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Precast Gelatin-Based Molds for Tissue Embedding Compatible with Mass Spectrometry Imaging

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Abstract

Preparation of tissue for matrix-assisted laser desorption ionization mass spectrometry imaging (MALDI-MSI) generally involves embedding the tissue followed by freezing and cryosectioning, usually between 5–25 μm thick, depending on the tissue type and the analyte(s) of interest. The brain is approximately 60% fat; it therefore lacks rigidity and poses structural preservation challenges during sample preparation. Histological sample preparation procedures are generally transferable to MALDI-MSI; however, there are various limitations. Optimal cutting temperature compound (OCT) is commonly used to embed and mount fixed tissue onto the chuck inside the cryostat during cryosectioning. However, OCT contains potential interferences that are detrimental to MALDI-MSI, whilst fixation is undesirable for the analysis of some analytes either due to extraction or chemical modification (i.e. polar metabolites). Therefore a method for both fixed and fresh tissue compatible with MALDI-MSI and histology is desirable to increase the breadth of analyte(s), maintain the topographies of the brain and provide rigidity to the fragile tissue whilst eliminating background interference. The method we introduce uses precast gelatin-based molds in which a whole mouse brain is embedded, flash frozen and cryosectioned in preparation for mass spectrometry imaging (MSI).

Nobel laureate Koichi Tanaka along with Franz Hillenkamp and Michael Karas are famed for developing the matrix-assisted laser desorption ionization (MALDI) technique.^{1,2} This ionization approach has developed rapidly over the last few decades giving rise to novel applications, including mass spectrometry imaging (MSI) of analytes in intact tissue.^{3,4} MALDI mass spectrometry imaging (MALDI-MSI) uses specially prepared tissue sections thaw mounted onto glass slides.⁵ It is a rapidly growing imaging technique applicable to a variety of endogenous biomolecules, including polar and non-polar metabolites and proteins.^{3,6,7} MALDI-MSI eliminates the need for chemical labels while expanding the

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SUPPORTING INFORMATION

The full scan range between m/z 150–2000 and the metabolite region between m/z 150–1000.

knowledge gained from a single tissue section and is therefore a novel analytical tool for visualizing the molecular distribution of many biomolecules across a sample.

Although many aspects of histological sample preparation are directly transferable to MALDI-MSI, there are still limitations to consider before proceeding. The quality of both histological and MALDI-MSI data depend heavily on the quality of the sectioned tissue. The tissue can be either fixed or fresh depending on the application and the analyte(s) of interest. Fixation is commonly used in histological preparation to preserve cellular information for microscopic analysis. It increases the rigidity of the tissue, making sectioning easier as the tissue is less susceptible to structural deformation.⁸ Detection of lipids is possible from fixed tissue, as shown in the analysis of eye tissue following paraformaldehyde (PFA) fixation.⁹ Peptides have also been detected from formalin-fixed paraffin-embedded (FFPE) tissue following enzymatic digestion.^{10, 11} However, small polar molecules are generally not reported due to losses during the fixation process likely due to extraction into the fixative. To avoid this potential loss of molecules, most MALDI-MSI studies use fresh frozen tissue sections. Ly *et al.* report a 72% overlap between FFPE fixed tissue and fresh tissue in the m/z 50–1000 range using a high-mass-resolution matrix-assisted laser desorption ionization Fourier-transform ion cyclotron resonance mass spectrometry imaging (MALDI-FT-ICR-MSI) platform.¹² Therefore fresh frozen tissue imaging is still important for metabolomic studies using MALDI-MSI.

Tissue embedding and mounting onto the cryostat chuck is commonly achieved using OCT, although it is not recommended for MALDI-MSI.¹³ OCT contains ~4% polyvinyl alcohol, ~10% polyethylene glycerol and benzalkonium chloride (an antifungal agent), which together produce a polymeric background signal in MALDI-MSI.^{14, 15, 16} This background signal can interfere with analyte signal (i.e. isobaric interference). Therefore, in an effort to avoid such deleterious effects, mass spectrometrists commonly avoid using an embedding medium altogether. However, since embedding helps increase the rigidity of fragile tissue samples, without an embedding medium, maintaining the structural topographies becomes challenging, specifically when working with fresh tissue samples. Indeed, it is not uncommon to use water to freeze mount the tissue onto the chuck inside the cryostat instead of using embedding medium.¹⁷ This approach, however, does not hold the tissue in place as sturdily as using embedding medium, and ice can cause cell damage, reducing histological resolution if serial sections are to be used for histology.

In order to be considered a suitable embedding medium for MALDI-MSI, the material must present a low background in the mass spectra and not interfere with the morphological features of the tissue. Recent research provides alternative embedding media that are compatible with MALDI-MSI. Strohalm *et al.* reported that pol[N-(2-hydroxypropyl)methacrylamide] was a safe embedding medium, with proof-of-concept shown using mouse lungs and bumblebee slices.¹⁸ Altelaar *et al.* successfully demonstrated the use of 10% gelatin to embed cerebral ganglia.¹⁹ Other examples of MALDI-MSI safe embedding material include carboxymethyl cellulose and tragacanth gum with proof-of-concept shown using whole mice and rabbit eyes respectfully.^{20, 21} Precast OCT based molds, have been reported for embedding brain tissue.²² Such OCT based molds are incompatible with MALDI-MSI; however precast gelatin-based molds are also available for

embedding intact mouse and rat brains. They are sold commercially as the Brain Block One Concept (or Brain Blocker One Concept), typically used to section tissue for histopathology. The gelatin-based molds include left and right precast cavities to house a whole mouse or rat brain. Essentially, the brain is encased within a gelatin-based medium so that the morphology of the brain remains intact during freezing and cryosectioning.

To the authors' knowledge, precast molds have not been considered for mass spectrometry (MS) applications. Here, we assess the suitability of applying the novel design to mouse brain tissue in preparation for MALDI-MSI.

Materials and Methods

Materials

The Brain Block One Concept was purchased from Electron Microscope Sciences (Hatfield, PA, USA). The dehydrated blocks were stored in the refrigerator at 4°C until ready to use. Prior to using, the precast gelatin-based molds were soaked in phosphate-buffered saline (PBS) at room temperature for 4–6 hours and monitored for expansion to the appropriate extent. The length of time required for expansion depends on the mouse brain (i.e. adult or adolescent).

Tissue

The Institutional Animal Care and Use Committee (IACUC) of the University of Florida approved all procedures (#20148382). Animals were housed with a 12h light-12h dark schedule, and were provided food and water *ad libitum*. Adult C57BL/6 mice were sacrificed and whole brains were harvested immediately from the skull either (a) after transcardiac perfusion using PBS followed by 4% PFA at 4°C for 2 days or (b) fresh, immediately post sacrifice, without perfusion.

Embedding

Fresh brains were first coated with a 15% gelatin solution (w/v in PBS) (Sigma Aldrich), and then embedded in the precast gelatin-based molds. Fixed brains were embedded and flash frozen using liquid nitrogen directly with no coating. Once encased the anterior, posterior, superior and inferior orientations of the brain were marked for later identification. A control fresh mouse brain was wrapped in aluminum foil and flash frozen in liquid nitrogen using no embedding procedure.

Cryosectioning

The precast mold was mounted onto the chuck inside the cryostat using OCT and sectioned at 25 µm using a Microm HM505E Cryostat at –20°C (Figure 1). Prior to thaw mounting onto glass slides the gelatin-based precast material was removed from around the tissue and discarded. Upon visual inspection, there was no residue from the precast. However, the 15% gelatin was thaw mounted along with the fresh tissue (Figure 1A).

MALDI-MSI

A Thermo Scientific LTQ XL linear ion trap mass spectrometer (MALDI LTQ XL) was used for MALDI-MSI experiments.²³ The MALDI source on this instrument is an intermediate-pressure source (7.5×10^{-2} Torr) with a pulsed nitrogen laser at 337.7 nm, a frequency of 60 Hz and energy of 250 μ J per pulse at 100% laser power. Sectioned tissue was dried in the desiccator for ~1 hour under vacuum and spray coated with dihydroxybenzoic acid (DHB) matrix at 40 mg/ml in 70:30 MeOH:H₂O with 10mM sodium acetate and 0.1% trifluoroacetic acid (TFA) using a Meinhard nebulizer. MALDI data were acquired using a laser energy and step size of 2.5 μ J and 100 μ m, respectively. To assess background interference a section of the gelatin-based mold was mounted onto a glass slide and coated with matrix under the same conditions as the tissue. In addition a control slide was also coated with DHB matrix for comparison. All imaging data was analyzed using ImageQuest Software (Thermo Scientific).

Results and Discussion

Sample Preparation

The application of precast gelatin-based molds as an embedding procedure for MALDI-MSI was assessed using mouse brain tissue. A fresh flash frozen mouse brain using no embedding procedure showed deformation of the native structure (Figure 2A). This occurred between the point of harvesting the brain and preparing it for flash freezing. However, the fresh brain tissue flash frozen once embedded within the precast cavity maintained its native structure (Figure 2B & C). This observation can be attributed to the brain being harvested and immediately encased within a precast cavity. Furthermore, a PFA fixed mouse brain tissue was embedded within the precast gelatin-based mold showing potential for a variety of analyte(s) to be investigated (Figure 2D). The fresh brain tissue was coated with 15% gelatin prior to embedding to compensate for over swelling of the cavity and subsequent crevice formation. Unlike non-embedding methods the precast mold does not curl during cryosectioning making mounting easier.

Following cryosectioning the remaining fixed and fresh tissue was stored, embedded within the precast gelatin-based mold in the freezer held at -80°C . After 10 months visual inspection presented no loss of its native structure or any visual damage to the mold itself.

Imaging

The gelatin-based material did not present any background interference in the MALDI signal (Figure 3A & B). Low signal intensity may correspond to poor matrix crystallization onto the gelatin-based mold. However, possible ion suppression or background interference was not considered an issue because the sturdy precast mold peeled away from the tissue after sectioning leaving the tissue free to be thaw mounted onto the glass slide, reducing smearing concerns. No sign of the gelatin-based mold was observed upon visual inspection of the tissue sections. A solution of 15% gelatin was used to eliminate crevice formation inside the cavity and offer additional support to the fragile tissue. A small amount of the 15% gelatin is thaw mounted onto the slide with the tissue (Figure 1A). This 15% coating can also be peeled away from the mounted tissue prior to matrix coating thus presenting no immediate

concerns. This effect is not seen for the fixed tissue, as 15% gelatin is not required and the precast mold peels away on sectioning. Furthermore, mounting the tissue onto the chuck inside the cryostat was possible using OCT because the medium does not come into contact with the tissue, only the precast mold, which was discarded after sectioning; no OCT polymeric contamination was observed in the MALDI-MSI (Figure 4A & B).

MSI data were successfully acquired; phosphatidylcholine [PC 38:4 + Na]⁺ at m/z 832, phosphatidylcholine [PC 36:1 + Na]⁺ at m/z 810, and phosphatidylcholine [PC 38:6 + Na]⁺ at m/z 828 were selected to produce spatially resolved images normalized to the total ion current (TIC) (Figure 4A and Figure S-1/S-2 of the supporting information). The pseudo-colored images for the given m/z values are colored according to the spectral intensity of that particular analyte. Overall, the structure is safeguarded within the precast mold from deformation offering a new alternative to the current embedding and cryosectioning methodologies for MALDI-MSI.

Conclusion

This study reports the use of precast gelatin-based molds for MALDI-MSI. Precast molds were successfully applied to both fixed and fresh mouse brain tissue to assist in freezing and sectioning of the fragile samples. The gelatin material did not present any spectral contamination unlike previously reported OCT embedding methods. Overall sectioning was smooth and the precast mold breaks away from the tissue prior to thaw mounting. However, the cost may pose a disadvantage depending on the application and the number of precast molds required per experiment.

Precast molds cannot only safeguard fragile tissue sample but could also improve sectioning reproducibility due to the orientation of the tissue within the molds. This could improve chemical visualization of specific brain regions for clinical investigations. Furthermore, similar precast molds could be produced for other fragile tissue samples such as the lungs or heart.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Fresh mouse brain tissue (A) thaw mounted onto a glass slide along with 15% gelatin and (B) the precast mold mounted onto the chuck inside the cryostat using OCT.

Figure 2. Mouse brain tissue (A) flash frozen in liquid nitrogen with no embedding procedure, (B) flash frozen in liquid nitrogen and embedded inside one half of the precast mold, (C) a fresh mouse brain inside the precast mold with added 15% gelatin and (D) a PFA fixed mouse brain inside the precast mold.

Figure 3. MALDI mass spectra of (A) DHB matrix only (m/z 273 $[2\text{DHB}+\text{H}-2\text{H}_2\text{O}]^+$).²⁴ and (B) a section of the precast mold coated in DHB showing poor spectral intensity.

Figure 4.

MALDI-MSI of a mouse brain coronal section (A) the phospholipid region and (B) MSI images of phosphatidylcholine [PC 38:4 + Na]⁺ at *m/z* 832, phosphatidylcholine [PC 36:1 + Na]⁺ at *m/z* 810, and phosphatidylcholine [PC 38:6 + Na]⁺ at *m/z* 828.