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Tip Enhanced Laser Ablation Sample Transfer for Biomolecule Mass Spectrometry

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Abstract

Atomic force microscope (AFM) tip enhanced laser ablation was used to transfer molecules from thin films to a suspended silver wire for off-line mass spectrometry using laser desorption ionization (LDI) and matrix-assisted laser desorption ionization (MALDI). An AFM with a 30 nm radius gold-coated silicon tip was used to image the sample and to hold the tip 15 nm from the surface for material removal using a 355 nm Nd:YAG laser. The ablated material was captured on a silver wire that was held 300 μ m vertically and 100 μ m horizontally from the tip. For the small molecules anthracene and rhodamine 6G, the wire was cut and affixed to a metal target using double-sided conductive tape and analyzed by LDI using a commercial laser desorption time-of-flight mass spectrometer. Approximately 100 fg of material was ablated from each of the 1 μ m ablation spots and transferred with approximately 3% efficiency. For larger polypeptide molecules angiotensin II and bovine insulin, the captured material was dissolved in saturated matrix solution and deposited on a target for MALDI analysis.

Introduction

Mass spectrometry imaging is a powerful technique that can be used to determine the distribution of specific biomolecules in tissue sections and their relative abundance, providing a mass-resolved image for each of the detected compounds [1-7]. In the current state of the art for imaging mass spectrometry it is possible to obtain mass spectra of large biomolecules such as peptides and proteins using matrix-assisted laser desorption ionization (MALDI) mass spectrometry imaging at a spatial resolution of 5 to 200 μ m and it is possible to obtain mass spectra of smaller molecules and atoms with spatial resolution less than 1 μ m using secondary ionization mass spectrometry (SIMS) and laser ablation inductively coupled mass spectrometry (LA-ICP MS). This micrometer size range is important because it is in the regime of single cells and the ability to perform mass spectrometry at this level can enable single cell imaging [8]. However, a generally applicable method for biomolecule sampling on this scale is not available.

The main technological impediment limiting the general application of mass spectrometry to submicrometer resolution imaging is the ability to transfer a sufficient quantity of material to the mass spectrometer and create enough ions for detection of the desired components. Mass

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spectrometry is highly sensitive, but samples must be ionized and delivered to the mass spectrometer with high efficiency for this level of performance to be realized. The general approach to mass spectrometry on small scales is to remove material from a small region using a focused laser or particle beam. The material is removed from the sample and ionized directly or transported to the mass spectrometer for ionization. The challenges in these sampling approaches are focusing the laser or particle beam on a sub-micrometer region of the target, transporting ions or neutrals to the ion source, and forming ions with high efficiency and without fragmentation.

The widespread use of laser-based biological mass spectrometry imaging began with the development of imaging matrix-assisted laser desorption/ionization in the late 1990s [9]. MALDI imaging involves the collection of mass spectra in a regular patterned array across an approximately 10 μm thick section that is deposited on a conductive microscope slide. Because MALDI creates ions with limited fragmentation, its use in imaging is applicable to a range of biomolecules from drugs and their metabolites to peptides and proteins [2, 5, 10-12]. The spatial resolution is approximately 50 μm and is limited by the spot size of the focused laser and ranges from 20 to 200 μm , although the detectable signal and throughput also must be considered [5]. The use of specialized optics can improve the spatial resolution further. For example, an objective lens with a central hole for ion transmission in a custom time-of-flight mass spectrometer achieved 1 μm spatial resolution for MALDI in vacuum [13]. A similar system adapted to a Fourier transform ion cyclotron resonance mass spectrometer achieved a lateral resolution of 500 nm, but required a diameter of 8 μm to produce sufficient ions for detection [14]. Modification of the optical configuration of commercial MALDI mass spectrometers can be used to achieve spatial resolution down to 5 μm [15, 16].

Secondary ion mass spectrometry (SIMS) imaging uses a focused ion beam to create ions with a spatial resolution less than a micrometer, but with a mass range below 1000 m/z [1, 17, 18]. SIMS can be used in a low beam intensity static mode or high beam intensity dynamic mode. Static SIMS is used for qualitative imaging whereas dynamic SIMS is used for quantitative elemental imaging and depth profiling. With its excellent spatial resolution, SIMS imaging can be used for the analysis of biomolecules at the sub-cellular level [1, 3, 8], and the spatial resolution for SIMS imaging can be as low as 50 nm for atomic and small molecular ions [19]. The use of cluster ions for the primary ion beam can improve the mass range, however, this comes at the expense of spatial resolution [4, 20].

The spatial resolution of MALDI imaging is ultimately restricted by the diffraction limit and typical optical configurations are far above this limit. However, near-field optics can be used to localize electromagnetic radiation below the diffraction limit, which is roughly half of the wavelength of the light [21]. Near-field desorption and ablation reduces the spatial resolution of laser-based mass spectrometry imaging to the micrometer size range and below and was first used for mass spectrometry of molecules using electron ionization [22]. Here, a reflective-coated optical fiber probe and a 349 or 355 nm UV laser is used to ablate material from a solid sample at a spatial resolution of 200 nm. Neutral molecules are sampled into vacuum through a capillary tube and directed into an electron ionization source of the mass spectrometer. The sampling efficiency for this approach is around .01% due to the 10%

transfer efficiency and 0.1% ionization efficiency [23]. Here, efficiency is defined as fraction of the solid material removed that is converted to detectable ions in the mass spectrometer. With this method, a 5 μm resolution was achieved for imaging of anthracene [24], limited by detection sensitivity. The desorbed material may not remain in the gas phase and much as 70% of the desorbed or ablated material may be redeposited close to the sample surface [23, 25].

Near-field laser ablation can be used to generate plasmas for atomic optical spectroscopy or mass spectrometry [7, 26]. Ablation can be achieved in aperture mode using a pulled optical fiber or in apertureless mode using a metal needle acts as an antenna for the electromagnetic radiation and the localized field at the AFM tip [27]. Apertureless laser ablation with a thin silver needle and 532 nm Nd:YAG laser has been used to sample material for inductively coupled plasma mass spectrometry (LA-ICP-MS) with sub-micron spatial resolution [28]. Although there is tip enhancement effect for the ablation, it is not clear that it is a near-field effect since the tip was ca. 100 nm from the surface [25]. We use the term “tip enhanced” to indicate that the process requires the presence of an AFM tip and may result either from near-field effects or other effects such as tip heating. A similar AFM tip-enhanced laser desorption ionization has been used for molecular imaging mass spectrometry [29]. Here, a 532 nm wavelength and 5 ns pulse width Nd:YAG laser was focused onto an atomic force microscope tip. The AFM tip was rastered across a pattern of rhodamine 6G dye and the molecular cation of the dye molecule was monitored with an ion trap mass spectrometer. A near-field LDI image was recorded at 2 μm spatial resolution that was limited by the limit of detection rather than the ability to minimize the ablation spot size.

The use of tip-enhanced or near field ablation for the detection of large biomolecules requires efficient coupling to a soft ionization method such as MALDI or electrospray. In particular, the method must be efficient at forming ions from clusters and small particles that are produced by near-field ablation [25]. One approach is to merge the ablated material with an electrospray source [30-32]. This technique has been used with a unique etched optical fiber configuration for infrared laser ablation imaging [33]. The spatial resolution observed was 20 μm and, although an optical fiber was used, ablation conditions were not in the near-field regime. An alternative method that does not require merging of the ablation plume with the electrospray is laser ablation sample transfer [34-37]. In this approach, the ablated material is captured in static or flowing solvent or on a solid surface, after which, MALDI or electrospray can be performed. Decoupling the sampling and ionization steps allows both to be optimized independently and has the potential for high efficiency sample utilization.

In the present work, we report tip enhanced laser ablation sample transfer using an atomic force microscope to position a conductive probe for UV laser ablation. A 355 nm pulsed laser was focused onto a gold-coated silicon AFM tip. The desorbed or ablated material was captured on a silver wire suspended above the AFM tip and the wire was removed for analysis directly by LDI or with the addition of a matrix for MALDI using a commercial time-of-flight mass spectrometer. Removal of material with submicron level spatial resolution, topographical images of the ablation craters, LDI, and MALDI detection of the captured material were demonstrated. The system is able to transfer amol quantities of molecules as large as peptides and small proteins with little to no fragmentation.

Experimental

A custom atomic force microscope (Anasys, Santa Barbara, CA) was used for sample imaging and tip enhanced laser ablation sample transfer. The system is based on a commercial system (afm+) that was modified to allow open access to the AFM stage but is otherwise identical. A gold-coated silicon probe (ACCESS-NC-GG, Applied Nanostructures, Mountain View, CA) with a radius of curvature of 30 nm was used for the ablation experiments. The nominal values of spring constant and resonant frequency were 78 N/m and 300 kHz, respectively. The probe geometry allows a direct optical path to the tip due to its 109° angle with respect to the cantilever axis.

The ablation laser is a 355 nm frequency tripled pulsed Nd:YAG laser (Powerlite 8000, Continuum, San Jose, CA) with a repetition rate of 10 Hz and 5 ns pulse width. The 355 nm wavelength was chosen for comparison to several of the previous near field ablation studies [22-25]. The polarization of the beam was adjusted using a half wave plate and polarization perpendicular to the tip axis was used in all of the experiments described below. Although optimum tip enhancement typically requires polarization parallel to the tip axis of a pointed probe [27, 38], the tip used in this work is a triangular pyramid that could favor enhancement at the pyramid edges, similar to what has been observed for triangular nanoprisms [39-42]. The beam was mildly focused using a 25 cm CaF₂ lens to irradiate the AFM probe at an angle approximately 80° from normal, nearly parallel with the laser table. The laser was directed toward the AFM tip at an angle of 45° in the horizontal plane and approximately 10° in the vertical plane. The focal point was approximately 4 cm beyond the probe and the resulting laser diameter where it intersected the tip was 600 μm. The efficiency of ablation was found to be highly dependent both on the laser focus and angle with respect to the AFM tip.

Tip-to-surface control for laser ablation was accomplished by engaging the tip in tapping mode with the sample at an amplitude set point of 1 V, where the amplitude sensitivity of the tip was 6-7 nm/V. The tip was operated in tapping mode with the tip-to-sample separation set to 15 nm, thus the tip was oscillating between 0 and 15 nm while being irradiated. Height control with a static tip was not possible with the current AFM control software. Ablation was performed using 30 laser shots over 3 s per ablation spot. The AFM conditions were the same for all of the material removal reported below. Figure 2 shows scanning electron microscopy images of AFM tips before use (Figure 2a) and after damage by irradiation (Figure 2b). The tips could be used for several days and approximately 1000 laser shots before tip breakage or removal of the gold coating rendered them ineffective at imaging or ablation.

The ablated material was captured on a silver wire with a diameter of 100 μm. The wire was flattened manually at the tip to produce a ribbon 400-500 μm in width. The wire was mounted on a three-axis mechanical stage and positioned above the AFM tip. The distance was viewed using the AFM video camera and the distance between the capture wire and the ablation spot was set to approximately 100 μm. The height of the wire above the sample surface was approximately 300 μm. For multiple spot collection, the wire was kept in the same position above the tip. A new wire was used for each set of collections.

The mass spectrometer used in this work is a MALDI TOF/TOF mass spectrometer (UltrafleXtreme, Bruker Corporation, Billerica, MA). The instrument was used in reflectron mode and mass spectra result from laser desorption ionization for anthracene and rhodamine 6G and MALDI for angiotensin II and insulin. After capture, the wire was removed from the positioning stage, cut to a 4 mm length and attached to a MALDI target using double-sided conductive tape. The laser spot diameter in the MALDI mass spectrometer was 100 μm and 500 shots were used to obtain the spectrum on each spot.

For MALDI mass spectrometry of angiotensin II and insulin, a saturated solution of α -cyano-4-hydroxycinnamic acid (CHCA, Sigma-Aldrich, St. Louis, Missouri) dissolved in a mixture of 85:15 ratio of acetonitrile (99.8%, EMD Chemicals, Gibbstown, New Jersey) and 0.1% trifluoroacetic acid (TFA, 99.0%, Fischer Scientific, Fairlawn, New Jersey) in water was used as the matrix. After capture, the silver wire was washed in 5 μl of the matrix solution and the matrix and extracted analyte was deposited on the MALDI target. The spectrum for angiotensin II was obtained in reflectron mode whereas linear mode was used for insulin. A 100 μm laser spot diameter and 500 shots were used to obtain the mass spectra in each spot. The reported spectra are a sum over the region of the deposit on the target where detectable signal was observed.

To prepare thin films for ablation, anthracene (99%, Sigma-Aldrich) was dissolved in acetonitrile (99.8%, EMD Chemicals) at a concentration of 1 mg/ml and a similar concentration solution of rhodamine 6G (99%, Sigma-Aldrich) was prepared in dichloromethane (99.9%, Sigma-Aldrich). Angiotensin II (Sigma-Aldrich) and bovine insulin (Sigma-Aldrich) were prepared in equal volumes of acetonitrile and 0.1% aqueous TFA. The thin film of the sample was prepared by slow evaporation of 3 drops of solution placed on glass cover slip.

Results and Discussion

Initial studies used tip enhanced transfer of material to a silver wire that was attached to a target for direct laser desorption ionization mass spectrometry analysis. The AFM system was first used to irradiate an anthracene thin film prepared from an acetonitrile solution. Figure 3a shows the AFM image of anthracene thin film before laser irradiation. The film roughness was found to be 3.4 nm, measured over the 1 μm area of Figure 3a. Material removal was performed by directing the laser onto the probe for 3 seconds with a repetition rate of 10 Hz at a fluence of 850 J/m² with the tip held 15 nm above the surface as described above. A representative image of the crater created using 30 laser shots is shown in Figure 3b. The spots were elongated in the dimension perpendicular to the laser beam with a dimension of *ca* 1 μm $\times$ 0.6 μm . The height profile of the crater along the line indicated in Figure 3b is shown in Figure 3c. The average depth of the ablation craters under the conditions used for Figure 3 was 200 nm and the average ablation spot area was 0.5 μm^2 . This corresponds to 40 fg of material removed from the thin film, assuming a conical crater. A 200 nm wide and 40 nm tall rim was formed around the anthracene ablation crater, which is attributed to the build up of molten ejecta.

Ablation craters for rhodamine 6G are shown in Figure 3d. The laser energy, focus, and number of shots were identical to that used to obtain Figure 3b. The rhodamine ablation craters were round with an average size of 900×650 nm. An image of a single crater is shown in Figure 3e and its depth profile is shown in Figure 3f. The average depth of the craters was 150 nm, which corresponds to 30 fg of material removed. The ejecta rim is 20 nm tall and 100 nm wide; the smaller crater rim may be related to the higher melting point of rhodamine 6G (290 °C) compared to anthracene (218 °C) and will also be affected by the film morphology and absorption of the two compounds.

The LDI mass spectrum of captured anthracene is shown in Figure 4. A total of 18 spots were irradiated under the conditions described above. Ablated anthracene from these spots was collected on a single flattened silver wire, which was cut to a length of 4 mm and mounted on a steel MALDI target with double-side conductive tape. Figure 4a shows the mass spectrum of the silver wire alone. The peaks at m/z of 107 and 109 arise from the abundant silver isotopes at approximately equal abundance. The peaks around m/z 216 and m/z 324 arise from Ag^{2+} and Ag^{3+} that are observed from direct irradiation of the silver surface, consistent with published results [43, 44]. Figure 4b shows the LDI mass spectrum dipped in a 1 mg/mL solution of anthracene in acetonitrile solvent. The $\text{M}^{+\bullet}$ peak for anthracene is observed at m/z 178. Figure 4c shows the mass spectrum of anthracene near field ablated and captured on the silver wire. Although the signal is two orders of magnitude lower, the anthracene peak is observed at m/z 178.

Mass spectra of rhodamine 6G are shown in Figure 5. Figure 5a shows a LDI mass spectrum obtained by dipping a silver wire in a 1 mg/mL dichloromethane solution of rhodamine. The prominent peaks at m/z 388, 415 and 444 are associated with rhodamine 6G LDI. The prompt fragmentation results from alkyl groups from the dye molecule and has been reported previously [45, 46]. Figure 5b shows the LDI mass spectrum of rhodamine 6G obtained by transfer from 18 ablation spots under the conditions reported for Figure 3. The mass spectrum has peaks at 388, 415 and 444 characteristic of rhodamine 6G, although the 388 peak is somewhat larger, suggesting that some fragmentation may have occurred during the ablation.

The efficiency of tip enhanced laser ablation sample transfer was estimated by comparison of the LDI signal of transferred rhodamine 6G with the signal obtained from a sample deposited from a solution of known concentration. To test the transfer efficiency, a 5 μL aliquot of rhodamine 6G from a 1 nM solution in dichloromethane was deposited on the MALDI target to form an approximately 2 mm diameter spot containing 5 fmol rhodamine 6G. LDI was performed exhaustively on the spot by irradiating each point on the deposit until the sample was depleted (several hundred laser shots) and the signal approached zero. The integrated signal for the base peak at m/z 444 was recorded and used as a calibration of signal per mol sample. The ablated rhodamine 6G spot dimensions were 900×650 nm and 150 nm in depth, yielding 70 amol material for a single conical crater. Comparison of the signal obtained from multiple ablated craters (for example Figure 5b) to the calibration gives a sample collection efficiency of 3% with an error of 0.5%. This is similar to the efficiency reported for droplet collection using far field ablation [36, 47].

We found that larger molecules could also be transferred without fragmentation using the tip-enhanced laser ablation sample transfer system. Here, the extra steps of matrix addition and deposition were necessary to detect the larger molecules. Figure 6 shows the ablation transfer of biomolecules for MALDI analysis. Figure 6a shows an AFM image of the laser-produced crater in a thin film of the peptide. The laser fluence was 1.1 kJ/m^2 and 30 shots were used to remove the material. The craters ranged from 500 to 800 nm deep with dimensions of 1100×650 , corresponding to 500 amol of peptide. Assuming a 3% transfer efficiency as determined above, this corresponds to 15 amol transferred to the wire. For the ablation of angiotensin II, the rim had a noticeable threefold rotational symmetry coincident with the edges of the triangular pyramid AFM tip, suggesting an edge enhancement of the electric field at these points (this can be observed to an extent in Figure 3, but is more pronounced here). The transferred material was dissolved in 5 μl of saturated CHCA in 1:1 acetonitrile and 0.1% TFA and deposited on the MALDI target for analysis. The mass spectrum in Figure 6b was obtained in positive ion reflectron mode. The most intense peak in the mass spectrum is protonated angiotensin $[M + H]^+$ with sodium and potassium adduct peaks visible to higher mass.

A linear mode MALDI mass spectrum of insulin is shown in Figure 6c. The material was transferred at a laser fluence of 1.1 kJ/m^2 and 30 shots from ten individual spots, dissolved in matrix, and deposited on a MALDI target for analysis as above. The base peak in the mass spectrum is protonated insulin with a doubly protonated peak also observed. Several other peaks are observed, for example at m/z 4305 and m/z 4820, and these are tentatively assigned to fragments of insulin. These peaks do not appear in MALDI spectra of an insulin standard obtained under comparable conditions and their intensity in relation to the insulin base peak varies from run to run. MALDI mass spectra of insulin standard obtained under comparable conditions do not contain fragments. The fragments were observed at the same m/z in multiple spectra, although the relative peak intensities varied slightly from spectrum to spectrum. A similar procedure was used to transfer the proteins cytochrome c, lysozyme, and myoglobin. Although comparable craters were formed with these samples, no high mass signal was detected in linear MALDI mass spectra with either CHCA or sinapinic acid matrix. This may be due to the limit of detection or to fragmentation of the larger proteins.

The tip enhanced material removal that we have observed may be a result of either local enhancement of the light field at the AFM tip or rapid heating of the tip with the thermal energy transferred to the sample. The observation that the optimum laser polarization is perpendicular to the tip axis rather than parallel is not consistent with a simple model of apertureless tip enhancement [27, 38], but as noted above, the triangular pyramid shape of the AFM tip may lead to near field enhancement at the pyramid edges with perpendicular polarization, similar to results from triangular nanoparticles [39–42]. The fact that the ablation crater is much larger than the tip radius is somewhat unexpected, but is consistent with field enhancement at the pyramidal tip edges. The 600 nm to 1 μm crater diameters are generally consistent with the crater sizes observed in previous studies using aperture [26] and apertureless tips [28]. The relative contributions of thermal and electric field effects remains an open question [48] and will be addressed in future studies.

Conclusions

Atomic force microscope tip enhanced laser ablation sample transfer to a suspended silver wire was demonstrated with direct off-line LDI MS of the collected material on the wire and MALDI MS of the material dissolved in matrix and deposited on target. When the AFM tip in contact with the sample is irradiated with a pulsed 355 nm ns pulse width Nd:YAG laser, craters between 600 nm and 1 μ m in diameter and between 150 and 800 nm deep are formed. The ablation craters are approximately ten times larger than the AFM tip diameter; however, relatively large craters have been observed previously in near-field laser ablation [25, 26]. Some craters have a three-fold symmetrical ablation and melting pattern, suggestive of enhanced material removal at the edges of the triangular pyramid AFM tip. This result is consistent with the observation that laser polarization perpendicular to the tip axis is optimum for material removal through a near-field process. However, it is possible that the material removal is driven by AFM tip heating and rapid heat transfer to the sample. We note that we have observed analyte melting under conditions that also result in the formation of intact protonated analyte molecules [49].

It is estimated that 3% of the material that is removed is redeposited on the suspended wire that was 300 μ m above the sample target. Previous studies have reported transport distances of 50 μ m or less for ablated material before it is deposited on the aperture probe [25]. Other studies have shown that jets of particulate are ejected to distances of more than 100 μ m [26]. It is possible that the angled AFM tip does not significantly obstruct the ablation plume so that a sufficient amount of material is transported to the capture wire. Alternatively, the wire may simply be capturing that fraction of the material that does not condense into particulate.

The largest molecule that was transferred intact was bovine insulin at 5733 Da. Some specific fragmentation was observed in the insulin mass spectrum that is most likely the result of the laser ablation sample transfer process. It was possible to ablate larger proteins, but these were not detected either due to fragmentation or insufficient sensitivity. Ongoing work is being directed at improving the amount of material collected and capturing material from cells and tissue. Because the capture is separate from analysis, tip enhanced capture of materials can be used with analysis methods other than mass spectrometry such as DNA/RNA sequencing and fluorescence assays.

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Figure 1.

Schematic representation of the experimental configuration for AFM tip-enhanced laser ablation sample transfer. The AFM tip was held 15 nm above the sample surface and the capture wire was displaced 100 μm from the probe and 300 nm above. The laser was at a 10° angle with respect to the plane of the sample.

Figure 2. SEM images of the AFM probe (a) before use and (b) damaged after use for laser ablation sample transfer.

Figure 3.

AFM images of thin film samples (a) anthracene before ablation and (b) after ablation, (c) depth profile. Rhodamine 6G ablation is shown for (d) four ablation craters, (e) crater with profile indicated, and (f) depth profile.

Figure 4. LDI mass spectra of (a) blank silver wire, (b) silver wire dipped in anthracene solution and (c) silver wire with tip-enhanced ablation transfer of anthracene.

Figure 5. LDI mass spectra of (a) silver wire dipped in rhodamine 6G solution and (b) tip enhanced laser ablation transfer from 18 spots.

Figure 6.

Tip-enhanced laser ablation sample transfer with MALDI: a) AFM image of an ablation crater of the angiotensin II peptide, b) MALDI mass spectrum of material transferred from a single angiotensin II spot, and c) MALDI mass spectrum of bovine insulin transferred from 10 spots.