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MPep MALDI Chips for high-sensitivity and high-throughput peptide analysis by MALDI-TOF MS

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In mass spectrometry, the need is rapidly growing for higher-sensitivity detection of low-abundance peptides, and for systems that use an automated, high-throughput workflow that includes offline liquid chromatography matrix-assisted laser desorption and ionization (LC-MALDI) coupling. SuNyx provides a new universal MALDI chip platform, which uses matrix spots deposited by vacuum sublimation through masks onto extremely low-wetting surfaces to provide superior control of liquid handling and sample spot preparation.

Over the last decade, MALDI time-of-flight mass spectrometry (MALDI-TOF MS) has evolved into a primary tool for biochemical analysis. Beyond the improvement of instrumentation, MALDI-TOF MS performance can also be increased 'upstream' in the proteomics workflow. This can be achieved by optimizing preparation techniques through the use of new plate surfaces and spot deposition techniques. We present the MPep chip, a plate for MALDI MS peptide analysis that incorporates predeposited matrix spots of α-cyano-4-hydroxycinnamic acid (CHCA). The spots are deposited by vacuum sublimation on extremely low-wetting (ultraphobic) surfaces, and the result is spots that are precisely defined with respect to size, shape and location. During sample preparation, the homogeneous structure of very small CHCA crystals is retained.

Predeposited matrix spots by vacuum sublimation

A thin contact mask, which is positioned at the surface of the sample plate during manufacturing, defines matrix spots and spot patterns. The mask is then coated by a film (typically 3-μm thick) of the matrix compound via vacuum sublimation, leaving spots on the plate surface at the openings of the mask. Simultaneously, vacuum sublimation is used to purify the matrix compound, yielding an extremely pure layer of small matrix crystals with typical diameters of less than 300 nm that are very homogeneously distributed within the spots. Due to the precision of the contact mask, this 'dry' deposition technique produces matrix spots that are precisely defined with respect to size, shape and location. This deposition technique is well established in the production of thin organic films in many areas (for example, for manufacturing of opto-electronic devices) and can be used with a variety of matrix materials, including CHCA, sinapinic acid, 3-hydroxypicolinic acid and 2,5-dihydroxybenzoic acid.

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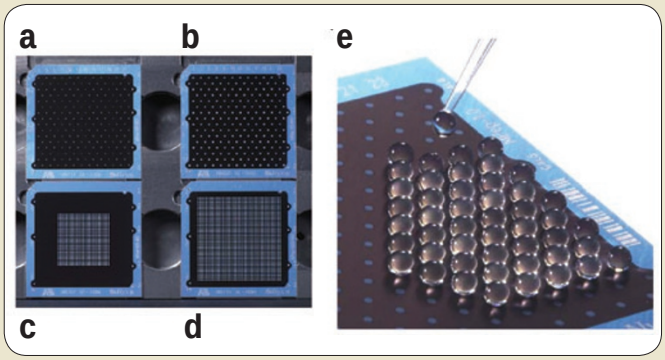


Figure 1 | Examples of MPep MALDI chips with different patterns of matrix. (**a**–**d**) Standard designs contain 192 spots of 300-μm (**a**) or 600-μm diameter (**b**). High-density patterns designed for offline LC-MALDI consist of 625 (25 × 25; **c**) or 1,600 (40 × 40; **d**) spots of 480-μm diameter each. All chips contain six mass calibration spots located in the pockets at the side of the array and four alignment spots. All examples shown are in the Opti-TOF format of Applied Biosystems MALDI-MS instruments. (**e**) Shown are 5-μl water drops on 600-μm diameter spots. The liquid drops are precisely located at the spots of the chip surface.

During sample deposition, only a fraction of the matrix is dissolved at each spot. The remaining solid matrix acts as a seed layer during recrystallization. This helps maintain the distribution and morphology of the crystals. This morphology is most likely the reason for the increased sensitivity and reproducibility of signal intensities (that is, the absence of 'sweet' spots) of these matrix spots relative to those obtained using conventional 'wet' deposition strategies.

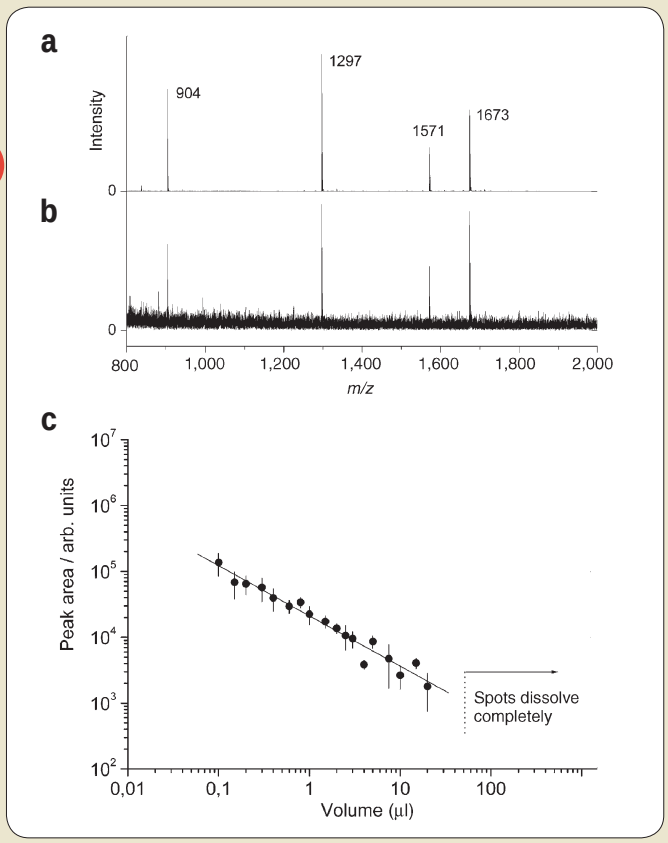
Matrix spots are located on an ultraphobic surface

The surface surrounding the matrix spots has ultraphobic wetting properties with respect to the solvents used for sample preparation. The term is generally used for contact angles greater than 150°; for

MPep chips, the water contact angle is more than 175°, but a more precise determination cannot be made owing to experimental errors at such high angles. On these surfaces, droplets of up to 50% (vol/vol) acetonitrile that are larger than 3-mm diameter will spontaneously roll off the surface at tilt angles greater than 5° without leaving liquid residues behind. In addition, the existence of a three-phase (solid-liquid-vapor) interface at the (geometrical) contact area of the liquid at the solid surface considerably decreases the true contact area of liquid and solid by orders of magnitude. For comparison: water on Teflon surfaces results in a contact angle of approximately 120° with a two-phase interface (solid-liquid) at the contact area. Therefore liquid-sample deposition on MPep chips is ultimately confined to the spots, as they are the only area where the liquid sample can be positioned (**Fig. 1**).

Product formats

MPep chips are currently available in the Opti-TOF format (Applied Biosystems Inc.) with spots of CHCA. Spot patterns include 192 spots (hexagonal pattern, 300-, 600- and 1,000-µm diameter), 625 spots (25 × 25 spots, cubic pattern, 480-µm diameter), or 1,600 spots (40 × 40 spots, cubic pattern, 480-µm diameter). The 625- and 1,600-spot patterns are predominantly designed for offline LC-MALDI applications (**Fig. 1a–d**). All versions are available with peptide standards for mass calibration purposes. Custom-made spot patterns are available upon request. MPep chips are individually sealed in bags under inert gas, with a guaranteed six-month shelf life. The single-use targets minimize the risk of cross contamination and eliminate time-consuming cleaning and regeneration procedures. Once an experiment is completed, the chips can be archived and revisited in further investigations. Test spots with calibration standards did not show any decrease in sensitivity for up to six months after sample preparation when protected from light and stored in a desiccator at room temperature. The range of chip formats is supplemented by a variety of accessories, including calibration standards, preparation reagents, and hardware such as sample holders for sample loading and data acquisition.



Sample preparation

Optimizing the performance of prespotted MPep chips requires certain measures during sample preparation. Generally it is preferred to incorporate the analyte into a minimal matrix volume, thereby increasing the concentration of the analyte in the doped matrix. This can be accomplished by using small-diameter spots. This preconcentration of the analyte increases the lateral surface area concentration of the analyte within the matrix. Alternately, the concentration of the analyte within the doped matrix volume can be increased 'vertically' by restricting the amount of matrix that is dissolved during sample loading and recrystallization. This can be achieved by using small sample volumes and a sample solution of appropriate composition (that is, a small amount of solvent that dissolves the matrix). Under these conditions, the analyte is incorporated into the uppermost layer of matrix crystals, yielding a high ratio of analyte to matrix. **Figure 2** shows the peak area for $m/z = 904$ ($(M+H)^+$, des-Arg^1-bradykinin) for 10-fmol samples of a peptide mixture loaded onto 600-µm diameter spots using different volumes of 20% (vol/vol) acetonitrile. The reduction of the sample volume from 20 µl to 0.1 µl increases the peak area nearly 100-fold owing to the higher concentration of analyte in the matrix near the surface. Depth profiles of analyte distribution (data not shown) reveal much deeper incorporation of analyte within the spot for the 20-µl sample compared to spots prepared from smaller volumes. Two sample spectra of 10-fmol samples prepared from different volumes (0.1 µl and 20 µl) demonstrate the considerably different data quality (**Fig. 2a**,**b**). After sample loading and drying, a final washing step with a solution containing $NH_4H_2PO_4$ is applied to subject the matrix to controlled, standardized recrystallization, yielding maximized sensitivity and reproducibility. Typical standard deviations of peak intensities from 10-fmol samples prepared from volumes of up to 2 µl are 10–30% for these peptides. The washing step also considerably reduces the yield of matrix cluster ions.

Detection of low-abundance peptides

Following these guidelines for sample preparation results in optimized detection sensitivity of peptides. **Figure 3** contains spectra from a dilution series of a small volume (4.2 nl) of peptide mix-

Figure 2 | Peak intensities of MALDI mass spectra depend on the liquid volume used for sample spot preparation. (**a**,**b**) Shown are MALDI mass spectra of a peptide mixture containing four peptides (des-Arg^1-Bradykinin, angiotensin I, Glu^1-fibrinopetide B and neurotensin; $m/z((M+H)^+)$ = 904, 1,297, 1,571 and 1,673, respectively). Samples were prepared from solutions in 20% (vol/vol) acetonitrile in 0.1% (vol/vol) trifluoroacetic acid using different sample volumes that contain 10 fmol of each peptide in all samples. Sample volumes are 0.1 µl (**a**) or 20 µl (**b**). (**c**) Peak area of m/z = 904 ($(M+H)^+$, des-Arg^1-Bradykinin) from samples containing 10 fmol in each sample volume. All samples were prepared by spotting the liquid sample volume on 600-µm spots. Data were obtained using a 4700 Proteomics Analyzer (Applied Biosystems Inc.) in reflector mode (50 shots at ten locations per spot). Each data point represents the average of 12 different spots. Error bars, s.d.

Figure 3 | Ultimate detection sensitivity of peptides using MPep MALDI chips. (**a**–**c**) MALDI mass spectra show peaks from angiotensin I, neurotensin and adrenocorticotropic hormone (clip 18–39) at m/z($(M+H)^+$) = 904, 1,297 and 2,467, respectively. Sample amounts were (**a**) 4.2 amol, (**b**) 1.3 amol or (**c**) 0.4 amol per spot prepared from 4.2-nl sample volumes (20% (vol/vol) acetonitrile in 0.1% (vol/vol) trifluoroacetic acid) on 280-µm sample spots. Concentrations were 1 fmol/µl to 0.1 fmol/µl. Data were obtained using a Reflex II MALDI mass spectrometer (Bruker Daltonics) in reflector mode from 20 shots at eight different locations on a single spot for each sample. Relative standard deviations of peak areas from ten different spots using 4.2 ± 1.2 nl are 34%, 41%, 66% for 4.2, 1.3, 0.4 amol/spot angiotensin I respectively and 41%, 47%, 32% for 4.2, 1.3, 0.4 amol/spot neurotensin, respectively.

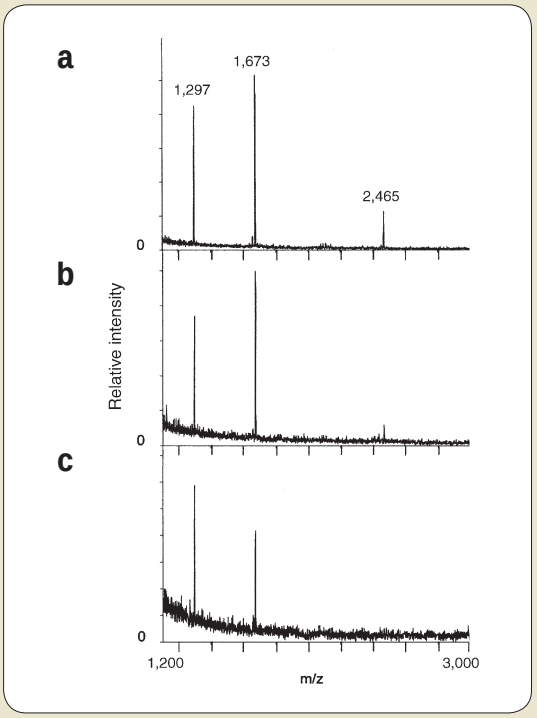
ture containing angiotensin I, neurotensin and adrenocorticotropic hormone (clip 18–39). Under these conditions, angiotensin I and neurotensin can be detected at levels of only 0.4 amol per spot, representing a considerable improvement of detection limit and reproducibility over conventional techniques.

LC-MALDI MSMS performance

MS and tandem MS (MSMS) MPep chips are ideally suited for offline LC-MALDI analysis. The benefits compared to conventional steel targets are demonstrated by the following performance test. A tryptic BSA digest was separated on a Vydac column (250 mm length, 150 µm inner diameter, C18 reverse phase, 5 µm, 300 Å) using a pump and an autosampler from Sunchrome. The capillary column was run using a gradient of 5% to 50% acetonitrile in 0.1% trifluoroacetic acid over 80 min. A splitter was used to reduce the flow rate to 1.5 µl/min for fraction collection. A total of 1,600 fractions were collected on an MPep chip (75 nl/fraction), and the stainless steel plate was spotted with 400 fractions (300 nl/fraction). After LC fractionation, 0.5 µl matrix solution was added to each dried spot on the stainless steel target using an optimized protocol.

MALDI mass spectra of positively charged ions were recorded on a 4700 Proteomics Analyzer (Applied Biosystems Inc.) in reflector mode. For the MPep chip, 400 (10 × 40) shots were taken in MS mode, and 800 (20 × 40) shots were taken in MSMS mode. Spectra from stainless steel targets were acquired from 1,000 (8 × 125) shots in MS mode, and 2,000 (16 × 125) shots in MSMS mode.

Spectra from spots of the MPep chip typically had intensities that were an order of magnitude higher in both MS and MSMS mode compared to the corresponding spots of the steel target. This is in spite of the fact that spectra from the steel target represent a four-fold greater quantity of peptides, and were acquired with 2.5-fold more laser shots. The amounts of peptides differ accordingly: for the MPep chip, 39 peptides matched in a Mascot database search (56% sequence coverage), whereas only 26 peptides were found for the steel target (45% sequence coverage). The most important difference, however, may be the larger number of spots on the MPep chip, which yield better resolution of peptide separation. In an earlier



application of a tryptic digest of lysozyme[1], we had demonstrated the detection of a low-abundance peptide after fractionation on a large number of spots.

Conclusions

High sensitivity analysis of peptides by MALDI-TOF MS can be obtained using spot arrays of CHCA on ultraphobic surfaces. Predeposited matrix spots manufactured via vacuum sublimation of CHCA with lithographic masks allow the control of the shape, size and location of each spot on the chip. MPep chips are ready-made, easy to use, and provide superior reproducibility within individual spots and between spots owing to the homogeneous structure of the matrix. The single-use, disposable targets reduce cross-contamination, and allow archiving and revisiting of samples. With an optimized protocol for MALDI-TOF sample preparation, sensitivities down to the low attomolar range can be reproducibly obtained. This technology is ideally suited for offline LC-MALDI-TOF MS.

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