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Time-of-flight Mass Spectrometry of Bioorganic Molecules by Laser Ablation of Silver Thin Film Substrates and Particles

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A laser desorption/ionization (LDI) technique, which uses laser ablation of a thin silver film substrate under vacuum conditions to desorb and ionize bioorganic molecules, was developed for molecular mass and structural reactivity analysis in time-of-flight mass spectrometry (TOF-MS). After a sample overlayer is deposited by solvent evaporation on a thin silver film substrate, it is subjected to 355 or 532 nm Nd:YAG laser light by back-irradiation. Photoablation of the silver film substrate occurs with sufficient laser fluence, producing Ag_n^+ ($n = 1$–9) cluster cations which can react with the desorbed bioorganic molecules in the gas phase to form M^+ or $[M + H]^+$ and $[M + Ag]^+$ ions for TOF-MS analysis. This LDI technique has been successfully applied to dithizone, benzo[*e*]pyrene, 1,4,8,11-tetraazocyclotetradecane, dicyclohexyl-18-crown-6, [5]-helicene dendrimer, gramicidin S, substance P and melittin. One advantage of this method over conventional LDI techniques is that the sample does not need to have appreciable spectral absorption at the laser wavelength. The use of silver in thin-film substrates affords analyte-dependent efficiencies that may serve for the direct and accurate mass analysis of specific groups of bioorganic molecules in sample mixtures. In a new sample preparation method, gramicidin S is added to a Tollen's reagent mixture for direct impregnation on to silver particles during their formation and growth in the colloidal solution. These silver particles provide a silver matrix for the analyte molecules, which can enhance the LDI efficiency to produce greater $[M + H]^+$ and $[M + Ag]^+$ signals. © **1998 John Wiley & Sons, Ltd.**

KEYWORDS: laser desorption ionization; time-of-flight mass spectrometry; ablation; silver film substrate; silver particles

INTRODUCTION

The study of biomacromolecules by mass spectrometry (MS) has been an active field of research in recent years. Time-of-flight mass spectrometry (TOF-MS), Fourier transform ion cyclotron resonance mass spectrometry (FTMS) and ion trap mass spectrometry (ITMS) have all been used in combination with a variety of ionization methods. Among the laser desorption/ionization (LDI) techniques, matrix-assisted laser desorption/ionization (MALDI) can provide molecular mass analysis up to well over 1 MDa.[1] In MALDI, a major function of the matrix is to absorb energy from the laser to fuel a dynamic process that brings sample molecules into the gas phase.[2] For this reason, effective matrix compounds must be chromophores for the laser wavelength used. Other than their spectral properties, the desorption effectiveness of these matrices is not always intuitively apparent. A matrix that performs very well for a certain class of biomolecules might not be applicable to other types, probably owing to unfavourable chemical or photochemical reactions between the matrix and the analyte during sample preparation or MALDI.[3,4] In addition, the molar ratio of matrix to analyte is a critical experimental parameter which is difficult to adjust correctly, especially when the amount of analyte is not known or when a mixture contains different amounts of several analytes. Furthermore, interferences may occur between matrix ion signals and analyte fragment ion signals in MALDI spectra. These facts suggest that non-matrix-assisted LDI-MS may be more suitable, particularly for the analysis of those compounds which have high optical absorption, relatively high volatility and sufficient ionizability to enhance the analytical sensitivity. Recently, mass spectra have been obtained for retinyl esters by LDI that are similar in quality to those obtainable by MALDI but without the interference of matrix ion signals.[5]

Since the decades of research in LDI-MS from the pre-MALDI era, LDI of large organic molecules from metal films is still an area of current activity.[6–8] The photodesorption of molecules by laser light is of considerable scientific interest in view of its application to metal film patterning and nano-structuring, as accomplished by various physical mechanisms.[9–11] Cationization in LDI experiments was first investigated in detail by Kistemaker and co-workers.[12] The exploitation of transition metal ions as chemical ionization (CI) reagents for aliphatic molecules was studied in gas-phase ion–molecule chemistry by Burnier *et al.*[13] Louris

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et al.[14] used a simple laser desorption experiment to examine the chemistry of gold ions with benzene. Cromwell *et al.*[15] observed transition metal cationization of perfluorinated polyethers in the gas phase by FTMS, using a low-fluence pulsed laser for polyether desorption and a high-fluence pulsed laser for metal ion formation from a 0.25 mm foil. A silver nitrate method was demonstrated by Kahr and Wilkins[16] for reasonably general LD-chemical ionization (CI) analysis of hydrocarbon polymers by FTMS. Dean and O'Malley[17] studied bulk silver probe tips covered with polymer samples by FTMS, using 1064 nm laser light to produce mass spectra that showed metal ion adducts to polymer molecules. Wu and Brodbelt[18] used laser ablation of pure metals to create metal ions in ITMS for comprehensive studies of the gas-phase complexation of metal ions with organic ligands. Gill *et al.*[19] created reagent metal ions (Cr^+, Cu^+, Fe^+ and Ni^+) selectively by tuning the laser to the appropriate '2 + 1' (photons to resonance + photon to ionize) transition. This resonant excitation process allowed the reactions of excited-state ions with acetone to be studied in ITMS. They proposed metal ion CI as an alternative strategy for the analysis of biomolecules that undergo extensive electron impact fragmentation. Recently, Alvarez *et al.*[20] have undertaken a systematic ITMS study to evaluate the analytical utility of metal ionization, with laser-desorbed Cu^+, Ni^+ and Co^+ from salts and metal foils, in the generation of structurally diagnostic ions for the characterization of complex organic molecules such as quinolone antibiotics.[20] All of these ionization schemes based on a cationization reaction are known to be analyte dependent.

Here we describe the development of a technique that utilizes laser ablation of thin silver film substrates or particles as a vehicle to bring a sample overlayer into the gas phase for TOF-MS analysis. The purpose of this work was to investigate the ionization scheme based on a cationization reaction of bioorganic analytes with silver ions, even though the bioorganic analytes do not have appreciable spectral absorption at the laser wavelength (355 or 532 nm). The ultimate goal will be to have a technique with select attributes (analyte-dependent efficiencies) that may serve for the direct and accurate mass analysis of specific groups of bioorganic molecules in sample mixtures.

EXPERIMENTAL

Thin metal films

Thin Ag, Au and Al film substrates were prepared at Lumonics Optics Group (Nepean, ON, Canada) by thermal evaporation of 99.9% pure metals at a pressure of 10^{-7} Torr (1 Torr = 133.3 Pa) and deposition on one face of clean microscope glass slides. Their nominal thicknesses, as given by the quartz crystal oscillator in the evaporation system, were 40, 40 and 27 nm respectively. These thin metal film substrates have optical absorbance values of 0.74, 1.49 and 1.46 at 355 nm, and also 1.22, 1.04 and 1.80 at 532 nm. They were stored in a vacuum desiccator until use.

Chemicals

NaOH, $AgNO_3$, aqueous NH_3, benzaldehyde, carbon tetrachloride, dithizone (or diphenylthiocarbazone), benzo[*e*]pyrene, 1,4,8,11-tetraazocyclotetradecane and dicyclohexyl-18-crown-6 were purchased from Aldrich (Milwaukee, WI, USA). Gramicidin S hydrochloride, substance P acetate and melittin were obtained from Sigma (St Louis, MO, USA). HPLC-grade solvents were purchased from Caledon (Georgetown, ON, Canada). All chemicals were used as received.

Sample overlayer

Sample solutions at a concentration of 0.15–4.5 mg ml^{-1} were prepared by dissolving dithizone in carbon tetrachloride, benzo[*e*]pyrene in toluene, 1,4,8,11-tetraazocyclotetradecane in ethanol, dicyclohexyl-18-crown-6 in methanol, gramicidin S hydrochloride in methanol, substance P in methanol, melittin in methanol and [5]-helicene polymer in chloroform. A 50 µl aliquot of each sample solution was allowed to dry on a 2.5 × 2.5 cm thin metal film substrate in air. The dry sample overlayer on the substrate was placed in the ion source chamber of a TOF-MS instrument for LDI experiments.

Silver particles

Conventional Tollen's reagent chemistry was adapted for the preparation of silver particles. Specifically, 0.5 ml of 5% $AgNO_3$, 12.5 µl of 10% NaOH and 80 µl of 50% aqueous NH_3 were added dropwise into a Pyrex test-tube and 4.4 ml of water were then added, followed by 23 mg of gramicidin S and 2.5 µl of benzaldehyde. No silver was observed to plate on the test-tube, but the mixture turned milky and slightly purple. The colloidal suspension was centrifuged and filtered. The brown residue, which contained silver particles impregnated with gramicidin S, was deposited on a thin silver film substrate for LDI/TOF-MS analysis.

LDI

The second or third harmonic ($\lambda = 532$ or 355 nm) output of a Lumonics (Nepean, ON, Canada) Nd:HyperYAG laser operated at a 10 Hz pulse repetition rate was used to ablate the thin metal film substrate and desorb the overlayer sample from the film surface. The laser beam was focused by a lens ($f = 20$ cm) to a spot size of ~0.5 mm in diameter on the back side of the thin metal film substrate. The laser energy was variable, depending on the signal intensity and mass spectral resolution desired. An optimum range was from 0.45 to 0.65 mJ per pulse (FWHM = 7 ns), as measured using a Scientech (Boulder, CO, USA) Model 365 power/energy meter equipped with an MC250 volume absorbing calorimeter. The laser fluence at the ablation spot therefore varied from 2 to 4 mJ mm^{-2}, which was slightly above the threshold fluence required for the photoablation of the different thin metal film substrates. These fluences

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generated a small population of silver ions inside the ablation plume, which reduced the degradation of mass resolution due to space-charge effects and ion–ion collisions. Nonetheless, keeping the laser beam at a fixed spot would yield signals for only a few laser shots as the thin silver film substrate was damaged by the photoablation process. A beam-steering prism was therefore placed inside the ion souce chamber; it could be engaged in translational and rotational motions by stepper motors to move the laser spot across the thin metal film substrate. At the end of a typical 30 s beam scan across 0.5 cm of the thin metal film substrate, the signals produced by 300 consecutive laser shots were summed and averaged in order to enhance the quality of the resulting mass spectrum. The TOF-MS instrument could tolerate a ± 0.3 cm spatial spread of ions produced in a plane perpendicular to the spectrometer axis (the z-axis) without causing a large dispersion in the flight time.

TOF-MS

LDI experiments were performed on a linear TOF-MS instrument constructed in our laboratory (Fig. 1). For this work, the pressure in the ion source and detector regions was maintained between 2.5×10^{-7} and 1.5×10^{-6} Torr. The pulsed-LDI ions were extracted from the source region using an acceleration voltage of $+20$ kV on the thin metal film substrate which also served as the repeller plate (grid A). The other three grids in the 52 cm field-free drift tube were grounded. All the mass spectra shown were taken in the positive ion mode. Ions were detected with a Comstock (Oak Ridge, TN, USA) CP-625/50C multichannel plate detector (MCP) with dual 40 mm diameter channel plates in a chevron configuration, operated with a front-face voltage of -1.6 kV (gain $= 5 \times 10^4$) to provide some post-acceleration. The analogue detector signal was 50 Ω d.c. coupled to channel A of a 30 MHz LeCroy (Chestnut Ridge, NY, USA) 9310M digital storage oscilloscope. Each laser shot yielded a single sweep, during which 1024 ion signal intensities were sampled at 33 ns intervals. Three hundred sweeps were summed and averaged to improve the signal-to-noise ratio of the mass spectrum. A fast photodiode detector provided prompt triggering of the digital oscilloscope at the onset of each laser pulse through channel B, which was also used for monitoring the average laser energy over 300 pulses. Known-mass peaks from Na^+, Ca^+, Ag_n^+ ($n = 1$–9) and several peptides were used for external mass calibration. Conversion of the time base to a mass

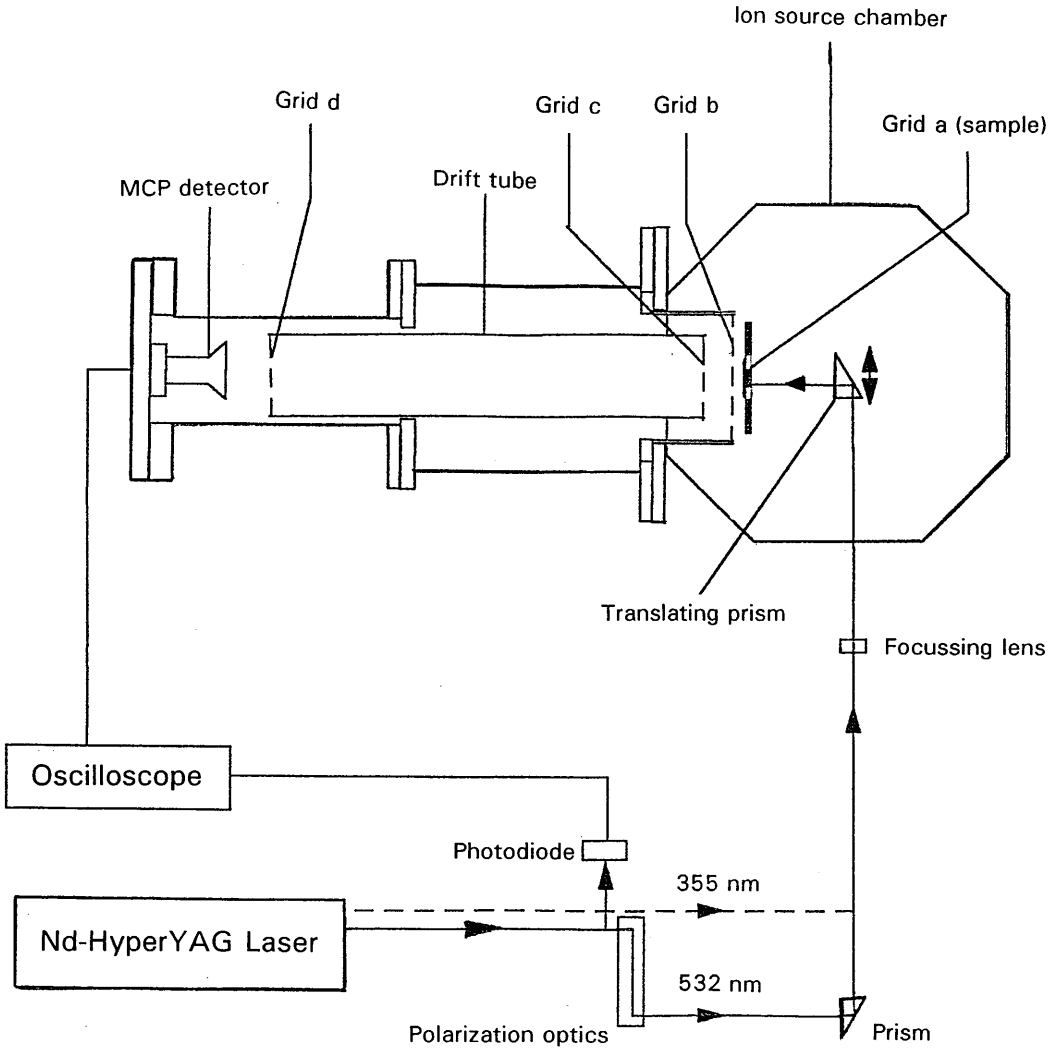

Figure 1. TOF-MS instrument for LDI of bioorganic molecules by laser ablation of thin metal film substrates.

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scale was accomplished with in-house Excel spreadsheet calculations using an empirical equation of $m/z = (kt)^2$, where k is a calibration constant.

RESULTS AND DISCUSSION

Thin silver film substrate

LDI/TOF-MS experiments were first carried out with thin silver film substrates using two different laser wavelengths. As shown in Fig. 2, five major peaks are seen at m/z 23 (1.41 μs), 40 (1.86 μs), 108 (3.05 μs), 216 (4.32 μs) and 324 (5.29 μs) in the mass spectra obtained under 20 kV acceleration. The first two signals may be assigned to Na^+ and Ca^+ ions, which originated from the glass slide underneath the silver film substrate. The other three signals are definitely Ag^+, Ag_2^+ and Ag_3^+ ions that were produced from the thin silver film substrate in a photothermal ablation process. Similar mass spectral peaks corresponding to gold and gold clusters (Au_n^+ with n up to 3) have been evident previously when a gold probe tip was tested in LDI/TOF-MS using 355 nm light from an Nd:YAG laser.[21] Noteworthy is the relative intensities of the Ag^+, Ag_2^+ and Ag_3^+ signals, which are approximately 100:10:20 for 532 nm but 100:50:10 for 355 nm. A thermodynamics and group theory explanation for the suppressed formation of Ag_2^+ by 532 nm LDI/TOF-MS of thin silver film substrates will be given in a separate report. Only a limited number of experiments involving UV laser ablation of metal surfaces have been reported in the literature; several studies of the ablation of transition metal surfaces by pulsed UV lasers under vacuum have been reviewed.[22] Whereas the main group dimers have been extensively studied in recent years, the dimers and trimers of transition and heavy metals, especially the role of the d-electrons in the chemical bonding, are still a challenging problem for experimentalists and theoreticians.[23] Finally, much weaker signals were observed in the mass spectra for higher silver clusters of general formula Ag_n^+ (n = 4–9). These results are presented in Table 1, which lists the film/compound tested, major peaks observed, flight times and corresponding m/z ratios.

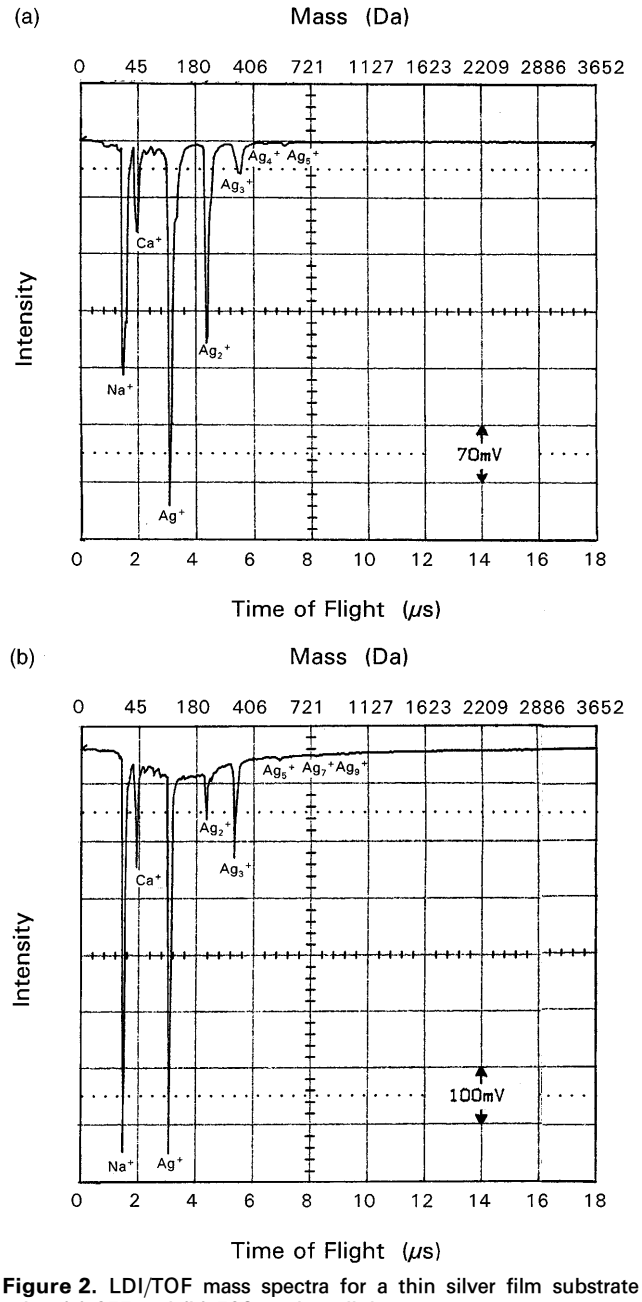

Figure 2. LDI/TOF mass spectra for a thin silver film substrate using (a) 355 and (b) 532 nm laser light.

Organic molecules

LDI/TOF-MS experiments at 355 nm were next conducted using dithizone as the first model compound to probe the chemical reactivity of bare Ag^+ ions (in the absence of a solvation shell or counterions) in the gas phase. Dithizone (M_r = 256 Da) is an efficient organic ligand which binds to Ag^+ in aqueous solution to form a neutral complex in classical solvent extraction. An overlayer sample of dithizone on a thin silver film substrate produced a mass spectrum which revealed no molecular ion signal but a moderate $[M + Ag]^+$ adduct ion signal at 5.69 μs, as shown in Fig. 3. The relative intensities of the Ag^+, Ag_2^+, Ag_3^+ and $[M + Ag]^+$ signals are ~80:50:10:20 for 355 nm. Apparently, the new $[M + Ag]^+$ signal intensity equals the decrease in Ag^+ signal intensity. This result confirms our hypothesis that bare Ag^+ ions generated by laser ablation are chemically reactive in the gas phase; they can be utilized for the LDI/TOF-MS analysis of dithizone when no molecular ion signal is observed. Bare metal ions are known to be reactive toward bond activation and transition metal ions exhibit a variety of reactivities with hydrocarbons, carbonyl compounds, alkyl halides and amines.[24] Furthermore, metal ions in their excited states have a larger reaction cross-section than do metal ions in their ground state. The enhanced reactivity of excited-state metal ions can form both metal-containing adducts and fragment ions. The strong signal observed at 1.86 μs in Fig. 3 is possibly a CS^+ fragment ion (m/z 44) from dithizone superimposed on Ca^+ (m/z 40).

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Table 1. LDI/TOF-MS results listing the flight times (μs) and corresponding *m/z* ratios (in parenthesis) of the major peaks observed for bioorganic compounds (M) deposited on different thin metal film substrates (S)

Substrate	Compound	Na^+	Ca^+	S^+	S_2^+	S_3^+	M^+	$[M+H]^+$	$[M+S]^+$	$[2M+S^+]$
Ag		1.41	1.86	3.05	4.32	5.29				
		(23)	(40)	(108)	(216)	(324)				
Ag	Dithizone	1.41	1.86	3.05	4.32	5.29			5.69	
		(23)	(40)	(108)	(216)	(324)			(364)	
Ag	Benzo[*e*]pyrene	1.41	1.86	3.05	4.32	5.29	4.66		5.58	
		(23)	(40)	(108)	(216)	(324)	(252)		(360)	
Ag	1,4,8,11-Tetraazocyclotetradecane	1.41	1.86	3.05	4.32	5.29	4.18			6.76
		(23)	(40)	(108)	(216)	(324)	(200)			(518)
Ag	Dicyclohexyl-18-crown-6	1.41	1.86	3.05	4.32	5.29	5.88		6.48	
		(23)	(40)	(108)	(216)	(324)	(373)		(481)	
Ag	[5]-Helicene dendrimer	1.41	1.86	3.05	4.32	5.29	10.86		11.26	
		(23)	(40)	(108)	(324)	(1343)			(1451)	
Ag	Gramicidin S hydrochloride	1.41	1.86	3.05	4.32	5.29		10.08	10.46	
		(23)	(40)	(108)	(216)	(324)		(1124)	(1249)	
Au (Cu)	Gramicidin S hydrochloride	1.41	1.86	2.34					10.29	
		(23)	(40)	(64)					(1205)	
Al	Gramicidin S hydrochloride	1.41	1.86	1.53					10.18	
		(23)	(40)	(27)					(1168)	
Ag	Substance P acetate	1.41	1.86	3.05	4.32	5.29		10.86	11.28	
		(23)	(40)	(108)	(216)	(324)		(1348)	(1455)	
Ag	Melittin	1.41	1.86	3.05	4.32	5.29	15.76		16.10	
		(23)	(40)	(108)	(216)	(324)	(2846)		(2954)	
Ag particles	Gramicidin S hydrochloride	1.41	1.86	3.05	4.32	5.29		10.08	10.46	
		(23)	(40)	(108)	(216)	(324)		(1142)	(1249)	

Benzo[*e*]pyrene ($M_r = 252$ Da) is a polycyclic aromatic hydrocarbon (PAH) which consists of five aromatic rings sharing ring edges. It was chosen as a model compound with an extensive π electron delocalization to probe the chemical reactivity of bare Ag^+ ions in the gas phase in the second 355 nm LDI/TOF-MS experiment. An overlayer sample of benzo[*e*]pyrene on a thin silver film substrate produced a mass spectrum which exhibits a strong M^+ molecular ion signal at 4.66 μs, as shown in Fig. 4. A weak $[M + Ag]^+$ adduct ion signal is also observed at *m/z* 360 (5.58 μs). The relative intensities of the Ag^+, Ag_2^+, M^+, Ag_3^+ and $[M + Ag]^+$ signals are ~100:40:70:3:2. Apparently, direct LDI of benzo[*e*]pyrene by the 355 nm laser light to produce M^+ is highly efficient owing to its UV absorption property. In addition, the strong interaction of Ag^+ with delocalized π electrons over the extensively conjugated molecular orbital of benzo[*e*]pyrene results in the formation of the $[M + Ag]^+$ adduct ion.

1,4,8,11-Tetraazocyclotetradecane ($C_{10}H_{24}N_4$; $M_r =$

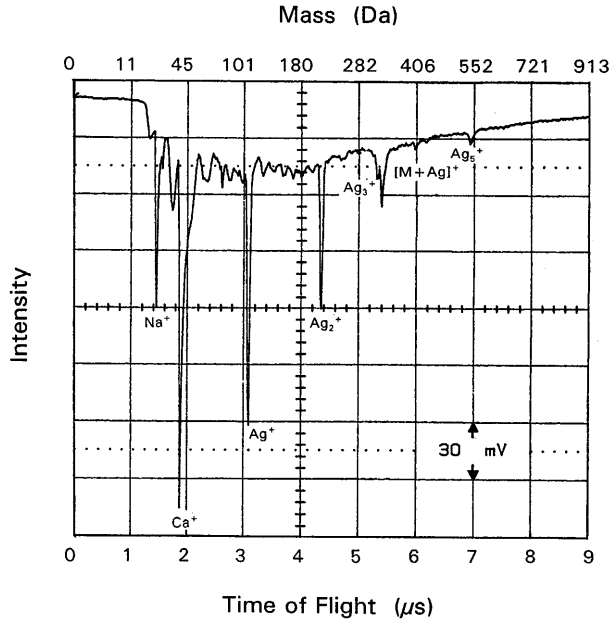

Figure 3. 355 nm LDI/TOF mass spectrum for dithizone on a thin silver film substrate.

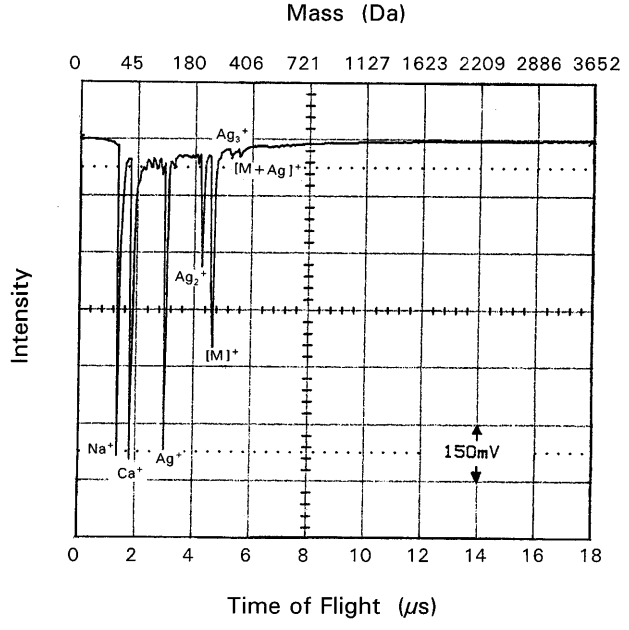

Figure 4. 355 nm LDI/TOF mass spectrum for benzo[*e*]pyrene on a thin silver film substrate.

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200 Da), also known as cyclam, is an ionophore which binds the Ag^+ ion to form a tight complex in aqueous solution.[25] In the third 355 nm LDI/TOF-MS experiment, an overlayer sample of 1,4,8,11-tetraazocyclotetradecane on a thin silver film substrate produced a mass spectrum which displays a weak M^+ molecular ion signal at 4.18 μs in partial overlap with the Ag_2^+ peak at 4.32 μs, as shown in Fig. 5. However, a strong signal is observed at 6.76 μs, which corresponds to m/z 518 from the $[2M + Ag]^+$ adduct ion. This result suggests that one Ag^+ ion is sandwiched between two 1,4,8,11-tetraazocyclotetradecane molecules, to establish a coordination structure in which the positive Ag^+ ion and eight lone pairs of nitrogen electrons are electrostatically attracted. The formation of such a complex is not totally unexpected, considering that the ring size of a tetraazocyclotetradecane framework is smaller than the Ag^+ ion (ionic radius = 126 pm). In a way, this coordination structure resembles that of the K^+–nonactin complex. The LDI/TOF-MS spectrum thus contains new information on the chemical properties of the analyte to facilitate its structural analysis and confirmation.

Dicyclohexyl-18-crown-6 (M_r = 373 Da) is a member of the crown ether family, which have the ability to form stable coordination complexes with particular metal ions. These compounds are cyclic ethers containing a number of regularly spaced oxygen atoms. The oxygens, using their unshared electron pairs, together coordinate the electron-deficient metal ion in the cavity of the crown. In the fourth 355 nm LDI/TOF-MS experiment, an overlayer sample of dicyclohexyl-18-crown-6 on a thin silver film substrate produced a mass spectrum which presents a strong M^+ molecular ion signal at 5.88 μs, as shown in Fig. 6. An $[M + Ag]^+$ adduct ion signal is also observed at 6.48 μs. The relative intensities of the Ag^+, Ag_2^+ and $[M + Ag]^+$ signals are ~66:10:34. As in the case of dithizone discussed above, a decrease in the Ag^+ signal intensity is found in the new $[M + Ag]^+$ signal intensity. Apparently, the Lewis acidity of Ag^+ ions, the lone electron pairs of the oxygen atoms and the cyclic structure of the 18-crown-6 all play an important role in the effective binding of Ag^+ to the polyether.[26] Such chemical reactivity of Ag^+ ions generated in the gas phase by laser ablation may prove to be useful for the LDI/TOF-MS analysis of other crown ethers with the appropriate crown ring size for the Ag^+ ion to fit within.

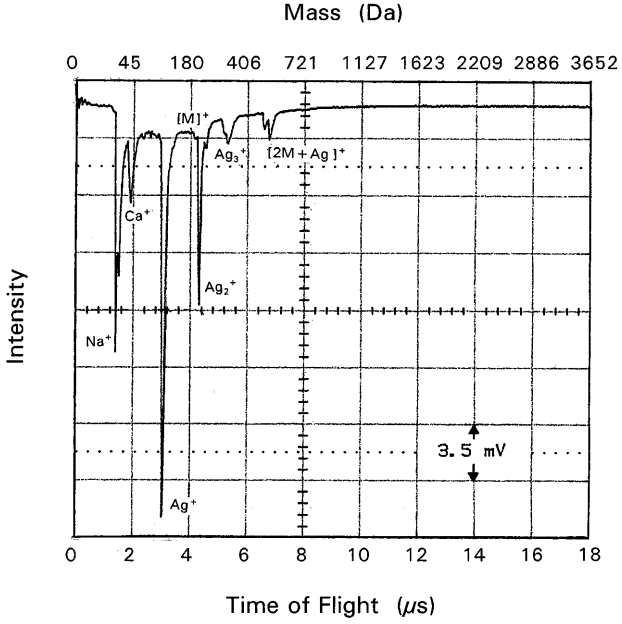

Figure 5. 355 nm LDI/TOF mass spectrum for 1,4,8,11-tetraazacyclotetradecane on a thin silver film substrate.

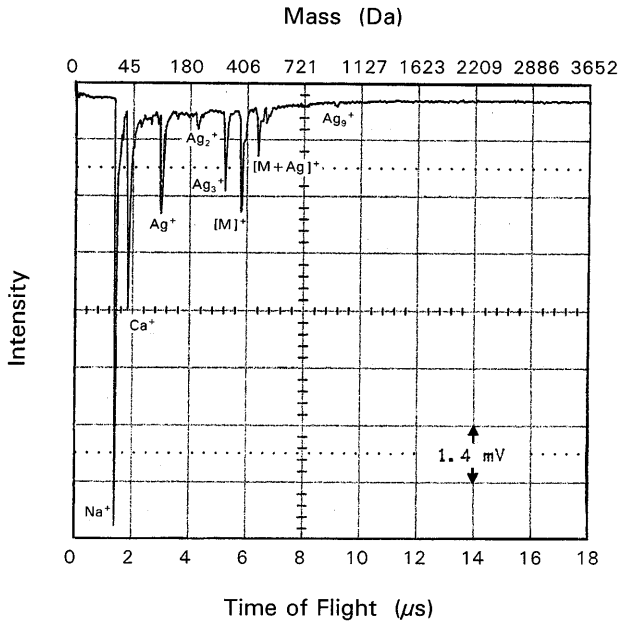

Figure 6. 355 nm LDI/TOF mass spectrum for dicyclohexyl-18-crown-6 on a thin silver film substrate.

A new dendrimer ($C_{87}H_{63}O_{12}N_3$; M_r = 1342.5 Da), which has recently been synthesized from [5]-helicene dicarboxylic anhydride by the polymer chemistry group at Carleton University, was used as the fifth compound to test the versatility of this LDI/TOF-MS technique. An overlayer sample of the dendrimer on a thin silver film substrate produced a 355 nm mass spectrum which consists of an M^+ molecular ion signal at 10.86 μs and an $[M + Ag]^+$ adduct ion signal at 11.26 μs, as shown in Fig. 7. Their masses are calculated to be 1340 ± 4 Da and 1446 ± 5 Da, respectively. These mass analyses have a 0.25% error, which is limited mainly by an uncertainty of ±17 ns in our TOF measurements. Hence LDI/TOF-MS is useful for confirming the chemical composition of the synthetic dendrimer by a fairly accurate mass analysis. Previously, silver-attached molecular ions of five phenylacetylene dendrimers have been reported for both infrared LD/CI (ranging in mass up to 3600 Da) and UV MALDI/CI using *trans*-retinoic acid as the matrix (ranging up to 15 kDa), when the $AgNO_3$ method was applied.[27] Peaks corresponding to $[M + Ag]^+$ and $[M + matrix + Ag]^+$ appeared in those mass spectra. MALDI/TOF mass spectra of the five phenylacetylene dendrimers could also be obtained without CI by $AgNO_3$. It was clear that those were true MALDI spectra because no dendrimer ions could be detected if samples were irradiated with 355 nm light in the absence of matrix. One drawback is that those MALDI/TOF-MS spectra all showed additional $[M_n]^+$

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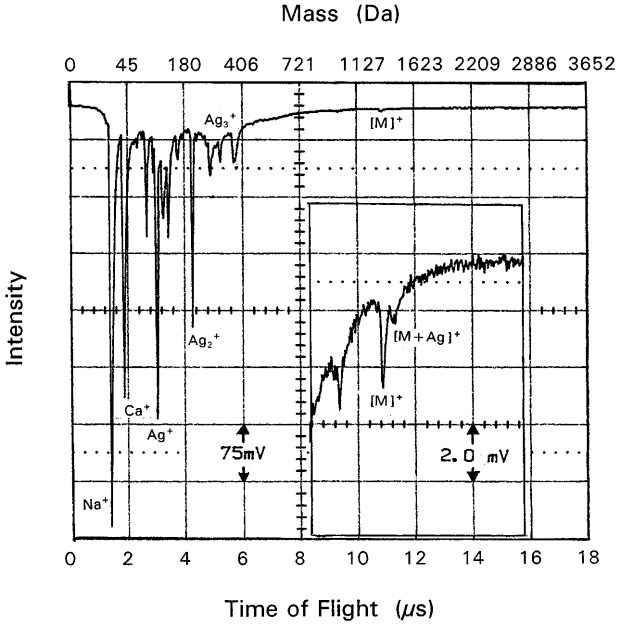

Figure 7. 355 nm LDI/TOF mass spectrum for a synthetic dendrimer on a thin silver film substrate.

peaks corresponding to dimer, trimer and tetramer cluster ions. The dimer cluster ion was often the most abundant, causing confusions in definitive molecular mass determination. By comparison, the present LDI/TOF-MS technique based on 355 nm laser ablation of a thin silver film substrate can perform direct LDI for dendrimer characterization, without any matrix or a pulsed IR laser, to show the M^+ molecular ion only but no higher mass cluster ions.

Biomolecules

The desorption of small molecules (under 293 Da) deposited on gold and irradiated with 351 nm UV laser light have been investigated previously by Li *et al.*[28] They found that desorption can occur through a thermal pathway, in which valence electrons of the gold substrate are radiatively stimulated and a rapid temperature jump is produced at the metal surface. This thermal energy is transferred to the adsorbed species, resulting in vaporization and desorption of those small molecules. However, similar experiments with larger molecules (oligonucleotides and proteins) did not yield an analyte signal.[21] Hence the performance of the present LDI/TOF-MS technique was evaluated using several standard peptides of intermediate molecular mass. The challenge in this study lay in getting these biomolecules on a thin silver film substrate into the gas phase and ionizing them without fragmentation. First, gramicidin S ($M_r = 1141$ Da) is a cyclic decapeptide which plays an important ionophorous role in biochemical ion balance processes. An overlayer sample of gramicidin S hydrochloride produced a 532 nm mass spectrum which exhibits an $[M + H]^+$ protonated molecular ion signal at m/z 1142 (10.08 μs), as shown in Fig. 8(a). Apparently, the hydrochloride may play a role in forming the $[M + H]^+$ ion by providing a source of H^+ for ionization. Formation of the protonated molecular ion has been reported previously for LD/CI with excited-state metal ions.[29] Since the flight path of our TOF-MS instrument has a short length of 52 cm, the resolution ($\Delta m/m$) is ~62 using 20 kV for ion acceleration and 140 at 30 kV acceleration. A weak $[M + Ag]^+$ adduct ion signal is also found at m/z 1249 (10.46 μs) for the first time, even though gramicidin S has been known to form $Li^+/Na^+/K^+$-cationized quasi-molecular ions readily under various LDI, MALDI and electrospray ionization (ESI) conditions.[30–32] Formation of the $[M + Ag]^+$ adduct ion implies that several collisions occurred between Ag^+ ions in the ablation plume and desorbed neutral/protonated gramicidin S molecules.[33] Such adduct ion formation by chemical ionization should be inefficient, considering that the reaction time is extremely short in the strong accelerating field used in this study. However, a great advantage of this LDI technique lies in its versatility. The silver film acts as an energy transfer medium which absorbs either 355 nm UV or 532 nm visible laser light to cause desorption of the sample overlayer during the photoablation process. Basically any sample overlayer, including non-absorbing materials, can be desorbed to produce molecular and adduct ions from the analyte based on the gas-phase ion chemistry that occurs in the ablation plume. Of course, the desorption could have resulted from a shock wave or pressure wave which is generated by the photoablation process.[34] Whether the desorption of gramicidin S occurs through a photothermal pathway or results from a shock wave, no significant fragmentation ion products of the cyclic decapeptide molecule due to thermal or pressure-wave degradation is evident in the mass spectrum. Since UV laser light is not necessarily required by this LDI technique, the chances are that significant fragmentation of the biomolecule due to photodecomposition can be prevented. In contrast, traditional LDI from bulk metal near the surface requires high laser fluences to operate the rapid heating event, owing to the high thermal conductivity of silver. Also, the doubly charged molecular ion $[M + 2H]^+$ is not seen, as would normally be expected from ESI via protonation.[35] These features of LDI greatly simplify the interpretation of mass spectra for analyte signal assignments.

Confirmation of the mechanistic role of Ag^+ in the ionization and cationization of desorbed gramicidin S molecules became necessary. Under the postulate that the nature of the metal ion might be of primary importance, thin-film substrates of different metals were tested in LDI/TOF-MS experiments. Kahr and Wilkins[36] had performed LDI experiments in the 10.6 μm far-infrared and 355 nm UV range with polybutadiene and polystyrene in which a 0.1 mm thick gold foil served as a substrate. Also, 10 nm thin gold film-assisted LDI/FTMS of gramicidin S deposited as a thick slurry in methanol–KBr solution had previously been achieved by Wahl *et al.*[37] using 1064 nm laser light. In the present study, an overlayer sample of gramicidin S hydrochloride on a 40 nm thin gold film substrate produced a 532 nm mass spectrum which displays a strong Na^+ ion signal, a Ca^+ ion signal and a Cu^+ ion signal at m/z 63.5 (2.34 μs) from impurities in the gold. Unlike thin silver film substrates, the thin gold film substrate does not generate peaks corresponding to Au_n^+ (with

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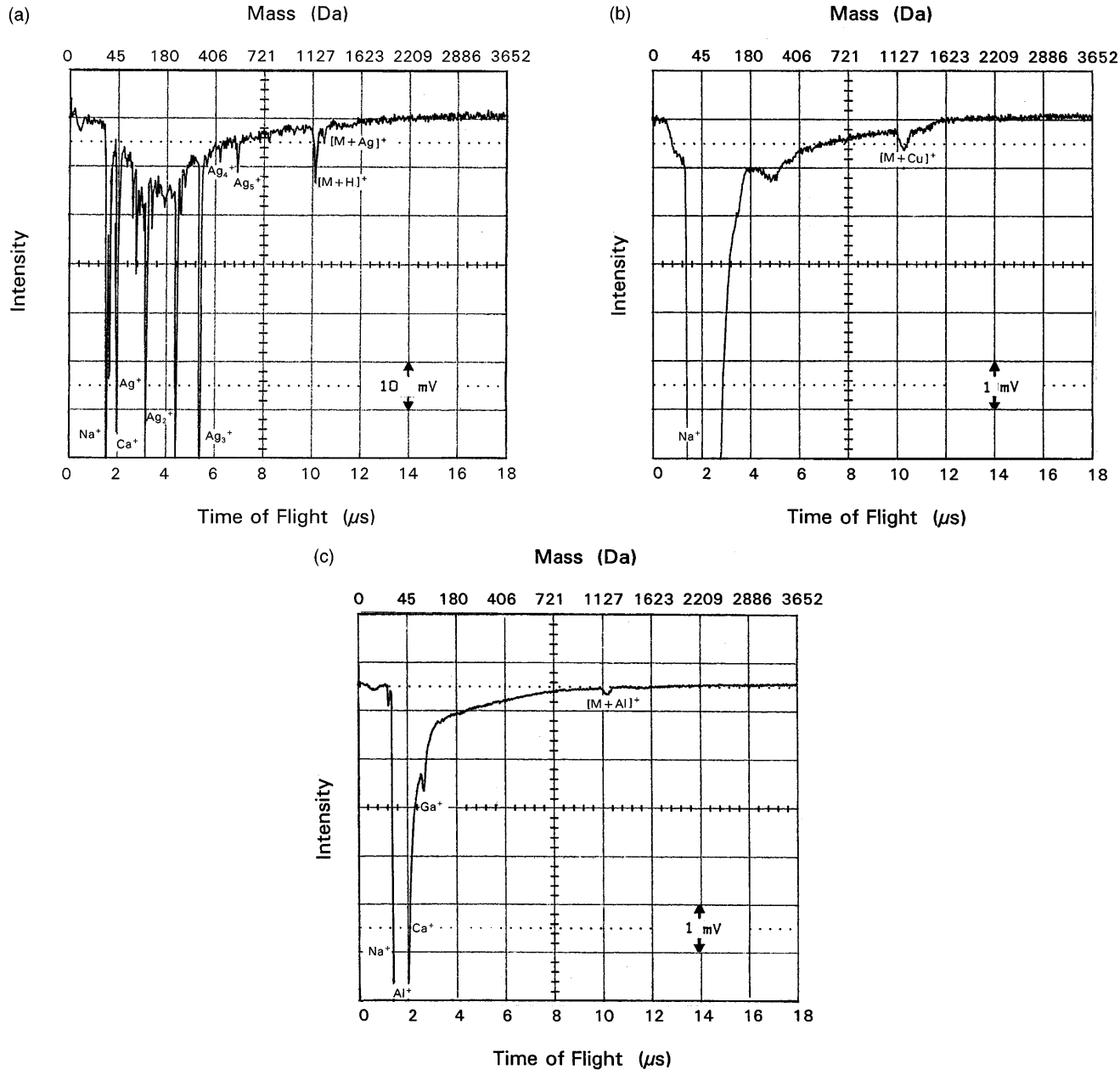

Figure 8. 532 nm LDI/TOF mass spectra for gramicidin S hydrochloride on (a) a thin silver film substrate, (b) a thin gold film substrate and (c) a thin aluminium film substrate.

$n = 1$–3) at laser energies up to 1.0 mJ per pulse (higher energies would cause a large dispersion in the flight times and hence serious degradation in mass resolution). One plausible explanation is that Au has a first ionization energy of 890 kJ mol^{-1} (or 1.47×10^{-18} J per atom), which is significantly higher than the 731 kJ mol^{-1} (or 1.21×10^{-18} J per atom) for Ag. Four-photon absorption is therefore necessary for the photoionization of Au at the 532 nm wavelength (3.73×10^{-19} J per photon). The probability of this multi-photon ionization for Au is definitely lower than that of the three-photon ionization process required of Ag. As shown in Fig. 8(b), an $[M + Cu]^+$ adduct ion signal is observed at m/z 1205 (10.29 μs). This result is consistent with a previous report that amino acids with non-polar side chains are more reactive towards gas-phase Cu^+ than amino acids with polar side chains.[38] Similar metal ionization with laser-desorbed Cu^+ to form $[L + Cu]^+$, where L is quinolone, has also been reported previously.[20] In comparison with thin silver film substrates, the thin gold film substrate also produces protonated molecular and adduct ions for the mass determination of gramicidin S. It seems that Ag^+ is not absolutely needed in the ionization and cationization of desorbed gramicidin S molecules. However, the analytical signals (or signal-to-noise ratios) produced by the thin gold film substrate are not as strong. One advantage of the use of gold film substrates is that no Au_n^+ gold cluster ion signals appear in the mass spectrum, which provides a spectral window above m/z 64 for the detection of any fragmentation products and sample matrix components if needed.

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Aluminium is another metal that had previously been used as the sample probe tip material in LDI studies.[39] The first ionization energy of Al is 578 kJ mol^{-1} (or 0.96×10^{-18} J per atom), which makes multiphoton ionization relatively easy. As shown in Fig. 8(c), an overlayer sample of gramicidin S hydrochloride on a thin aluminium film substrate produced a 532 nm mass spectrum which presents an Na^+ ion signal, a Ca^+ ion signal, a strong Al^+ ion signal at m/z 27 (1.53 μs) and a Ga^+ ion signal at m/z 70 (2.47 μs) from impurities in the aluminium. No $[M + H]^+$ protonated molecular ion signal is revealed but an $[M + Al]^+$ adduct ion signal is observed at m/z 1168 (10.18 μs). In comparison with thin silver and gold film substrates, the thin aluminium film substrate also produces adduct ions for the mass determination of gramicidin S. However, the analytical signal (or signal-to-noise ratio) produced by the thin aluminium film substrate is weak. In summary, the best LDI efficiencies are afforded by the thin silver film substrate under similar laser energy conditions. This is particularly noteworthy, considering that silver has intermediate optical absorbance, density, specific heat, melting point, heat of fusion, heat of vaporization and first ionization energy that fall between those values for gold and aluminium. Under the assumption of insignificant heat flow out of each thin metal film substrate on a nanosecond time-scale during the pulsed laser ablation process, our calculations show that the quantity of incident laser energy stored in the sample overlayer is also intermediate for silver. Besides its unique chemical reactivity, the only distinct property of silver is that its boiling point (952 °C at 10^{-6} Torr) is lower than those of gold (1211 °C) and aluminium (1063 °C). Other thin metal film substrates should be used as thermometers to provide more temperature information for a better understanding of the laser ablation process.[7]

The utility of thin silver film substrates for LDI/TOF-MS analysis was extended to other types of peptides. Substance P (M_r = 1347 Da) is a linear peptide which had previously been analyzed by MALDI using sample addition to a thin layer of a α-cyano-4-hydroxycinnamic acid matrix.[40] An overlayer sample of substance P acetate on a thin silver film substrate produced a 355 nm mass spectrum which consists of an $[M + H]^+$ protonated molecular ion signal at m/z 1348 (10.86 μs) and an $[M + Ag]^+$ adduct ion signal at m/z 1455 (11.28 μs), in addition to the usual Na^+, Ca^+, Ag^+, Ag_2^+ and Ag_3^+ peaks as shown in Fig. 9. It is noteworthy that the $[M + Ag]^+$ signal is 2.5 times more abundant than the $[M + H]^+$ signal. When the laser wavelength is switched to 532 nm, the same ion signals are seen again, although the $[M + Ag]^+$ signal is only 0.7 times as abundant as the $[M + H]^+$ signal. These high abundances of adduct ions clearly demonstrate that the Ag^+ affinity to substance P is significantly enhanced by a sulfur atom in the methionine residue. The coordination of the lone electron pair(s) of the sulfur atom can play an important role in Ag^+ binding. In comparison, no intact molecular ion could be observed by 118 nm photoionization of substance P in a previous study, probably owing to facile backbone cleavage of the linear peptide.[41]

It is well known that useful LDI mass spectra of peptides above 2000 Da are difficult to obtain.[36,40] Using

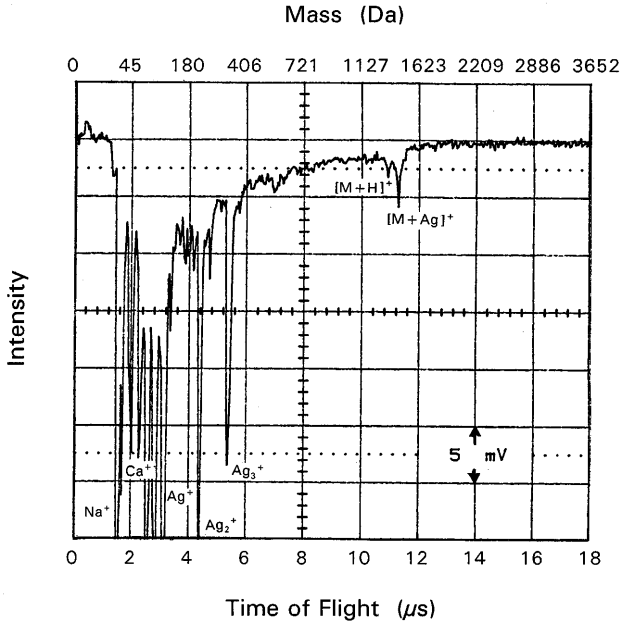

Figure 9. 355 nm LDI/TOF mass spectrum for substance P on a thin silver film substrate.

the present LDI/TOF-MS technique, the largest peptide that can be successfully analyzed is melittin (M_r = 2846 Da), which is a bee venom. As shown in Fig. 10, an overlayer sample of melittin on a thin silver film substrate produced a 532 nm mass spectrum which exhibits an M^+ molecular ion signal at m/z 2846 (15.76 μs) in addition to the usual Na^+, Ca^+, Ag_n^+ (n = 1–9) peaks. An $[M + Ag]^+$ adduct ion signal at m/z 2954 (16.10 μs) can barely be observed above the background noise. This successful ionization of a peptide with a molecular mass higher than 2800 Da indicates that less internal energy is associated with the present technique than conventional LDI techniques. The detection of greater masses is limited, however, because the binding energy

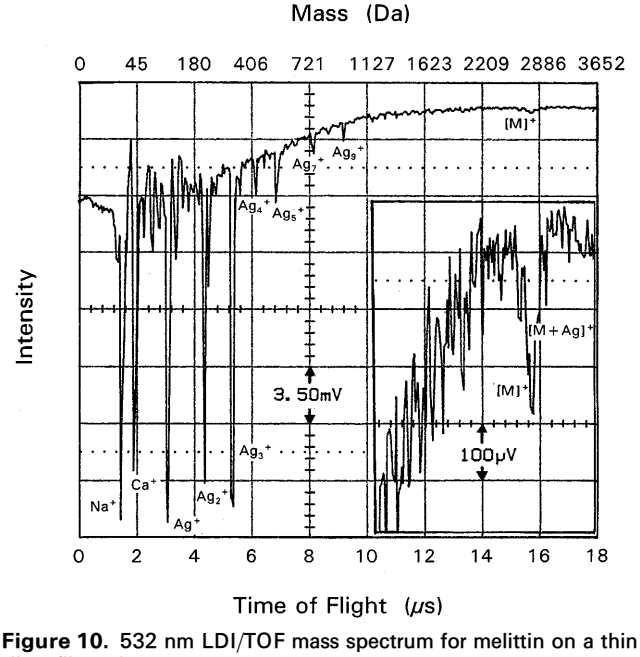

Figure 10. 532 nm LDI/TOF mass spectrum for melittin on a thin silver film substrate.

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of peptides to the substrate surface increases with more polar functionalities in larger peptides through substantial hydrogen bonding. In addition, analyte–analyte interactions become stronger. If desorption occurs for a considerable laser energy input, the internal energy per bond will be high. This causes instability, resulting in fragmentation of the peptide molecules during LDI.

Silver particles

The search for novel silver substrates to extend the capabilities of the present LDI/TOF-MS technique continues to evolve. In a new sample preparation method, gramicidin S is added to a Tollen's reagent mixture for direct impregnation on silver particles during their formation and growth in the colloidal solution. These silver particles provide a silver matrix for the analyte molecules, which can potentially enhance the LDI efficiency to produce greater $[M + H]^+$ and $[M + Ag]^+$ signals. A sample of gramicidin S-impregnated silver particles deposited on a thin silver film substrate produced 355 and 532 nm mass spectra which display an $[M + H]^+$ protonated molecular ion signal at m/z 1142 (10.08 μs) and an $[M + Ag]^+$ signal at m/z 1249 (10.46 μs), as shown in Fig. 11. The $[M + H]^+$ signal was stronger at 532 nm, whereas the $[M + Ag]^+$ signal was larger at 355 nm. Note that these two signals are both much more intense than the previous ones obtained from the sample overlayers of gramicidin S on thin silver, gold and aluminium film substrates. Apparently, the silver particles can hold an increased amount of gramicidin S to produce intense $[M + H]^+$ and $[M + Ag]^+$ signals. The silver matrix may even provide for more efficient LDI to form abundant molecular and adduct ions. This sample preparation method can be compared and contrasted with the ultra-fine metal plus liquid matrix method reported by Tanaka *et al.*[42] in 1988. In their method, an ultra-fine cobalt powder (UFP, diameter = 30 nm) and glycerol were dissolved in ethanol or acetone. A mixture of this solution and the aqueous sample solution (1 mg ml^{-1}) was vacuum dried on the sample holder for a few minutes before TOF-MS analysis. UFP has the features of high photoabsorption, low heat capacity and extremely large surface area per unit volume. Heating of the UFP by laser light of a wavelength longer than the particle diameter enhanced the speed of sample heating by laser irradiation and molecular ions were formed more easily. By the addition of glycerol to the sample, molecules of the sample were replenished to the laser beam irradiation position. This allowed the molecular ion formation to continue for the accumulation of 500–2000 single-shot spectra over a total data acquisition time of 2–4 min. Laser ionization mass spectra were obtained for lysozyme (M_r = 14 306 Da) and chymotrypsinogen (M_r = 25 717 Da), where broad peaks due to $[M + Na]^+$, $[M + K]^+$, $[M + H]^+$ and metastable ion contributions were observed. A major drawback of the UFP method is that $[nM + \text{cation}]^+$ and $[nM + 2 \text{ cations}]^{2+}$ (n = 1–7) were also detected. These molecular cluster signals can create confusion in the interpretation of molecular mass and fragmentation information, especially when mixture samples are analyzed. By comparison, our sample preparation method using Tollen's silver particles does not exhibit any molecular cluster signals. Larger peptides and proteins will be tested in order to evaluate its full potential. Improvement will also require a further scale-down in the Tollen's reagent mixture chemistry.

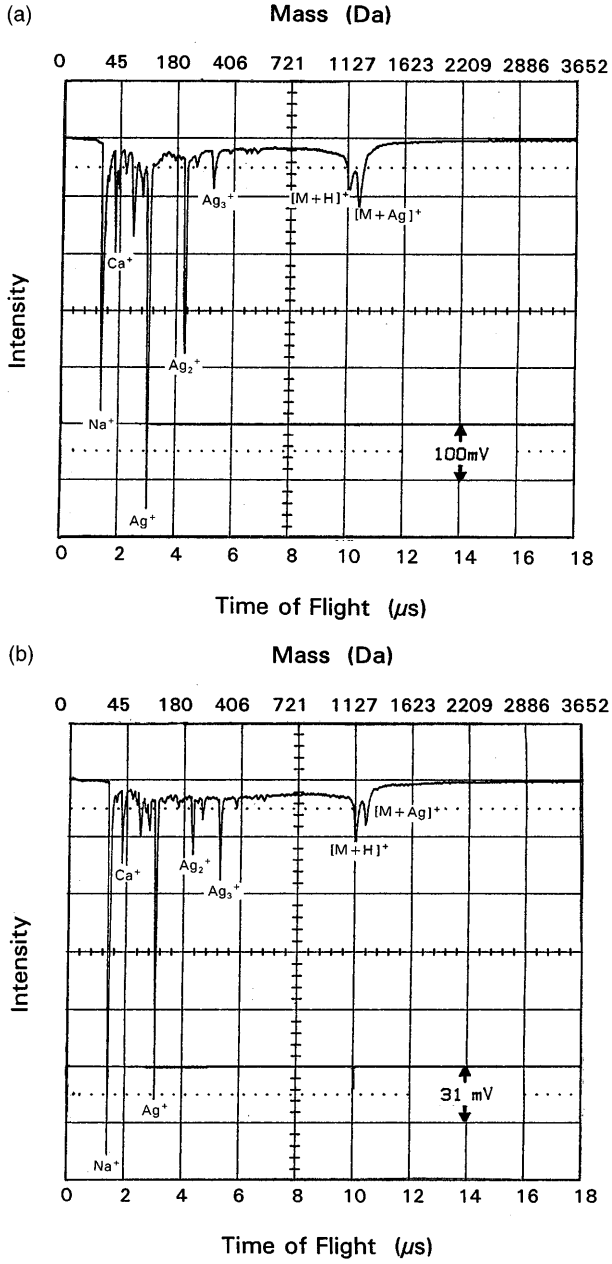

Figure 11. LDI/TOF mass spectra for gramicidin S on silver particles using (a) 355 and (b) 532 nm laser light.

CONCLUSION

LDI/TOF-MS based on photoablation of thin silver film substrate and particles has been investigated as a technique of mass analysis for bioorganic molecules. The thin silver film acts as a selective substrate which is good for the molecular mass analysis of bioorganic substances with intrinsic Ag^+ binding properties. Low-

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energy laser pulses can provide sufficiently high fluences to vaporize the thin silver film substrate and bring single or clustered atoms of metal into the gas phase along with the analyte for LDI. This technique has been successful for selected bioorganic molecules in the mass range from 200 to 3000 Da, where good protonated molecular and adduct ion signals were obtained. One major advantage of the technique is that a 532 nm visible laser can be used, under modest laser fluence conditions, to avoid resonant absorption which may lead to extensive fragmentation of the analyte molecules. Another advantage is that sample preparation is simple, without any matrix addition. A wider range of bioorganic molecules will be tested in order to understand better the full potential usefulness of the LDI chemistry. Also, the use of Tollen's reagent solution for the impregnation of silver particles with analyte has proved successful. Enhancement of the mass spectral signals provides a greater sensitivity for the analysis of low concentrations and small amounts of bioorganic compounds. Further development of this sample preparation method may facilitate the routine analysis of biomedical samples by LDI/TOF-MS.

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