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APPLICATION NOTE

A Simple Sonication Improves Protein Signal in Matrix-Assisted Laser Desorption Ionization Imaging

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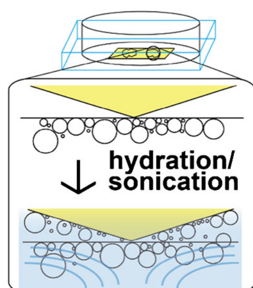

Abstract. Proper matrix application is crucial in obtaining high quality matrix-assisted laser desorption ionization (MALDI) mass spectrometry imaging (MSI). Solvent-free sublimation was essentially introduced as an approach of homogeneous coating that gives small crystal size of the organic matrix. However, sublimation has lower extraction efficiency of analytes. Here, we present that a simple sonication step after the hydration in standard sublimation protocol significantly enhances the sensitivity of MALDI MSI. This modified procedure uses a common laboratory ultrasonicator to immobilize the analytes from tissue sections without noticeable delocalization. Improved imaging quality with additional peaks above 10 kDa in the spectra was thus obtained upon sonication treatment.

Keywords: Matrix-assisted laser desorption ionization, Mass spectrometry imaging, sample preparation, protein

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Introduction

MALDI MSI provides spatial distributions of molecules on biological sample sections [1–3]. It has been largely used as a tool for cancer diagnosis, disease biomarker searching, drug metabolism investigation, and microbial natural product discovery [4–8]. The qualify of MALDI MSI highly relies on the appropriateness of matrix application. Matrix is deposited onto the sample surfaces in direct or indirect manner. For example, saturated matrix solutions are sprayed pneumatically to form crystallines on sample surfaces [9]. Higher spatial resolution to about 10–20 μm is achieved if cycles of matrix spraying–drying are carefully implemented to prevent coagulation between droplets [10]. For wet samples such as bacterial colonies, matrix powders are spotted directly onto the sample surfaces through test sieves [11]. Spotting techniques have limited spatial resolution as the crystal sizes of the matrix are usually larger than the sprayed ones. Moreover, the diffusion of extracted analytes in matrix solution could lead to a decreased spatial resolution for tissue imaging [12].

On the other hand, although commercialized robotic sprayers are accepted as a standard protocol for MALDI MSI, solvent-free sublimation has been demonstrated as an inexpensive and reproducible method. Compared with home-built ones, sublimation approach allows homogeneous coatings of the matrix onto the sample surfaces and thus minimize the so-called "hot spot" effect [13]. Meanwhile, sublimation usually leads to a poor protein extraction efficiency. By incorporating an additional step of hydration after matrix application, the efficacy of ionization for proteins in MALDI-TOF (time-of-flight) analysis improves considerably [14]. Notably, a combination of hydration after sublimation was found to extend the observed mass range up to 30,000 *m/z* [14]. In this contribution, sample pretreatment of six-step rinsing before matrix application and hydration procedures has been established to provide high-quality tissue MSI using 2,5-dihydroacetophenone (2,5-DHA) as matrix for robust protein signals [15].

Herein, we demonstrated that by adding a simple sonication step after sublimation and hydration (Figure 1), an improved MALDI-TOF spectrum was obtained. Remarkably, protein signals at *m/z* >10,000 were significantly enhanced, allowing us to visualize more than a dozen of new ions that were not observed in the serial section without a sonication treatment. Such anomalous phenomenon is explained by a mechanism of sonication-assisted extraction of large molecules in tissue sections. Details of the experimental procedures, spectrometry characterization, mass spectrometry imaging, and the proposed mechanism are elaborated in the following sections.

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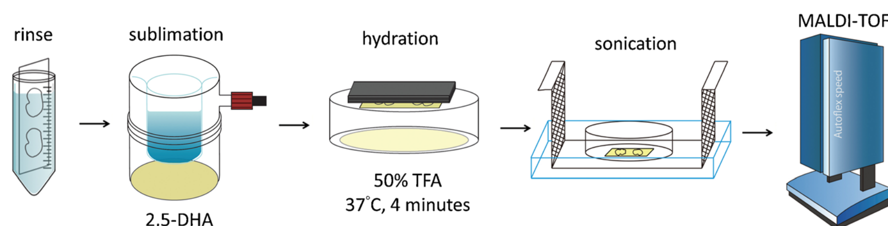

Figure 1. Schematic illustration of the sample preparation workflow incorporating a sonication step

Experimental

Details of materials and tissue preparation are described in Supporting Information.

Matrix Sublimation/Hydration

The sublimation apparatus was purchased from Singlong (Taichung, Taiwan). Details of matrix sublimation and hydration procedures for thin tissue sections are described elsewhere [14]. The sublimation apparatus was coupled to a vacuum pump and placed in a sand bath on a hot plate. The matrix was dissolved in methanol in the sublimation apparatus and allow to dry so as to create a homogeneous layer of matrix on the bottom of the apparatus. Sublimation was carried out using 150 mg of 2,5-DHA at 105 °C with a 0.7 Torr vacuum for 10 min. The amount of sublimated matrix onto the tissue section was controlled by exposure time.

A hydration chamber was made by Petri dish, filter paper, and stainless steel plates. The sample slide was attached to the cover lid of a petri dish with tape. A filter paper was placed in the bottom of the Petri dish with rehydration solution (500 μL of water and 500 μL of TFA) pipetted onto it. The Petri dish and cover lid were reassembled and sealed with parafilm to create a hydration chamber. Packed stainless steel plates (originally at 25 °C) were placed on the top of the cover lid right above the sample to work as a heat sink and assist the condensation of hydration solution onto the tissue section. The hydration chamber was then placed into the incubator and remainbed there for 4 min. The incubation temperature was set to 37 °C.

Sonication

After the sublimation and hydration steps, the stainless steel plates were removed from the top of the hydration chamber. The chamber was kept sealed to maintain humidity. It was then placed upside down in the Elmasonic S 30 H ultrasonicator at the center of the water bath. The sonication was operated under conituous mode with frequency of 37 kHz for 2 or 5 min. The temperature of the sonication bath was monitored by alcohol thermometer. The temperature rose from 24 °C to 26 °C after 2 min of sonication and to 30 °C after 5 min of sonication.

Mass Spectrometry

All MALDI-TOF MSI was performed on Bruker Autoflex Speed mass spectrometer in the positive ion linear mode via lexControl 3.3 software. Accumulated 1000 laser shots per pixel were acquired with a 1 kHz repetition rate by Smartbeam II Nd:YAG laser with attenuator offset at 80%. Laser operating power was at 90% with a raster size of 100 μm and beam size set at 3_medium. The detector gain was set at 2710 V (4.0x). The measurement order was set at Fast (from top to bottom). The imaging data was processed by top hat baseline subtraction. Image acquisition was carried out using FlexImaging 3.0, and spectral analysis was performed with FlexAnalysis 3.4.

A schematic overview of the sample preparation workflow is shown in Figure 1 and Supporting Information Video.

Results and Discussion

To test the efficacy of the new protocol, serial mice brain sections were treated with different extents of sonication. As shown in Figure 2a, significant alteration of the mass spectral profiles in the tissue sections was observed upon modification of the way it was prepared. Similar to what has been reported in the literature, there were not many ion peaks at the mass range of *m/z* >2500 on the sample analyzed directly after sublimation without any further treatment [16].

On the other hand, numerous ion peaks at 2500–10,000 *m/z* were found in the hydrated section, where the Petri dish was opened immediately without sonication (Figure 2a, red). Furthermore, in sections treated with 2 min of sonication, a more informative spectrum was obtained, especially in the range of *m/z* > 5000 (Figure 2a, green). Interestingly, relative intensities of the peaks in the lower mass range, e.g., below *m/z* 5000, were found to decrease, whereas peaks above *m/z* 5000 increased considerably. Such shift of ion distribution to the larger *m/z* region was also observed in the section treated with 5 min of sonication (Figure 2a, blue). The effect of sonication treatment on MSI was not increased significantly after lengthening the sonication time from 2 min to 5 min.

These results indicated that ions of large biomolecules are more prone to be produced after sonication. The most striking feature is that quite a few low abundance ion species were notably observed when a simple sonication was performed following the standardized hydration step. This provided us an efficient way to reveal the low abundance biomolecules on biological tissue sections in a spatial manner. In Figure 2b, we demonstrate the MALDI MSI of several representative ions that were "induced" by sonication in the brain section. For example, *m/z* 7749, 8565, 11798, and 3563 were revealed clearly in the hippocampus, whereas ions of *m/z* 9203 and 12309 were found specifically in the white matter of the brain section. Sonication not only allowed

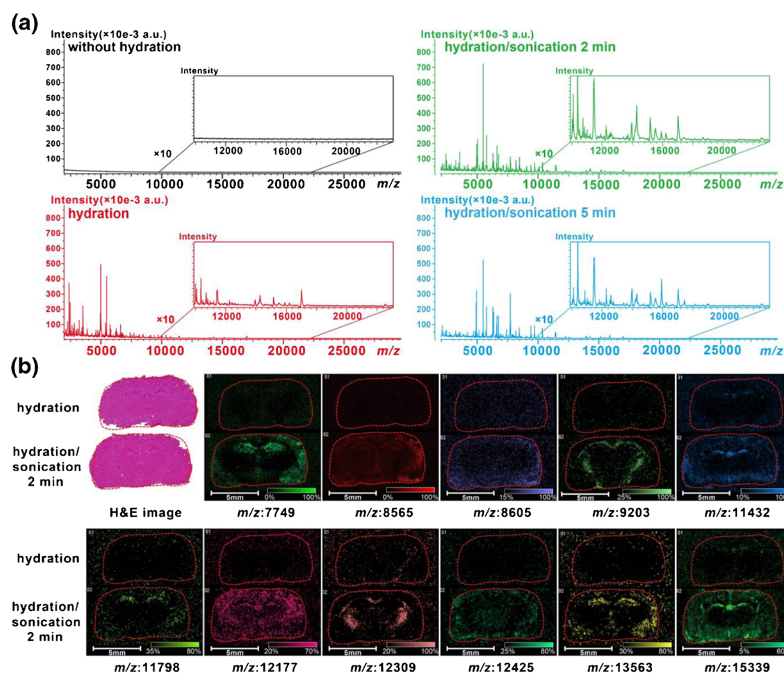

Figure 2. Enhancement of ion signals by sonication on MALDI MSI. **(a)** Overall spectra of the mass spectrometry imaging with different sample preparation methods. **(b)** Imaging of unique ions after sonication for 2 min compared with hydration-only sample. The two sections were sublimated with equal amount of 2,5-DHA at the same time and were side-by-side serial sections. Putative assignments of the protein identities are listed in Supplementary Table S1

us to discover lower abundance molecules originally buried within the noise but also help us to visualize the biomolecules expressed at higher level in greater detail. As shown in Figure 3, sonication gave improved imaging of protein ions such as cytochrome *c* oxadase polypeptides VIII (*m/z* 8324), histone h4 (*m/z* 11367), myelin basic protein (*m/z* 14127), and histone h3 (*m/z* 15332). The assignments of ions are based on the MALDI MSI data of previously identified proteins [17].

To rationalize the above results, an acoustic wave-induced microscale mixing of matrix with analytes is thus proposed (Supplementary Figure S1). This is not surprising as ultrasonic bath is used daily by graduate students in the laboratory to resuspend their samples. Furthermore, ultrasound-assisted extraction of plant oil and proteins has been widely studied in food chemistry [18, 19]. It is thought that the implosion of cavitation bubbles, derived during the propagation of ultrasonic

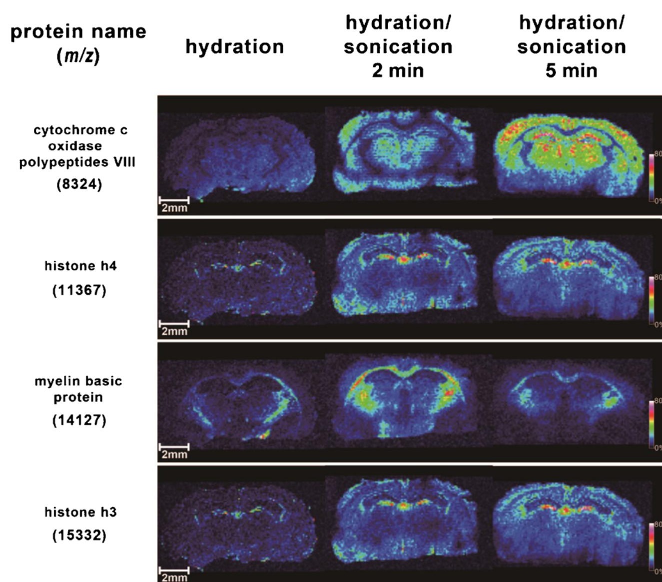

Figure 3. MALDI-TOF MSI of the ions with improved quality without losing spatial resolution after 5 min of sonication treatment. The effect of sonication differed from protein to protein. The assignments of ions are based on the MALDI MSI data of previously identified proteins [17]

pressure waves, creates micro-jets near the surface of the liquid-solid interface [19]. Such high-velocity local liquid stream and particle collision may help to release large biomolecules from the collapsed lipid rafts of the tissue sections. Meanwhile, relatively small and hydrophilic compounds were extracted efficiently by the hydration solution and then transferred to the surface of the matrix crystal, regardless of the subsequent sonication. After sonication treatment, the larger molecules were mobilized from the tissue section, competing for the surface of the matrix crystals with the smaller ones. The overall effect leads to the enrichment of spectra at higher *m/z*.

The small-scale turbulence created by sonication is especially useful when stirring of the sample is impractical. By irritating the collision in a microscale environment, spatial resolution can thus be preserved. It is evidenced by the fact that there is no obvious delocalization of the analyte after sonication (Figure 3). Another evidence is provided with the microscopy snapshots of matrix crystal before and after sonication, in which the matrix crystals remained the same size after the hydration/sonication processes, indicating that the effect of ultrasonic waves is at microscopic scale (Supplementary Figure S2).

Conclusions

We describe in this Application Note a simple, fast, and effective preparation protocol of tissue sections to enhance the performance of MALDI MSI in detecting large biomolecules. This protocol incorporates the use of a common laboratory ultrasonicator following the matrix sublimation and hydration steps. A molecular imaging of improved quality can be achieved without analyte delocalization. The subsequent MALDI MSI shows a drastic increase in signal intensity at higher *m/z* in response to the additional sonication step. We suggest the optimal sonication time of 2 min for the Elmasonic S 30 H ultrasonicator. Unique peaks were observed after sonication and the spatial resolution of the MSI was well preserved. Further utilization and application of sonication during sample preparations are worthwhile to explore for MALDI MSI.

Acknowledgments

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