

Matrix vapor deposition/recrystallization and dedicated spray preparation for high-resolution scanning microprobe matrix-assisted laser desorption/ionization imaging mass spectrometry (SMALDI-MS) of tissue and single cells

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Matrix preparation techniques such as air spraying or vapor deposition were investigated with respect to lateral migration, integration of analyte into matrix crystals and achievable lateral resolution for the purpose of high-resolution biological imaging. The accessible mass range was found to be beyond 5000 u with sufficient analytical sensitivity. Gas-assisted spraying methods (using oxygen-free gases) provide a good compromise between crystal integration of analyte and analyte migration within the sample. Controlling preparational parameters with this method, however, is difficult. Separation of the preparation procedure into two steps, instead, leads to an improved control of migration and incorporation. The first step is a dry vapor deposition of matrix onto the investigated sample. In a second step, incorporation of analyte into the matrix crystal is enhanced by a controlled recrystallization of matrix in a saturated water atmosphere. With this latter method an effective analytical resolution of 2 μm in the x and y direction was achieved for scanning microprobe matrix-assisted laser desorption/ionization imaging mass spectrometry (SMALDI-MS). Cultured A-498 cells of human renal carcinoma were successfully investigated by high-resolution MALDI imaging using the new preparation techniques. Copyright © 2010 John Wiley & Sons, Ltd.

Laser microprobe mass spectrometry (LMMS) has been a well-established method for micrometer-resolved chemical analysis of surfaces and biological samples for almost 30 years.^{1,2} In the 1980s several limitations of microprobe techniques were overcome. In particular, the sensitivity was improved by several orders of magnitude and an extension of the mass range to ≤ 1000 u allowed the characterization of organic molecules.^{3–5} The LMMS technique,^{6,7} however, is applicable to selected biological problems only. Successful LMMS analysis depends on the characteristics of the individual biological sample (e.g. spectral absorption, ionization energy or volatility^{8,9}) and, as a result, LMMS has never become a standard technique in biological analysis.

When matrix-assisted laser desorption/ionization (MALDI) was developed, this new method became the preferred choice for solid-state-based bioanalytical mass spectrometry.^{10,11} Over the past 20 years, MALDI-MS has routinely been used to analyze non-volatile substances

of higher molecular weight, such as peptides,¹² proteins,¹³ DNA,¹⁴ oligosaccharides,¹⁵ polymers¹⁶ or fullerenes.¹⁷

The investigation of biological material such as tissues, micro-dissected tissues or single cells recently came into focus in the life sciences. In such experiments, cell lysates or single cells are mixed with matrix and analyzed using MALDI-MS.^{18–20} Alternatively, tissues are blotted on a membrane and analyzed after matrix preparation.²¹ No time-consuming work-up is necessary for this direct characterization of biological material. Furthermore, the high tolerance of MALDI-MS to contaminants and detergents is a significant analytical advantage.

Secondary ion mass spectrometry (SIMS) has been used for the microprobe analysis of inorganic and organic substances.²² The focus size of the ion beam and thus the spatial resolution ranges from 50 nm to several μm .^{23,24} In contrast to MALDI-MS, where the sample ablation depth per analysis is in the range of several nm to 1 μm , the SIMS ablation depth is in the range of only a few monolayers per analysis. In order to obtain a sufficient number of analyte ions per sample spot and to keep sufficient mass resolving power, the ion beam diameter in SIMS analysis is mostly increased to considerably more than 50 nm. Due to the desorption/ionization process, the mass range of SIMS is limited to ≤ 1000 u. Matrix-enhanced SIMS (ME-SIMS) may

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partly overcome this limitation in the future, but its current sensitivity is still low.²⁵

Scanning near field microscopy (SNOM) is a method of achieving a laser spot smaller than the wavelength of light. With this method, an irradiated area of a few nanometers can be obtained for the desorption of ions.²⁶ The mass spectrometric sensitivity is, however, rather low and only small molecules can currently be detected.

MALDI imaging has been mostly employed with a lateral resolution of approximately 25 μm or more. The lower limit in spot size of these instruments has mainly been defined by the chosen matrix preparation method and by the optical design of the instrument. Structures in the dimension of cells cannot be resolved with such instruments.^{27–31} Combining LMMS and MALDI-imaging-MS has led to high spatial resolution imaging of biomolecular substances. Instrumentation for micrometer-resolved scanning microprobe MALDI (SMALDI)-MS and first experimental results have been reported by our group.^{32,33} Alternative instrumental approaches for atmospheric pressure (AP)-MALDI imaging at about 10 μm spatial resolution were developed recently.³⁴ At such level of lateral resolution, the application and crystallization of matrix material are becoming critical parameters with respect to the effective lateral resolution obtainable from the resulting distribution images.^{35,36} Various matrix preparation methods have been described.^{37–42} Vapor deposition was investigated by Hankin *et al.* but showed only poor sensitivity, due to a lack of incorporation of the analyte into the matrix.⁴³ A recrystallization step was suggested by Monroe *et al.*, without specifically focusing on the application to imaging mass spectrometry and this gave a lateral resolution of 50 μm only.⁴⁴ In an approach to improve this situation, we describe herein new dedicated preparation methods for high-resolution imaging such as nebulizing with a gas stream or vapor deposition/recrystallization.

EXPERIMENTAL

Instrumentation

The dedicated laboratory-built 'Lamma 2000' instrument was described earlier.³² The laser spot size on the sample for high-resolution distribution images was 0.7 μm at 337 nm wavelength of the nitrogen laser (STR337ND; LSI, Franklin, MA, USA). Concentration distributions of sample components were determined for multiple substance classes over the mass range up to 6200 u with this instrument.³⁵ For the following image analyses, areas of 100 \times 100 μm were scanned with a lateral resolution of 1 μm , and 10 000 mass spectra were acquired per imaging experiment. All mass spectra were stored and evaluated automatically and image formation was performed automatically by home-built software. Mass signals were selected manually or automatically prior to SMALDI analysis. All mass spectral images presented are either grey scale images or are converted into RGB overlay images using a common image processing program. The time-of-flight (TOF) instrument was operated in the linear mode with an acceleration voltage of 13 kV.

As an alternative approach another laboratory-built reflectron TOF instrument, 'Aladim II', was employed, featuring a reduced lateral resolution of approx. 10 μm .¹²

Optical imaging

Microscopy bright field images were acquired with a commercial microscope (BX41; Olympus, Hamburg, Germany) in reflection geometry.

Material

For the investigation of matrix preparation techniques, mixtures of three peptides were used, substance P (Sigma Aldrich, Buchs SG, Switzerland, M = 1346.73 u), melittin (Serva Electrophoresis, Heidelberg, Germany, M = 2844.75 u), and human insulin (Sigma Aldrich, M = 5803.64 u). Synthetic peptides 'K2' (NH₂-RKF_WLLMPAV-NH₂, M = 1258.73 u) and 'K2B' (biotinyl- ϵ -aminocaproyl-RKF_WLLMPAV-NH₂, M = 1597.63 u) were obtained from Institute of Biochemistry, RWTH Aachen, Aachen, Germany. Red dye coating prepared with a felt-tip pen was used as a standard sample.

To optimize the preparation procedure and the sensitivity, three of the most common matrices were tested; 2,5-dihydroxybenzoic acid (DHB, Fluka-Riedel de Haen, Buchs SG, Switzerland), α -cyano-4-hydroxycinnamic acid (CHCA, ACS Sigma-Aldrich) and sinapinic acid (SA, Fluka-Riedel de Haen).

Carcinoma cell lines (A-498⁴⁵) were obtained from the Center of Medical Science, University of Tübingen, Tübingen, Germany. Microstructured target surfaces were obtained by the Fraunhofer Institute for Interfacial Engineering and Biotechnology Stuttgart, Stuttgart, Germany.⁴⁶

Solvents of HPLC grade (acetone, ACS Merck, Nottingham, UK; isopropanol, Licosolv Merck; water, ACS Sigma-Aldrich; ethanol, Uvasol Merck; trifluoroacetic acid, Fluka-Riedel de Haen) were used to clean the target prior to preparation and as analyte solvents.

Preparational techniques

Pump spray

Various pumping spray flacons from standard personal hygiene products were used as preparation devices for matrix application. The spray flacons obviously differed in droplet size. Small flacons with a volume of approximately 20 mL were found to be optimal in terms of practical handling. Liquid was sucked in through the riser from the storage vessel by manual pressing, nebulized by a nozzle and sprayed onto the sample. The distance between nozzle and sample was varied over a wide range for optimization.

Nebulizer with gas stream

To obtain a finer and more homogeneous spray of the matrix droplets, a home-built nebulizer with a high gas flow at the withdrawal of the liquid was used. The matrix solution was taken up with a syringe (25 μL , 180 μm inner needle diameter; Hamilton) and screwed into a tapering glass tube. The tip of the syringe ended in the opening of the glass tube, whose diameter was approximately twice the diameter of the syringe needle. A second opening of the glass tube was used

as a propellant gas inlet. Air, nitrogen or helium was used as the nebulizing gas at a pressure of 500 hPa. The high gas flow at the tip of the syringe needle resulted in a very fine spray of the matrix solution. The matrix solution was sprayed at a distance of 2 cm perpendicular to the sample surface. The volume of a filled syringe was sufficient to spray for approximately 15 s and to moisten a surface of 1.5 cm².

Vapor deposition/recrystallization method

To minimize migration of analyte on the sample surface during matrix application the deposition of matrix was performed in a solvent-free procedure. A regular vapor deposition system (JEE-4B; Jeol, Tokyo, Japan) was used for matrix transport. A temperature controller was attached to the material reservoir during the matrix sublimation process. Heating of the reservoir was achieved by placing it in contact with two electrodes, establishing an electric current of up to 40 A. The pressure within the system was varied for optimization between atmospheric pressure and 1×10^{-3} Pa and was finally set to 100 Pa.

Matrix powder was sublimed under vacuum conditions in order to produce a thin matrix layer on the sample surface. A reservoir of dry matrix powder was placed 15 to 30 mm below the sample target, facing down towards the reservoir. The reservoir was heated slowly to 45–50°C at a pressure of 100 Pa. If the chosen temperature was too high, an explosive disintegration of matrix powder resulted in the inhomogeneous deposition of chunks of matrix on the sample. By keeping the matrix reservoir at a constant temperature for 5 min a thin layer of matrix was produced on the sample surface.

After deposition of a matrix layer the integration of the analyte from the surface into the layer crystals was found to be necessary for a successful SMALDI analysis. As a second preparation step, the layer was recrystallized by applying a saturated water atmosphere. The sample was positioned in a desiccator providing a humidified water atmosphere. A reservoir of water soaked up in a tissue paper was placed under the sample (0.5–1 mL, H₂O). To keep the saturated water atmosphere stable over 72 h the desiccator was sealed with a resin foil (Parafilm M; Roth, Karlsruhe, Germany). The desiccator was heated to a temperature of 65–85°C to recrystallize the deposited matrix material. After between 24 and 72 h, depending on temperature, no further recrystallization was visibly observed.

RESULTS AND DISCUSSION

In order to minimize the migration of biomolecular components being prepared in e.g. tissue samples, it is necessary to develop dedicated methods of matrix application. Methods for preparing a sample for high lateral resolution imaging are presented in the following.

In the case of SMALDI imaging one has to be concerned about micrometer migrations. Various methods of matrix application were tested, four of which are schematically shown in Fig. 1. The key to an optimized preparation is to find a feasible compromise between analyte incorporation into matrix crystals and minimized analyte migration. The standard dried-droplet preparation is known to result in an

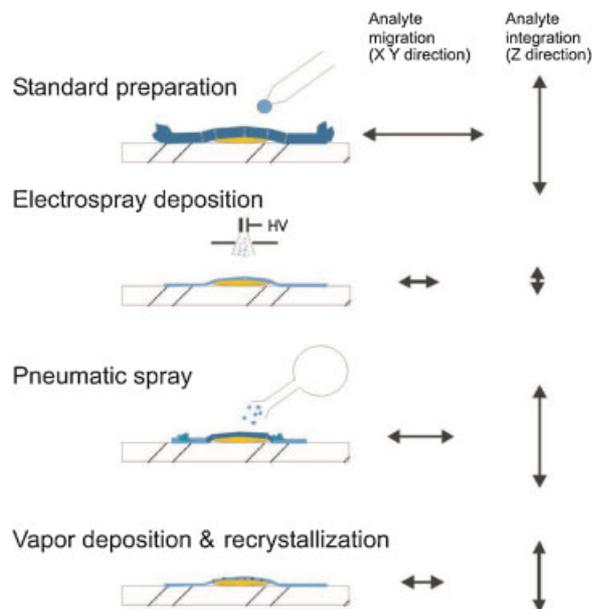


Figure 1. Methods of matrix application for SMALDI-MS.

effective incorporation of analyte into matrix crystals and in very high signal intensities. Migration of analytes within the liquid phase, however, was found to be strong. Consequently, the spatial information of the sample composition is lost with this technique. Electrospray deposition of matrix, on the other hand, resulted in minimized analyte migration if microdroplets were almost dry upon deposition, but analyte incorporation was found to be insufficient due to a strongly reduced liquid-phase interaction. We found a good compromise in the pneumatic spraying of matrix under optimized conditions, leading to sufficient analyte incorporation under reduced migration and high spatial resolution conditions. Controlling the optimal conditions, however, is difficult for pneumatic spraying. A more reproducible method was found by separating the preparation into two steps, deposition and recrystallization. The matrices were vapor-deposited onto sample surfaces using a modified standard vacuum deposition system. In a second step, the matrix-covered sample was processed in a humidified atmosphere under controlled conditions for recrystallization.

Pump spray experiments

For the matrix solution various solvent mixtures were used, such as ethanol, acetone and water in different mixing proportions.

It was found that a high proportion of water leads to very long drying times and visible turbulences during drying. Strong migration of the analyte is expected under such conditions. The best results were obtained with pure acetone or ethanol as solvents. Direct spraying onto the sample from a short distance (<10 cm) was found to be unfavorable. Due to the short distance a large quantity of liquid was applied to a small area of the sample surface, leading to a liquid droplet on the sample and strong turbulent migration.

The best homogeneous matrix layers were obtained by horizontal spraying in parallel to the sample surface. A distance of approximately 30–40 cm between nozzle and

sample center with the sample being positioned 10 cm below the spray axis was found optimal. In this configuration large droplets, which would moisten the sample too much, could not reach the sample surface but were caused to deviate by gravity. Figure 2 shows a sample sprayed five times with a DHB solution (20 mg/mL in ethanol). On the surface, regions with differently crystallized matrices are visible. The form and size of the crystals depend strongly on the size of the liquid droplets deposited on the sample. The upper right picture shows a magnification from an area where large droplets impinged on the surface. In this region the crystals had a size of approximately $20\ \mu\text{m} \times 5\ \mu\text{m}$ and showed a clearly three-dimensional structure. Between the crystals, matrix-free regions of approximately the same size were obtained, so that only 50% of the sample was actually covered by matrix. Such deposition conditions are clearly unfavorable for high-resolution imaging. The lower part of the sample was covered with smaller droplets showing different crystallization behavior. In the lower right picture of Fig. 2, the matrix was found to be homogeneously distributed. Drying and crystallization took place much faster here, than in the upper area. Due to the fast crystal growth no noticeable material transport from the liquid film into individual larger crystals was obtained. Uncovered gaps therefore are only in the range of $1\text{--}2\ \mu\text{m}$ in size, in this area. Furthermore, the crystals appear to be flat and have a maximum size of $10\ \mu\text{m} \times 5\ \mu\text{m}$.

Sinapinic acid (SA) and α -cyano-4-hydroxycinnamic acid (CHCA) were sprayed onto a gold-plated sample holder using the same procedure (data not shown). A saturated SA solution in ethanol was used. The resulting crystals were substantially smaller than with the DHB matrix. Again the distribution of matrix on the surface was strongly inhomogeneous. The matrix crystals had a size of approximately $5\ \mu\text{m}$ and they often formed as elevated heaps. Between the crystals, very large uncovered areas ($20\text{--}30\ \mu\text{m}$) were obtained. A similar situation was found for CHCA crystals after spraying with the pumping sprayer. Here, a matrix solution of 20 mg/mL CHCA in ethanol was used.

The mass spectrometric investigation of pump-sprayed matrix crystals did not exhibit differences from normal

dried-droplet preparations in spectral characteristics with any of the three matrices used. The preparation method, however, was found to be of limited reliability in routine applications. The large inhomogeneity produced by the different sizes of the droplets led to insufficient controllability of wetting, migration and crystallization on the surface. For imaging mass spectrometry in a lateral resolution range above $50\ \mu\text{m}$ this preparation method led to good results and it can be considered as a simple alternative. Uncontrollable droplet sizes, however, led to unquantifiable results, as known from regular dried-droplet preparations. For smaller laser focus diameters, on the other hand, the obtained gaps between the matrix crystals result in unusable mass spectra.

Nebulizer experiments

The distance between sample and nebulizer was found to considerably influence the crystallization behavior of the matrix on the surface. The formed droplets were smaller than the pump spray droplets, but they were already partly crystallized in flight, due to evaporation of the solvent, impinging on the sample as nanocrystalline dry matter. Integration of analyte into the matrix crystals is expected to be reduced under these conditions. Nanocrystalline layers were obtained with distances between nebulizer and surface of more than 40 mm, resulting in poor analyte sensitivity.

At a distance of approximately 20 mm to the nebulizer, however, a homogeneous microcrystalline matrix layer was formed on the sample surface. Microdroplets impinging on the surface still contained solvent under these conditions, allowing integration of analyte into the matrix crystals. Such layers led to a better signal intensity of the analyte. The matrix layer was obtained using a matrix solution of 20 mg/mL DHB in ethanol. The same kind of matrix layers was produced with SA and CHCA. While the matrix layers from CHCA looked similar to the DHB layers, the crystals generated with SA solution were significantly smaller. The SA layer, however, had a surface coverage of 70–80% only, with gaps in a size of $5\text{--}10\ \mu\text{m}$ between the crystals. The mass spectra of the matrix layers did not differ in quality from those in the normal dried-droplet preparations as long as the distance between nebulizer and sample surface was less than 30 mm.

Using air as a propellant, a yellow staining of the matrix layers was observed. This was interpreted as a degradation reaction of the matrix, similar to degradation of matrix solutions after long storage. Degradation is assumed to be promoted by atmospheric oxygen in the fine droplet fog. The function of the matrix can be impaired by oxidation, and oxidation of the analyte can also not be excluded. In addition, an increased occurrence of larger DHB clusters and an intensity reduction of analyte signals were observed in the spectra of these preparations (Fig. 3). This behavior is of interest not only for MALDI imaging techniques, but also for MALDI sample preparation in general. The use of air as propellant in spray preparation techniques should be completely avoided according to our findings.

Consequently, all the subsequent gas-nebulizing experiments were performed with nitrogen as a propellant gas, in order to avoid the degradation reactions described above.

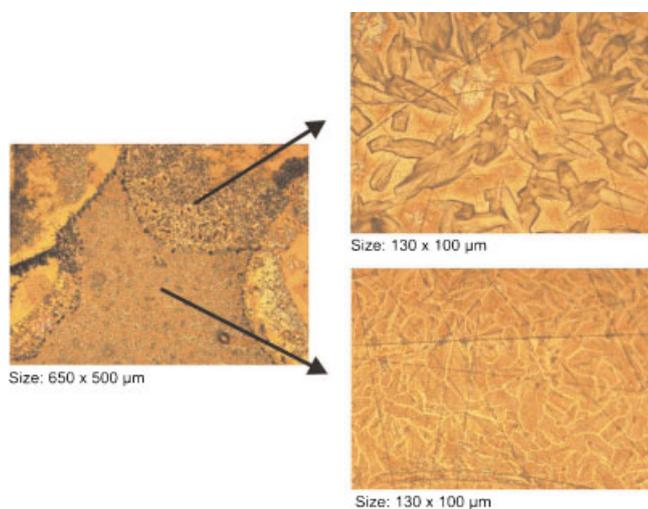


Figure 2. DHB crystals prepared by the pump spray method.

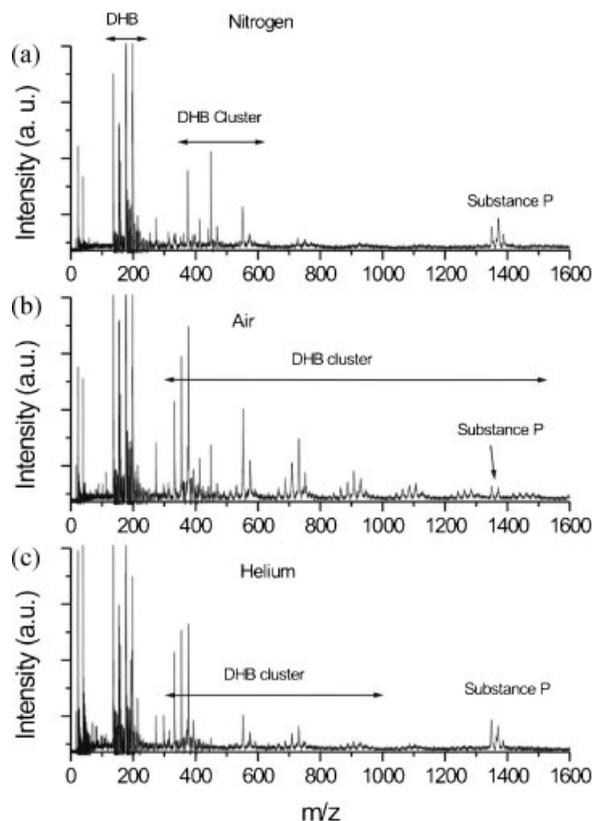


Figure 3. Formation of DHB clusters with different gases: (a) nitrogen, (b) air, and (c) helium.

Cultured single cells were scanned with a step size of $1\ \mu\text{m}$ to show the prospects of the nebulizing methods in SMALDI-MS. For this experiment human carcinoma cells of the cell line A-498 (diameter approx. $20\ \mu\text{m}$) were transferred onto a sample holder (1000 cells in $20\ \mu\text{L}$). The cells were incubated for 4 h at 37°C with 5% CO_2 for adhesion. Detergents and culture medium were eliminated from the sample using several washing steps with phosphate buffer (pH 7.4). Fixation of the cells was achieved by adding $100\ \mu\text{L}$ of acetone (Fig. 4, left image). In a last step the sample was covered with matrix using the nitrogen nebulizer spray method with DHB in 100% acetone (Fig. 4, right image). MALDI mass spectra were obtained with the Aladim II instrument in a lower mass range up to $700\ \text{u}$ (reflector mode) and in a higher mass range up to $16000\ \text{u}$ (linear mode) to characterize the cell surfaces (Fig. 5). Respective ion

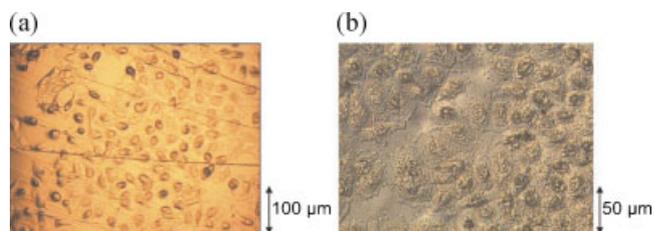


Figure 4. Optical Images of cells (a) prior to and (b) after preparation with a nebulized DHB layer. See text for further explanation. Image size was (a) $650\ \mu\text{m} \times 500\ \mu\text{m}$ and (b) $320\ \mu\text{m} \times 250\ \mu\text{m}$.

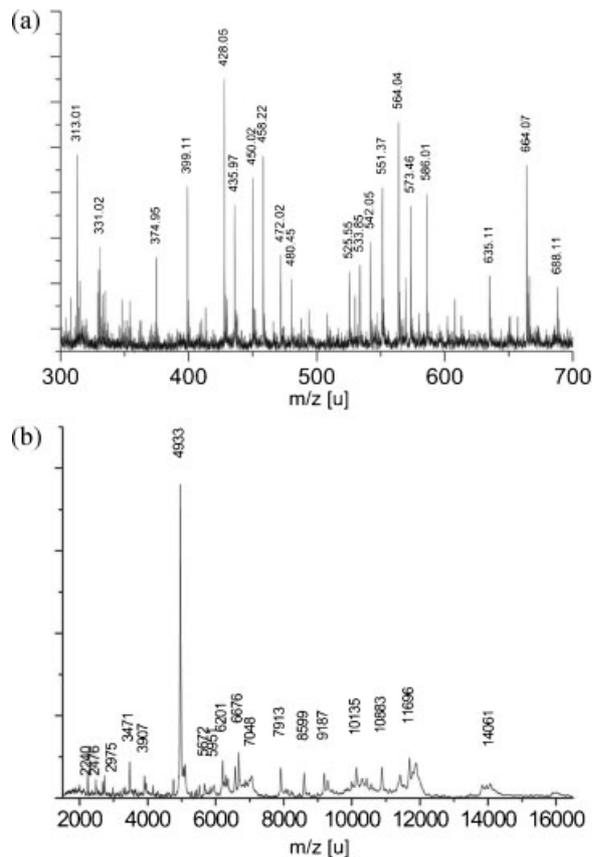


Figure 5. MALDI spectrum of carcinoma cells (A-498) in the low-mass range (a) and in the higher-mass range up to $16000\ \text{u}$ (b).

distribution images of single cells produced by (single shot per pixel) SMALDI mass spectra are shown in Fig. 6. Three example signals ($551\ \text{u}$, $564\ \text{u}$ and $4933\ \text{u}$) image the shapes of the cells and illustrate different ion distributions within and between cells ($551\ \text{u}$ and $564\ \text{u}$). By subtraction of pixel intensity data, visualization of the analytical information was improved. Subtraction of grey scale images indicated that the component with $m/z = 551\ \text{u}$ was localized in certain cells or cell compartments only, while the component with $4933\ \text{u}$ was equally dispersed over all cells. A number of cells were obviously damaged during the sample preparation procedure, limiting effective spatial resolution for these cells. This explains the observed unstructured distribution of certain other components.

Vapor deposition/recrystallization experiments

Another method of matrix application tested was sublimation of solid matrix and vapor deposition on the sample surface, as described. Three matrices, DHB (melting point at atmospheric pressure: $236\text{--}238^\circ\text{C}$), SA ($203\text{--}205^\circ\text{C}$) and CHCA (252°C), were used for these investigations. All three matrices were first examined for their stability at a temperature close to the melting point. First attempts at atmospheric pressure had already shown that it is not necessary to heat the matrices up to the melting point to obtain a matrix layer on the sample. Sufficient material sublimed at temperatures between 80°C and 130°C and formed a fine-crystalline layer on the sample (Fig. 7, right

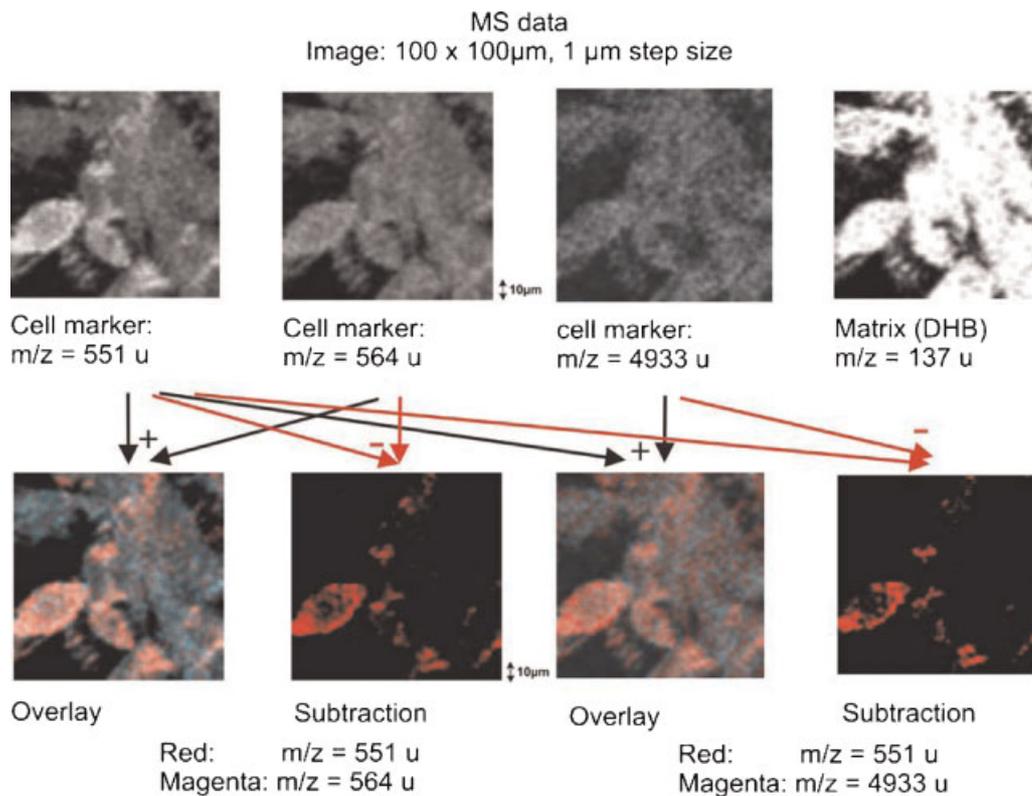


Figure 6. SMALDI images of human carcinoma cells (A-498) after DHB matrix preparation using a nitrogen gas-assisted nebulizer. A single laser pulse per pixel was used for mass spectral data acquisition.

image). The layers formed by careful heating were analyzed with the Lamma 2000 mass spectrometer to confirm their chemical stability. The first DHB layer was formed by heating the matrix to 130°C for 12 min under atmospheric pressure. Measurements of the matrix layer on the sample holder are shown in Fig. 8(a). Degradation products of DHB in the low-mass range and DHB clusters in the higher-mass range were obtained, different from MALDI spectra after the standard dried-droplet preparation. Further experiments showed that the formation of degradation products and matrix clusters can be

suppressed by sublimation of the matrix at a reduced pressure and concomitantly at a reduced temperature (Fig. 8(b)). A pressure of 1 hPa and a temperature of 48°C were found to be optimal for the sublimation of DHB. The sample holder was covered with matrix within 5 min under these conditions.

The other two matrices tested (SA and CHCA) showed a similar behavior. SA started to sublime under atmospheric pressure at approximately 90°C and with reduced pressure of 1 hPa at 45°C. CHCA sublimed at approximately 110°C under atmospheric pressure and at 53°C at a pressure of

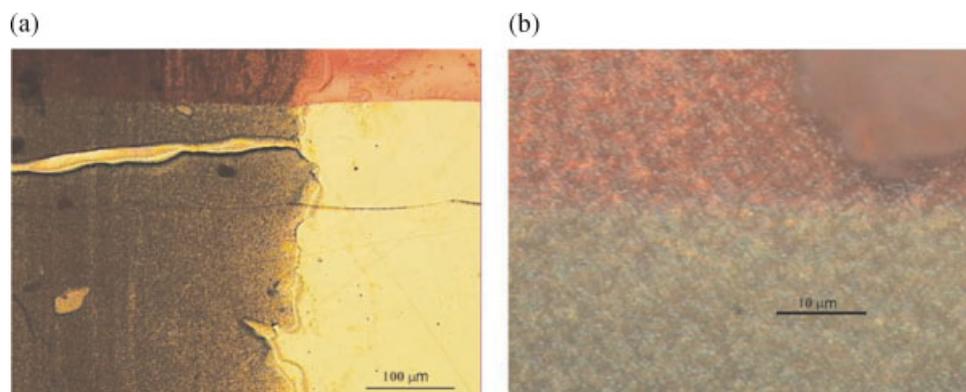


Figure 7. (a) Optical image of a gold-plated sample holder with dye from a felt-tip pen (upper part) and DHB matrix deposition (left part). (b) Magnification (10 \times) of the edge of the felt-tip pen coating.

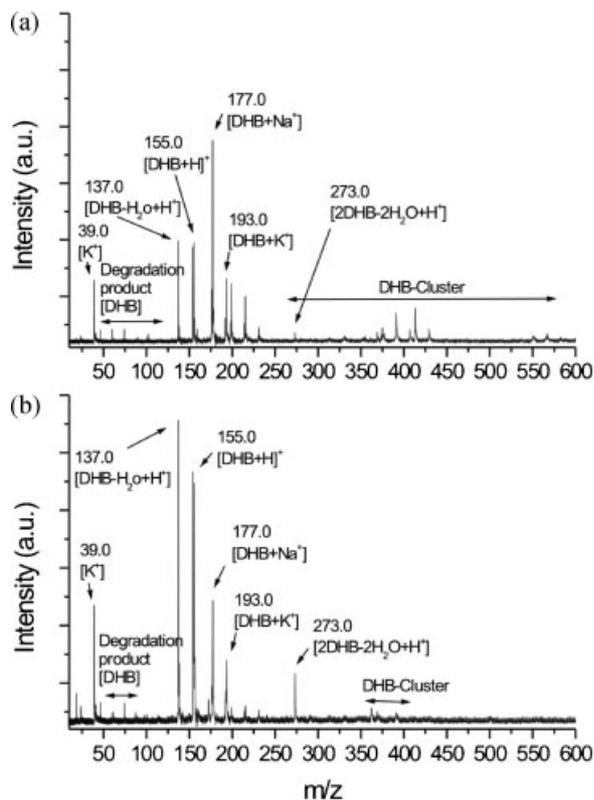


Figure 8. Mass spectra of vapor-deposited matrix (DHB) sublimed at (a) 130°C and (b) 48°C.

1 hPa. The mass spectra also showed degradation products and matrix clusters at atmospheric pressure.

Matrix layers deposited on the sample holder this way were found to be unstable against mechanical stress (Fig. 7(a)). Irradiation with the high-focused laser beam was sufficient to eject larger chunks from the layer. This was predominant with SA and CHCA, but also observed to some extent when using DHB.

With a laser energy close to the threshold of ion detection, the mechanical damage of the DHB layers could be reduced and measurements with a laser focus diameter of 1 μm were possible (data not shown). The sharp border between the red dye of a felt-tip pen coating and the gold surface remained after vapor deposition of the matrix and showed migration of less than 1 μm in the mass spectral images. The produced matrix layers were examined with respect to their quality in measuring MALDI mass spectra from the surface. Therefore, a mixture of three peptides (substance P, melittin and human insulin) was applied to the sample holder prior to matrix vaporization. The components were diluted in ethanol/water (1:1) with a concentration of 1×10^{-5} mol/L and mixed in the molar ratio of 1:2:4 (substance P/melittin/human insulin). This mixture was tested under normal dried-droplet MALDI preparation conditions and was found to result in high signal intensities at 1 μm laser focus beam. For the matrix vaporization test, 1 μL of the peptide mixture was applied to the sample holder and dried. After approximately 10 min the droplet had completely dried, so that

vaporization with the matrix could be accomplished. After preparation with matrix, the mass spectra showed only very small signal intensities of the three substances at increased laser irradiances. With a laser irradiance typically used for MALDI measurements, no mass signals of the peptides were detected. Only by summation of at least 200–400 single spectra at several sample positions and increased laser irradiance, did the weak mass signals become recognizable. At higher irradiance, degradation products and matrix clusters were observed. The low intensity of the peptide signals can be explained by an incomplete incorporation of analyte in the matrix nanocrystals. Incorporation of the analyte is not an absolute prerequisite for MALDI but strongly increases signal intensities.^{47,48} Due to the small laser focus in our experiment, parallel desorption of matrix and analyte is rather unlikely, if incorporation is missing. With consecutive laser pulses applied to the same sample position, spectra were found to vary. With the first laser pulses only matrix signals were obtained. After 5 to 10 laser pulses analyte signals became visible in the mass spectra, for only 2 to 3 additional pulses. This behavior documents an insufficient mixing of matrix and analyte layers. Only when the vaporized matrix layer was almost ablated, analyte and matrix were desorbed in parallel for a few laser pulses. After that, the sample was used up completely.

Reduction of the matrix layer thickness to less than 1 μm was not applicable in this configuration, because matrix layers evaporated after only a few minutes under high-vacuum conditions in the instrument. Imaging analysis could not be performed with these layers since the total measurement period was in the range of 10–90 min. Such layers might be feasible for atmospheric pressure ion sources, however, where no major evaporation of matrix takes place.³⁴

Figure 9 shows that the matrix is chemically intact after sublimation and vapor deposition. The produced matrix layers covering the deposited peptides were re-dissolved

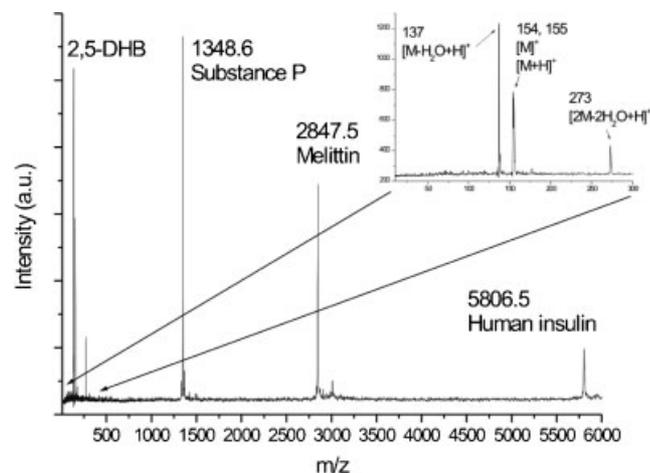


Figure 9. MALDI mass spectrum of a peptide mixture covered with vapor-deposited matrix DHB and subsequently re-dissolved by 1 mL ethanol/water solvent.

with 1 μL ethanol/water (1:1) and dried by a warm air flow. The generated matrix/analyte crystals resulted in a normal MALDI spectrum typical for dried-droplet preparations, with a signal-to-noise ratio increased by two orders of magnitude compared with analysis of the layered sample. No matrix clusters or degradation products were observed in this case (Fig. 9, inset).

From these results it was concluded that a localized re-dissolution of the matrix/analyte layers after vapor deposition could be an advantageous strategy to increase signal intensities. The matrix preparation procedure was thus separated into two steps, a matrix deposition step and a matrix/analyte integration step. Confinement of the re-dissolution/integration process to the size of the aimed lateral resolution was achieved by exposing the sample to a humid environment, in order to induce recrystallization.

The vaporized samples were put into a closed glass box as described in the Experimental section. First attempts were accomplished with 1 mL distilled water and a temperature of 65°C. Later studies were accomplished with 0.5 mL water and temperatures up to 85°C. Reducing the quantity of humidifying water limits condensation under the lid of the box and thus limits the problem of falling droplets. In the first attempts a recognizable recrystallization of the matrix layers was observed after 2–3 days of incubation (Fig. 10(b)). While DHB exhibited optically obvious changes, the visible effects for SA and CHCA were rather small. The mechanical stability of the DHB matrix layers also improved, depending on the degree of recrystallization. The incubation of SA and CHCA layers led to a mechanical destabilization of the layers instead.

The incubated DHB layers recrystallized substantially faster than SA or CHCA. After a few hours, first changes in the DHB layer became visible. After 14 days the process was complete. The different behavior of the three matrices is probably caused by their different water solubility. While SA and CHCA are poorly water-soluble, DHB has a high solubility in water.

The crystallization process could be accelerated by increasing the temperature of the saturated atmosphere. With a temperature of 85°C sufficient recrystallization was obtained after 24 h. After 3 days no further change in the sample surface appearance was observed. Shorter incubation times will become possible by optimizing physicochemical parameters such as temperature gradients, pressure, and the solvents used. This will be investigated in future studies in order to prevent chemical or biological changes in the sample during the recrystallization step. Another test demonstrated that peptides can be prepared with a high lateral resolution using the described sublimation/recrystallization procedure (Figs. 11 and 12). A volume of 1 μL of a 1×10^{-5} mol/L solution of substance P was deposited and dried on a gold-plated sample holder which had been coated previously with a diluted red dye. Afterwards the sample was vapor-deposited for 5 min with DHB as described above (48°C, 100 Pa; incubated at 85°C for 24 h). The border of the deposited droplet was still recognized clearly after incubation of the sample with water (Fig. 11).

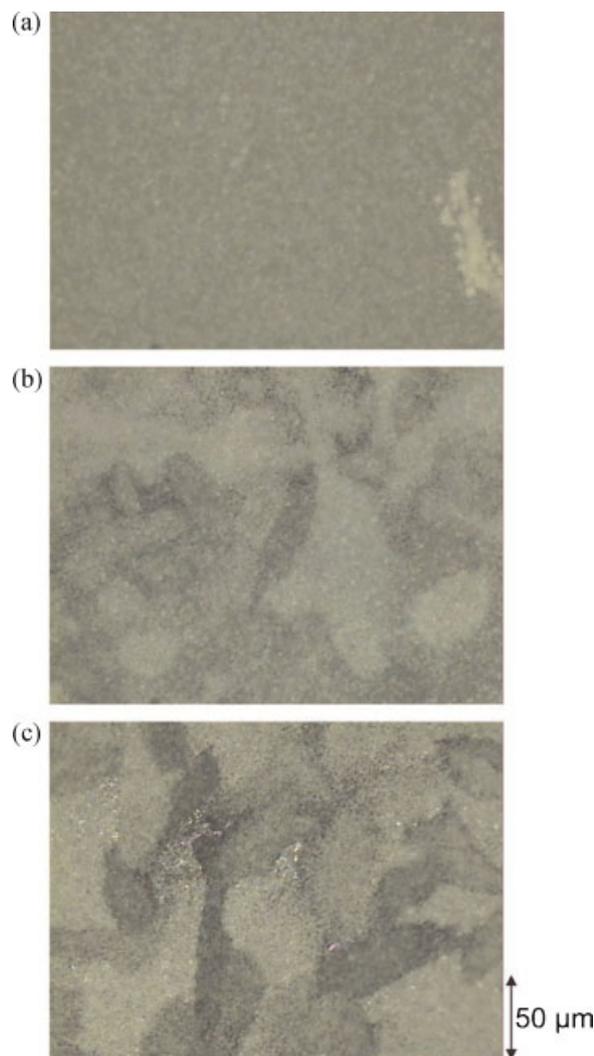


Figure 10. Incubation of a vapor-deposited DHB layer with water. Optical images taken (a) prior to incubation, (b) 3 days after incubation, and (c) 14 days after incubation.

Furthermore, recrystallization of the matrix was visibly observed.

Figure 12 shows that an effective lateral resolution of approximately 2 μm was achieved with the described preparation method by SMALDI-MS. Two consecutive measurement scans from the same sample position were recorded. An area of 100 $\mu\text{m} \times 100 \mu\text{m}$ was scanned with a step size of 1 μm . In each case one laser pulse per step was applied to the sample and one mass spectrum per step was measured. Three mass signals were imaged in Fig. 12. The upper row shows the mass signal of the DHB layer ($[2\text{DHB}-2\text{H}_2\text{O}+\text{H}]^+ = 273 \text{ u}$). The middle row represents the red dye coating on the bottom of the sample ($[\text{M}+\text{H}]^+ = 450 \text{ u}$). The bottom row shows the signal of the peptide substance P ($[\text{M}+\text{H}]^+_{\text{avg}} = 1348.6 \text{ u}$). The distribution of the components shows that the matrix and the red dye were homogeneously distributed, in contrast to the substance P image which shows the edge of the analyte deposit. The signal gradient at the edge of the analyte deposit was found to be approximately 2 μm wide. Signal intensities obtained

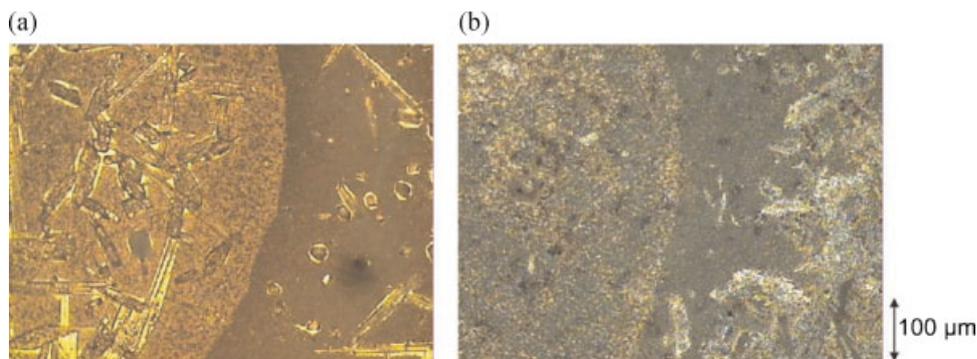


Figure 11. Analyte deposit covered with a DHB layer. Optical image taken (a) before and (b) after incubation with a water atmosphere. Analyte was 1 μL of a 1×10^{-5} mol/L solution of substance P in water/ethanol (1:1), dried in air prior to matrix deposition.

after vapor deposition/recrystallization were considerably higher than those obtained with the spraying method and at least one order of magnitude higher than with matrix vapor deposition alone. The second scan of the same sample position shows that the thin matrix layer is already partly ablated after the first scan. Effective lateral resolution is obviously retained, demonstrating that laser ablation by the first scan does not cause additional migration or sample relocation.

A structured silica target was used to demonstrate the quality of the preparation method. Hydrophobic spots of 120 μm were created. The target was then

incubated with a mixture of peptides (hydrophobic and hydrophilic peptides) for 10 min and washed with 0.05% Triton X-100, 15 times with 30 mM Tris-HCl and 3 times with 3 mM Tris-HCl to remove unbound peptides. The sample was then prepared by vapor deposition and recrystallization as described before. An area of 100 $\mu\text{m} \times 100 \mu\text{m}$ was scanned by SMALDI-MS with 1 μm step size and a single laser shot per step. In Fig. 13 an optical image of the scanned area shows the excavated parts of the spots. The matrix layer was removed by the laser beam. The two hydrophobic peptides K2 and K2B ($[\text{M}+\text{H}]^+ = 1259.6$ u and 1598.8 u) were bound to the spots, revealed by the resulting ion distribution images, in contrast to the hydrophilic peptides removed by the washing procedure. For these peptides no mass signal could be generated.

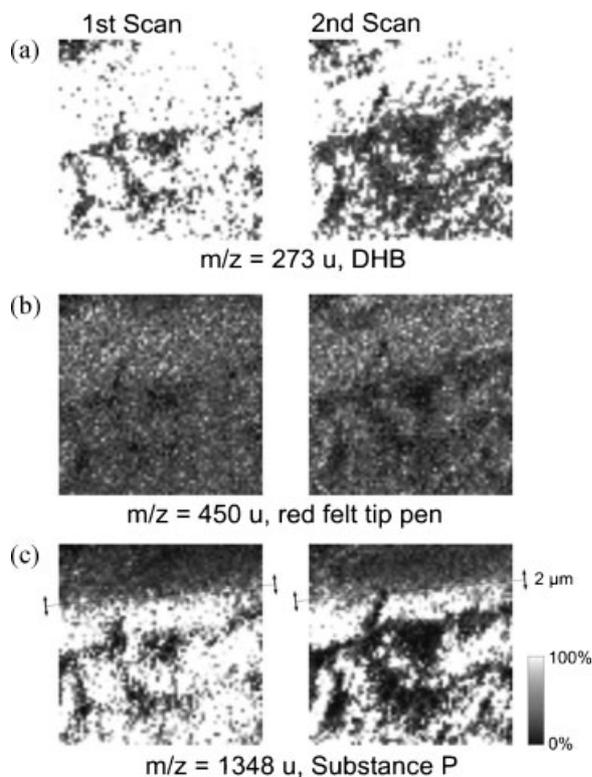


Figure 12. SMALDI-MS ion distribution images taken from the edge of the analyte deposit of Fig. 11 (scanning area 100 $\mu\text{m} \times 100 \mu\text{m}$, 1 μm step size).

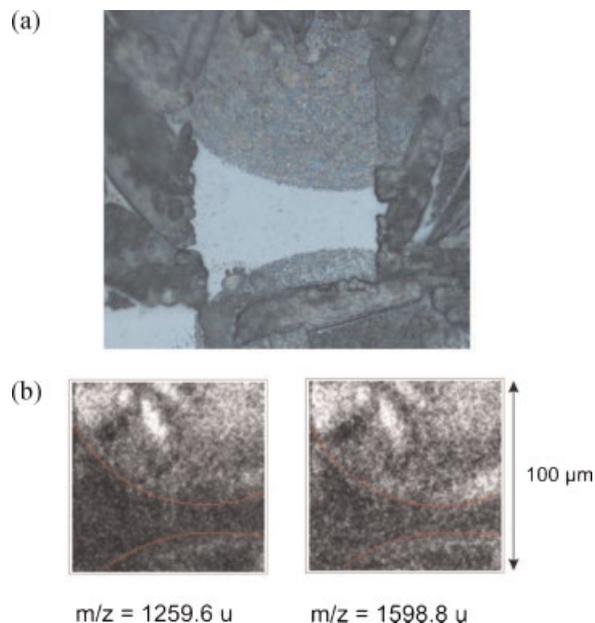


Figure 13. (a) Optical image of structured silica target scanned by SMALDI-MS. (b) Two SMALDI-MS ion distribution images of the scanned area of hydrophobic peptides K2 ($[\text{M}+\text{H}]^+ = 1259.6$ u) and K2B ($[\text{M}+\text{H}]^+ = 1598.8$ u).

CONCLUSIONS

The results reported above demonstrate properties of different matrix preparation methods for complex biological or synthetic surfaces. The pump spray method was found to be useful in the laser focus range above 50 μm . With such lateral resolution, the crystal size, inhomogeneities of the matrix, and analyte migration effects play a minor role in obtaining mass spectrometric images of biological samples. For high-resolution imaging mass spectrometry the pump spray method was found to be insufficient. The nebulizer method gave better results in terms of homogeneity and effective lateral resolution, but the signal intensities were only average due to insufficient incorporation of analyte into matrix crystals. Degradation reactions were another limitation when reactive propellant gases were used. The first SMALDI-MS results demonstrated here for the case of cultured cancer cells show very promising results for high-resolution imaging MS on the cellular and subcellular level. Vapor deposition/recrystallization of the matrix resulted in the highest effective lateral resolution with high signal intensities. Separation of matrix preparation into two independent steps led to a superior controllability of preparation conditions and significantly improved sensitivity and spatial resolution. A reduction in necessary recrystallization time has to be developed to avoid chemical or biological changes in the sample. Possible influences of matrix preparation on biological sample integrity will be investigated and optimization of the preparation procedure will lead to a broader applicability in the future. At the current status of the described method a lateral analytical resolution of 2 μm is available for high-resolution SMALDI imaging mass spectrometry.

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