MATRIX-ASSISTED LASER DESORPTION FOURIER TRANSFORM
MASS SPECTROMETRY FOR BIOLOGICAL COMPOUNDS*

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Matrix-Assisted Laser Desorption Fourier Transform Mass Spectrometry for Biological Compounds*

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INTRODUCTION

The recent development of matrix-assisted UV laser desorption (LD) mass spectrometry has made possible the ionization and detection of extremely large molecules (with molecular weights exceeding 100,000 Daltons) [1,2]. This technique has generated enormous interest in the biological community for the direct examination of large peptides and oligonucleotides. Although this matrix-assisted ionization method has been developed and used almost exclusively with time-of-flight (TOF) mass spectrometers, research is currently in progress to demonstrate this technique with trapped ion mass spectrometers, such as Fourier transform ion cyclotron resonance mass spectrometry (FTMS). The potential capabilities of FTMS for wide mass range, high resolution measurements, and ion trapping experiments (for ion-molecule reactions and MS^n experiments) suggest that this instrumental technique should be useful for the detailed structural characterization of large ions generated by the matrix-assisted technique. We have recently demonstrated that matrix-assisted ultraviolet laser desorption, with either 266 or 355 nm radiation, can be successfully used with FTMS for the ionization of small peptides [3]. The objective of this report is to summarize the application and current limitations of matrix-assisted laser desorption FTMS for the characterization of peptides and oligonucleotides at the isomeric level.

EXPERIMENTAL

All experiments were performed with an Extrel FTMS-2000 Fourier transform mass spectrometer (3 Tesla magnet) equipped with a Quanta Ray DCR-11 Nd:YAG pulsed laser. The laser was operated in the Q-switched mode (donut-shaped beam, 8 nanosecond pulse width) to generate radiation at 1064, 355, and 266 nanometers. The laser beam was focussed onto the probe tip in an area of approximately 250 x 250 microns, giving an estimated power density of low 10^6 W/cm² for 266 nm. Increasing the laser power density above this value resulted in increased fragmentation, in accord with results obtained by other research groups [2]. The experiment was conducted by firing the laser at the beam event to ionize the sample. Immediately following the laser pulse, an ejection pulse (150 millisecond duration) was used to eliminate either the $(M+H)^+$ or $(M-H)^-$ ions of the matrix compound, depending on whether negative or positive ions were examined. This event was followed by broad band excitation and detection of the ion cyclotron signal. A complete mass spectrum could be obtained from a single laser shot, although signal averaging was used to obtain better spectra.

The samples were prepared by mixing one microliter of an aqueous analyte solution (approximately 10^{-4} M) with a few microliters of an aqueous matrix solution (approximately 10^{-2} M), based on experimental conditions outlined for the TOF experiments [1,2]. A few microliters of this resulting solution was then dried onto the tip of a stainless steel solids probe tip used for the laser desorption experiments. Although the detection limit of the technique was not examined in detail, generally 10-100 picomoles of analyte was placed on the probe tip, although this amount certainly exceeds that required for the laser desorption process.

Even though both TOF and FTMS are pulsed techniques and can be interfaced with pulsed laser desorption/ionization, there are several important differences between these two mass spectral techniques. First, the time scales of the two techniques are dramatically different. Ion detection in FTMS occurs tens to hundreds of milliseconds after ion formation, whereas the TOF instruments utilize much shorter time scales (typically microseconds). More important is the fact that fragmentation of a large ion in the flight tube of a TOF does not affect its detection, because the fragment ions arrive at the detector at the same time that the undissociated parent would have arrived. Thus ion fragmentation is more readily observed in the FTMS experiments as compared to TOF measurements. Second, because the FTMS is an ion trap technique, it has a limited dynamic range (approximately 10^6). This implies that the analyte cannot be too dilute in the presence of the excess matrix, or the analyte ions will not be observed. Third, ion detection by FTMS is accomplished by monitoring ion image current on the receiver plates, as opposed to ion detection by an electron multiplier in TOF. This difference implies that while FTMS cannot count single ions, it does not suffer from multiplier insensitivity to large ions. However, the successful detection of large ions ($m/z > 50,000$) by FTMS has not yet been realized, and may be currently limited by instrumental factors of the FTMS [4].

RESULTS AND DISCUSSION

Peptides

Following protocols outlined by Karas and Hillenkamp [1], 266 nm laser desorption FTMS using nicotinic acid (3-pyridine carboxylic acid) as the matrix was examined for peptides. This matrix-assisted ionization technique dramatically improved the abundance of the intact molecular $(M+H)^+$ or adduct $(M+Na)^+$, $(M+K)^+$ ions. Even for compounds like leucine enkephalin and methionine enkephalin which yield $(M+H)^+$ ions under laser desorption conditions without using a matrix, the addition of a nicotinic acid matrix significantly enhanced the abundance of molecular ions while reducing the amount of fragmentation observed. While laser desorption of larger peptides like Substance P without a matrix present yields virtually no $(M+H)^+$ ions, the use of a nicotinic acid matrix provides remarkable signal enhancement for $(M+H)^+$ and $(M+Na)^+$ ions. Laser desorption using 266 nm and a nicotinic acid matrix was useful for generating molecular ions for peptides with molecular weights up to at least 2000 Daltons, as shown in Table 1. This table lists the UV laser desorption of peptides using a variety of matrices (+ indicates that $(M+H)^+$ or $(M+Na)^+$ ions were observed, - indicates that no molecular ions were observed). Initial investigations of large peptides, such as bovine insulin A-chain (MW = 2535) and bovine insulin (MW = 5733.5), failed to reveal molecular or fragment ions using the experimental conditions outlined above.

Table 1. Effect of laser wavelength and matrix composition on laser desorption ^a

	MW	266 nm				355 nm
		SS ^b	NA ^b	PA ^b	PDA ^b	SA ^b
Leucine enkephalin	555.6	+	+	+	+	+
Methionine enkephalin	573.5	+	+	+	+	+
Gramicidin S	1141.5	+	+	+	-	+
Substance P	1347.7	-	+	+	-	+
Gramicidin D	1882	-	+	+	-	+
Bovine insulin A-chain	2735	-	-	-	-	-
Bovine insulin	5733.5	-	-	-	-	-

^a + indicates that $(M-H)^+$ or $(M+Na)^+$ were observed, - indicates that no molecular ions were observed.

^b SS - stainless steel with no matrix, NA - nicotinic acid, PA - 2-pyrazine carboxylic acid, PDA - 2,3 pyrazine dicarboxylic acid, SA - sinapinic acid.

These results may imply that ion detection with conventional FTMS hardware under these experimental conditions may be the limiting factor rather than ion formation. Extensive research is underway in our laboratory as well as many other FTMS labs to address FTMS ion detection limitations for biological compounds [4].

Chait and coworkers have examined a variety of matrices and have determined that 2-pyrazine carboxylic acid can also be used for 266 nm laser desorption experiments [2]. As shown in Table 1, this matrix works as well as nicotinic acid for the FTMS experiments, and reveals the same mass limitation. The ionization enhancement is strongly dependent on the chemical structure of the matrix compound. For example, 2,3-pyrazine dicarboxylic acid was found not to provide matrix enhancement for the 266 nm laser desorption FTMS experiments, even though this compound is structurally quite similar to pyrazine carboxylic acid. This experiment supports results obtained by TOF experiments [2] and verifies matrix-assisted enhancement of the FTMS experiments as opposed to differences in sample preparation.

Because many compounds absorb radiation at 266 nm, laser desorption at this wavelength usually excites both the matrix and the analyte, often resulting in fragmentation of the analyte. To avoid this problem, alternate wavelengths and appropriate matrices can be chosen to minimize spectra absorption by the analyte. Chait and coworkers have reported that sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) provides an excellent matrix for the 355 nm laser desorption of peptides [2]. Results obtained with FTMS confirm the success of this method, shown in Table 1. No significant differences in fragmentation were observed for the peptides examined here at the two different wavelengths. This wavelength dependence should be more pronounced for compounds which absorb strongly at 266 nm, such as oligonucleotides. The absence of $(M+H)^+$ ions for 355 nm laser desorption FTMS of peptides with MW > 2500 supports the 266 nm results and once again alludes to the current instrumental limitations of FTMS.

Based on the success of matrix-assisted laser desorption FTMS for peptides, we decided to examine normal and modified oligonucleotides. Molecular ions from these compounds are quite difficult to obtain by laser desorption due to the ease of breaking the weak glycosidic bonds of the oligonucleotide. Examination of these oligomers without using the matrices indicated extensive fragmentation to produce the nucleic base ions, with virtually no fragment ions indicative of the sequence of the oligomer. However, matrix-assisted laser desorption using 266 nm and nicotinic acid was useful for generating (M-H)⁻ ions for small oligonucleotides. Even though the oligomers absorb at this wavelength, characteristic negative ions such as (M-H)⁻ and fragment ions were observed which provided detailed sequence information. The negative ion spectra were the most informative for these compounds. Matrix-assisted 355 nm laser desorption FTMS has not been investigated for oligonucleotides at the present time.

Oligonucleotide dimers can be easily characterized with this technique. Figures 1 and 2 illustrate the negative ion spectra obtained for two isomeric dimers, d(GA) and d(AG). Although (M-H)⁻ ions at *m/z* 579 are observed for each isomer, fragment ions are observed which result from loss of the nucleic bases as well as preferential cleavage of the phosphate ester linkage from the 3' end of the oligomer. This latter fragmentation pathway generates *m/z* 346 as the most abundant sequence ion from d(AG), while *m/z* 330 is the most abundant sequence ion from d(GA). These ions provide the information necessary to identify the 3' end of the oligomers and differentiate the isomers. A modified oligonucleotide dimer d(TpT) containing ethylation of the phosphate linkage was also examined with this method. An (M-H)⁻ ion was observed at *m/z* 574 as well as fragment ions at *m/z* 546 (M-C₂H₄)⁻ and *m/z* 349 (cleavage of the phosphate linkage). Isolation and collision-induced dissociation of the *m/z* 349 ion generated several fragment ions, including *m/z* 125 which was presumed to be the thymine nucleic base. However, close examination of the ion at nominal *m/z* 125 revealed two ions, *m/z* 125.03565 (C₅H₅N₂O₂⁻ of thymine) and 125.00092 (PO₄C₂H₆)⁻. In this case, a resolution of greater than 3500 (FWHM) was needed to resolve these two isobaric ions and verify the position of ethyl substitution on the phosphate linkage for this dimer.

Examination of oligonucleotide trimers also provided molecular weight and sequence information. Two trimers, d(GGT) and d(TGT), were examined with this matrix-assisted technique. For d(GGT), a molecular ion (M-H)⁻ at *m/z* 899 was observed along with abundant fragment ions at *m/z* 650 and 321, which provided the sequence information for the oligomer. Note that these ions result from fragmentation from the 3' end and would provide differentiation of d(5'-GGT-3') from d(5'-TGG-3'). Examination of d(GGT) under the same experimental conditions (i.e. laser wavelength, energy, sample concentration) without nicotinic acid as the matrix indicated almost complete loss of sequence information. The only ion observed in this case was the *m/z* 321 ion, corresponding to the terminal thymidine phosphate residue of the oligomer. Isolation and collisional dissociation of the (M-H)⁻ at *m/z* 899 for d(GGT) gave the same fragment ions as observed in the laser desorption experiment. Laser desorption of the trimer d(TGT) yielded characteristic negative ions corresponding to (M-H)⁻ at *m/z* 874 and the 3' cleavage ions at *m/z* 650 and 321, which provided the sequence information for this trimer.

A series of oligonucleotide tetramers, including four d(CGCG) isomers, were examined to ascertain the level of sequence information available from the fragment ions. The isomeric d(CGCG) set included not only positional isomers such as d(CGCG) and d(CCGG) but also compounds differing only in the location of the 3'

Figure 1 Negative ion FTMS spectrum of d(GA) using 266 nm laser desorption with a nicotinic acid matrix.

Figure 2 Negative ion FTMS spectrum of d(AG) using 266 nm laser desorption with a nicotinic acid matrix.

end such as d(5'-CGCG-3') and d(5'-GCCG-3'). Characteristic (M-H)⁻ ions as well as ions corresponding to fragmentation predominantly from the 3' end of the tetramer were observed, as shown in Figure 3 for d(CGCG). Substantial fragmentation of the tetramer is observed under these experimental conditions, as might be expected since these oligonucleotides absorb readily at the 266 nm laser desorption irradiation. Even though many different fragment ions are observed, examination of the four spectral regions outlined in Figure 3 were sufficient to determine the sequence of this tetramer. Note that (M-G)⁻ at m/z 1022 is observed while (M-C)⁻ is completely absent. This nucleic base elimination is assumed to originate from the 3' end of the tetramer, as evident in Table 2. This fragment ion, along with the sequence ions, enables isomeric differentiation of oligomers which are identical in sequence and differ only in the assignment of the 3' end. Sequence ions resulting from fragmentation of the 5' end of the tetramers were observed in minor abundance, especially when a cytosine residue was at the 3' end of the oligomer, and provided additional sequence information. The negative ions obtained for a oligonucleotide containing all four DNA bases, d(TGCA), are listed in Table 2. An (M-H)⁻ ion at m/z 1172 was observed as well as sequence ions at m/z 948, 618, and 330 (from 3' end). For this tetramer, no characteristic (M-base)⁻ ion was observed. Examination of a modified version of this tetramer, d(TG^{*}CA) where G^{*} is O⁶-methyl guanine, indicated an (M-H)⁻ ion at m/z 1186 and characteristic fragment ions as shown in Table 2. The first two sequence ions (m/z 330 and 618) are identical to those observed for the tetramer d(TGCA); however, the 3rd sequence ion at m/z 962 is 14 amu higher than the analogous ion for d(TGCA), verifying that methyl adduction occurs at this position.

Figure 3 Negative ion FTMS spectrum of d(CGCG) using 266 nm laser desorption with a nicotinic acid matrix.

Table 2. Oligonucleotide tetramer sequence ions obtained by 266 nm laser desorption.

Tetramer	(M-H) ⁻	(M-base) ⁻	Sequence ions
d(CGCG)	1173	(M-G) ⁻	964, 635, 346 (3' end)
d(GCGC)	1173	(M-C) ⁻	924, 635, 306 (3' end) 964, 635, 346 (5' end)
d(CCGG)	1173	(M-G) ⁻	964, 675, 346 (3' end)
d(GGCC)	1173	(M-C) ⁻	924, 595, 306 (3' end) 964, 675, 346 (5' end)
d(TGCA)	1172	-	948, 618, 330 (3' end)
d(TG [*] CA)	1186	-	962, 618, 330 (3' end)

G^{*} = O⁶-methyl guanine

The largest oligonucleotide observed to date by matrix-assisted laser desorption FTMS is the hexamer d(GCTAGC) with a molecular weight MW = 1791. The negative ion spectrum revealed an (M-H)⁻ ion at m/z 1790 as well as several sequence ions. The most abundant fragment ions observed for this oligomer were due to cleavage of every second phosphate linkage from the m/z 1790 ion, forming m/z 1252 and 635. Examination of larger oligonucleotides failed to reveal molecular ions, implying that ion detection factors may currently limit the useable mass range. Further research is in progress to investigate these limitations for large oligonucleotides.

CONCLUSIONS

Matrix-assisted UV laser desorption FTMS has been demonstrated for both peptides and oligonucleotides with molecular weights up to 2000 Daltons. The choice of matrices and laser wavelengths for the FTMS experiments agree very well with results obtained by TOF experiments and verify the matrix enhancement effects for FTMS. Either 266 or 355 nm can be used with the appropriate matrices to generate (M+H)⁺ ions for small peptides. Examination of oligonucleotides using 266 nm and a nicotinic acid matrix revealed (M-H)⁻ ions as well as many fragment ions, which provided the information necessary to determine the oligomer sequence at the isomeric level for oligonucleotide dimers up to hexamers. The lack of observation for peptide and oligonucleotide ions at m/z > 2500 may imply that current FTMS ion detection factors under these experimental conditions limit the detection of large ions. Further work is in progress to investigate and reduce these limitations using larger magnets, alternate cell designs, and ion manipulation techniques.

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8

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