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MALDI Mass Spectrometry Imaging of Lipids and Primary Metabolites on Rat Brain Sections

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Abstract

Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry Imaging (MALDI MSI) enables the localization and the structural identification of a large set of molecules on a surface with ten to twenty micrometer resolution. MALDI is often coupled to time-of-flight (TOF) tandem analyzers which remain a versatile instrument for the detection and the structural analysis of molecular ions. This technique can be used to locate either large biomolecules, such as peptides/proteins, or to small endogenous or exogenous ones. Among them, lipids and primary metabolites are of high interest because they can reflect the cell state. This chapter will thus be dedicated to the analysis, on rat brain tissue sections, of lipids in positive and negative ion modes, and primary metabolites in the negative ion mode. A particular attention will be paid to the structural characterization of lipids using lithium cationisation in the positive ion mode.

Keywords: Matrix-Assisted Laser Desorption/Ionization, time-of-flight, mass spectrometry imaging, tandem mass spectrometry, lipid, primary metabolite.

1. Introduction

Mass spectrometry imaging (MSI) has gained of importance since the last 15 years. Matrix-Assisted Laser Desorption/Ionization (MALDI) MSI was firstly described in 1994 by Spengler¹ and by the group of Caprioli in 1997 for the localization of peptides and proteins on tissue surface.^{2,3} Since these first demonstrations, the technique was hardly improved in terms of robustness, sensitivity, spatial resolution and data analysis. Nevertheless, the identification of the peptide/protein peaks in the m/z range between 1 500 and 25 000 remains highly challenging and requires either insensitive top-down approaches⁴ or extraction/digestion/separation steps.⁵ In parallel, MALDI MSI was also used for the localization of lipid^{6,7} and primary metabolite⁸ ion species in the m/z range between 250 and 1 500. In fact lipids are major constituents of cells and partially reflect the tissue state. Moreover, due to the development of instruments, such as time-of-flight (TOF)⁹ or OrbitrapTM,¹⁰ exact mass measurements can be performed directly on tissue sections. MS/MS capabilities of most of the mass spectrometers dedicated to MSI allow the rapid structural identification of the lipid species without requiring extraction or separation. In this chapter sample preparation for MALDI MSI of lipids^{11,12} and primary metabolites⁸ on rat brain sections will be described in positive and negative ion modes. In fact rat brain can be considered as a good model sample due to its high availability, its easy sample preparation and its lipid composition depending on the different anatomical areas. Moreover, lithium cationisation of the lipid species leads to better structural characterization in the positive ion mode.¹³ For matrix deposition, several automated technologies are commercially available. Most of them are based on the nebulization of the matrix solution on the tissue section using a robot and allow reproducible sample preparation. Because of its ease of use and robustness, the methods presented here were developed using the TM-SprayerTM from HTX-Imaging.

2. Materials

2.1 Chemicals

1. α -Cyano-4-hydroxycinnamic acid (CHCA)
2. 9-aminoacridine (9-AA)
3. Lithium trifluoroacetate
4. Trifluoroacetic acid
5. HPLC-grade water
6. Ethanol
7. Acetonitrile
8. Sodium pentobarbital
9. Optimal Cutting temperature (OCT) embedding medium
10. Dry ice

2.2 Instruments and materials

1. TM-Sprayer™ from HTX-Imaging (Carrboro, NC, USA) or equivalent robot for matrix deposition
2. 4800 MALDI TOF/TOF mass spectrometer from AB Sciex (Les Ulis, France) equipped with 4000 Series Imaging software (www.maldi-msi.org, M. Stoeckli, Novartis Pharma, Basel, Switzerland) and Tissue View software (AB Sciex, Les Ulis, France) or equivalent.
3. Cryostat (model CM3050-S; Leica Microsystems SA, Nanterre, France or equivalent)
4. Optical microscope (Olympus BX 51 fitted with x1.25 to x50 lenses (Olympus France SAS, Rungis, France) equipped with a Color View I camera, monitored by Cell^B software (Soft Imaging System GmbH, Münster, Germany) or equivalent
5. Desiccator
6. Sonicator device

7. Vortex mixer

8. Sample plate for MALDI instrument (conductive glass slide coated with indium-tin-oxide or stainless steel plate)

3. Methods

3.1. Tissue sectioning

All experiments on rat brains were performed in accordance with the protocols approved by the National Commission on animal experimentation and by the recommendations of the European commission DGXI.

1. Male Wistar rats of typical 400 g weight are euthanized by an intraperitoneal injection of sodium pentobarbital ($> 65 \text{ mg.kg}^{-1}$).

2. The trimmed tissue blocks were immediately frozen in dry ice to prevent crack formation during freezing and stored at $-80 \text{ }^{\circ}\text{C}$ prior to MS experiments (Note 1).

3. To fix the frozen tissue block to the cryostat adapter, you add a few drops of OCT embedding medium and quickly deposit the tissue block over it. The transparent OCT embedding medium is slowly becoming white when hardening.

4. Cut tissue section of $12 \text{ }\mu\text{m}$ thickness using the cryostat at -20°C .

5. Deposit two successive tissue sections on either a conductive glass slide or stainless plate adapted to the MALDI holder. One is needed for the optimization of the instrumental parameter whereas a second one will be used for the MALDI MSI experiment (Note 2).

6. Store the samples at -80°C in a box to prevent degradation by ice.

7. Before MS analysis, warm the sample at room temperature for few seconds and dry it under vacuum at a pressure of a few hPa during 30 min in a desiccator. Calibrants can be deposited on the tissue section required for the instrumental optimization.

8. Take pictures of the sample using a microscope. It is important to keep the same orientation of the sample between the microscope observation and the MALDI MSI acquisition in order to easily correlate the images.

3.2. Lipid MALDI MSI in the positive ion mode

1. Prepare 10 mL of the CHCA matrix solution at 10 mg.mL^{-1} in acetonitrile/water/trifluoroacetic acid (70/30/0.1, v/v/v). The solution needs to be vortexed during 1 min and then sonicated during 5 min before being sprayed.

2. Fill the reservoir loop of the TM-Sprayer™. Five mL are enough for the matrix deposition over four different sample plates.

3. Fix the flow rate of the isocratic pump at $240 \text{ }\mu\text{L.min}^{-1}$, the temperature of the nozzle/air spray at 120°C and the velocity of the sample stage at 120 cm.min^{-1} .

4. Verify that the nozzle is not blocked and that the matrix is correctly deposited using a blank plate. If the matrix surface looks homogeneous, place the sample plate and deposit the matrix. The matrix deposition is done in less than 2 minutes. Rinse the complete system with an acetonitrile/water/trifluoroacetic acid (70/30/0.1, v/v/v) mixture for 30 min.

5. Introduce the sample plate in the vacuum chamber of the mass spectrometer. The time needed to reach the pressure limit allowing the plate admission in the analysis chamber can be longer than for classical dried-droplet MALDI sample.

6. A first tissue section will be used to optimize the instrumental parameters, *i.e.* the laser intensity, the extraction delay and the number of laser shots. Laser intensity is usually fixed 10 % higher than the ionization threshold whereas a typical value for the extraction delay is 450 ns. The number of laser shot is chosen by firing the sample at a fixed position and monitoring the signal-to-noise ratio of the lipid signals. Typical number of laser shots per pixel is about 150.

7. Calibrate the mass spectrometer. Two ways can be chosen. The first consists in the deposition of calibrants at a higher concentration on the tissue section before the matrix deposition. In that case, the calibrant ions are generated at the same position as the lipid species and thus a 10 ppm internal calibration can be achieved. The second methods consists in using the signal of known lipid species, for example the ions corresponding to three glycerophosphatidylcholines (PCs) at m/z 758.5700 ($[\text{PC } 34:2+\text{H}]^+$), m/z 782.5676 ($[\text{PC}34:1+\text{Na}]^+$) and m/z 798.5416 ($[\text{PC}34:1+\text{K}]^+$).

8. Determine the tissue area which will be imaged on the second tissue section.

9. Chose the pixel size. Minimal pixel size can be determined by the dimension of the crater formed after 150 laser shots at a fixed position on the tissue sample. Depending on the instrument, pixel size can be from 5 μm to 100 μm (Note 3). Oversampling can also be considered. In that the pixel size is smaller than the crater size. Sensitivity is usually lower when using this acquisition mode.

9. Start the acquisition. The total acquisition time is calculated by the 4000 Series Imaging software. Typical acquisition time per pixel is between 1 and 2 seconds depending on the number of laser shots per pixel.

10. Remove the sample from the mass spectrometer. The sample can be several days in a desiccator without significant degradation. It can be useful to keep the sample if structural characterization of lipid species is required.

11. Analyze the data using Tissue View software or equivalent ones. A list of available data treatment software is provided on the website <http://www.maldi-msi.org/> . Typical brain lipid images are presented in reference 11. Similar lipid distribution should be obtained following the above procedure.

3.3. Structural characterization of lipids using lithium cationization in the positive ion mode

1. Prepare 10 mL of the CHCA matrix solution at 10 $\text{mg}\cdot\text{mL}^{-1}$ in acetonitrile/water/trifluoroacetic acid (70/30/0.1, v/v/v). Add a solution of lithium trifluoroacetate at 2 $\text{mg}\cdot\text{mL}^{-1}$ (1/1, v/v). The final solution needs to be vortexed during 1 min and then sonicated during 5 min before being sprayed.

2. Fill the reservoir loop of the TM-Sprayer™. Five mL are enough for the matrix deposition over four different sample plates.

3. Fix the flow rate of the isocratic pump at 300 $\mu\text{L}\cdot\text{min}^{-1}$, the temperature of the nozzle/air spray at 150°C and the velocity of the sample stage at 120 $\text{cm}\cdot\text{min}^{-1}$.

4. Verify that the nozzle is not blocked and that the matrix is correctly deposited using a blank plate. If the matrix surface looks homogeneous, place the sample plate and deposit the matrix. The matrix deposition is done in less than 2 minutes. Rinse the complete system with an acetonitrile/water/trifluoroacetic acid (70/30/0.1, v/v/v) mixture for 30 min.

5. Introduce the sample plate in the vacuum chamber of the mass spectrometer. The time needed to reach the pressure limit for introducing the sample in the analysis chamber can be longer than for classical dried-droplet MALDI sample.

6. A first tissue section will be used to optimize the instrumental parameters, *i.e.* the laser intensity, the extraction delay and the number of laser shots. Laser intensity is usually fixed 10 % higher than the ionization threshold whereas a typical value for the extraction delay is 450 ns. The number of laser shot is chosen by firing the sample at a fixed position and monitoring the signal-to-noise ratio of the lipid signals. Typical number of laser shots per pixel is about 150.

7. Calibrate the MS mode. In the case of lithium adduct, calibration must be done with known lipid signals such as those at m/z 740.5782 ([PC 32:0+Li]⁺) and m/z 766.5938 ([PC 34:1+Li]⁺).

8. Calibrate the MS/MS mode using specific fragments of the ion at m/z 766.5938 ([PC 34:1+Li]⁺): 707.6 (loss of triethylamine), 583.6 (loss of phosphocholine), 577.6 (loss of phosphocholine+lithium), 190.2 (phosphocholine cationized by lithium) and 86.1 (dehydroxycholine).¹³

9. Acquire MS/MS spectra of lithium-cationized lipids using the “metastable suppressor ON” mode. Selection window need to be optimized in order to select a single lipid species without a too significant loss of sensitivity. Spectra can be compared to those previously published.¹³

10. If MALDI MS/MS imaging is required, same procedure than described in paragraph 3.2. can be followed.

3.4. Lipid and primary metabolite MALDI MSI in the negative ion mode

1. For lipid analysis, prepare 10 mL of the 9-AA matrix solution at 10 mg.mL⁻¹ in ethanol/water (70/30, v/v). For primary metabolite analysis, add 1% of trifluoroacetic acid to the matrix solution. The solution needs to be vortexed during 1 min and then sonicated during 5 min before being sprayed.

2. Fill the reservoir loop of the TM-Sprayer™. Five mL are enough for the matrix deposition over four different sample plates.

3. Fix the flow rate of the isocratic pump at 240 µL.min⁻¹, the temperature of the nozzle/air spray at 80°C and the velocity of the sample stage at 120 cm.min⁻¹.

4. Verify that the nozzle is not blocked and that the matrix is correctly deposited using a blank plate. If the matrix surface looks homogeneous, place the sample plate and deposit the matrix. The matrix deposition is done in less than 2 minutes. Rinse the complete system with an ethanol/water (70/30, v/v) mixture for 30 min.

5. Introduce the sample plate in the vacuum chamber of the mass spectrometer. The time needed to reach the pressure limit for introducing the sample in the analysis chamber can be longer than for classical dried-droplet MALDI sample.

6. A first tissue section will be used to optimize the instrumental parameters, *i.e.* the laser intensity, the extraction delay and the number of laser shots. Laser intensity is usually fixed 20 % higher than the ionization threshold whereas a typical value for the extraction delay is 450 ns. The number of laser shot is chosen by firing the sample at a fixed position and monitoring the signal-to-noise ratio of the lipid signals. Typical number of laser shots per pixel is about 200 for lipid analysis and 80 for primary metabolite analysis.

7. Calibrate the mass spectrometer. Calibration must be done with known lipid signals such as those at m/z 700.5281 (plasmalogen phosphatidylethanolamine 34:1, [PE-p34:1-H]⁻), m/z 788.5442 (glycerophosphatidylserine 36:1, [PS 36:1-H]⁻) and m/z 888.62351 (sulfatide C24:1, [ST 24:1-H]⁻) or with known primary metabolite signals such as those at m/z 346.0553 ([adenosine monophosphate -H]⁻), m/z 426.02164 ([adenosine diphosphate -H]⁻) and m/z 505.98799 ([adenosine triphosphate -H]⁻).

8. Calibrate the MS/MS mode using the previously described fragments of either the ion at m/z 888.62351 (sulfatide C24:1, [ST 24:1-H]) or the ion at m/z 427.02947 ([adenosine diphosphate -H]).

9. Acquire MS images or MS/MS spectra as described above in paragraph 3.2. and 3.3..

4. Notes

1. Formaldehyde fixation is always avoided when possible in order not to chemically modify the lipid species.¹⁴

2. Classical histochemistry (hematoxylin and eosin staining for example) is usually performed on adjacent tissue sections in order to visualize the different anatomical parts of the rat brain. It must be noticed that histochemistry can also be performed on the same tissue section that was used for MALDI MSI experiments.^{15,16}

3. 4800 MALDI TOF/TOF from AB Sciex is working with a fixed laser focus. It must be noted that high value of laser power is related to larger amount of ablated material, *i.e.* larger crater size. Other MALDI instruments allow the modification of the laser focus depending on the applications (proteomics for which high sensitivity is required *versus* MSI requiring high lateral resolution).

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