Desorption/Ionization of Biomolecules from Aqueous Solutions at Atmospheric Pressure Using an Infrared Laser at 3 μm

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A new atmospheric pressure (AP) infrared (IR) matrix-assisted laser desorption/ionization (MALDI) ion source was developed and interfaced with a Thermo Finnigan LCQ ion trap mass spectrometer. The source utilized a miniature all-solid-state optical parametric oscillator (OPO)-based IR laser system tunable in the λ = 1.5–4 μm spectral range and a nitrogen ultraviolet (UV) laser (λ = 337 nm) for use in comparative studies. The system demonstrated comparable performance at 3 μm and 337 nm wavelengths if UV matrices were used. However, AP IR-MALDI using a 3 μm wavelength showed good performance with a much broader choice of matrices including glycerol and liquid water. AP IR-MALDI mass spectra of peptides in the mass range up to 2000 Da were obtained directly from aqueous solutions at atmospheric conditions for the first time. A potential use of the new AP IR-MALDI ion source includes direct MS analysis of biological cells and tissues in a normal atmospheric environment as well as on-line coupling of mass spectrometers with liquid separation techniques. (J Am Soc Mass Spectrom 2002, 13, 354–361) © 2002 American Society for Mass Spectrometry

Infrared (IR) matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) [1] was introduced shortly after the discovery of conventional ultraviolet (UV) MALDI-MS [2, 3]. In many cases IR-MALDI was shown to be superior to UV-MALDI mostly because of its softness and as a result lesser analyte ion fragmentation [4–7]. In addition, the search for new effective matrices in the UV region is difficult because only a small number of potential matrices absorb on UV laser wavelengths and the correlation between matrix absorption properties and the MALDI ionization mechanism is not clearly understood [8, 9]. In contrast, there are many more potential matrices for IR-MALDI because of the strong absorption of molecular compounds in the IR spectral region; the correlation between ion formation and matrix absorption in IR-MALDI is also under discussion [10]. The IR radiation penetrates much deeper into the sample compared to UV, an effect that may be important in some cases like the analysis of *Bacillus* spores [11].

One promising matrix material for IR-MALDI is water in liquid or ice form, the latter providing compatibility with the vacuum requirements of a mass spectrometer. Water is a native environment for most biological molecules and is attractive with regard to IR-MALDI because of its strong O–H stretching mode absorbing near 3 μm [12]. Several other IR matrices, which have given promising results, provide a physiological, non-denaturing environment for the analytes. Among them are glycerol, often used as a (co)solvent for large biomolecules, and Tris/HCl, a commonly used biochemical buffer [12, 13].

The concept of laser ablation from frozen aqueous solutions of DNA mixtures was first exploited at the visible wavelengths of a dye laser (λ = 578 or 589 nm) [14, 15]. It was found that for a sufficiently thin frozen aqueous solution on a copper substrate, the copper itself acts under high-power irradiation as the chromophore resulting in the transfer of energy to the ice film by shock heating. Using a similar technique, Tanaka, in the first MALDI experiment, introduced a separate chromophore in the form of tiny 300 Å cobalt particles dispersed in glycerol [3]. In another approach [16] DNA oligomers were embedded in thin films of

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frozen aqueous solutions containing photoabsorbing substituted phenols and desorbed using a Nd:YAG laser at 266 and 355 nm. Sensitivity and mass resolution are approaching that obtained using a crystalline UV-MALDI matrix (3-hydroxypicolinic acid). In the case of IR-MALDI (λ = 2.94 μm), mass spectra of superior quality and reproducibility were obtained for proteins up to 30 kDa [12]. Best results were obtained when the water remained with the protein in the form of hydration in the temperature range of 170–200 K. Good results were obtained using a 2.94 μm laser with cryogenically cooled matrices based on mixtures of water, glycerol, and other additives [17, 18] as well as frozen alcohol [19]. It is worthwhile to note that during analysis of whole bacterial spores using an IR laser, molecular ions with the masses up to 19,050 Da can be obtained without adding any chemical matrix [20].

In a different approach, analyte ions are desorbed from a thin jet of analyte aqueous solution injected directly into a vacuum through a 10 μm nozzle by means of an IR laser pulse tuned to a vibrational resonance of the water [21]. Water in the jet flow (typically 0.1–1 ml/min) is supercooled considerably below its freezing point due to fast, evaporative cooling in the vacuum. A few millimeters past the nozzle, in the laminar flow regime, the liquid jet is crossed with an IR laser pulse (Nd:YAG-pumped optical parametric oscillator with amplifier; energy 20–30 mJ/pulse; irradiance 10^6–10^8 W/cm^2). Ions formed were orthogonally extracted into time-of-flight mass spectrometer (TOFMS) for analysis. To maintain a high vacuum the liquid jet was finally trapped and frozen out on a liquid nitrogen cool trap. This method is soft enough to detect intact hemoglobin from an aqueous solution [22].

In contrast to conventional vacuum MALDI, a new atmospheric pressure (AP) MALDI technique [23] allows analyzing samples in a normal atmospheric environment resulting in no requirements of vacuum compatibility for a sample. Recently, AP MALDI has been interfaced with an ion trap [24]. So far this techniques has been applied to regular crystalline matrices using a UV laser. The real benefit of using liquid matrices including volatile ones, such as water, can be demonstrated by utilizing an IR laser for desorption/ionization. Recently we coupled a Thermo Finnigan LCQ ion trap mass spectrometer with an AP MALDI ion source equipped with an IR laser tunable in the 1.5–4 μm spectral range. Here we report initial results obtained at a 3 μm wavelength using water and other matrices.

Experimental

AP IR-MALDI-MS Interface

Experiments were carried out on a Thermo Finnigan (San Jose, CA) LCQ Classic ion trap mass spectrometer integrated with a modified MassTech Inc. (Burtonsville, MD) Model AP/MALDI-110 ion source as shown in Figure 1. The AP/MALDI source housing consisting of two aluminum cap-like flanges replaces an LCQ electrospray ionization (ESI) source. An optical flange bolted to the LCQ stage holds major optical elements for directing and focusing a laser beam and viewing a sample using a CCD camera. A target flange contains a target plate with multiple samples on it and x and y linear translational stages for moving samples into a working position. The optical flange is mounted on the LCQ inlet flange using standard bolt holes and the target flange is placed onto the LCQ sliding platform so that the two flanges can be connected to each other in the operational position and slide apart for sample target change or maintenance.

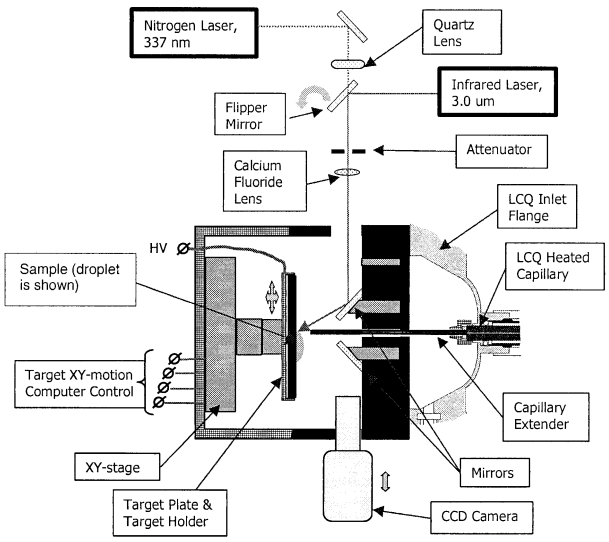

Figure 1. Scheme of interfacing an AP IR/UV-MALDI ion source with a Thermo Finnigan LCQ ion trap mass spectrometer (sample droplet is not in scale).

In previous AP MALDI experiments [23, 24] the sample was located in close proximity (usually 1–2 mm) to the inlet hole of the atmospheric pressure interface of the mass spectrometer. In the AP/MALDI design the optical flange mounted on the LCQ inlet flange includes an extended capillary to transfer ions formed on the target plate toward an LCQ atmospheric pressure interface capillary inlet hole. The design allows two capillaries to be sealed at the place of the connection using a Teflon gasket. The extended capillary is made of a 2 in. long stainless steel tubing having a 0.06 in. o.d. and a 0.0325 in. i.d. that is about two times larger than the LCQ inlet capillary i.d. Due to the larger i.d., the extension capillary does not introduce a substantial pressure drop to the inlet gas flow. Thus, the gas pressure at the LCQ inlet hole remains approximately the same as before the LCQ modification; this arrangement ensures undisturbed operation of the modified LCQ atmospheric pressure interface. Ion losses due to the extension of the inlet capillary were estimated by using capillaries of different lengths. We observed about a 30% decrease of the ion signal when the extension capillary length was increased from 2 to 4 in.

That is in a good agreement with the published experimental and theoretical data on transfer of ions through long capillaries [25]. The extra space provided by the extended capillary is required for mounting the optical lens and mirrors since the AP/MALDI design uses a large (about 1.5 in. × 1.5 in.) target plate for analyzing multiple (up to 64) samples. The tip of the extended capillary is placed normal to the target surface at a distance of about 2 mm.

Two lasers, a Thermo Laser Science Inc. (Franklin, MA) Model 337 Si nitrogen UV laser (wavelength λ = 337 nm, laser pulse duration is about 4 ns) and a built-in-house Yb:YAG-pumped optical parametric oscillator (OPO) infrared laser system have been used. A periodically poled lithium niobate (PPLN) crystal is used as the OPO medium. The wavelength of the IR laser can be tuned in the 1.5–4.0 μm range but it was fixed at λ = 3 μm throughout this study. The duration of the IR laser pulse was about 40 ns. The same optical channel that includes an iris diaphragm used as a laser energy attenuator, a 100 mm focal length calcium fluorite lens and an aluminum-coated turning mirror (see Figure 1), is used for directing and focusing both the IR and UV laser beams onto the target surface. The beams are focused onto the target area closest to the extended capillary inlet tip. The beam incidence angle is 45°. Since the focal length of the CaF_2 lens for the UV laser is about 8% shorter compared to that for the IR laser, an additional negative quartz lens having a focal length F = −250 mm is inserted into the UV beam channel to get it focused on the target without moving the CaF_2 lens. Switching between UV and IR laser is achieved by flipping a high-reflective dielectric mirror in the IR laser optical channel. The irradiated sample and laser spots are observed on a video monitor using a CCD camera with total magnification of about ×100. The viewing angle is about 45°. The viewing channel consists of a turning mirror and a 38-mm relay lens for imaging the sample onto the CCD camera's sensitive area. The size of a laser spot was measured on the video monitor using dark traces left after matrix depletion on the target surface covered with the CHCA matrix (see below). The spot shape is close to elliptical and is about 125 × 220 μm for the IR laser and 130 × 250 μm for the UV laser. The maximum laser energy on the target measured directly at the target using a Molectron Detector (Portland, OR) Model EM-400 laser energy meter equipped with a Model J8LP pyroelectric probe, is about 140 μJ/pulse for the UV laser and 200 μJ/pulse for the IR laser. During operation, the laser energy attenuated by the iris diaphragm was at the level of 6–60 μJ/pulse and 140–200 μJ/pulse in the case of the UV- and IR-MALDI, respectively.

The gold-plated steel target plate in the AP/MALDI source is placed atop the crossed x and y linear motorized stages controlled via a computer serial RS-232 port using MassTech's software. This software allows full control of the target plate position relative to the extended capillary tip. When the sample is consumed by laser-induced desorption, the software allows one to continuously move the sample along the spiral line to provide a position with a fresh sample for every laser shot. In order to facilitate the migration of ions toward the extended capillary inlet, the target plate was placed at a high voltage in the 1.5–3 kV range (provided by the LCQ instrument—the needle voltage in the ESI ion source). In order to lower ion losses while they are transferred to the mass analyzer, the AP MALDI chamber is filled with dry nitrogen (not shown in Figure 1, supplied from the LCQ).

Operation and Sample Preparation

Experiments were carried out in a repetitive laser shot mode (laser frequency 5–10 Hz). The laser trigger times were not synchronized with the LCQ operation. The AP MALDI ions effectively formed a quasi-continuous ion beam at the exit of the LCQ inlet capillary. The temperature of the LCQ capillary was held at 200 °C unless otherwise specified. Typical high voltage applied to the target plate was 2000 V. The spectra presented have been accumulated during a time interval from 30 s to 3 min depending on the analyte concentration. The experiments described in this work were all obtained in a positive ion mode.

The samples for mass spectrometric analysis were prepared on a gold-plated target plate by mixing 0.5–1 μl of 0.1% TFA aqueous analyte solution with 0.5–1 μl of a matrix solution. In the case of crystalline matrices the samples were dried under forced air at room temperature. Two different crystalline matrix solutions were used for the UV MALDI analysis. 2,5-Dihydroxybenzoic acid (DHB) and α-cyano-4-hydroxycinnamic acid (CHCA) were prepared as saturated solutions in a mixture of water and acetonitrile (volume ratio 1:1). These matrices were also used in IR-MALDI analysis as well as glycerol and pure water, HPLC grade. The matrices and peptides were purchased from Aldrich (Milwaukee, WI) and Sigma (St. Louis, MO), respectively, and were used without any further purification.

Results and Discussion

While AP UV-MALDI quadrupole ion trap mass spectrometry (QITMS) has been the subject of study in several research laboratories [24, 26, 27], an AP IR-MALDI ion source has never been interfaced with any mass spectrometer including QITMS. Since our experimental setup is coupled with both UV and IR lasers, we concentrated first on a comparative study of AP IR- and UV-MALDI-QIT mass spectrometry.

Comparative Study of Ion Trap Mass Spectrometry of Peptides Using AP IR- and UV-MALDI

The results of our first experiments with Angiotensin I (FW 1296.5 Da) obtained in normal MS mode with the

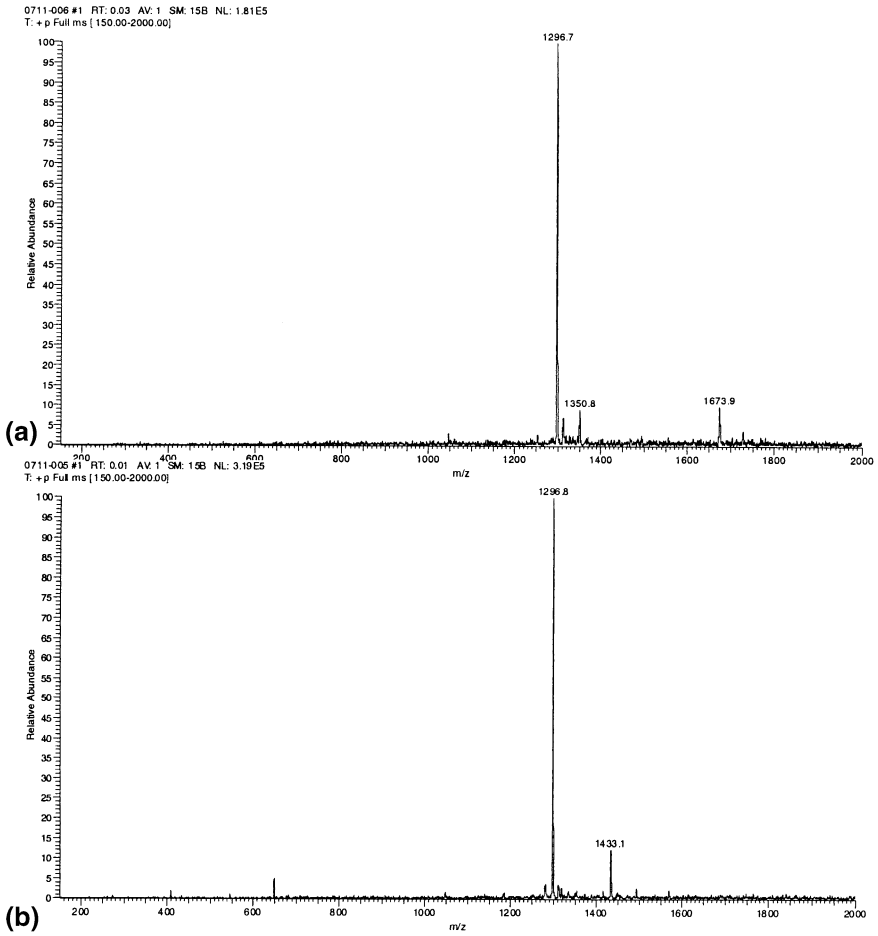

Figure 2. AP UV-MALDI mass spectra of Angiotensin I using CHCA (**a**) and DHB (**b**) as a matrix (10 pmol loaded).

AP UV- and IR-MALDI sources are shown in Figure 2a and b and Figure 3a–d, respectively.

All spectra taken using both UV and IR lasers are of comparable high quality. In Figures 2 and 3 the molecular ions corresponding to the sodium-cationized ions (*m/z* 1318) are seen as small satellites to the main protonated ion peaks at *m/z* 1296. In contrast to conventional vacuum MALDI in QITMS, where fragment ions are common in mass spectra [28–30], no fragments are observed at atmospheric pressure in the case of either AP UV- or IR-MALDI. Mostly singly-charged molecular ions are observed in the spectra that distinguish MALDI from ESI where ions are predominantly multiply-charged. Only small peaks at *m/z* 649 corresponding to doubly-charged protonated ions are observed in AP MALDI spectra obtained with DHB and glycerol matrices (see Figure 2b and Figure 3b and c). A cluster ion of analyte with two CHCA matrix molecules is observed with the UV laser at *m/z* 1674 (Figure 2a) which disappears if the capillary temperature is raised to 300 °C (data not shown). No such cluster ions were observed in the case of AP IR-MALDI. The peak at *m/z* 1350 is present in every spectrum and probably can be attributed to Mn cations originated from target steel substrate used in our experiments. The appearance of the spectra is not very sensitive to the laser energy if it is over an ion formation threshold.

As in vacuum IR-MALDI, the sample in AP IR-MALDI using crystalline matrices (CHCA and DHB) is consumed after several laser shots. In contrast to conventional vacuum UV-MALDI the same can be observed in AP UV-MALDI if high enough energy is applied. The laser energy threshold for ion formation in the case of AP IR-MALDI was much higher compared to AP UV-MALDI (about 150 μJ/pulse of the IR energy for desorption of ions from the water sample compared to 3 μJ/pulse of the UV energy required in the case of the CHCA matrix).

At the experimental conditions set for this study a water droplet of a few μl volume naturally dries on the target plate during 2–3 min which limits applications for this method. This drawback can be overcome by switching to continuous flow sample introduction techniques that are planned for future study. In contrast to the experiments with water, the samples prepared with glycerol as a matrix can be used many hours without substantial drop in the ion signal. This means that the sample consumption using a glycerol matrix is negligi-

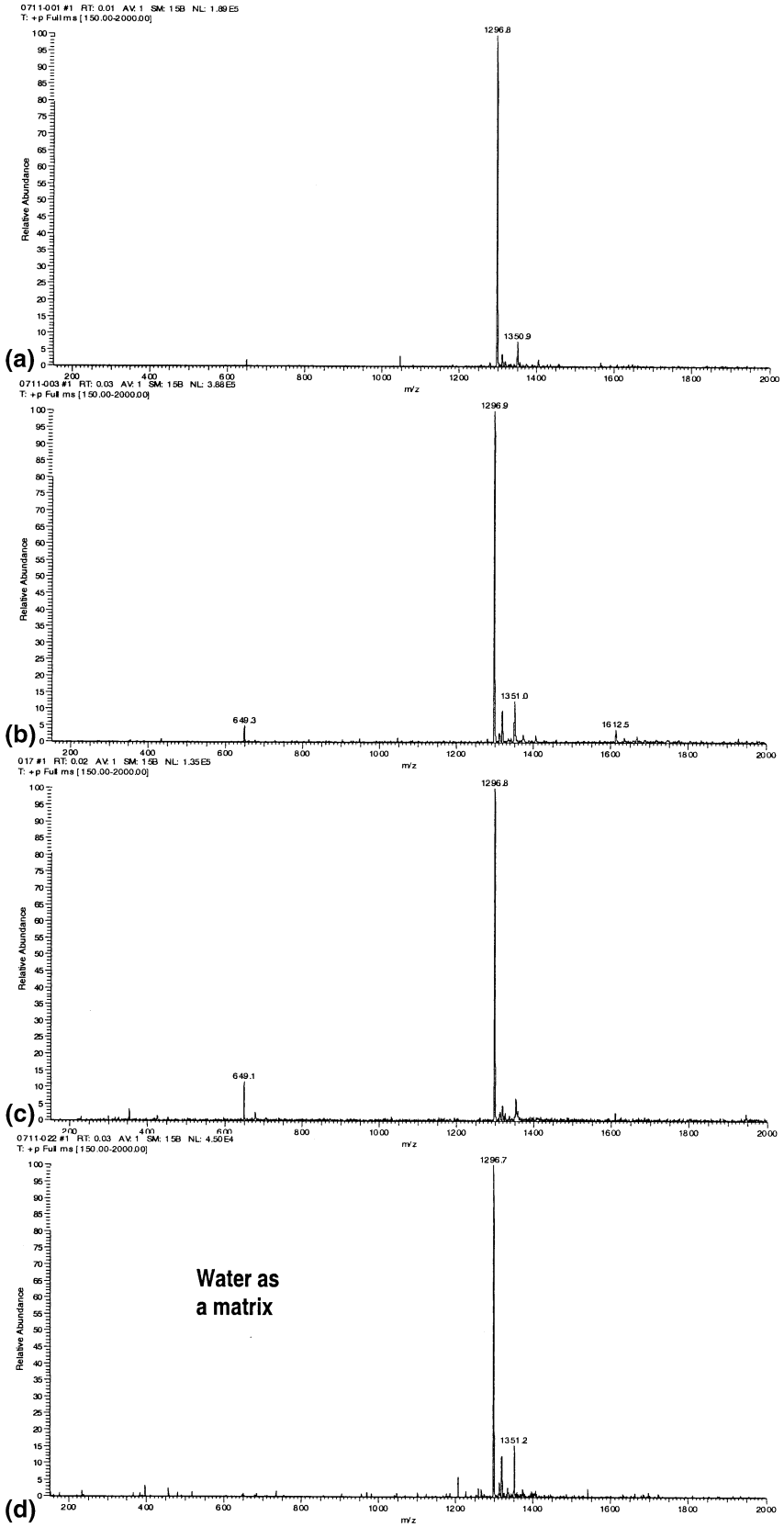

Figure 3. AP IR-MALDI mass spectra of Angiotensin I using CHCA (**a**), DHB (**b**), glycerol (**c**), and water (**d**) as a matrix (10 pmol loaded).

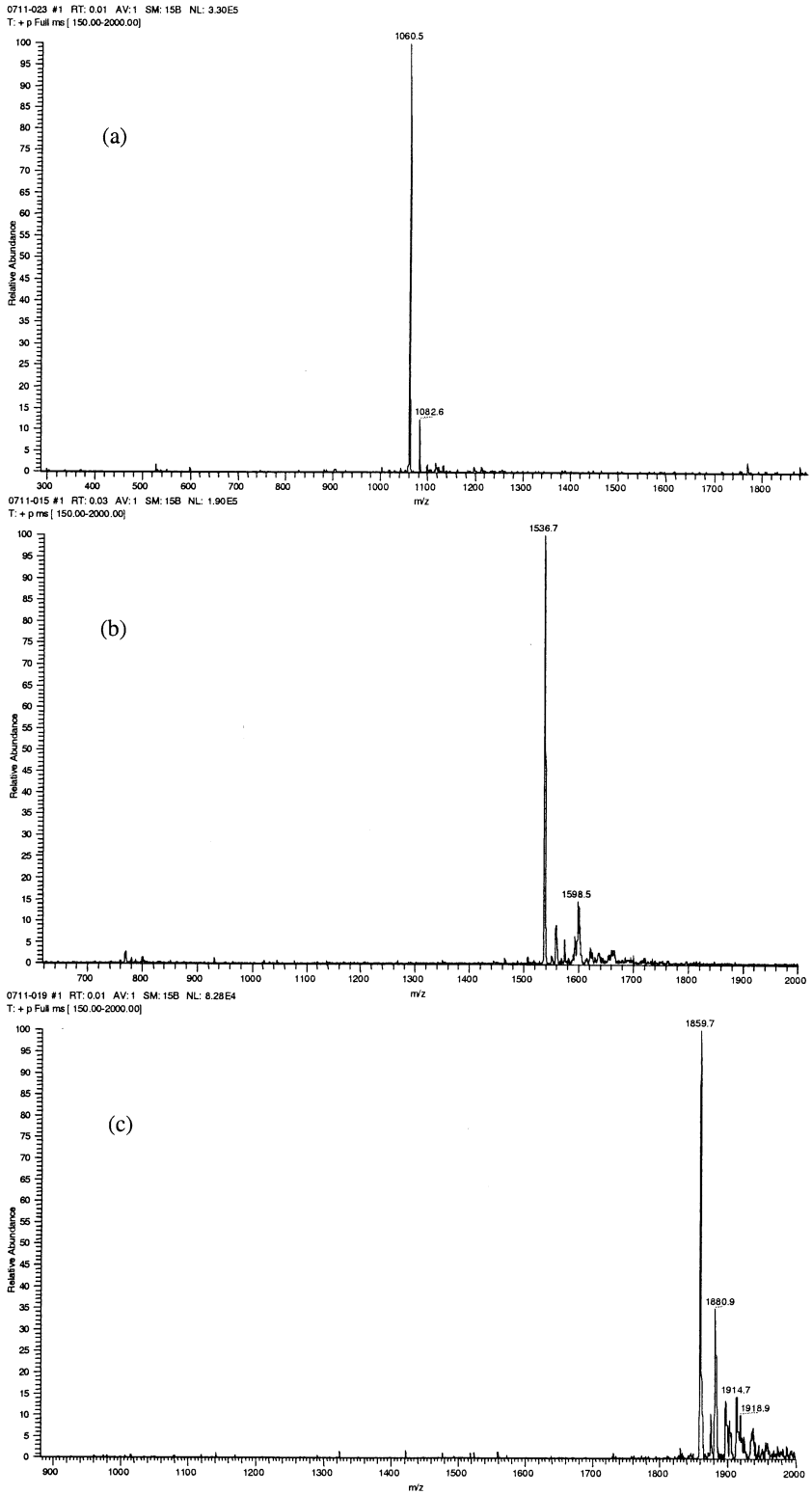

Figure 4. AP IR-MALDI mass spectra of peptides obtained using water as a matrix: (**a**) Bradykinin (FW 1060.2 Da); (**b**) Fibrinopeptide A (FW 1536.8 Da); (**c**) γ-Endorphin (FW 1859 Da). 10 pmol of each peptide loaded.

ble. This is quite different from AP MALDI experiments where crystalline matrices (such as CHCA and DHB) are used. In those experiments the sample (at a large level of laser energy) can be consumed during several laser shots (in both cases of atmospheric pressure UV- and IR-MALDI) forcing the user to constantly move the

target to have a stable ion signal. The extra time feature of the glycerol matrix can be fully exploited in the off-line experiments since it provides extra time for MS/MS analysis.

Use of Liquid Water as a Matrix in AP IR-MALDI Ion Source

The most striking result obtained with the IR laser is shown in Figure 3d where the AP IR-MALDI spectrum obtained using liquid water as a matrix is shown. To the best of our knowledge this is the first MALDI-MS spectrum obtained directly from a natural liquid solution of a biopolymer compound in the atmospheric environment. So far, ESI was the only choice for generation of ions directly from aqueous solutions. In a laser spray [31, 32], one of the numerous types of ESI sources, ions are generated using an explosive evaporation of effusing aqueous solution by focusing a 10.6 μm radiation from a CW CO_2 laser on a tip of the spraying capillary. This atmospheric method of ionization using a continuous laser results in formation of multiply-charged ions, like in normal ESI. As one can see from the data in Figure 3d the spectrum taken using water as a matrix looks like a true MALDI spectrum without any sign of multiply-charged ions. More spectra using water as a matrix are illustrated in Figure 4a–c where the spectra of Bradykinin (FW 1060.2 Da) (Figure 4a), Fibrinopeptide (FW 1536.8 Da) (Figure 4b), and γ-Endorphin (FW 1859 Da) (Figure 4c) are shown. As in any MALDI spectrum, only protonated and cationized molecular ions are observed mostly in the AP IR-MALDI spectra using water as a matrix.

The formation of ions using water as a matrix (as well as using glycerol as a matrix) takes place at conditions different from that typical for crystalline matrices. While ions from crystalline matrices can be generated from any point on the sample spot, the ion formation from water and glycerol samples in our experiments took place only at the droplet boundary area closest to the direction where the laser beam comes from (see Figure 1 for an illustration of the relative position of the droplet, the laser beam, and the capillary). Since other droplet areas did not generate ions and the laser energy threshold for water matrix (150 μJ/pulse) were close to the maximum energy supplied by our laser to the target (about 200 μJ/pulse), we explain this phenomenon by different laser energy thresholds for ion generation on different areas of the droplet due to its curvature. A typical droplet had about 2 mm diameter and 1 mm height. Location of the droplet on the vertical target surface did not significantly disturb its spherical shape. Because of this shape, the incidence angle for the laser beam interacting with the droplet will depend on the incidence area. The largest incidence angle, and thus the smallest laser energy threshold, should be observed for the boundary areas adjacent to the direction of the laser beam. We expect the area where ions can be formed to increase when we increase the laser energy in future experiments. Another interesting feature is a vibrational motion of droplets observed on the video monitor. With a frequency of 5 Hz used for operation of the IR laser, a heart-beating picture was clearly seen on the screen. Whether this phenomenon contributes to the ion formation is not yet clear.

Ionization of biologically-important species directly from the solutions opens new ways of analysis of biochemical processes under natural conditions, e.g., in cells, tissues, etc. This is in addition to the potential of using this technique for direct MS analysis of analyte solutions used in widely-utilized separation devices such as HPLC, CE, and even gel electrophoresis. In our preliminary test, we tested a 2:1 mixture of water-acetonitrile as a matrix and obtained spectra similar to those obtained with pure water (data not shown). Although this off-line experiment may be affected by fast evaporation of acetonitrile, it indicates the potential of using solutions other than pure water as a matrix.

Conclusions

A new AP IR-MALDI ion source using an IR laser operated at a 3 μm wavelength has been interfaced with a commercial LCQ ion trap MS instrument. In comparison to AP UV-MALDI, the AP IR-MALDI produces no analyte-matrix clusters. This result cannot be directly extended to the potential use of AP IR-MALDI on TOFMS instruments because of a much longer lifetime of ions in a QITMS (50–100 μs in the case of TOFMS and 100–1000 ms in QITMS). Cluster formation in an AP IR-MALDI-TOFMS needs further study.

The most striking result with an IR laser was obtained using water as a matrix. This is the first time that MALDI-MS spectra were obtained directly from a natural liquid solution of a biopolymer compound in the normal atmospheric environment. Although our preliminary study shows that AP IR-MALDI is at least as sensitive as AP UV-MALDI, this issue requires additional efforts and studies that will include (but is not limited to) the influence of experimental parameters (such as temperature of the inlet capillary and the wavelength and energy of the IR laser) on the ion signal. Improvements in sample preparation techniques can further increase the sensitivity. The issue of the AP MALDI mass range limit still remains open. So far AP MALDI has been interfaced with commercially-available ESI instruments which all have limited mass/charge range.

For a long time, ESI was the only choice for generation of biological ions directly from aqueous solutions. This study opens a way for using AP IR-MALDI for direct on-line coupling of liquid separation techniques such as HPLC and CE to a mass spectrometer. Use of glycerol as a matrix in AP IR-MALDI was shown to lead to very long times (many hours) of a stable ion signal that can be useful for off-line MS/MS analysis.

Acknowledgments

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