A Self-Assembled Matrix Monolayer for UV-MALDI Mass Spectrometry

Stéphane Mouradian, Christine M. Nelson, and Lloyd M. Smith*

Contribution from the Department of Chemistry, University of Wisconsin, Madison, Madison, Wisconsin 53706

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Abstract: Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry has in recent years significantly advanced the field of polymer analysis. However, the mechanisms of the desorption and ionization processes, and in particular the critical role played by the matrix, remain unclear. In the present work, the usual matrix is replaced with a self-assembled monolayer consisting of a UV absorbing matrix-like compound covalently linked to a gold surface. Analytes such as proteins or oligonucleotides are directly deposited on the covalently modified probe tips and mass analyzed by laser desorption time of flight (TOF) mass spectrometry. Several types of monolayers were investigated and tested for their ability to produce positive and negative analyte ions. Molecular ion signals were obtained for dT_{10} oligonucleotides and proteins as large as cytochrome C on monolayers of methyl *N*-(4-mercaptophenyl)carbamate (MMPC). The amenability of this model system to characterization with established physical and chemical methods should help investigate the processes involved in MALDI.

Introduction

The development of matrix-assisted laser desorption/ionization (MALDI) mass spectrometry has significantly advanced the analysis of large synthetic and biological polymers.[1,2] In this technique, analyte molecules are incorporated in a matrix which absorbs UV or IR radiation. A pulsed laser beam is used to desorb and ionize the analyte molecules which are then mass analyzed. Analytes such as proteins, polysaccharides, and nucleic acids may be analyzed in a few seconds using a time of flight mass spectrometer.[3−13] In spite of this notable success, the critical role of the matrix in the laser desorption/ionization process remains unclear. Although many compounds have been screened as possible matrices, only a handful have proven useful.[1,5,10,14−22] In many cases, the chemical differences between an effective or ineffective matrix are extremely subtle. For example, simply changing a substituent position on an aromatic ring can dramatically affect signal quality. Although a proper matrix is crucial to the success of the technique, the present limited understanding of desorption/ionization mechanisms and of the parameters critical to an effective matrix has limited matrix selection to an empirical screening process.

A major difficulty in studying the role of the matrix in MALDI is the inhomogeneous and variable nature of the samples. These typically consist of small crystals, varying in shape and size, formed by air drying solutions containing both the matrix and analyte. Work by Savickas et al.[23] suggests that an uneven crystallization pattern causes variations in analyte concentration on the probe tip, yielding spot to spot variations in signal intensity. This inhomogeneity of the samples makes it difficult to determine localization of the analyte within matrix crystals,[21,24] and to examine parameters related to the role of matrix in the MALDI process. To address this, several methodologies have been proposed to increase the reproducibility of the sample preparation. Weinberg et al.[25] reported that more homogeneous crystals could be obtained by drying the analyte−matrix mixture in vacuum. Speir et al.[26] showed that small peptides could be analyzed using a layer preparation method where sinapinic acid was electrosprayed as a substrate layer on top of which an electrosprayed film of analyte was applied. This technique, referred to as "substrate enhanced desorption", produced uniform samples but was only successful for the analysis of peptides smaller than ten amino acids. Perera

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et al.[27] reported more uniform samples and a general improvement of the quality of mass spectra by spin coating the probe tips with the analyte−matrix mixture. Another methodology developed by Beavis and Xiang[28] used a first layer of matrix crystals that were crushed mechanically and used as a seed layer for the analyte−matrix mixture applied subsequently. This allowed the formation of uniform films which produced high ion currents and increased the tolerance to contaminants. Using fast solvent evaporation, Vorm et al.[29,30] also reported an improvement in sample homogeneity as well as an increase in resolution and sensitivity. Higher sensitivity was also noted by two groups when first coating the sample probe tip with a nitrocellulose film before applying matrix and analyte.[31,32] An alternative method using a frozen aqueous desorption medium was reported by Nelson et al.[33] and showed interesting capabilities. However, the noted poor reproducibility of this technique limited its general usefulness.

In a different approach, Hutchens and Yip[34] described the use of a monolayer of matrix molecules covalently bound to agarose beads, upon which analyte was deposited. Using this method, referred to as SEND (surface enhanced neat desorption), the authors noted an improvement in sensitivity and mass accuracy. Although these results have not been confirmed by our or others' laboratories,[27,29] the basic idea of covalently binding matrix molecules to the surface remains of interest. Such a system would allow the preparation of homogeneous, uniform desorption media which would be amenable to careful physical characterization and study.

Self-assembled monolayers (SAMs) on gold have been extensively used in a variety of fields.[35−40] They are typically formed by treating a gold covered surface of interest with a solution of monolayer monomers containing a thiol group. Spontaneous covalent bonding occurs between the gold and sulfur atoms resulting in formation of the monolayer. While studies of SAMs formed from long-chain alkanethiols have been most common,[35−39,41−43] a few studies reported formation of SAMs of aromatic compounds similar to MALDI matrices.[44−50] A variety of spectroscopic[35,51−55] and electrochemical[46,56−60] surface analytical techniques have been utilized to characterize these monolayers. Mass spectrometric techniques such as secondary ion mass spectrometry (SIMS)[61−64] or laser desorption (LDMS)[65−68] have proven to be useful tools for such analyses by virtue of their ability to desorb molecules deposited on clean metal surfaces. SAM monomers bound to a surface have been directly analyzed by SIMS[69−72] and surface induced dissociation (SID).[73−75] In addition, Hemminger et al. demonstrated that SAMs can be analyzed by UV laser desorption mass spectrometry.[44,76,77]

We report here a novel method for MALDI sample preparation which uses a SAM of matrix molecules covalently linked

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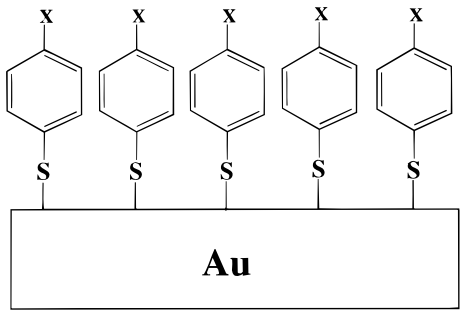

Figure 1. Schematic representation of a SAM matrix. The orientation of the molecules was arbitrarily chosen for this figure.

to a gold surface (Figure 1). In this technique, the monolayer units are "matrix-like" in that they are small substituted aromatic molecules that absorb in the ultraviolet. Additionally, they contain a thiol group that allows them to self-assemble on gold. This homogeneous, planar, and relatively well defined model system is likely to facilitate investigation of the processes involved in MALDI.

Experimental Section

Monolayer monomer solutions (1 mM) were made in ethanol using the following compounds without further purification: methyl *N*-(4-mercaptophenyl)carbamate (MMPC), 6-mercaptonicotinic acid (MNA), α-mercapto-*p*-toluic acid (MPTA), 2-mercapto-5-nitropyridine (MNP), 4-acetamidothiophenol (ATP), all purchased from Aldrich (Milwaukee, WI). The gold probe tips (2.2 mm in diameter, 10.8 mm long, 99.99% pure gold wire, obtained from Aldrich) were polished using subsequent slurries of 5 and 0.3 μm alumina powder (Buehler, Lake bluff, IL). They were cleaned in "piranha" solution (1:4 H_2O_2/H_2SO_4) and rinsed with deionized water and ethanol. They were then soaked at least 12 h in the monolayer solution. After soaking, they were rinsed with ethanol and allowed to air dry. **Caution:** "Piranha" solutions react violently with many organic materials and should be handled with extreme care. For control experiments, tips were prepared in a similar manner but were soaked in neat ethanol instead of the monolayer solution. Analytes, either proteins or oligonucleotides, were then directly deposited on the tips, air dried, and inserted into the instrument. The peptide luteinizing hormone releasing hormone (LHRH, 1180 Da) and proteins insulin (5734 Da) and cytochrome C (12327 Da) were bought from Aldrich (Milwaukee) and used without purification. The oligonucleotides $d(ACGT)_2AC$ (3012 Da) and dT10 (2980 Da) were made by the University of Wisconsin Biotechnology Center on a DNA synthesizer model 394 (ABI-Perkin Elmer, Foster City, CA) and purified by passage over a C18 SEP-PAK cartridge (Waters, Milford, MA). Analytes were deposited on the sample probe tip as 1-μL aliquots of the following aqueous solutions: LHRH (20 pmol/μL), insulin (20 pmol/μL), cytochrome C (50 pmol/μL)), oligonucleotides $d(ACGT)_2AC$ (50 pmol/μL) and dT_{10} (50 pmol/μL). For conventional MALDI spectra, saturated solutions (ca. 0.1M) of 2,5-dihydroxybenzoic acid (DHBA, Aldrich, Milwaukee, WI) and MMPC were prepared in 9:1 and 8:2 water−acetonitrile mixtures, respectively. Matrix (1 μL) was mixed with 1 μL of the previously described analyte solutions. No ion exchange beads were used in the monolayer solution, the analytes or the conventional MALDI matrices. All probe tips were inspected using a bench microscope (stereomicroscope 80X, Leica) prior to mass spectrometric analysis.

The samples were mass analyzed using a modified Vestec VT 2000 laser desorption time-of-flight mass spectrometer (PerSeptive Biosystems, Boston, MA) described previously.[6] Briefly, analytes are desorbed and ionized using a 355-nm Nd:YAG laser (Lumonics HY 400, Kanata, ON, Canada) and accelerated in two stages to 30 KV before entering the 2 m long flight tube. Ions are detected by a 20-stage focused mesh electron multiplier Model MM1-1SG (Becton Dickinson, Sparks, MD). Each sample was analyzed in both positive and negative ion mode. Signal was recorded by a Tektronix TDS 520 oscilloscope (Beaverton, OR) for acquisition times corresponding to 10 mass spectra.

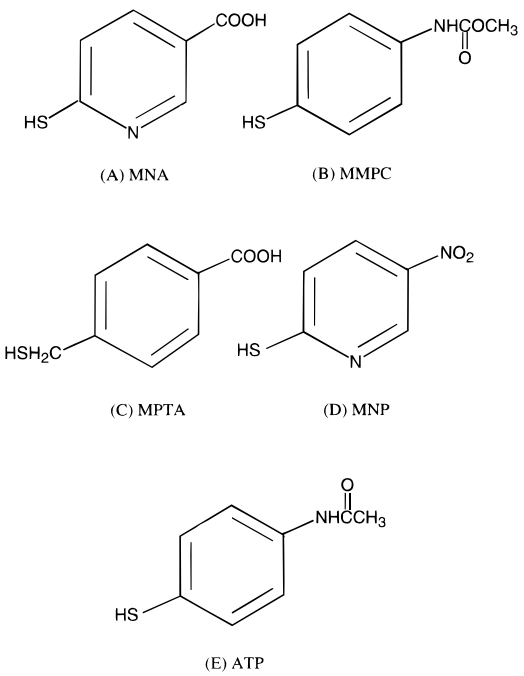

Figure 2. SAM matrix monomers. (A) 6-mercaptonicotinic acid (MNA), (B) methyl *N*-(4-mercaptophenyl)carbamate (MMPC), (C) α-mercapto-*p*-toluic acid (MPTA), (D) 2-mercapto-5-nitropyridine (MNP), and (E) 4-acetamidothiophenol (ATP).

The formation of self-assembled monolayers on gold was evaluated using polarization modulation Fourier transform infrared spectroscopy[78] (PM-FTIR) on a spectrophotometer model 740 (Nicolet, Madison, WI). Thin gold films were vapor deposited on microscope cover slips as described elsewhere.[36] Briefly, microscope slides are first thoroughly cleaned in concentrated sulfuric acid and then silanized with (3-mercaptopropyl)trimethoxysilane (MPS, Aldrich). Gold (D. F. Goldsmith, 99.99%, Evanston, IL) is vapor deposited at room temperature and the gold/glass samples are subsequently annealed at 300 °C for 1 h. These gold-coated slides are then soaked overnight in the monolayer solution. UV absorbances of the matrix compounds were either measured on a UV−vis spectrophotometer model 8452 (Perkin Elmer, Palo Alto, CA) or obtained from the literature.[79]

Results and Discussion

Figure 2 shows the five monolayer monomers used in this work. Each of them was prepared in solution as described above and allowed to form on the gold probe tips. Analyte was then added on top of the derivatized tip to evaluate the ability of a given monolayer to assist production of analyte molecular ions. Each sample, LHRH, insulin, cytochrome C, $d(ACGT)_2AC$, and dT_{10} were tested with each monolayer.

In all control experiments with underivatized gold tips, no discernible analyte signals could be obtained and only low mass peaks corresponding to gold (197 Da) and gold clusters Au_n with n up to 3 were evident in the mass spectra (see Figure 5 in later discussion). Among the five SAMs systematically tested, we observed analyte signal only when the gold tip was derivatized with the MMPC monolayer. In positive ion mode, LHRH, insulin, and cytochrome C could be observed using this matrix-like compound (Figures 3A, 3B, and 3C, respectively). The best peak resolution obtained for LHRH was 340 while it was 480 for insulin and 25 for cytochrome C. In negative ion mode, the oligonucleotide dT_{10} was detected with a resolution of 310 (Figure 3D) but no signal was observed from the mixed base oligonucleotide $d(AGCT)_2AC$. In these experiments, far

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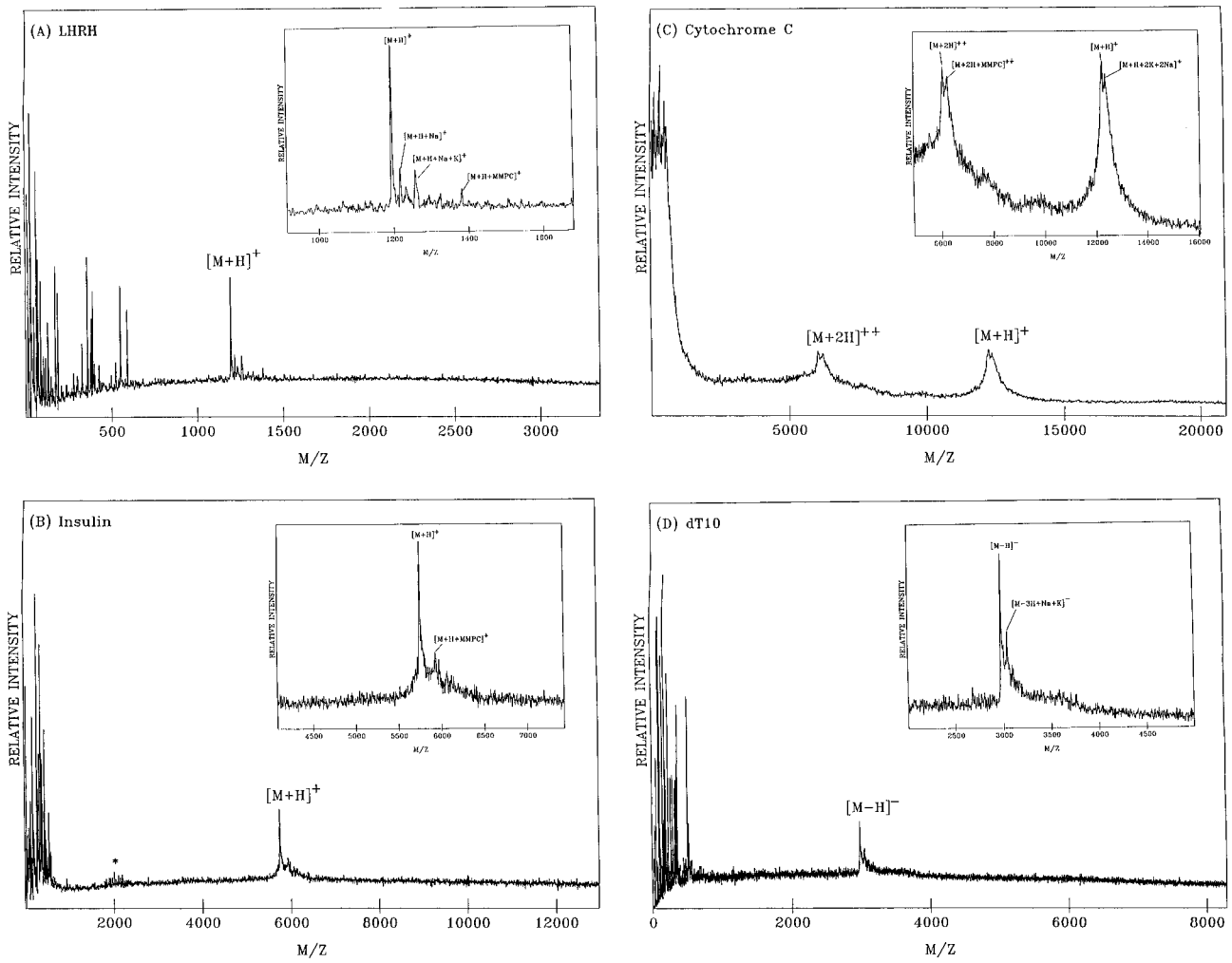

Figure 3. Mass spectra obtained by LD/TOFMS from MMPC monolayers on gold. (A) LHRH (1180 Da), (B) insulin (5734 Da), and (C) cytochrome C (12327 Da) in positive ion mode; and (D) dT10 oligonucleotide (2980 Da) in negative ion mode. Peak resolutions (fwhm) in parts A−D were 340, 480, 25, and 310 respectively. Spectra A, B, and D were collected at a sampling rate of 2 ns and spectrum C was collected at a rate of 4 ns.

fewer individual spectra were obtainable from a given region of the sample than in conventional MALDI. Therefore, fewer sequential mass spectra were averaged (typically 10). We attribute this effect to a rapid depletion of the SAM matrix from the probe tip, a not unexpected result given the thinness of the monolayer compared to conventional MALDI crystals. As monolayer monomer ions are observed in the mass spectra (see below), this depletion effect is thought to be caused by gradual removal of the monolayer from the surface. Figures 4A−C show mass spectra of the same analytes prepared as conventional MALDI samples with the matrix 2,5-DHBA. These data also are averages of 10 spectra, and required about 45% less laser power to obtain a comparable analyte signal intensity than did the monolayer samples. This explains the noticeable higher intensity of the matrix peaks when using the SAM system. Under these conditions, a slightly lower signal/noise was obtained from the MMPC-SAM system than in conventional MALDI using 2,5-DHBA, while mass resolutions were at least as high (see legends of Figures 3 and 4).

Matrix SAM formation was evaluated by PM-FTIR for the five compounds. Four solutions of each were prepared and allowed to bind to gold-coated slides overnight. Spectral peak intensities for major peaks (Table 1) were measured to check the reproducibility of the surface coverage. Intensities were very reproducible for the five matrix/monolayer systems (coefficient of variation <5%), consistent with the formation of a self-assembled monolayer.[48] For the mass spectrometry experiments, the gold-probe tips carrying SAM and analyte were inspected with a bench microscope as is conventionally done in MALDI to examine sample morphology. In contrast to the conventional preparation, no crystals were observed in any samples and no visible difference was apparent between the derivatized and underivatized probe tips. This does not rule out the presence of a microcrystalline structure that could be elucidated under higher magnification. Moreover, it is likely that the analyte itself forms multilayers on top of the matrix SAM. For example, Vorm et al.[30] used an atomic force microscope (AFM) to identify particles in the 1 μm size range in their sample preparation although it appeared homogeneous under a microscope. In this work, the multiplicity of functional groups carried by the analyte considerably complicates the IR signal and PM-FTIR analysis was accordingly not performed when the analyte was present on top of the matrix.

In order to observe an analyte signal as shown in Figures 3A−3C, the analyte molecules must be desorbed and ionized. Both the gold substrate and the monolayer matrix could play roles in this process. Several groups[44,65,68] have previously investigated the desorption of small molecules directly deposited on gold and irradiated with UV light. Depending on the conditions, they found that desorption could occur through either thermal or non-thermal pathways. In the thermal pathway,[44,65,68] valence electrons of the gold substrate are radiatively stimulated and a rapid temperature jump is produced at the metal surface. This thermal energy is then transferred to the adsorbed species

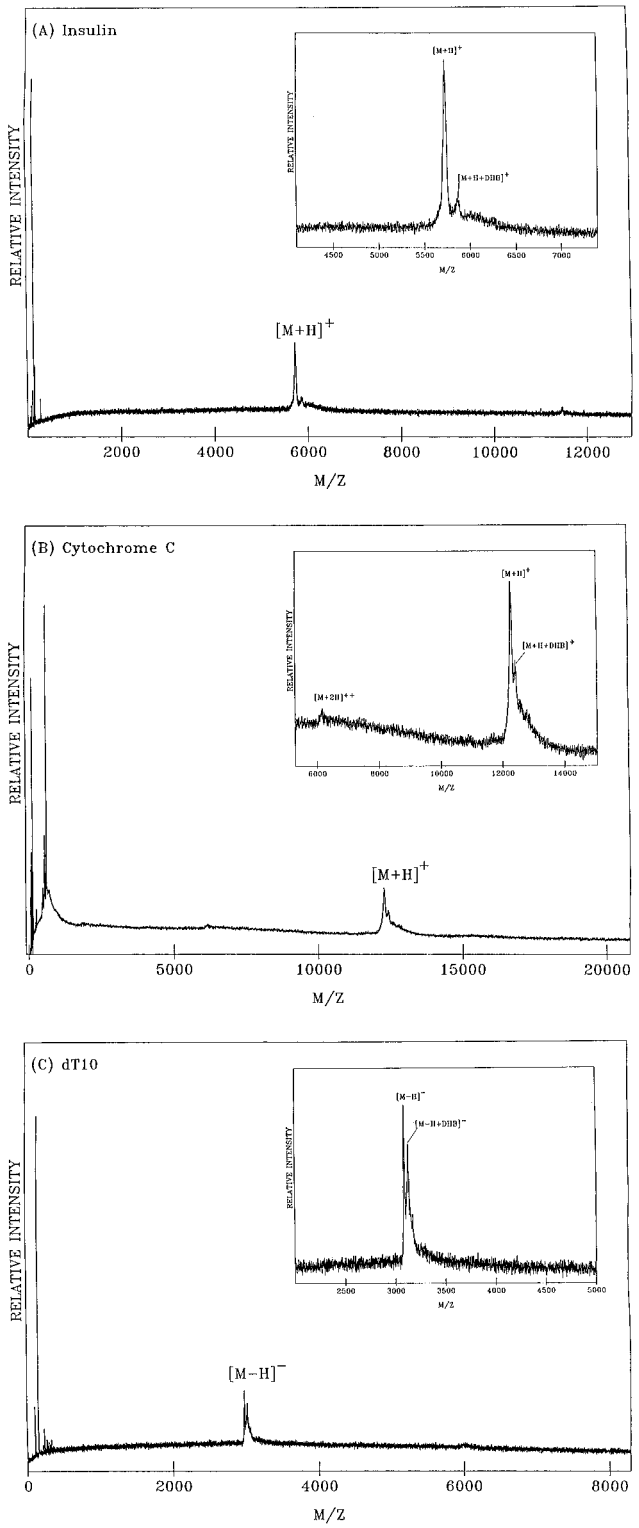

Figure 4. Mass spectra obtained using the matrix 2,5-DHBA on gold-probe tips: (A) insulin, (B) cytochrome C, and (C) dT10. Peak resolutions (fwhm) in parts A−C were 140, 90, and 250, respectively. Spectra A and B were collected at a sampling rate of 2 ns and spectrum C was collected at a rate of 4 ns.

resulting in vaporization. Li et al.[68] showed that this mechanism was responsible for the desorption of small molecules (<293 Da) with laser excitation at 351 nm. Since a similar wavelength (355 nm) was employed in the present study, the same mechanism may be involved.

Alternatively, non-thermal mechanisms may also occur in which the laser energy is absorbed directly by the monolayer species rather than by the gold surface, as in conventional MALDI. In this case, one would expect the monolayer monomers to be able to absorb the 355-nm radiation to a certain extent. The bulk molar extinction coefficients of the five matrix monolayers and the matrices 2,5 DHBA and 3-HPA (3-hydroxypicolinic acid) are listed in Table 2. These values should be considered as approximate guides, rather than as exact values, as bulk extinction coefficients may not correspond exactly to those applicable in the self-assembled monolayer environment. It is evident from these values that MMPC is not the strongest absorber of the panel, indicating that strong absorption is not the determining factor in efficacity of the SAM matrix. This is similar to matrix behavior in conventional MALDI, where, for example, 3-HPA is a very effective matrix although it is a rather weak absorber ($\epsilon = 350$ L mol^{-1} cm^{-1}).

Table 1. Infrared Frequencies and Vibrational Assignment for the Monolayers in the Mid-IR Region

monolayer	freq, cm^{-1}	assignment[a]	
MNA	1583	$\nu_{C=C}$	ring stretch vibration
	1727	$\nu_{C=O}$	carboxylic acid stretch[b]
MMPC	1599	$\nu_{C=C}$	ring stretch vibration
	1537	$\nu_{O=C-N}$	amide II
MPTA	1607	$\nu_{C=C}$	ring stretch vibration
	1723	$\nu_{C=O}$	carboxylic acid stretch[b]
MNP	1592	$\nu_{C=C}$	ring stretch vibration
	1520	ν_{NO_2}	asymetric stretch[c]
ATP	1595	$\nu_{C=C}$	ring stretch vibration
	1533	$\nu_{O=C-N}$	amide II

[a] Assignments were taken from refs 78 and 83 unless otherwise noted. [b] From refs 36 and 84. [c] From ref 85.

Table 2. Extinction Coefficients Obtained at 355 nm for Matrix Compounds in Bulk Solution

compds	ϵ (L mol^{-1} cm^{-1})[a]	compds	ϵ (L mol^{-1} cm^{-1})[a]
MNA	8880	ATP	170
MMPC	570	2,5-DHBA	1750[b]
MPTA	490	3-HPA	350[b]
MNP	4200		

[a] All extinction coefficients were measured in our laboratory except as otherwise noted. [b] From ref 79.

These considerations suggest three possible routes for desorption/ionization from the MMPC monolayer: (1) a purely thermal mechanism where the gold substrate absorbs the laser energy and is responsible for direct desorption and ionization of the analyte; (2) the gold substrate absorbs the laser energy and thermally desorbs MMPC, which then acts as a propellant and ionizer for the analyte; and (3) a purely non-thermal mechanism where the monolayer matrix absorbs the light and desorbs and ionizes the analyte. Control experiments on underivatized gold as well as on four of the five SAMs examined did not yield analyte signal. The matrix monolayer thus appears to play an important role in analyte desorption and/or ionization. The low mass regions of the mass spectra were examined to determine whether matrix species were desorbed from the surface during irradiation. Figures 5A−F show that the five compounds exhibit characteristic negative ion RS$^-$ signals (here R refers to the parent aromatic ring species), as previously noted by Hemminger et al.[44,76] Depletion of the monolayer with time was clearly observed in these laser desorption experiments, as keeping the laser at a fixed spot yielded signal for only a few laser pulses. The presence of spectral peaks corresponding to matrix ions demonstrates that for all five of the aromatic thiols examined, the matrix monolayer is ablated during the laser desorption process. However, analyte signal could be observed only with one of these thiols, MMPC. This demonstrates that the chemical nature of the monolayer "matrix" is critical in the

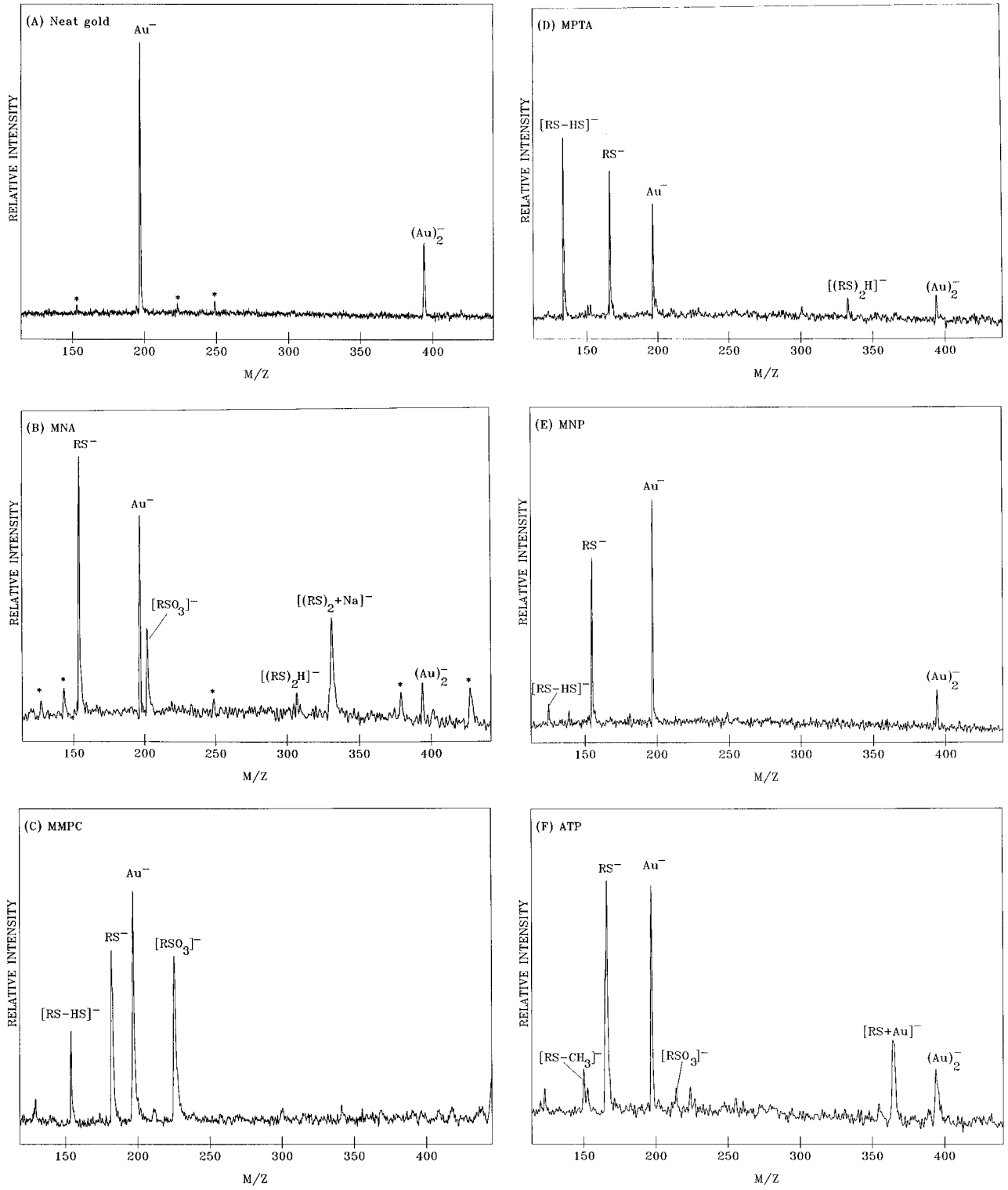

Figure 5. Mass spectra of SAM matrices on gold-probe tips: (A) 6-mercaptonicotinic acid (MNA, 155 Da), (B) methyl *N*-(4-mercaptophenyl)-carbamate (MMPC, 183 Da), (C) α-mercapto-*p*-toluic acid (MPTA, 168 Da), (D) 2-mercapto-5-nitropyridine (MNP, 156 Da), and (E) 4-acetamidothiophenol (ATP, 167 Da). The RS^- monomer parent ion is indicated on each spectrum. RSO_3^- corresponds to the oxidized derivate of the thiol. Peaks marked with an asterisk correspond to unidentified compounds.

desorption/ionization process, consistent with hypotheses (2) or (3) above, and similar to the behavior observed in conventional MALDI.

In order to further study the non-thermal pathways, we investigated whether MMPC could also function as a conventional MALDI matrix. To this end, a saturated solution of MMPC (*ca.* 0.1 M) in 80:20 acetonitrile/water was prepared. One microliter of this MMPC solution was deposited on stainless steel probe tips along with 1 μL of the protein or oligonucleotide analytes using the same amounts of analytes as were used in the monolayer experiments. The final matrix/analyte ratio in these experiments was 5000:1 as is typical in conventional MALDI. A thick white noncrystalline mass was observed and we were unable to produce a crystal-like appearance by adjusting the solvent ratio. No analyte peaks were observed in MALDI mass spectra obtained from these samples. This rather unexpected result may be partially explained by the coarse nature of the dried sample since it is our experience that such samples

typically yield inferior results. When a 1-μL MMPC drop was allowed to dry on clean stainless steel probe tips before subsequent addition and drying of 1 μL of analyte, a similar white mass was obtained. However, in this case an analyte signal was obtained that was lower in intensity than was observed with the gold/monolayer system (data not shown). This suggests that MMPC monomers act in a matrix-like manner when a thin interface exists between the monomers and the analyte. In addition, this suggests that these monomers function in both ordered and disordered arrays.

Conclusions

These results show that a "matrix-like" self-assembled monolayer can be used in place of a conventional MALDI matrix, and that the chemical nature of this surface influences ion production. It is thus found that methyl *N*-(4-mercaptophenyl)carbamate can provide the conditions necessary for the desorption and ionization of moderate weight proteins and polythymidine oligonucleotides. Although simple thiophenol derivatives were used in this work, studies using more complex thiol derivatives such as biphenyl or terphenol mercaptans[46,48] may reveal other matrix−SAM systems of interest. This relatively homogeneous and defined monolayer system should prove much more amenable to study than conventional MALDI systems using various surface analysis techniques (e.g., scanning tunneling microscopy (STM)[80−82] and ellipsometry[41,53]). It is our hope that this system will prove useful for elucidation of the molecular mechanisms underlying the MALDI process.

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