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Dried-droplet probe preparation on AnchorChip™ targets for navigating the acquisition of matrix-assisted laser desorption/ionization time-of-flight spectra by fluorescence of matrix/analyte crystals

Henrik Thomas, Jan Havliš†, Jan Peychl and Andrej Shevchenko*
Max Planck Institute of Molecular Cell Biology and Genetics, Pfotenhauerstrasse 108, 01307 Dresden, Germany

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We have developed a dried-droplet probe preparation method for peptide mass fingerprinting by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS), which uses AnchorChip™ targets and α-cyano-4-hydroxycinnamic acid (CHCA) as a matrix. Upon drying of a matrix and analyte mixture on the AnchorChip, salts and low molecular weight contaminants were pooled at the hydrophilic metal anchor, whereas 10–50 μm matrix/peptide crystals firmly adhered at the surface of a hydrophobic polymer and the entire target could be subsequently washed by submerging it in 5% formic acid for 2–3 min. Epifluorescence microscopy suggested that peptides were completely co-localized with CHCA crystals at the AnchorChip surface. Fluorescent images of the probes were of good contrast and were background-free, compared with images taken by a video camera built into the ion source. CHCA/peptide crystals were easy to recognize at the surface and peptide mass maps were acquired from them without further adjustment of the position of the laser beam. These crystals were remarkably stable towards the laser depletion and almost no matrix-related ions were typically observed in the low *m/z* region of peptide mass maps. The sensitivity of the peptide mass mapping was at the low-femtomole level.

Identification of proteins by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry has become a cornerstone method in proteomics (reviewed in[1,2]). Recent developments in tandem mass spectrometry technology have enabled in-depth MS/MS investigation of peptide peaks using a variety of instruments, such as MALDI TOF/TOF,[3,4] MALDI QqTOF,[5,6] or MALDI ion trap[7,8] mass spectrometers. However, peptide mass mapping remains a hallmark strategy in MALDI-based proteomics because of its unmatched throughput, low femtomole to attomole sensitivity, relative simplicity of the instrumentation and sample preparation, and remarkable tolerance towards common low molecular weight contaminants. As in any protein identification method, mining a database with peptide mass fingerprints is a statistical process, in which the significance of hits depends on the number of detected peptides and on the accuracy with which their *m/z* values were determined.[9,10] Although a variety of methods for the preparation of MALDI probes has been developed (reviewed in[11]), further improvements in the sensitivity, speed, tolerance to impurities and sequence coverage of MALDI peptide maps remain the focus of persistent research efforts.

In dried-droplet probe preparations,[12] aliquots of the sample and the matrix solution are mixed and deposited onto the surface of a stainless steel target. Co-crystallization of the matrix and the analyte occurs during the evaporation of a volatile organic solvent, and low molecular weight contaminants such as salts are largely excluded from the analyte/matrix crystals. Although these crystals can be rinsed gently with cold dilute trifluoroacetic acid (TFA), the efficiency of de-salting is usually low. The method is rather simple and lends itself to complete automation, although at the femtomole level the sensitivity and the reproducibility of detected signals are poor. In thin layer probe preparations[13] a matrix is first deposited on a stainless steel support and then an acidified aliquot of the analyte is placed on top of a preformed matrix layer and allowed to co-crystallize with the matrix. An exposed layer of peptide/matrix crystals can be washed without affecting the quality of TOF spectra. Doping CHCA matrix with nitrocellulose[14] improves the sensitivity by reducing peptide losses that occur during the washing step. The method provides good sensitivity and resolution;

**Correspondence to*: A. Shevchenko, Max Planck Institute of Molecular Cell Biology and Genetics, Pfotenhauerstrasse 108, 01307 Dresden, Germany.
E-mail: shevchenko@mpi-cbg.de
†On leave from Masaryk University, Faculty of Science, Department of Analytical Chemistry, Kotlářská 2, CZ-611 37 Brno, Czech Republic.
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however, it is rather laborious and difficult to automate. Furthermore, so called 'sweet spots' (patches of matrix/analyte crystals from which high quality spectra can be acquired) are randomly distributed at the surface of the target, and are difficult to recognize using a video camera built into the ion source. Thin layer crystals are quickly depleted by laser pulses, and accurate adjustment of the laser fluence is required during the acquisition of spectra.[15]

Relatively large volumes of peptide solutions can be desalted and concentrated on self-made reversed-phase microcolumns[16] or on ZipTip™ tips,[17] and then eluted with a few microliters of the matrix solution directly onto a MALDI target. The procedure enables extensive washing since reversed-phase beads trap peptides much more efficiently than matrix crystals, although loss of peptides during the clean-up step has also been reported.[18]

It would therefore be advantageous to either trap peptides by reversed-phase beads[19] directly on a stainless steel target, or use pre-structured targets with a hydrophobic chemically inert polymer support (reviewed in[11]). AnchorChip™ targets marketed by Bruker Daltonics consist of 200 to 800 μm circular gold granules regularly arrayed on the surface of a stainless steel target covered by a strong water-repelling polymer.[20] According to the original dried-droplet recipe for use with these targets, protein digests are first cleaned up on a ZipTip and then are eluted directly onto the hydrophilic metal anchor with a solution of 2,5-dihydroxybenzoic acid (2,5-DHB). Evaporation of the organic solvent concentrates the matrix/analyte mixture at the metal anchor (Table 1). Alternatively, a layer of α-cyano-4-hydroxycinnamic acid (CHCA) can be pre-formed on the anchor and peptides are directly adsorbed onto this layer from acidified aqueous solutions[21] (Table 1). No ZipTip clean-up is required in this case, and the use of CHCA matrix increases the sensitivity of peptide detection and the spot-to-spot reproducibility of TOF spectra.

We note, however, that in both AnchorChip-based methods (Table 1) the hydrophobic surface is not actually used to trap peptides, but rather assists in concentrating droplets of the analyte/matrix mixture at the hydrophilic anchor. However, adsorption of peptides on hydrophobic surfaces promotes matrix/analyte co-crystallization and increases the sensitivity of MALDI analysis.[18,22] Furthermore, laser ablation from polymer surfaces increases the yield of positive ions, as it is thought that no photoelectrons would be emitted from these targets by a laser beam.[23]

In this paper we present an alternative method for the probe preparation on AnchorChip targets. The composition of the matrix solution and the sample buffer was adjusted in such a way that the bulk of the peptide material was retained and co-crystallized with the CHCA matrix at the surface of a hydrophobic polymer, while salts and other hydrophilic impurities were pooled at the hydrophilic metal anchor. The peptide/matrix crystals firmly adhered to the surface, and even very extensive washing did not affect the quality of subsequently acquired MALDI-TOF mass spectra, while the sensitivity of peptide detection remained at the low-femtomole level.

Table 1. Overview of sample preparation methods for AnchorChip™ targets

Method	Dried-droplet[20]	Thin layer[21]	Dried-droplet (this study)
Matrix	DHB	CHCA	CHCA
Desalting	ZipTip	On target	On target
Peptides localized	At the anchor	At the anchor	Mainly at the polymer surface
Washing	No	Yes	Yes

EXPERIMENTAL

Materials and reagents

Unless otherwise noted, all chemicals were purchased from Sigma Chemicals (St. Louis, MO, USA) and were of analytical grade. Porcine trypsin modified by reductive methylation was purchased from Promega (sequencing grade modified trypsin, cat. no. V5111, Mannheim, Germany). α-Cyano-4-hydroxycinnamic acid, the matrix for preparing MALDI probes, was purchased from Bruker Daltonik GmbH (Bremen, Germany). A fluorescent peptide, (TAMRA)-KKKPKCK (TAMRA denotes 6-tetramethylrhodamine), purified by HPLC, was purchased from the Bioanalytics and Synthesis laboratory, NMI at the University of Tübingen, Germany. Its molecular mass was checked by MALDI-TOF.

Instrumentation, software and acquisition of spectra

Mass spectrometry was performed using a Reflex IV (Bruker Daltonik GmbH) MALDI-TOF mass spectrometer, equipped with a Scout 384 ion source. Spectra were processed by XMass 1.5.1 and BioTools 2.1 software (Bruker Daltonik GmbH). AnchorChip™ 384/600 (Bruker Daltonics GmbH), with metal anchors of diameter 600 μm, was used as target. TOF spectra were acquired in the range m/z 830–3000 with deflection set at m/z 550. The instrument resolution (FWHM) was approximately 10 000. The diameter of the laser beam was approximately 50 μm, as estimated in a separate experiment from the diameter of a hole burned in a CHCA layer at high laser fluence.

Typically 200 laser pulses per spectrum were accumulated under operator control. The external video monitor was used to precisely focus the laser beam on the crystals. It is recommended to turn on the instrument a few hours before starting measurements because the optics are affected by temperature gradients and the position of a laser spot on the target drifts while the instrument is warming up. Laser pulses shot onto the AnchorChip coating did not damage its surface.

TOF mass spectra were calibrated internally using autolysis products of trypsin (m/z 1153.560 and 2163.056). Proteins were identified using the Mascot 1.8 (Matrix Science Ltd., UK) programme installed on a local server; database searches were performed against a non-redundant protein database MSDB downloaded from the European Bioinformatics Institute (EBI).

In-solution digestion

The stock solutions of bovine serum albumin (BSA) in 50 mM ammonium bicarbonate buffer, with protein concentration

3.7 and 0.95 fmol/μL, were digested in 0.25 μM tryspin. The digestion was performed overnight at 37°C.

In-gel digestion

Protein bands were visualized by Coomassie Brilliant Blue R 250 or silver staining and digested with trypsin, as described previously.[14,24] Proteins were reduced in-gel using 10 mM dithiothreitol, and alkylated with 55 mM iodoacetamide (optional), followed by a washing step that included dehydrating gel pieces with acetonitrile and rehydrating with ammonium bicarbonate buffer. Gel pieces were again dehydrated with acetonitrile and dried down in a vacuum centrifuge. Gel pieces were further rehydrated for at least 40 min at 4°C in a mini-thermostat (ThermoStat Plus, Eppendorf, Hamburg, Germany) in a 0.5 μM solution of trypsin in 50 mM ammonium bicarbonate buffer. After the rehydration step, the remaining trypsin solution was aspirated from the test tube and samples were digested overnight at 37°C in a closed thermostat to avoid condensation of water from the digestion buffer on the lid of the test tube. The digestion was performed in the minimal volume of the digestion buffer that just covered the gel pieces.

Sample preparation for MALDI analysis

CHCA (2 mg) was dissolved in 0.33 mL of 2.5% aqueous TFA and 0.66 mL acetonitrile. Note that the powder dissolves slowly and it can take a few hours before it is completed. The matrix solution is stable and could be used within several months if kept in a closed Eppendorf tube at room temperature.

A 1.2 μL aliquot withdrawn from an in-gel digest was pipetted onto the AnchorChip target. 0.6 μL of the matrix solution (prepared as described above) was spiked directly into the analyte droplet. The mixture was allowed to dry at room temperature (approximately 30 min). Upon drying the entire surface of the target was submerged in 5% formic acid for 2–3 min and then allowed to dry at room temperature for another 15–30 min.

Cleaning of AnchorChip targets

The AnchorChip targets were cleaned by washing them with an organic solvent (ethanol, methanol or acetonitrile) for a few minutes, and then rinsing them with water and, finally, with the same organic solvent. No memory effects were observed. No changes in the surface properties of AnchorChip targets were noticed in a time period of more than 1 year.

Epifluorescence microscopy

0.6 μL of a 40 μM stock solution of the fluorescent N-terminally labelled peptide (TAMRA)-KKKPKCK in 50 mM ammonium bicarbonate buffer was mixed with 0.6 μL of the in-solution trypic digest of 1.25 μM BSA, loaded on the AnchorChip target, and mixed with CHCA matrix as described above. A MALDI-TOF spectrum of the mixture was acquired to test whether the peptides successfully co-crystallized with the matrix. The target was further examined under an upright epifluorescent microscope Axioplan 2 (Zeiss, Germany) equipped with a mercury short arc photo-optic lamp (HBO 103 W, Osram, Germany) and Zeiss Plan-Neofluar 2.5×/0.075 objective. Two different filters were applied to selectively visualize either the peptide or the matrix. The fluorescence of the TAMRA-labelled peptide was visualized using the filter set which is routinely used for detection of rhodamine, i.e., excitation filter BP 550/20 nm (540–560 nm bandpass filter), dichromatic filter LP 550 nm (longpass), and emission filter BP 610/60 nm (580–640 nm bandpass filter). CHCA matrix was visualized using a filter set which is routinely used for detection of cyan fluorescence proteins, i.e., excitation filter BP 436/20 nm (426–446 nm bandpass filter), dichromatic filter LP 455, and emission filter BP 470/40 nm (450–470 nm bandpass filter). All filters were purchased from Chroma Technology Corp., USA. Eight-bit digital images with 1520 × 1080 pixels resolution were obtained with the use of a cooled CCD camera (SPOT RT Monochrome 2.1.0, Diagnostic Instruments Inc., USA) controlled by image acquisition software IPLab v. 3.5.5. (Scanalytics, USA). The images were converted into black-and-white pictures by Adobe Photoshop software (Adobe Systems, CA, USA).

RESULTS

Probe preparation protocol

Aliquots of a stock solution of acidified CHCA matrix and in-gel protein digest were mixed in 2:1 v/v ratio, and the sample was allowed to dry slowly at the surface of an AnchorChip target. The correct volume ratio of the matrix and analyte solutions is essential for successful co-crystallization and should be maintained if another type of AnchorChip target is employed or other sample volume is loaded. If stronger ammonium bicarbonate buffer is used for the digestion of proteins, the concentration of TFA in the matrix stock solution should be adjusted accordingly. However, using more than 5% of TFA in the stock solution often yielded background peaks spaced by 22 Da, which presumably originated from a contaminating polymer extracted from the tube material.

The selected concentrations of the matrix, as well as of water and organic solvent, provided a suitable environment for the co-crystallization of peptides with the matrix. Salts and low molecular weight impurities were pooled onto a hydrophilic metal anchor in the middle of the target, while a large fraction of matrix crystals adhered to a hydrophobic surface around the anchor (Figs. 1(A) and 1(B)). Peptide/matrix crystals at the anchor were usually smaller in size and often produced low-quality spectra dominated by intense peaks of matrix clusters.

We found it to be particularly important that the co-crystallization should proceed at a relatively slow pace and is completed in approximately 30 min. Accelerated drying (e.g., by using a heating fan) yielded crystals that were too small, and which were difficult to target with a laser beam. This considerably slowed down the acquisition of spectra, although the size of crystals did not directly affect the spectra quality. No correlation of the size of crystals with loaded amount of peptides was apparent.

It was originally suggested that re-crystallizing samples directly on AnchorChip targets might improve the quality of peptide mass maps.[21] However, we found that none of the suggested recipes were compatible with our protocol. Re-

crystallization only enhanced the intensity of matrix peaks, resulting in overall lower quality of TOF spectra.

Upon drying, the entire surface of the target was submerged for 2 min in 5% formic acid to wash out salts. Peptide/matrix crystals largely remained intact while salts were washed out from the anchor (Fig. 1(C)). If necessary, the crystals could be subjected to longer washing (up to 1 h), although the intensity of peptide signals in TOF spectra decreased (data not shown). Since the entire target plate was washed with a large volume of the diluted acid, there was no danger of cross-contaminating the samples by peptides deposited at neighbouring targets, which often happens during washing of thin layer targets prepared using conventional recipes.[13]

The protocol was applied with no adjustments to analyze in-gel digests of proteins separated on various types of polyacrylamide gels. The only exceptions were in-gel digests of proteins separated on low-percentage gels containing 5% or less of acrylamide, which are often used to separate high molecular weight proteins. Peptide/matrix crystals yielded from aliquots of these in-gel digests appeared normal on visual inspection; however, no useful peptide signals were detected in the acquired TOF mass spectra. Presumably, oligomers of acrylamide inhibited the desorption of peptides from the matrix crystals, since no peaks corresponding to trypsin autolysis products, usually abundant, were observed. In these cases we extracted peptides from the gel pieces with 5% formic acid and acetonitrile, and dried down the pooled extracts in a vacuum centrifuge.[14] The digests were re-dissolved in 5% formic acid, cleaned-up on a ZipTip, and eluted with 75% acetonitrile directly onto the target. The resulting crystals were mostly concentrated at the anchor, and good-quality spectra were acquired without further washing of the target.

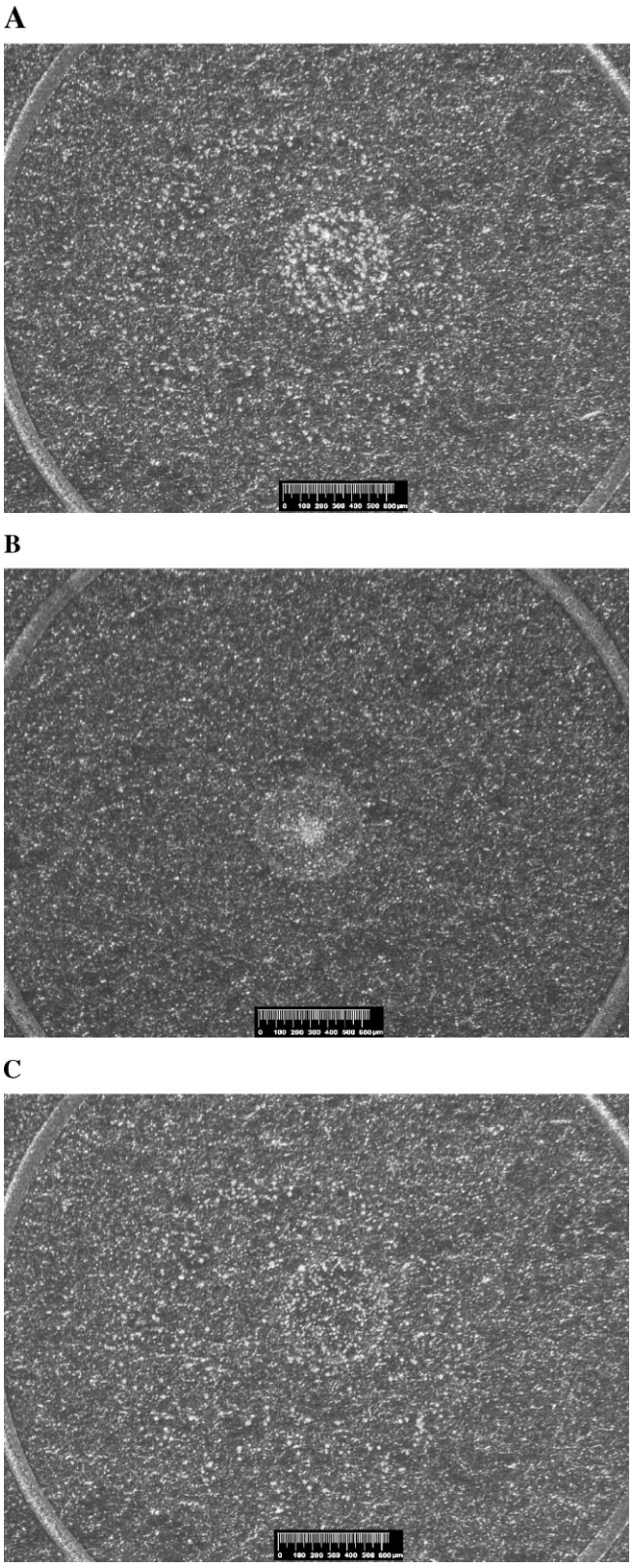


Figure 1. The AnchorChip target at different sample preparation steps examined under the optical microscope. Images from a built-in video camera had lower resolution and contrast, but otherwise looked identical. (A) The AnchorChip target with the dried mixture of the digest and matrix before the washing step. Salts and low molecular impurities mostly pooled at the metal anchor (in the middle). (B) An aliquot of the digestion buffer was mixed with an aliquot of 66% acetonitrile in 2.5% TFA (the solvent in which matrix stock solution was usually prepared); the sample was allowed to dry on the target, and the spot of salts was observed only at the metal anchor. (C) The same target as in (A) after 2 min washing in 5% formic acid; salts were completely removed from the polymer surface, while peptide/matrix crystals were scattered throughout the surface with larger crystals adhered to the hydrophobic polymer. Washing of the target presented in (B) removed salts completely, leaving a clean surface (image not shown).

Co-crystallization of peptides with CHCA matrix

The probe preparation protocol yielded crystals that were almost randomly scattered across the AnchorChip target (Fig. 1). However, it was not clear if all peptide material was completely absorbed into these crystals, or remained in 'matrix-free' patches at the anchor or at the hydrophobic surface. To visualize the distribution of peptides at the target, a peptide N-terminally labelled with the fluorescent rhodamine group 6-TAMRA was loaded onto the AnchorChip together with an aliquot of the in-solution digest of BSA. By using a very hydrophilic, strongly positively charged peptide, (TAMRA)-KKKPKCK, we first tested whether it could be trapped at the hydrophobic polymer surface, rather than

pooled at the metal anchor together with salts and other hydrophilic contaminants. Second, we tested whether it would co-crystallize with the relatively hydrophobic CHCA matrix, and third, whether the peptide would be washed away from at least some of matrix/analyte crystals during subsequent clean-up.

The probe was further processed as described above (although washing with 5% formic acid was omitted), and then analyzed by MALDI. A good-quality peptide map was obtained, which was almost identical to the map of a BSA digest obtained in a control experiment without spiking in the labelled peptide. The TAMRA-labelled peptide was also detected in the MALDI-TOF spectrum as a peak at m/z 1271.47. This confirmed that both the TAMRA-peptide and tryptic peptides of BSA co-crystallized with the CHCA matrix. The same target was further examined under an epifluorescent microscope. Using two different filter sets, which were chosen by considering the absorption and emission spectra of CHCA[25] and of 6-TAMRA, it was possible to selectively visualize either CHCA matrix or the TAMRA-labelled peptide, despite the fact that their amounts on the target differed by a factor of more than 100. The specificity of the optical detection was tested in a series of control experiments in which only the TAMRA-labelled peptide, or only the matrix solution, was loaded onto an AnchorChip, and the images were taken under the settings specific for the detection of the matrix and TAMRA-labelled peptides, respectively. We observed that the bulk of the TAMRA-peptide was associated with matrix crystals with no detectable patches comprising the 'free' peptide. At the same time, almost no peptide-free matrix crystals were detected (Fig. 2). Similar observations were previously reported for dried-droplet preparations that used 2,5-DHB and 2,6-DHB and organic solvents, in which every crystallite at the target surface incorporated a fluorescent-labelled protein.[26] CHCA matrix is most efficiently used in thin layer preparations[13,21] and it is thought that peptide analytes are not incorporated into crystals, but are rather adsorbed onto their surface.[26,27] The low resolution of our imaging system did not allow us to determine the exact localization of the TAMRA-peptide within individual CHCA crystals. However, the distribution of peptides within crystals might not be affecting the sensitivity since even a surface contact between grains of the matrix and the analyte would be sufficient for successful MALDI analysis.[26,28]

The image of an AnchorChip target (Fig. 2) demonstrated that peptide/matrix crystals were not specifically tethered to either the hydrophobic polymer surface or to the anchor, but were rather irregularly scattered throughout the entire target. In our experience it was possible to acquire peptide spectra from crystals located both at the anchor and at the hydrophobic surface. However, the latter spectra were usually of better quality, presumably because they were not in contact with salts during the crystallization, as were the crystals located at the anchor (Fig. 1).

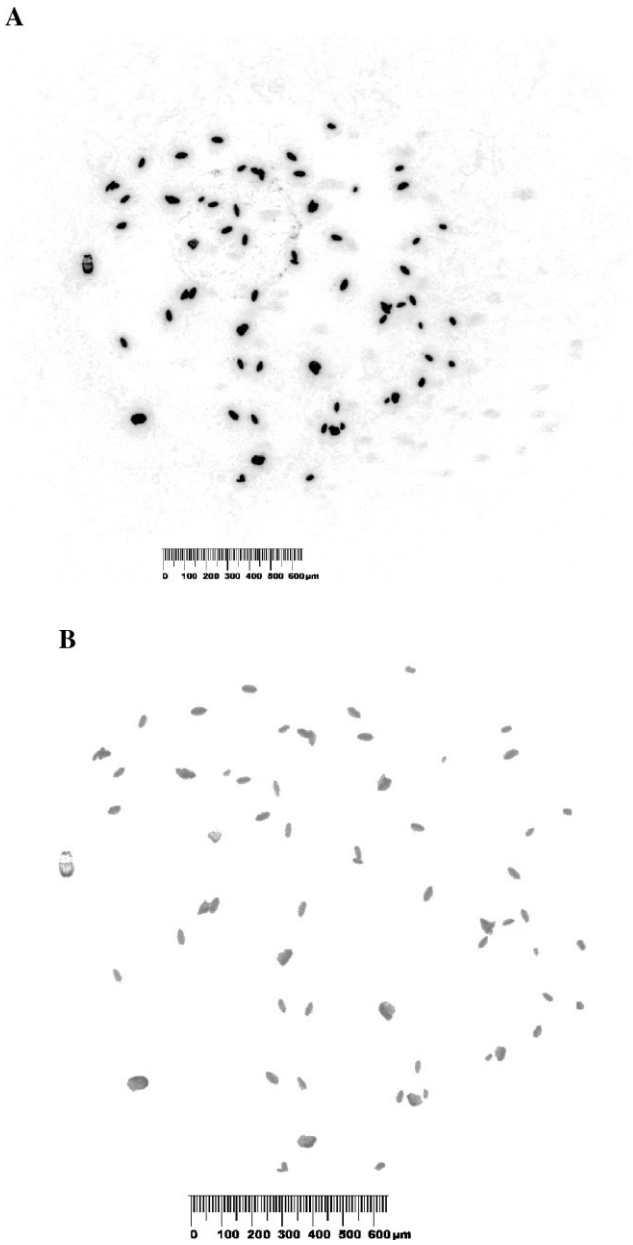


Figure 2. Fluorescence images of peptide/matrix crystals incorporating the N-terminally labelled fluorescent peptide (TAMRA)-KKKPKCK-COOH at the surface of the AnchorChip. Peptide concentration was 0.05 mg/mL. (A) Under rhodamine filter settings only the fluorescence of TAMRA-labelled peptide was detected; low-intensity shadowy patches originate from light scattering on the target surface. (B) Under cyan fluorescent protein (CFP) filter settings only the fluorescence of CHCA matrix was visible. No TAMRA-labelled peptide was detected under CFP settings and no matrix was detected under rhodamine settings in the corresponding control experiments, in which only peptide or only matrix was loaded (data not shown).

Sensitivity of peptide mass fingerprinting

The dried-droplet sample preparation method yielded sizable (ca. 10–50 µm), well-spaced crystals of peptides and CHCA matrix, and no prior clean-up of the sample was required. To test the sensitivity and the robustness of the method, two stock solutions of BSA with concentrations of 3.8 and 0.95 fmol/µL were digested with trypsin, and 1 µL aliquots of the digests were analyzed. Seven clearly detectable peaks corresponding to BSA peptides were observed in the

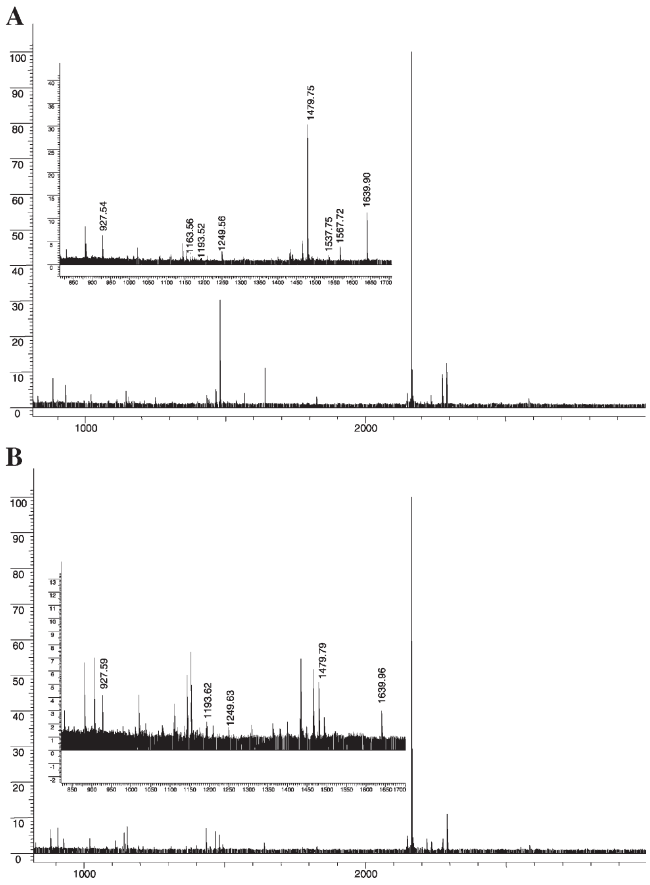


Figure 3. MALDI-TOF peptide mass maps of in-solution digests of BSA acquired using the described sample preparation procedure. Sample loading was: (A) 3.8 fmol and (B) 0.95 fmol of the digested protein. Peaks corresponding to BSA tryptic peptides are designated with their *m/z* values; unlabeled peaks are autolysis products of trypsin. No intense peaks due to matrix clusters were detectable.

peptide mass map when 3.8 fmol of the digest were loaded on a target, and five peaks were detected when the loading was 950 amol (Fig. 3). The *m/z* values of the peptide peaks matched the calculated values for corresponding BSA peptides within 50 ppm mass tolerance. Notably, even at the level of 1 fmol, the intensity of detected peaks or signal-to-noise ratio did not limit the protein identification. However, because of keratin peptides and trypsin autolysis products it was difficult to recognize low-abundance BSA peptides in the spectrum, and the overall score of database searching hits declined.

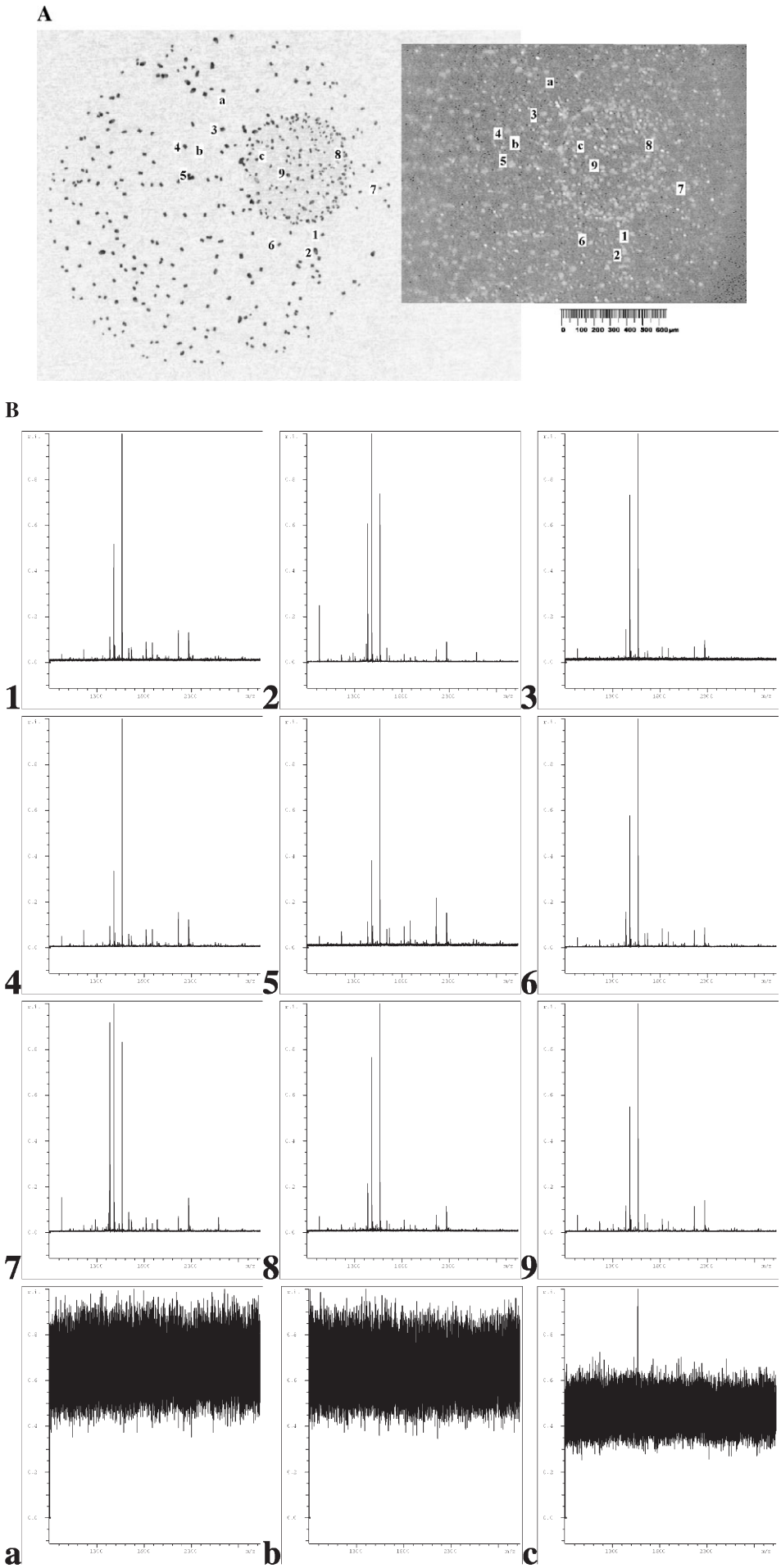
Fluorescence images navigate the acquisition of MALDI-TOF spectra

We demonstrated that the bulk of the loaded peptide material was fully associated with CHCA crystals, from which good-quality peptide mass fingerprints were acquired at the low-fmol level. At the same time, almost no matrix crystals void of the peptide analyte were observed. Therefore, MALDI peptide mapping would be much simplified if one could directly recognize and ablate only matrix/analyte crystals, rather than randomly searching for 'sweet spots' throughout the entire target surface. However, probes on AnchorChip targets, viewed through a video camera built into the ion source, looked rather 'messy' because of the irregularity of polymer and metal surfaces, reflection and scattering of light, as well as remnants of salts and low molecular weight impurities (Fig. 1). Many crystals were visible in the image, but not every visible spot was a much sought-after crystal. We reasoned that CHCA fluorescence might pinpoint matrix/crystals on the target, and thus facilitate navigation for the successive acquisition of MALDI-TOF spectra from multiple spots.

We prepared a probe with 500 fmol of the in-solution digest of BSA. Images of the AnchorChip target were taken using both a video camera from the Scout ion source and the epifluorescence microscope (Fig. 4). Once the images had been scaled to the same size using the diameter of the visible metal anchor as a reference, it was possible to match some of the spots on both images. Nine crystals were selected in the fluorescent image along with three apparently empty spots used as a control, and MALDI spectra were acquired at all 12 locations. Notably, areas around the control spots (a, b, c in Fig. 4) did not look entirely empty on the video camera image, but were rather patchy with a large number of diffuse low intensity spots. Good-quality MALDI peptide maps were acquired from all nine selected spots without further adjustment of the position of the laser beam. At the same time, no useful peptide signals were detected in the spectra acquired from the three control spots. Overall, the acquired spectra demonstrated good reproducibility. The same set of peptides was detected in all nine peptide mass maps, although relative intensities of peptide peaks varied considerably which, however, is commonly observed in MALDI peptide mapping. To estimate the reproducibility of absolute intensities of peptide signals, each spectrum was acquired using 200 laser pulses, the intensity of the most abundant peak (base peak) in each spectrum (panels 1–9) was determined, and the average intensity of all base peaks was calculated. Base peak intensities in the spectra acquired from nine crystals were within ±45% of the average intensity.

We concluded that a combination of our dried-droplet sample preparation protocol and fluorescence imaging provided higher confidence in identifying matrix/peptide crystals on the target, and could improve considerably the quality and the throughput of MALDI-MS and especially of MALDI-MS/MS investigations.

Figure 4. (A) Image from a built-in video camera (panel on the right-hand side) and the fluorescence image (panel on the left-hand side) of a sample of 500 fmol BSA digest on an AnchorChip target. The images were re-scaled to the same size and matched. Nine spots (1–9) and three empty areas (a–c) were selected on the fluorescence image, and matched with corresponding positions on the optical image. Note that it is unclear from viewing the optical image whether or not these areas are really void of crystals. (B) MALDI-TOF mass spectra were acquired from the spots (1–9) and empty areas (a–c) without further adjustment of the laser beam position. All spots detected by fluorescence (1–9) yielded good peptide mass fingerprints, but only background signals were detectable in the spectra acquired from the reference spots a–c.



DISCUSSION

We describe here a simplified dried-droplet probe preparation method utilizing CHCA matrix and pre-structured AnchorChip targets that enabled peptide mass fingerprinting at the low-fmol level. The CHCA/peptide crystals thus obtained were relatively large and stable towards laser depletion, so that high-quality peptide maps could often be acquired from a single crystal. Fluorescence images suggested that the incorporation of peptides into matrix crystals and/or their adhesion on the crystal surface was complete; no detectable peptides were found outside distinctly shaped crystallite areas, which was yet another factor enhancing the sensitivity.

As is typical for dried-droplet preparations, peptide and matrix solutions were directly applied onto the target and no precise pipetting, on-target washing, or re-crystallization was required. The method also enabled efficient de-salting of samples in two stages; first, salts (rather than peptides) were pooled at the hydrophilic anchor, and second, peptide/matrix crystals firmly adhered to the surface of the target and allowed very extensive washing. Therefore, we believe the procedure would be relatively easy to automate.

Peptide/matrix crystals were scattered throughout the target surface, and thus far best results have been obtained using manual navigation of the laser beam rather than a computer-controlled acquisition routine. However, since the crystals were clearly detectable at the surface, suitable raster algorithms for automated acquisition might be developed.

We have been using the described method for more than a year, and several thousands of in-gel digests have been analyzed. In our hands the method has been more sensitive and robust compared with alternative thin layer preparation recipes on stainless steel[14] or AnchorChip[21] targets. Since no intense matrix ions were observed in the low m/z region of MALDI-TOF spectra, it was much easier to recognize low-abundant peptides thus increasing substantially the statistical confidence of the protein identification.

We demonstrated that matrix fluorescence assisted us in targeting matrix/peptide crystals, since neither salts nor the polymer surface demonstrated any noticeable fluorescence under the examined optical settings. We note here that CHCA strongly absorbs the light at the emitting wavelength of a nitrogen laser (337 nm), and therefore the same laser could, in principle, be used to excite CHCA molecules. Strong emission of CHCA is observed in the broad range from 425–525 nm (maximum at 475 nm),[25] which is in the visible light region. We noticed that a built-in video camera registered a light flash every time the laser beam hit a CHCA crystal, if the built-in light source was turned off.

We see several possibilities for how CHCA fluorescence could assist in acquiring MALDI spectra. A UV-light source could be used to illuminate the target, and contrast images of fluorescent crystals could be used to guide the analysis from spot to spot. Interestingly, sometimes crystals (rather than surface irregularities) were better visible if the target was illuminated by light falling at a small angle to its surface. However, it might not be straightforward to match the position of crystals at the image with the actual position of a spot of the laser beam on the target. Alternatively, the laser beam could probe the surface of the target in an automated mode at reduced laser fluence. Once fluorescence of a CHCA crystal has been detected, the laser fluence could be increased up to the desorption threshold and start the automated acquisition without further moving the target. We speculate that the method might be most useful in MALDI-MS/MS, in which software and/or the operator typically spends considerable time in looking for a 'sweet spot' which would yield intense precursor signals together with low chemical noise, and the acquisition of many MS/MS spectra from a single sample is important.

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