9650): the inset shows the two well resolved single strands.

peaks with little or no fragmentation; a single peak representing the quasi-molecular ion $[M-H]^-$ is observed when analyzing HPLC purified oligonucleotides (data not shown).

Using ATT, short pieces of DNA were also examined. Figure 2 shows the spectrum of a double-stranded DNA fragment containing 16 bp (5 μM in water). From the spectrum it is possible to determine the MW of the two distinct single strands with good accuracy (calc. 4902.3 and 5013.3) achieved at low laser irradiance. The two peaks, each representing one DNA strand, show different intensities, likely arising from a different response to MALDI as discussed earlier (9). In addition, the peak representing the DNA duplex (expected MW 9916.6) is either absent or indistinguishable from background. When the laser intensity is increased to high power, a broad peak at *m/z* 9900 is observed, attributed to an unresolved triplet $[2M_a-H]^-$, $[M_aM_b-H]^-$ and $[2M_b-H]^-$ where a and b are the two strands, rather than a single peak due to the DNA duplex.

In conclusion, we have shown that ATT co-crystallized with ammonium citrate is an optimum matrix for the analysis of oligonucleotides and short DNA fragments by MALDI. The major advantages of ATT over the other matrices routinely used (e.g. 3′,4′,6′-trihydroxy acetophenone or 3-hydroxy picolinic acid) are that it is easy to obtain sharp peaks which can be mass assigned accurately, sample preparation is easy, and crude or just partially purified (e.g. by OPC) samples can be examined. Further work is in progress to investigate ATT use as a matrix in the analysis of restriction enzyme digested DNA fragments.

ACKNOWLEDGEMENTS

We thank LBC and LMB, NIDDK for their assistance in supplying the sample used in this work. We also thank Dr H. M. Fales for the use of the equipment and comments on the manuscript.

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