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Trypsin and MALDI matrix pre-coated targets simplify sample preparation for mapping proteomic distributions within biological tissues by imaging mass spectrometry

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Abstract

Prefabricated surfaces containing α -cyano-4-hydroxycinnamic acid and trypsin have been developed to facilitate enzymatic digestion of endogenous tissue proteins prior to matrix-assisted laser desorption/ionization (MALDI) imaging mass spectrometry (IMS). Tissue sections are placed onto slides that were previously coated with α -cyano-4-hydroxycinnamic acid and trypsin. After incubation to promote enzymatic digestion, the tissue is analyzed by MALDI IMS to determine the spatial distribution of the tryptic fragments. The peptides detected in the MALDI IMS dataset were identified by Liquid chromatography-tandem mass spectrometry/mass spectrometry. Protein identification was further confirmed by correlating the localization of unique tryptic fragments originating from common parent proteins. Using this procedure, proteins with molecular weights as large as 300 kDa were identified and their distributions were imaged in sections of rat brain. In particular, large proteins such as myristoylated alanine-rich C-kinase substrate (29.8 kDa) and spectrin alpha chain, non-erythrocytic 1 (284 kDa) were detected that are not observed without trypsin. The pre-coated targets simplify workflow and increase sample throughput by decreasing the sample preparation time. Further, the approach allows imaging at higher spatial resolution compared with robotic spotters that apply one drop at a time.

Keywords

trypsin pre-coated targets; bottom-up proteomics; imaging mass spectrometry; MALDI; enzymatic digestion; sample preparation; FTICR mass spectrometry

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Introduction

Matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI IMS) has been established as a powerful technology in biological research enabling imaging of biomolecules within thin tissue specimens.^[1–4] In a typical experiment, a tissue is cryosectioned into thin slices (3–12 μm) and placed onto a target suitable for MALDI analysis. The tissue section is washed to remove salts and then coated with a MALDI matrix that aids in the desorption and ionization of biomolecules. Analysis is performed by interrogating the tissue section with a laser, acquiring mass spectral data at individually defined x, y positions (pixels). Relative ion intensities are plotted for each ion of interest to generate two-dimensional maps of molecular distributions.

In a typical MALDI imaging experiment, signals from intact proteins up to 25 kDa in molecular weight can be obtained directly from a tissue sample. Signals from larger proteins are more difficult to measure as the sensitivity of microchannel plates used for ion detection declines as the mass of the ion increases^[5] and also because larger proteins are more difficult to ionize.^[6] These factors make the detection of higher molecular weight biological species difficult. As a result, alternative sample preparation methods have been developed to enhance the detection of proteins up to 70 kDa. In one approach, ferulic acid as a matrix and Triton X-100 and xylene were used to solubilize large proteins.^[7,8] Although this is an improvement, high molecular weight proteins remain a challenge to detect on a routine basis.^[9]

A general approach for measuring the distribution of high molecular weight proteins (>25 kDa) in a tissue sample by MALDI IMS involves the *in situ* tryptic digestion and subsequent imaging of the peptide fragments that are generated.^[10] In this bottom-up proteomics strategy, trypsin is locally spotted onto the tissue section to digest proteins. A robotic spotter or sprayer is used to control the conditions of trypsin applications (volume, area, concentration and location). In a second step using these or other related equipment, matrix is applied to the sample. Previously, these methods have been used in the analysis of both formalin-fixed^[11–15] and fresh-frozen tissues.^[10] The advantage of *in situ* digestion is improved confidence in protein identification through detection of multiple peptides for a given protein in a single imaging experiment^[10] and the ability to perform on-tissue fragmentation experiments while maintaining spatial fidelity. In contrast, liquid chromatography-tandem mass spectrometry (LC-MS/MS) approaches performed on tissue homogenates enable routine protein identification but with limited spatial information.^[16] Despite the advantages of *in situ* enzymatic digestion, the existing coating approaches are challenged by cumbersome experimental methods whereby the sequential application of trypsin and matrix onto the tissue sample can take many hours for each specimen.

In the present paper, we describe an approach that incorporates the MALDI matrix and proteolytic enzymes onto target surfaces prior to tissue mounting. This greatly simplifies tissue preparation for MALDI IMS and allows the end user to avoid time-consuming post-tissue deposition steps that can involve costly specialized equipment. Our previous work has shown that matrix pre-coated targets can provide simple, standardized, rapid methods for sample preparation. Tissue samples are applied directly onto the prefabricated matrix-

containing slides. Grove *et al.* used such pre-coated targets that consisted of thin matrix films for the imaging of lipids, peptides and pharmaceuticals from brain and pituitary tissues.^[17] Yang *et al.* relatedly demonstrated the successful imaging of lipid and protein distributions in brain and kidney tissues using such pre-coated slides.^[18,19] In the current work, we detail the development and use of targets pre-coated with both an enzyme (trypsin) and matrix [α -cyano-4-hydroxycinnamic acid (CHCA)] for bottom-up imaging of large proteins from frozen tissue samples.

Experimental section

Materials

Acetonitrile (ACN), trifluoroacetic acid (TFA), CHCA, diisopropylethylamine (DIEA), trypsin from bovine pancreas, cytochrome c from equine heart, n_α -benzoyl-L-arginine 4-nitroanilide hydrochloride, triethanolamine, calcium chloride, sodium chloride and hydrochloric acid were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as received. Conductive indium tin oxide (ITO)-coated microscope slides were purchased from Delta Technologies (Loveland, CO, USA). Frozen rat brain was obtained from Pel-Freez (Rogers, AR, USA) and stored at -80°C .

Digestion of protein standard

A test solution of ionic matrix consisting of $50\text{ }\mu\text{M}$ CHCA and $100\text{ }\mu\text{M}$ DIEA was prepared in water. Cytochrome c and trypsin at 10 and $0.5\text{ }\mu\text{M}$, respectively, were added to allow digestion. After incubation for 24 h at 37°C , a $1\text{-}\mu\text{l}$ aliquot was spotted onto a MALDI target. Cold 10% TFA in water (0°C) was spotted onto the sample to convert the ionic matrix into its crystalline form. After 1 min of contact, the acid solution was decanted leaving behind a crystalline film on the MALDI plate that was dried in a desiccator and then analyzed using a Bruker Ultraflextreme MALDI-time-of-flight in reflectron mode (Bruker Daltonics, Billerica, MA, USA).

Slide fabrication

Matrix and trypsin were sequentially spray-coated onto an ITO-coated glass slide using a HTX TM Sprayer (HTX Technologies, LLC, Chapel Hill, NC, USA). CHCA was dissolved in a $9:1$ ACN:water solution at 10 mg/ml . To deposit the matrix, the sprayer was operated at a 0.2 ml/min flow rate using a high-performance liquid chromatography pump, a 2 mm track spacing, a 1000 mm/min nozzle velocity, a 70°C nozzle temperature, a 6 psig nebulizer pressure and 90% ACN as the pushing solvent. The number of passes was varied between 10 and 20 to control the thickness of the matrix coating.

In a second step, trypsin as a 2 mg/ml solution in water was sprayed on top of the matrix coating. A syringe pump was integrated with the HTX TM Sprayer to accommodate the flow rate of $30\text{ }\mu\text{l/min}$ used for trypsin deposition. The sprayer conditions were 2 mm track spacing, 1000 mm/min nozzle velocity, 30°C nozzle temperature, a 6 psig nebulizer pressure and water as the pushing solvent. The number of passes was varied between 4 and 12 to control the density of trypsin on the slide. Slides were coated with CHCA at densities

of 0.3–1.0 mg/cm² and with trypsin at densities of 0–30 µg/cm² in order to determine optimal concentrations.

Stability of pre-coated slides

The activity of deposited trypsin on the pre-coated slides was measured colorimetrically using a substrate solution comprised of 800 µl of 200-mM triethanolamine buffer and 20-mM CaCl₂ (pH 7.8 at 25 °C), 500 µL of 0.1% (w/v) *N*_α-benzoyl-L-arginine-4-nitroanilide hydrochloride, and 100 µl of 0.9% (w/v) NaCl. In a cuvette, this solution was combined with 100 µL of a 1-mM HCl solution containing trypsin. The cuvette was placed in a microplate reader (SpectraMax M2e, VWR International, Radnor, PA, USA), and the trypsin activity was quantified by measuring the linear change in absorbance at 405 nm over a 1-min period. The rate of change was measured for trypsin solutions prepared at 500, 200, 100, 50, and 0 µg/ml in 1 mM HCl, and a calibration curve was generated.

In order to measure the activity of trypsin in a coated substrate, 1 ml of a 1-mM HCl solution was pipetted over the slide, and the resulting solution was collected. The process was repeated three times. The activities of these solutions were measured and their cumulative rates of change computed as a measure of overall activity for a pre-coated slide. Trypsin activity was measured for freshly prepared slides and those stored for extended periods at 4 or –20 °C.

Tissue mounting

Frozen rat brain tissue was sectioned to 4 µm at –20 °C using a Leica CM 3050S cryostat (Leica Microsystems GmbH, Wetzlar, Germany). A pre-coated slide in the cryostat chamber was warmed for 2 s using a gloved finger, and then, the slide was placed directly on top of the sectioned tissue. The tissue adhered to the slightly warm slide in preference to the cold metal plate. Tissue sections were then thaw mounted on the pre-coated target and stored in a vacuum desiccator prior enzyme activation and analysis by IMS.

In situ tryptic digestion

The mounted tissue section was placed in a chamber saturated with DIEA for 5 min to convert CHCA into its ionic liquid form. The slide was then placed in a plastic Petri dish, sealed with tape and kept in an oven for 3 to 16 h at 37 °C. After cooling to room temperature, the slide was immersed in a solution of 10% TFA at 0 °C for 1 min to return the matrix to its crystalline form. The slide was dried in a desiccator before MALDI analysis.

Matrix-assisted laser desorption/ionization imaging mass spectrometry

A Bruker rapifleXTM MALDI Tissue typerTM (MALDI-time-of-flight, Bruker Daltonics, Billerica, MA, USA) was used in the protocol development to evaluate slides with different densities of trypsin and matrix. The instrument was equipped with a scanning smartbeam 3D 10 kHz laser (355 nm, frequency-tripled Nd: YAG laser) and operated in positive ion reflector mode at 20 kV. The laser focus setting was set to ‘single’ (laser beam diameter ~5 µm), with a scan size of 1000 µm and a pixel size of 1000 µm in both the *x* and *y*-directions. Each pixel is an average of 500 laser shots.

Imaging experiments were performed using a 15 T MALDI Fourier transform ion cyclotron resonance (FTICR) mass spectrometer (Bruker Daltonics, Billerica, MA, USA). The instrument is equipped with an Apollo II dual MALDI/electrospray ionization ion source and a Smartbeam II 2 kHz Nd:YAG (355 nm) laser. All images were collected using the small laser setting (beam diameter: ~50 μm) with a pixel spacing of 100 μm in both x and y directions. Data were collected from m/z 500–6000 with a spectral resolving power of 130 000 at m/z 1000. External calibration was performed prior to analysis using cesium iodide clusters. FlexImaging 5.0 (Bruker Daltonics) was used to visualize ion images.

In-gel tissue extraction and liquid chromatography-mass spectrometry analysis

Polyacrylamide hydrogels were fabricated using the procedures described by Harris *et al.* and Taverna *et al.*^[20,21] A cylindrical piece of polyacrylamide gel measuring 2 mm in diameter and 2 mm in height was completely dried, immersed in 1 $\mu\text{g}/\mu\text{l}$ solution of trypsin and placed onto the cerebellum region of a brain tissue section for 4 h. The gel was removed from the tissue and placed in an Eppendorf tube into which an aqueous extracting solution containing 50% ACN and 5% formic acid was pipetted to fully cover the hydrogel. After 15 min of gentle agitation, the solution was collected and replaced by a 100-mM ammonium bicarbonate solution. The Eppendorf tube was agitated for 15 min to re-swell the gel. This process of extraction and re-swelling was repeated two additional times. The extracts were dried down and reconstituted in 0.1% formic acid. The solution was analyzed using LC-MS/MS, and the results were analyzed using Scaffold 4.2.1 (Proteome Software, Portland, Oregon).

Identification of tryptic peptides

From each LC-MS/MS experiment, the 20 most abundant proteins in the tissue sample were found based on total spectrum count. The tryptic peptides were sequenced using mMass^[22] allowing for up to 2 missed cleavages. (mMass is an open source mass spectrometry tool free available at www.mmass.org.) Peak picking from the average FTICR spectrum was performed in DataAnalysis 4.2 (Bruker Daltonics, Billerica, MA, USA), and this peak list was compared with the expected masses for tryptic peptides resulting from the 20 most abundant proteins allowing up to 5 ppm error. Ion images were generated for selected matched peptides using FlexImaging 4.1 (Bruker Daltonics, Billerica, MA, USA).

Results and discussion

Digestion of a protein standard in the ionic matrix

The optimal pH for trypsin activity is between 7.5 and 8.5.^[23] As such, the ability to fabricate a functional pre-coated slide containing both matrix and trypsin presents a challenge as most MALDI matrices used for peptide analysis (such as CHCA and 2,5-dihydroxybenzoic acid) are acidic and would thereby reduce trypsin activity. To address this issue, previous studies involving *in situ* digestion by trypsin for MALDI IMS have employed separate steps to apply trypsin and matrix to tissue samples.^[13,24] In order to avoid a step to deposit the matrix on the tissue after digestion, we pursued an approach whereby both trypsin and matrix would be present on the slide, and a base is added to neutralize the acidity of CHCA used as matrix. We selected DIEA for the base because its bulky structure yields

an ionic liquid upon reaction with CHCA.^[25,26] We hypothesized that its liquid state and hygroscopic properties would provide a favorable environment for the extraction of peptides and proteins from the tissue and yield suitable conditions for trypsin activity. Aqueous solutions of the ionic liquid formed from CHCA and DIEA exhibited a pH of 8 and buffering capacities similar to those for ammonium bicarbonate solutions of similar concentration. We note that DIEA offered specific processing benefits in that it is volatile and could be readily added to CHCA in the vapor phase.

We evaluated the activity of trypsin in the CHCA-DIEA ionic liquid medium using cytochrome c as a convenient protein of known sequence having readily identifiable fragments. Although various ionic matrices have shown promise for the detection of lipids, proteins and peptides by MALDI IMS from tissue samples,^[25,27] we only obtained low MS signals for these species directly from the CHCA-DIEA ionic matrix. Conversion of the liquid CHCA-DIEA matrix to crystalline CHCA by a rinse with 10% TFA was necessary to obtain more intense peptide signals. Figure 1 compares the mass spectral results for trypsin digestion of cytochrome c in the CHCA-DIEA ionic matrix after its conversion to the crystalline CHCA matrix with control experiments performed without trypsin or cytochrome c. In the case of CHCA-DIEA with no trypsin or cytochrome c (Fig. 1(a)), only matrix signals for CHCA were observed, as expected. When trypsin was included in the ionic matrix (Fig. 1(b)), various trypsin autolysis peaks were present including those highlighted at m/z 659.4, 2163.6 and 2273.2. When both trypsin and cytochrome c were present, signals corresponding to tryptic fragments from cytochrome c were observed in the mass spectrum (Fig. 1(c)). As examples, the peaks highlighted in Fig. 1(c) by asterisks were not present in the control spectra and represent tryptic fragments resulting from cytochrome c. Additionally, the autolysis peaks observed in Fig. 1(b) are much less prominent when both cytochrome c and trypsin were present (Fig. 1(c)). The conclusion drawn from these results is that the neutral pH medium provided by CHCA-DIEA is a suitable matrix-containing environment for trypsin digestion.

Fabrication of pre-coated slides

The processing advantage provided by pre-coated slides for imaging tissue samples is that the matrix and enzyme can be applied prior to mounting of the tissue. As a result, pre-coated slides could be prepared in advance so that multiple tissue samples could then be processed concurrently. This approach requires no treatment steps or access to specialized equipment to apply trypsin or matrix to the tissue. In a typical preparation, we sequentially spray-coated matrix and then trypsin onto an ITO-coated glass slide using a robotic sprayer as shown schematically in Fig. 2(a). With this sprayer, a solution of matrix (20-mg CHCA/ml in 90% ACN(aq)) flows through a nozzle and is then converted into a fine mist by a pressurized stream of nitrogen. We attempted to apply these coatings in a single step; however, the peptide signals from tissue samples were significantly lower when the enzyme and matrix were mixed and sprayed in one step than when the enzyme and matrix were sprayed in separate sequential steps (data not shown). As a result, we sprayed CHCA and trypsin in separate steps to form the pre-coated substrates.

In optimizing the coating process, we calculated the expected amount of deposited CHCA on a slide from the sprayer conditions by the equation

$$mass\ deposited = \frac{(\#\ of\ passes) \times (concentration) \times (flow\ rate) \times (slide\ area)}{(track\ spacing) \times (stage\ velocity)} \quad (1)$$

In practice, a fraction of the aerosolized matrix did not deposit on the slide. Table 1 compares the ratio of the actual deposited amount of CHCA, as calculated by weighing the slides before and after spraying, with that calculated using Eqn (1). We defined the ratio between the actual and calculated amounts as a deposition efficiency, and this was dependent on both the nozzle temperature and the nebulizing pressure. For example, the deposition efficiency decreased with increasing nebulizer pressure, decreasing from 74% to 44% as the pressure was increased from 6 to 13 psig with the nozzle temperature at 30 °C (cases 1 and 2) and from 47% to 35% as the pressure was increased from 10 to 13 psig with the nozzle temperature at 70 °C (cases 3 and 4). The higher nebulizing pressure is hypothesized to generate a finer, less focused mist, resulting in more of the matrix being deposited away from the slides. The nozzle temperature affected the deposition efficiency with greater deposition (44% vs 35%) being obtained at 30 °C than at 70 °C using a common nebulizer pressure of 13 psig. These data suggest that lower temperatures and lower pressures provided conditions where more of the sprayed matrix was collected on the slides. However, wetter films of the deposited matrix resulted at these spraying conditions (lower nebulizing pressures and lower nozzle temperatures) that yielded inhomogeneous coatings with large matrix crystals on the slides. To achieve uniform films with small crystal sizes while maintaining reasonable deposition efficiencies, we used a nozzle temperature of 70 °C and a nebulizer pressure setting of 10 psig for spray-coating CHCA onto the slides. These conditions produced CHCA films with crystal sizes ranging from 10 to 30 µm.

Films of trypsin were deposited in a second step onto the matrix coated slide using an HTX TM sprayer. Here, trypsin was dissolved in water at 2 mg/ml and deposited using a nozzle temperature of 30 °C in order to prevent protein denaturation. A nebulizer pressure of 6 psig was used to ensure that the majority of the ejected trypsin was deposited onto the slide. Because of the small amounts of trypsin deposited, its mass on a slide could not be accurately measured. Instead, based on the employed flow rate, nozzle temperature and nebulizing pressure, a deposition efficiency around 74% was expected based on the results in Table 1; however, its value may be different as water was used as solvent for trypsin. As the deposition efficiency for trypsin was not determined, the reported values given in the next sections for trypsin densities on a slide represent those determined by Eqn (1) and do not take into account a deposition efficiency.

We also examined the level of trypsin activity in the pre-coated slides using a colorimetric assay to monitor the hydrolysis rate of a nitroanilide (BAPNA). In our experiment, we compared the rates of BAPNA hydrolysis from solutions formed from trypsin on pre-coated slides with those obtained from solutions of fresh trypsin having concentrations that would have been produced if the deposition efficiency was 100%. We found that the trypsin activity

in solutions prepared from the pre-coated slides was about 60% that of the fresh solutions, representing the product of losses from the deposition efficiencies (~75%) and events such as denaturation. Using this colorimetric assay, we also compared the activity of trypsin in these freshly prepared slides with that from slides that had been stored for 10 months at -20°C . We observed minimal losses in activity, suggesting that the trypsin/matrix pre-coated slides can be prepared well in advance and then stored over an extended period of time prior to their use.

Application of pre-coated slides

The multi-step method by which a pre-coated slide would be used to prepare a tissue for MALDI IMS analysis is shown schematically in Fig. 2(b). A thin tissue section is thaw mounted directly on top of a pre-coated slide. The CHCA is converted into its ionic liquid form by placing the slide in a chamber saturated with DIEA and water vapor (with 5 min being a preferred duration). The slide is then incubated in a sealed Petri dish at 37°C to allow protein digestion. In practice for most preparations, tryptic peptides could be observed using incubation times as short as 3 h and overnight incubation yielding more digestion as evidenced by greater signal intensities. After incubation, immersion of the slide in cold (0°C) 10% TFA for 1 min is used to remove DIEA and return CHCA to its crystalline state as needed for MALDI IMS.

Effect of matrix concentration on the peptide signal from tissue

Yang *et al.* found that the amount of matrix on a pre-coated substrate affected the MALDI signal intensities for both proteins and lipids from tissue samples.^[18,19] For proteins, matrix densities of 1.1 mg/cm^2 or higher were required to yield good MALDI MS signals, with slides having lower matrix densities exhibiting reduced signal intensity.^[19] For lipids, optimal matrix densities were 0.3 to 0.4 mg/cm^2 , with higher amounts yielding weaker signals.^[18] As the tryptic peptides of interest would range in mass between those for proteins and lipids, we anticipated that the optimal matrix density for the CHCA/trypsin slides would be between 0.3 and 1 mg/cm^2 based on these prior observations.

Figure 3 shows average mass spectra obtained from $4\text{-}\mu\text{m}$ thick rat brain sections using CHCA concentrations ranging between 0.3 and 1 mg/cm^2 . The trypsin concentration was held constant at $15\text{ }\mu\text{g/cm}^2$ in all three cases. In all samples, strong lipid signals were observed between m/z 600 and 800. At higher masses where peaks from peptides would appear, the strength of these signals varied with matrix density. We specifically observed that CHCA concentrations between 0.3 and 0.4 mg/cm^2 yielded the strongest peptide signals with their intensities decreasing rapidly using CHCA concentrations above these values. At these greater amounts of CHCA, we hypothesize that the additional matrix reduces the effective trypsin concentration generating fewer peptide fragments. Alternatively, the thicker CHCA coatings may not fully convert to the ionic liquid form during the 5-min exposure to DIEA and provide an overly acidic environment for digestion. Any CHCA remaining in its solid form would be less effective for analyte extraction.

Based on the results in Fig. 3, we hypothesized that further reductions in CHCA density might provide further improvements in signal intensity. Instead, we found that CHCA

concentrations below 0.3 mg/cm² yielded incomplete coatings of matrix on the slide during the conversion step back to the crystalline matrix form. To avoid this situation as well as the reduced signal obtained at higher CHCA concentrations, we recommend the use of pre-coated slides with CHCA densities between 0.3 and 0.4 mg/cm² for producing strong peptide signals from a tissue.

Effect of trypsin concentration on peptide signal from tissue

The amount of trypsin is an important parameter in controlling the rate and extent of digestion. Previous studies employing robotic spotters to deposit matrix and trypsin on tissue samples found that the optimal trypsin concentration was between 2 and 5 µg/cm².^[28] In these studies, multiple passes of trypsin spotting were employed to enable optimal extraction. Because repeated extraction operations would not be possible with pre-coated slides, we anticipated that higher concentrations of trypsin would be needed.

To determine the desired concentration of trypsin in the pre-coated slides, we fabricated slides with trypsin concentrations ranging from 0 to 30 µg/cm² and a constant matrix concentration of 0.37 mg/cm². Serial sections of rat brain tissue were mounted on these slides with varying trypsin concentrations, and the tissue sections were incubated for 4 h at 37 °C to allow digestion. Average FTICR MS spectra from rat brain sections are presented in Fig. 4, showing the spectral changes affected by trypsin concentration. The spectrum in Fig. 4(a) represents the result from a control experiment in which trypsin was absent; the MS signals in this spectrum correspond to lipids and endogenous peptides present in the brain tissue. In particular, signals at *m/z* 700–900 correspond to lipids and signals at *m/z* 1400–1700 correspond to lipid dimers. We note that the greater intensity for the lipid dimer signals relative to those for lipid monomers was due to having tuned the instrument for optimized detection in the *m/z* range (*m/z* > 1000) for the tryptic peptides of interest.

As the trypsin concentration is increased (Fig. 4(b)–(d)), tryptic fragments are observed particularly from *m/z* 1700–3500. The intensity of peptide signals increases in this range as the trypsin concentration increases. These changes occur with a reduction in signals from lipids as compared with the control experiment (Fig. 4(a)). Based on these data, a trypsin concentration of 30 µg/cm² is recommended for generating intense peptide signals. Further increases in trypsin concentration are not recommended as they produce disproportionate increases in autolysis signals that complicate measurement of peptide signals from the tissue (data not shown).

Fourier transform ion cyclotron resonance imaging of peptides in Rat brain (coronal section)

Having established a method to conduct protein digestion with trypsin in the ionic matrix and optimized coating densities for trypsin and matrix in the pre-coated slides, we examined the utility of these slides for MALDI IMS. In these experiments, frozen rat brain tissue sections (4 µm thickness) were thaw mounted onto pre-coated slides and prepared using the procedure illustrated in Fig. 2(b). The tryptic digestion step was conducted at 37 °C for 4 h. Analysis of the tissue by MALDI FTICR IMS yielded an average spectrum that contained over 2200 ions in the *m/z* 1000–5000 region with S/N > 4 as shown in Fig. 5(a). The richness

and quality of the spectral data provided by the enzymatic digestion using the pre-coated slides are illustrated in the expanded spectra presented in Fig. 5(b) and (c).

Matrix-assisted laser desorption/ionization Fourier transform ion cyclotron resonance image data (~13 500 pixels) were collected from rat brain tissue sections prepared on pre-coated slides at a spatial resolution of 100 μm (pixel spacing). To minimize interference from overlapping isotopic patterns, images were generated by selecting only the highest intensity isotope for each peptide. The high mass resolving power (130 000 at m/z 1000) and mass accuracy (<5 ppm) of the FTICR IMS experiment enabled many of the detected signals to be matched to tryptic fragments of proteins identified in rat brain from LC-MS/MS analysis. Performing molecular imaging experiments with high performance mass analyzers (e.g. FTICR and Orbitrap) enables mass matching between imaging data and orthogonal proteomics experiments with high confidence.^[29–31]

A number of proteins in the FTICR IMS dataset were readily correlated with the LC-MS/MS results using mass accuracy. The list of proteins identified by LC-MS/MS was sorted in order of abundance based on total spectrum count. Tryptic fragments were generated for the 20 most abundant proteins using mMass^[22] allowing for up to two missed cleavages. The molecular weights of these fragments were calculated in mMass and searched against the list of peaks in the IMS dataset yielding a number of matches. For example, thirteen peptide signals were matched within 1 ppm of the tryptic fragments expected to result from myelin basic protein, an essential protein in the central nervous system involving neuronal transmission.^[32] Ion images for all of these peptides showed a common localization pattern providing further verification of their identification and their common origin. Selected images from these data are presented in Fig. 6, showing the predominant localization of myelin basic protein in the corpus callosum and thalamus of rat brain. These results are in agreement with previously reported images of myelin basic protein using MALDI IMS.^[10] The present approach offers a simpler workflow and allows imaging at improved spatial resolution relative to this previous work.

Purkinje cell protein 4 (PEP-19) and neurogranin were also identified, and their distributions determined in rat brain using data obtained by the described pre-coated approach and a similar procedure of mass matching. Figure 7 shows images for two ions identified by this process as corresponding to tryptic fragments from either neurogranin or PEP-19. In Fig. 7(a), neurogranin is shown to be localized in the cerebral cortex and hippocampal region of the rat brain. In Fig. 7(b), PEP-19 appears highly localized in the thalamus and to a lesser degree in the cerebral cortex. Both of these distributions are in agreement with the results of previous work.^[30]

Table 2 summarizes the relevant sequence data for the detected tryptic fragments from these three proteins including experimental and calculated masses. A total of 13 peptides are listed for myelin basic protein and two each for neurogranin and PEP-19. There was excellent agreement between the calculated and experimental masses with differences of 1 ppm or less for most ions. The excellent agreement between experimental and theoretical masses and the presence of multiple peptides for each protein ensured a high level of confidence in the identifications listed in Table 2.

Fourier transform ion cyclotron resonance imaging of peptides in Rat brain (horizontal section)

In order to test the ability of this pre-coated approach in resolving finer tissue structures, a horizontal rat brain section was used as this plane presents a number of smaller anatomical features including the cerebellum, which has a number of fine features (e.g. white matter, molecular layer and granular layer). Figure 8 shows the images for signals identified in Table 3 to be tryptic fragments from five different proteins in a horizontal rat brain section. The image data were collected at a spatial resolution of 100 μm (pixel spacing) with ~16 000 pixels. To minimize interferences from overlapping isotopic patterns, images were generated by selecting only the highest intensity isotope for each peptide. Even at this modest resolution, substructures within the brain (e.g. the white matter and the molecular layer in the cerebellum) are clearly resolved. For each of these five proteins, the identified tryptic fragments show the same co-localization. For example, myelin basic protein was localized in the corpus callosum and the white matter in the cerebellum region. Neurogranin was present in the cerebral cortex and absent from the cerebellum region. Brain acid soluble protein 1 was present at low concentration in the cerebellum and at higher concentrations elsewhere in the brain. Myristoylated alanine-rich C-kinase substrate was present at high concentration in the molecular layer, at lower concentration in the cerebral cortex and thalamus, and absent from the white matter and corpus callosum. Together, these images show the utility of this pre-coated approach in determining the distributions for proteins, here varying in molecular weight from 7 to 284 kDa. These images also confirm the ability of the pre-coated slides to resolve fine structures down to 100 μm .

In the LC-MS/MS experiments, additional proteins were identified that yielded only a single tryptic fragment in the MALDI IMS data. Although co-localization studies were not possible to confirm identifications, signals from these fragments could be used to generate images that represented the distributions for their parent proteins. For the IMS data obtained from the rat brain tissue, images for majority of the 20 most abundant proteins could be obtained. Figure 9 shows a collection of images for an additional 11 proteins from a single peptide. Although further validation is needed to improve identification confidence, these tentative results demonstrate the capabilities of this method to provide a way to map large proteins that otherwise would not be detected by imaging intact proteins.

Fourier transform ion cyclotron resonance imaging of lipids in rat brain

In a typical post-coated preparation of a tissue for protein or peptide analysis, organic washes are used to remove lipids.^[33] After these washing steps, lipids signals in the mass spectra from the tissue are greatly reduced. Because no organic washes are performed on the tissue in the pre-coated approach, the mass spectra from the tissue includes both signals from lipids and peptides, allowing imaging of both species to be conducted from a single tissue sample.

Figure 10 shows selected ion images for some of the most abundant lipids present in rat brain. Tentative identifications for these lipids are based on mass accuracy. Although the images were obtained under conditions tuned to $m/z > 1000$, tissue structures such as corpus callosum and the cerebellum region are clearly observed. We note that the lipid images

presented in Fig. 10 were obtained from the same tissue section and same dataset that was used to generate the peptide images in Fig. 8, 9. Together they illustrate that the pre-coated approach enables imaging of different analyte classes from the same tissue section in a single experiment.

Conclusions

Deposition methods to pre-coat matrix and trypsin onto slides prior to tissue availability provide a simplified approach for the imaging of proteins and lipids from biological tissues using MALDI IMS. Using the developed pre-coated strategy, ion images for tryptic fragments from various proteins in rat brain tissue could be generated. These images were acquired at a higher resolution (100 μm pixel size) than the spotting density provided by robotic spotters used in current post-treatment methods. This higher resolution allowed delineation of the molecular and granular layers in the cerebellum. The spatial resolution demonstrated using the pre-coated slides was comparable with homogenous post-coating approaches.

The collected MALDI IMS signals for multiple tryptic fragments from the same parent protein enabled additional confidence in identifying the fragments and parent proteins. For example, nine tryptic fragments from myelin basic protein were spatially mapped and found to be localized in same regions of the tissue. Tryptic fragments from additional large proteins in the brain tissue section were mapped. These included myristoylated alanine-rich C-kinase substrate (29.8 kDa), spectrin alpha chain, non-erythrocytic 1 (284 kDa) and a number of other proteins that could not be detected in their intact form.

In addition to providing a convenient route for producing and mapping tryptic peptides, the pre-coated slides enabled the detection and imaging of lipids from the investigated tissue section. Both peptides and lipids were mapped in a single experiment from the same tissue section. The lipid and peptide distributions could be readily correlated as their simultaneous measurement from the same tissue eliminates issues associated with co-registering images obtained from serial tissue sections.

The developed pre-coated method reduces the processing required for a tissue for MALDI IMS after sample acquisition. As such, the steps for depositing matrix and trypsin could be performed elsewhere and in advance, enabling more efficient handling and analysis of biological samples. By this method, tissue sections are simply mounted on the pre-coated substrate and undergo simple hydration and washing steps prior to IMS analysis. No matrix or trypsin deposition steps onto the tissue are required.

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Figure 1.

Matrix-assisted laser desorption ionization (MALDI) time-of-flight spectra show digestion of cytochrome c in an ionic matrix medium. The mass spectra for (a) α -cyano-4-hydroxycinnamic acid-diisopropylethylamine (CHCA-DIEA), (b) CHCA-DIEA and trypsin and (c) CHCA-DIEA, trypsin and cytochrome c are shown. The samples were incubated for 24 h at 37 °C. Autolysis products are marked with circles, and tryptic fragments of cytochrome c are marked with asterisks. In all cases, 1 μ l of the solution was spotted on the MALDI plate, and then immersed into cold 10% trifluoroacetic acid solution to convert the matrix into its crystalline form before MALDI analysis.

Figure 2.

(a) Matrix and trypsin are sequentially sprayed onto a conductive substrate. (b) General procedure for preparing a tissue sample for matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI IMS) analysis using a pre-coated slide. The tissue sample is placed onto a pre-coated slide, exposed to vapors of diisopropylethylamine and water for 5 min, and then incubated in a sealed chamber for 3 to 16 h at 37 °C to allow digestion. An immersion in 10% trifluoroacetic acid for 1 min removes the diisopropylethylamine and returns the matrix to its crystalline form for matrix-assisted laser desorption/ionization imaging mass spectrometry.

Figure 3.

Effect of matrix concentration on peptide signal from rat brain tissue sections. Trypsin concentration was $15 \mu\text{g}/\text{cm}^2$ in each case, and the tissue thickness was $4 \mu\text{m}$. Digestion was performed for 3 h at 37°C . Data were collected on a Bruker rapifleXTM MALDI Tissue typerTM.

Figure 4.

Effect of trypsin concentration on mass spectra obtained from rat brain tissue sections prepared using the pre-coated slides. Trypsin concentrations were as follows: 0, 3, 15 and 30 $\mu\text{g}/\text{cm}^2$; α -cyano-4-hydroxycinnamic acid density was 0.37 mg/cm^2 . Digestions were conducted at 37 $^{\circ}\text{C}$ for 4 h. Data provided are Fourier transform ion cyclotron resonance imaging mass spectrometry average spectra from rat brain tissue.

Figure 5.

Average matrix-assisted laser desorption ionization Fourier transform ion cyclotron resonance mass spectrum acquired from a rat brain tissue section using the pre-coated slide from m/z 500–5500 (Panel A). Tissue thickness was 4 μm , and digestion was conducted for 4 h at 37 $^{\circ}\text{C}$. Panels B and C are provided to highlight the overall spectral quality and richness of the ions observed from tissue using pre-coated trypsin targets.

Figure 6.

Selected ion images for tryptic fragments of myelin basic protein from rat brain. Imaging data were acquired using matrix-assisted laser desorption ionization Fourier transform ion cyclotron resonance mass spectrometry from α -cyano-4-hydroxycinnamic acid/trypsin pre-coated targets at 100 μ m spatial resolution. All ions have similar spatial distributions and were matched to tryptic peptides from myelin basic protein within 1 ppm.

Figure 7. Matrix-assisted laser desorption ionization Fourier transform ion cyclotron resonance images for tryptic fragments resulting from (a) neurogranin (7.5 kDa) and (b) PEP-19 (6.8 kDa) in rat brain. Neurogranin is localized in the cerebral cortex and hippocampal region of the brain. PEP-19 is localized in the thalamus and cerebral cortex.

Figure 8.

Matrix-assisted laser desorption ionization Fourier transform ion cyclotron resonance ion images of horizontal rat brain tissue section. (a) Serial section stained using Hematoxylin and Eosin (H&E). Peptides corresponding to (b) myelin basic protein (c) neurogranin (d) brain acid soluble protein 1 (e) myristoylated alanine-rich C-kinase substrate and (f) spectrin alpha chain, non-erythrocytic 1. Digestion using α -cyano-4-hydroxycinnamic acid/trypsin pre-coated targets was conducted at 37 °C for 15 h.

Figure 9. Matrix-assisted laser desorption ionization Fourier transform ion cyclotron resonance ion images of horizontal rat brain tissue section for proteins with only one tryptic peptide. Ig gamma-2A chain C region has two tryptic peptides.

Figure 10.

Matrix-assisted laser desorption ionization Fourier transform ion cyclotron resonance lipid ion images acquired simultaneously to peptide images using the pre-coated slides for a rat brain horizontal tissue section. Tentative identifications were based on mass accuracy.

Table 1

Comparison of spray-coating conditions used for generating pre-coated CHCA slides. The ratio between the actual and calculated amounts of deposited matrix is defined as a deposition efficiency. The flow rate was 0.2 ml/min in all cases

Nozzle temperature (°C)	Nebulizer pressure (psig)	Deposition efficiency (%)
30	6	74
	13	44
70	10	47
	13	35

CHCA, α -cyano-4-hydroxycinnamic acid.

Table 2

Proteomics data for several proteins detected in the coronal section of the rat brain by MALDI FTICR IMS, including the molecular weights of the intact proteins, the molecular weights for the observed tryptic peptides and the sequences determined using accurate mass matching.

Protein detected in the tissue	Tryptic peptides from <i>in situ</i> digest			
	$[M+H]^+_{\text{exp}}$	$[M+H]^+_{\text{cal}}$	(ppm)	Sequence
Myelin basic protein (14.2 kDa)	861.4358	861.4360	-0.2	r.SGSPMARR.
	1019.5651	1019.5646	0.5	r.HGFLPRHR.d
	1067.5023	1067.5017	0.6	r.FSWGGRDSR.s
	1336.6318	1336.6314	0.3	k.YLATASTMDHAR.h
	1460.7168	1460.7169	-0.1	r.TQDENPVVHFFK.n
	1502.7709	1502.7710	-0.1	r.TTHYGSLPQKSQR.t
	1745.8385	1745.8388	-0.1	r.HGSKYLATASTMDHAR.h
	1755.8643	1755.8660	-1.0	r.DTGILDSIGRFFSGDR.g
	2141.1123	2141.1138	-0.7	r.TQDENPVVHFFKNIVTPR.t
Neurogranin (7.5 kDa)	1064.5233	1064.5232	0.1	k.GPGPGPGGAGGAR.g
	1192.6200	1192.6181	1.5	r.KPGPGPGGAGGAR.g
PEP-19 (6.8 kDa)	1923.8266	1923.8277	-0.6	k.VQEEFDIDMDAPETER.a
	2051.9231	2051.9226	0.2	k.KVQEEFDIDMDAPETER.a

Table 3

Proteomics data for several proteins detected in the horizontal section of the rat brain by MALDI FTICR IMS, including the molecular weights of the intact proteins, the molecular weights for observed tryptic peptides and the sequences determined using accurate mass match.

Protein detected in the tissue	Tryptic peptides from <i>in situ</i> digest			
	$[M+H]^+_{exp}$	$[M+H]^+_{cal}$	(ppm)	Sequence
Myelin basic protein (14.2 kDa)	1081.5472	1081.5425	4.4	r.FFSGDRGAPK.r
	1502.7742	1502.7710	2.1	r.TTHYGSLPQKSQR.t
	1745.8407	1745.8388	1.1	r.HGSKYLATASTMDHAR.h
	2141.1096	2141.1138	-2.0	r.TQDENPVVHFFKNIVTPR.t
Neurogranin (7.5 kDa)	2933.5194	2933.5268	-2.5	r.TQDENPVVHFFKNIVTPRTPPPSQGK.g
	1192.6125	1192.6181	-4.7	r.KGPGPGGPGGAGGAR.g
	1781.8427	1781.8460	-1.9	k.SGECGRKGPGPGGPGGAGGAR.g
	1904.8947	1904.8958	-0.6	r.KGPGPGGPGGAGGARGGAGGPGSGD.
Brain acid soluble protein 1 (21.8 kDa)	2132.9216	2132.9214	0.1	k.AGEASAESTGAADGAPQEEGEAK.k
	2673.3394	2673.3366	1.1	k.APAPAAPAAEPQAEAPVASSEQSVAVKE.
	3011.3897	3011.3865	1.1	k.SEGAAEEQPEPAPAPEQEAAAPGPAAGGEAPK.a
	3283.5230	3283.5197	1.0	k.AGEASAESTGAADGAPQEEGEAKKTEAPAAGPEAK.s
	3565.6542	3565.6565	-0.6	k.AEPEKSEGAAEEQPEPAPAPEQEAAAPGPAAGGEAPK.a
Myristoylated alanine-rich C-Kinase substrate (29.8 kDa)	2432.0937	2432.0848	3.6	r.EAEAAEPEQPEQPEQPAEEPR.a
	2799.2164	2799.2048	4.1	k.DEAAAAAGGDAAPGEQAGGAGAEGAEGGESR.e
	3558.6081	3558.5991	2.6	r.EAEAAEPEQPEQPEQPAEEPRAEPPSEAVGEK.a
Spectrin alpha chain, non-erythrocytic 1 (284 kDa)	1676.7818	1676.7762	3.3	k.HEDFEKSLSAQEEK.i
	1774.8493	1774.8541	-2.7	k.LIQNNHYAMEDVATR.r
	2271.1482	2271.1490	-0.3	k.HQKHQAFEAEELHANADRIR.g