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Measuring salty samples without adducts with MALDI MS

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Abstract

Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) has been used for the discovery of hundreds of novel cell to cell signaling peptides. Beyond its advantages of sensitivity and minimal sample preparation requirements, MALDI MS is attractive for biological analyses as high quality mass spectra may be obtained directly from specific locations within prepared tissue sections. However, due to the large quantity of salts present in physiological tissues, these mass spectra often contain many adducts of cationic salts such as sodium and potassium, in addition to the molecular ion $[M+H]^+$. To reduce the presence of cation adducts in MALDI mass spectra obtained directly from tissues, we present a methodology that uses a slow condensation procedure to enable the formation of distinct regions of matrix/analyte crystals and cation (salt) crystals. Secondary ion mass spectrometric imaging suggests that the salts and MALDI matrix undergo a mutually exclusive crystallization process that results in the separation of the salts and matrix in the sample.

Keywords: MALDI; Salt; Tissue; Mass spectrometric imaging; Secondary ion mass spectrometry

1. Introduction

Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) has become a mainstream method in bioanalytical chemistry as it enables the direct measurement of a variety of analytes in a wide range of biological samples from lipids [1] to peptides [2] and intact proteins [3]. The analysis of small volume and chemically complex samples such as brain sections and single cells has allowed the discovery and identification of numerous novel peptides in a range of animals, including invertebrates [4,5], amphibians [6], and mammals [7]. Sample preparation in such cases consists of three steps: sample isolation, transfer onto a sample plate, and the application of a MALDI matrix solution.

In the course of the past decade, single-cell and direct tissue measurement with MALDI MS has become an effective tool for proteome and peptidome investigation. An early application of MALDI MS was the analysis of individual, isolated nerves and cells [8]. Prohormone processing of a number of neuropeptide precursors has been successfully elucidated with this approach in several neurobiological models [5,9]. MALDI MS has also been used to study post-translational modifications of peptides [10], measure the peptide profiles of subcellular structures such as neuronal processes [2], and assay the contents of single secretory vesicles [11]. However, tissues and cells contain high levels (130 mM up to hundreds of mM) of various salts, the presence of which hinder and/or complicate analyses.

High concentrations of sodium and potassium salts can inhibit the formation of crystalline MALDI matrix crystals and form adducts with analyte molecules during the desorption/ionization process [12]. In the latter case, these adducts spread the signal of a single compound into multiple peaks in the mass spectrum which may reduce the qualitative sensitivity of analyses. The formation of several peaks from a single compound also increases the complexity of the resulting spectra, making data analysis much more difficult, particularly when dealing with signals of an unknown identity.

Previous methods to address the problem of salts in MALDI MS analyses have consisted of developing new, more salt tolerant matrices [13,14], sample washing protocols [3,12,15], or extraction procedures [16–20]. The development of matrices tolerant to salts often does not reduce adduct formation but rather

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allows the formation of matrix crystals from high-salt solutions. Additionally, washing protocols may cause the loss of some analyte during the rinsing procedure. Protocols to eliminate the need for the application of a matrix entirely, such as desorption/ionization on silicon [21] and surface-enhanced laser desorption/ionization [22], are also being developed.

Here, we present a matrix application and preparation strategy that reduces the formation of salt adducts in mass spectra obtained via MALDI MS. By controlling the conditions of matrix crystallization and extending the period of time over which the sample crystallizes, adducts of sodium and potassium are reduced in peptide standard solutions containing 100 mM sodium and potassium, as well as nervous tissue samples from the sea slug *Aplysia californica*. In addition, the mechanism of salt adduct reduction has been studied using secondary ion mass spectrometry (SIMS) imaging [23] by observing the distribution of MALDI matrix and salts within a MALDI sample at micron spatial resolutions.

2. Experimental

2.1. Peptide standards

A solution of peptide standards containing bradykinin, substance P, and luteinizing hormone-releasing hormone (all Sigma–Aldrich, St. Louis, MO) was prepared in deionized (DI) water or 100 mM NaCl and KCl (both Sigma–Aldrich) to study the level of salt adduct formation and the reduction of adducts following the condensation procedure. Glass slides were used as MALDI targets and were prepared by first adhering a thin layer of Parafilm M (Pechiney, Neenah, WI) to the surface of the slide by stretching the parafilm across the slide and then placing the slide in a 70 °C oven for 30 s. Small holes, ~200 μm in diameter, were then made in the Parafilm M surface using a pair of forceps. This allows for the creation of small hydrophilic sample spots on the otherwise hydrophobic target as has previously been shown to be beneficial for mass-limited MALDI analyses [13,24,25]. To cause the rapid crystallization of the samples and simulate standard tissue preparation protocols, 0.5 μL of the peptide solution (100 fmol of each peptide) was applied to the slide followed by 0.5 μL of a 10 mg/mL solution of 2,5-dihydroxybenzoic acid (DHB) (Sigma–Aldrich) in 4:1 acetone:H_2O and then placed in a drying oven for 45–60 s until dry.

Some samples were then placed into a homemade, humidity-controlled environmental chamber equipped with a system to allow the adjustment of the sample temperature as previously reported [26]. Briefly, the chamber consists of a 10 cm × 10 cm × 10 cm Plexiglas box with a Peltier device (Melcor, Trenton, NJ) and a thermocouple in the center of the chamber to allow the temperature of the sample to be tracked and adjusted via a CN77000 temperature controller (Omega, Stamford, CT). The relative humidity in the chamber was increased to 85–90% by passing nitrogen gas through a water-filled bubbler and samples were placed onto the Peltier device. The samples were then cooled until droplets of approximately 50 μm in diameter were observed on the surface via a CCD camera (DFW-X700, Sony, Tokyo, Japan) attached to a 7× zoom microscope (Edmund Optics, Barrington, NJ). The Peltier was then turned off and the sample allowed to return to room temperature while the droplets slowly evaporated. This process of thermal cycling and condensation was repeated three times for each "condensed" sample.

2.2. Tissue preparation

A. californica (200 g) were obtained from Charles Hollahan (Santa Barbara, CA) and kept at 15 °C in an aquarium containing continuously circulating, aerated and filtered seawater prepared according to manufacturer instructions using Instant Ocean salts (Aquarium Systems Inc., Mentor, OH). Animals were anesthetized by injection of isotonic $MgCl_2$ (~30–50% of body weight) into the body cavity. The abdominal ganglia were dissected and placed in artificial sea water (ASW), containing (in mM) 460 NaCl, 10 KCl, 10 $CaCl_2$, 22 $MgCl_2$, 6 $MgSO_4$, and 10 HEPES, pH 7.8, prior to sectioning.

For sectioning, the abdominal ganglion was rapidly frozen on dry ice and then affixed to the stage of an HM 550 ultramicrotome (Microm International, Waldorf, Germany) with a small quantity of water. This provides the mechanically stable mounting of small ganglia on the metal stage and eliminates the need for an embedding media (e.g., TissueTek), which can otherwise cause significant suppression of analyte signals acquired from biological samples [15,27]. Thin tissues sections (20 μm) were prepared at −20 °C and immediately placed onto clean glass slides.

MALDI matrix was applied in one of two manners to allow for the comparison of several traditional preparative strategies. DHB was either manually spotted in 0.5 μL aliquots of a 10 mg/mL solution in 1:1 acetone:H_2O (as in profiling experiments) or applied in a thin layer via a commercial airbrush (Badger, Franklin Park, IL) from a 30 mg/mL solution from 3:1 acetone:water (as in MALDI mass spectrometric imaging experiments). The airbrush nozzle was positioned at a distance of ~15 cm from the sample surface and the mixture was sprayed onto the sample with multiple passes for ~2 min. This resulted in a relatively uniform coverage of DHB across the sample surface while limiting the time the tissue remains wet to reduce the likelihood and/or extent of analyte redistribution. Some coated samples were placed into the humidity-controlled chamber and thermally cycled as noted in the previous section.

2.3. SIMS samples

Samples were prepared on hydrophobic silicon shards. Raw silicon wafers (Montco, Spring City, PA) were cut to 1 cm × 1 cm squares and etched in a piranha solution (3:1 conc. H_2SO_4:H_2O_2) for 1 h. (Extreme caution must be exercised as piranha is a strong oxidant and reacts violently with organic impurities. An explosion-proof hood should be used.) The shards were then washed and dried before silanizing in a 1% solution of octadecyltrichlorosilane (Sigma–Aldrich) in 3:1 hexane:chloroform for approximately 15 min. Shards were then washed in hexane, dried, and stored under nitrogen until needed.

A solution containing 10 mg/mL DHB, 1 mM acetylcholine, 250 mM NaCl, and 10 mM KCl was prepared in 3:1 acetone:H_2O and applied to the silicon shards with the airbrush as described above. The solution was applied essentially dry as observed by visual inspection immediately following application. As before, samples were then placed into the humidity-controlled chamber and cycled through three condensation cycles. Samples were inserted into the mass spectrometer without any further treatment within hours of preparation to minimize any analyte redistribution between preparation and analysis.

2.4. MALDI MS

MALDI mass spectra were acquired using an UltraflexII ToF-ToF mass spectrometer (Bruker Daltonics, Billerica, MA) with a solid-state UV laser capable of a variable repetition rate. Positive-ion mass spectra were collected at 50–100 Hz in the reflectron mode with a delay time of 20 ns and an acceleration voltage of +25 kV. Each unsmoothed mass spectrum is the sum of 500–1000 laser shots. All mass spectra were externally calibrated and data analysis was performed with FlexAnalysis (Bruker Daltonics) and Excel (Microsoft, Redmond, WA).

2.5. SIMS

Experiments were performed on a TRIFT III time-of-flight secondary ion mass spectrometer (Physical Electronics, Chanhassen, MN) equipped with a gold liquid metal ion cluster source operating at 22 keV. The primary ion beam was randomly rastered in a 256 × 256 pixel region at 8 kHz with a 25-ns pulse width. Total ion doses were kept below the static limit of 1×10^{13} primary ions cm^{-2}. Ion image resolution was not limited by the diameter of the focused beam but rather by the spacing between the raster positions. No charge compensation was used and no sample charging was observed. Individual mass spectra were obtained over the *m/z* 0–2000 range for each pixel and later analyzed using WinCadence software (Physical Electronics).

3. Results

3.1. Standards

The effect of the condensation procedure was studied using a series of peptide standards to observe the presence of salt adducts in samples prepared in 100 mM salt solutions. As presented in Fig. 1A, significant sodium and potassium adducts $[M+Na]^+$ and $[M+K]^+$, respectively, are observed. The presence of these adducts is, however, diminished in relation to the protonated ion after the sample has undergone the condensation procedure as shown in Fig. 1B. Although not completely eliminated, the adduct peaks appear greatly reduced in the condensed sample.

The reduction of salt adduct formation has been quantified for both the $[M+Na]^+$ and $[M+K]^+$ signals. For each of the conditions, five separate samples were prepared and analyzed. The intensity of the protonated ion signal $[M+H]^+$, and that of the $[M+Na]^+$ and $[M+K]^+$ signals were collected and used to allow comparisons between the untreated samples and those that underwent the condensation procedure. As shown in Fig. 2, the condensed samples provided an $[M+H]^+/[M+\text{adduct}]^+$ ratio comparable to that of the sample that was prepared in DI water. This effect is most significant for the sodium adduct while the reduction of the potassium adducts occurs to a lesser extent.

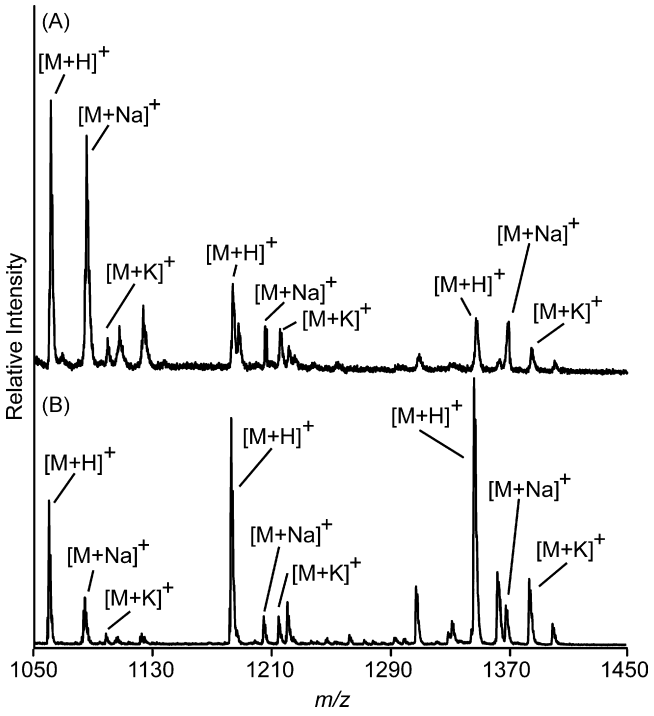

Fig. 1. Mass spectra of peptide standards with and without the condensation procedure. (A) Mass spectrum from an untreated sample shows large $[M+Na]^+$ and $[M+K]^+$ signals. (B) The salt adducts are reduced in a mass spectrum from a condensed sample.

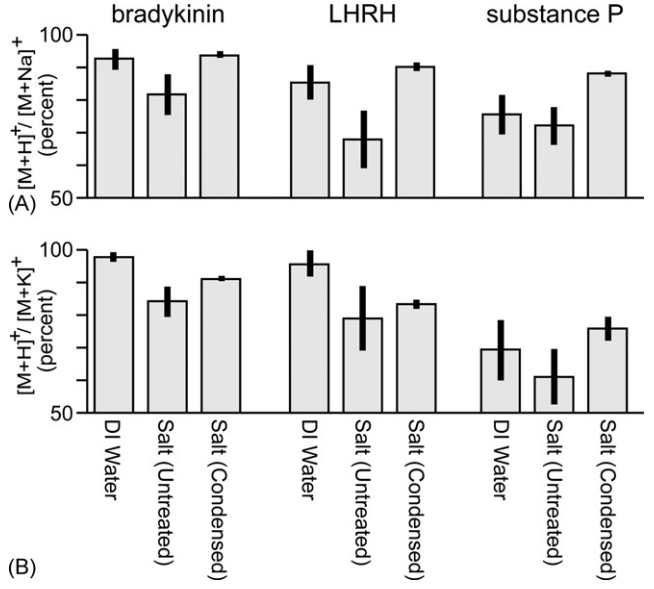

Fig. 2. Semi-quantitative analysis of (A) sodium adducts and (B) potassium adducts from peptide standards prepared in DI water and 100-mM salt solutions with and without the condensation process. Error bars represent the standard deviation from five analyses.

The $[M+H]^+/[M+Na]^+$ ratio for bradykinin and LHRH are not significantly different between the condensed sample and the sample prepared in deionized water ($p = 0.57$ and 0.8, respectively). In the case of substance P, the condensed sample was significantly improved over the DI sample ($p = 0.001$). The standard deviation of the protonated ion to the salt adduct ratio is also markedly lessened in the condensed samples (1.5% in condensed samples versus 7.1% in untreated salt samples and the DI water samples whose S.D. was 4.9%). This suggests that the slow crystal formation (>15 min) afforded by the condensation process allows for more uniform co-crystallization of DHB and analytes. In addition, the S/N of peaks observed in the condensed samples is markedly higher than the untreated salt sample (mean 480 versus 84, respectively, for the protonated ion for the three peptides examined). In other words, the spectra are of higher quality in terms of intensity, reproducibility, and simplicity.

3.2. Direct tissue measurements

A similar reduction in $[M+Na]^+$ and $[M+K]^+$ ion formation is observed in a 20-μm-thick section of the abdominal ganglion of *A. californica*. Fig. 3 shows the reduction of such adducts with acidic peptide (from the egg laying hormone prohormone). Mass spectra were obtained from similar regions of tissue prepared from serial sections using standard direct tissue MALDI MS protocols for matrix application via aerosol (Fig. 3A) and microspotting (Fig. 3B). Fig. 3C shows the mass spectrum from tissue that was coated in matrix via an aerosol and then prepared using the condensation methodology.

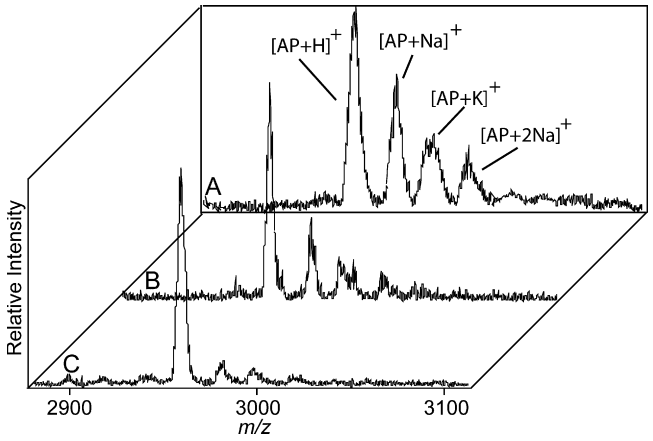

Fig. 3. Adduct reduction in tissue samples with various matrix preparation techniques. Mass spectra showing acidic peptide from the abdominal ganglion of *Aplysia* prepared by (A) aerosol application, (B) microdroplet, and (C) aerosol followed by the condensation protocol show varying levels of salt adduct formation with longer extraction periods reducing $[M+Na]^+$ and $[M+K]^+$ signals to a greater extent.

The greatest degree of adduct formation to acidic peptide was observed in the sample that was sprayed with matrix and thus, had the shortest period of time in which the sample was "wet." This is often the case in MALDI mass spectrometric imag-

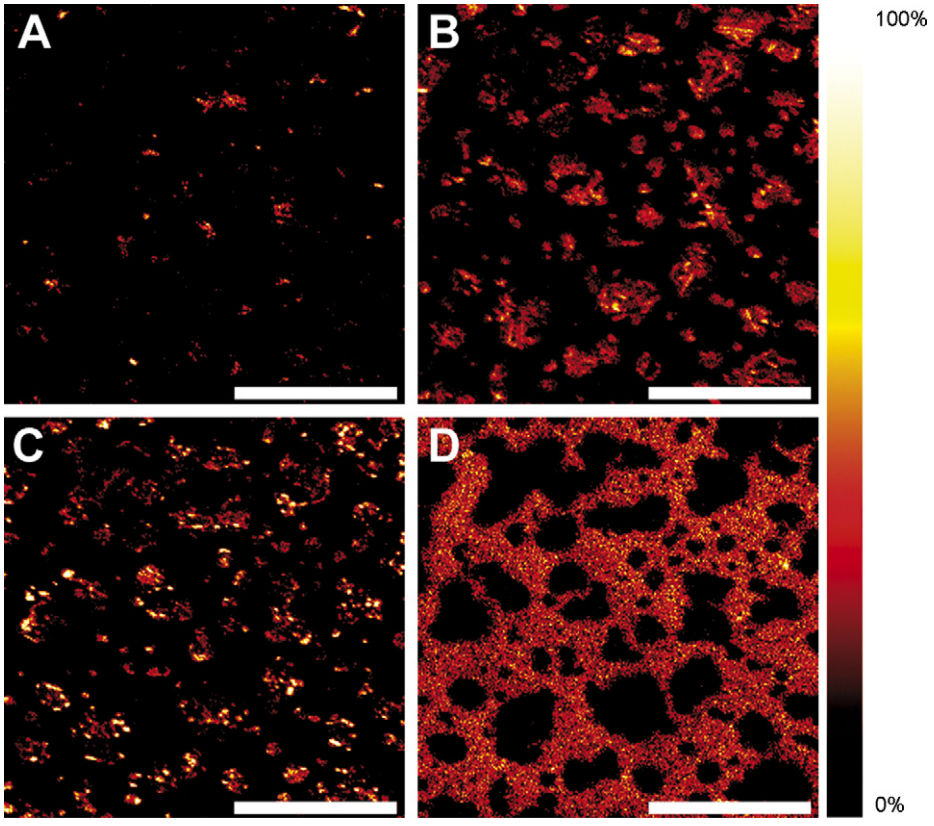

Fig. 4. SIMS imaging of a condensed sample shows the complementary distribution of (A) sodium (*m/z* 23) and (B) DHB (*m/z* 154). The ion image for (C) acetylcholine (*m/z* 146) shows that analytes do not undergo such partitioning while (D) silicon (*m/z* 28) from the substrate produces a negative image of where the sample crystals are located. Scale bar = 100 μm.

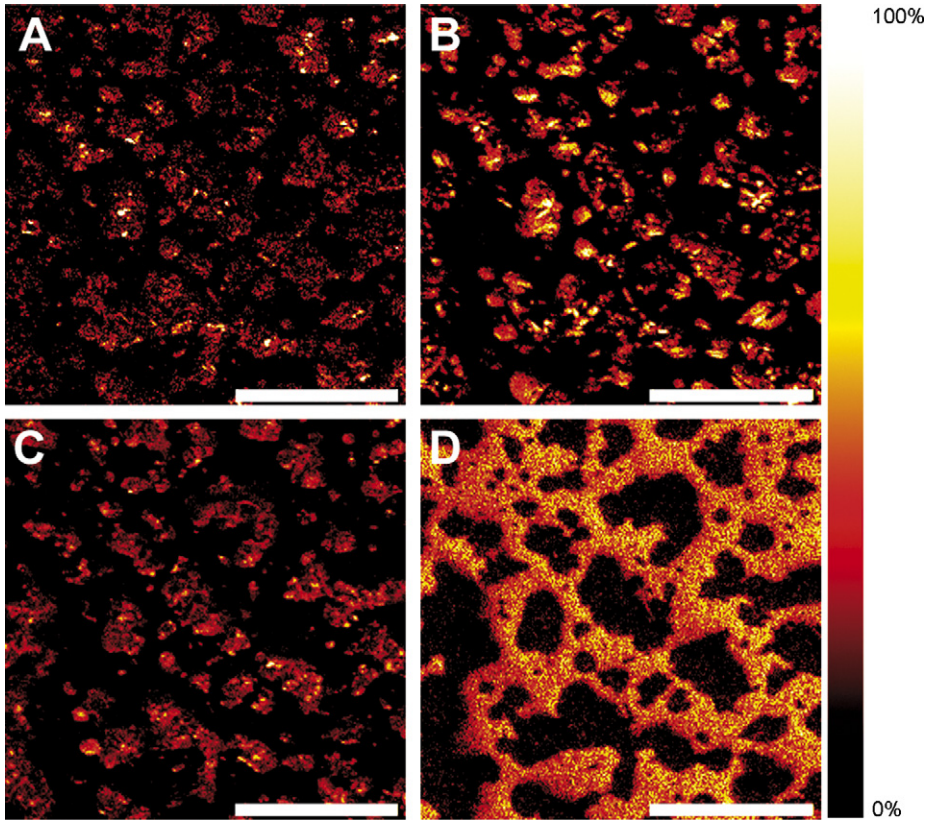

Fig. 5. SIMS imaging of an untreated sample illustrates similar distribution patterns for (A) sodium (*m*/*z* 23), (B) DHB (*m*/*z* 154), and (C) acetylcholine (*m*/*z* 146). (D) The image for silicon (*m*/*z* 28) arises from the substrate with no signal from regions of the sample where crystals are present. Scale bar = 100 μm.

ing experiments where analyte redistribution must be prevented. The application of droplets of matrix appears beneficial in profiling experiments and results in slower crystal formation and, as observed in Fig. 3B, a lower level of salt adduct formation. These salt adducts are minimized in the condensed sample (Fig. 3C). Such a reduction is rather remarkable as the *Aplysia* physiological solution contains approximately 500 mM Na^+ and often produces a large amount of $[M + Na]^+$ and $[M + K]^+$ signals even in single, isolated cell preparations [2].

3.3. SIMS

To understand the reasons for the reduction in salt adducts following the condensation procedure, SIMS has been used to produce chemical images of the prepared samples. As the sensitivity of SIMS for peptides such as acidic peptide is much poorer than for smaller molecules, the distribution of a small neurotransmitter, acetylcholine, was used as a marker of analyte distribution in a mixture of salts and DHB. Surprisingly, the ion images for sodium (Fig. 4A) and DHB (Fig. 4B) illustrate almost mutually exclusive distribution patterns. The ion signal for acetylcholine (Fig. 4C), however, is relatively homogenous in the crystalline regions of the sample as illustrated by the lack of silicon signal from the underlying silicon substrate (Fig. 4D).

This mutually exclusive crystallization is not observed when the sample has not undergone the condensation procedure as shown in Fig. 5. For this example, the sample has been prepared in an identical manner as the sample shown in Fig. 4 but did not undergo the condensation process. The ion images for sodium (Fig. 5B), DHB (Fig. 5B), and acetylcholine (Fig. 5C) show similar distribution patterns to each other across the entire sample. Again, silicon (Fig. 5D) produces a negative image of the matrix-salt-analyte particles.

4. Discussion

Using the condensation methodology, the appearance of $[M + Na]^+$ and $[M + K]^+$ signals in mass spectra obtained under salty conditions and from tissues is minimized. In standard solutions, these adducts are reduced to levels near or less than those from solutions prepared in DI water. The long periods of crystal formation lead to increased analyte incorporation and more homogenous samples, possibly due to the improved formation of a crystalline matrix, which results in the observed improvement in S/N values in condensed samples. Although it is still difficult to make signal intensity reproducible across multiple samples, the condensation methodology assists in minimizing ionization differences across samples as indicated by the reduced standard deviation of the $[M + H]^+/[M + \text{adduct}]^+$ signal ratio.

High resolution SIMS imaging experiments suggest that the MALDI matrix and the salts are physically isolated from

each other in condensed samples and undergo a differential crystallization process whereby the sodium is incorporated into one crystal type and the DHB matrix forms a distinct crystal, with the detectable analyte within the DHB crystals. The absence of detectable levels of Na^+ and K^+ within the DHB crystals can explain the reduction of salt adducts in condensed samples as few salts would be present in the ejection plume following the laser pulse, and so few adducts would be formed.

Previous publications have addressed salt adduct and poor crystal formation by first rinsing the tissue with cold ethanol [3]. While rather effective, such methods do not prevent these adducts from forming. The high-salt content of living tissues presents significant challenges with direct tissue MS. The methodology presented here allows the reduction of these salt adducts. Although the reduction of the $[M+K]^+$ signal is not as strong as the sodiated form, this adduct is less prevalent in most biological tissue samples. By inhibiting the effective dilution of an analyte signal, the sensitivity of a method should be increased.

The additional extraction period provided by the prolonged drying time may assist in increasing analyte sensitivity. Conceivably, this may also cause analyte redistribution during the condensation process, which is deleterious for imaging mass spectrometric experiments. One recently developed sample preparation strategy [26] addresses this challenge by separating the tissue section into thousands of discrete sections that can be individually probed. The condensation methodology will certainly aid MALDI MS studies in which samples have been isolated before being applied to the target and for samples that are present in a high-salt solution.

5. Conclusions

Recondensing samples on the surface of a MALDI matrix coated sample greatly reduces the observed level of $[M+Na]^+$ and $[M+K]^+$ adducts in mass spectra from both standards and tissue. SIMS imaging demonstrates that the reduction of salt adducts may be due to the differential crystallization of the salts and MALDI matrix. This physical separation of the salts and the MALDI matrix allows the formation of mass spectra with minimal signals arising from sodium and potassium adducts. Such adduct reductions may greatly benefit the sensitivity of direct tissue MALDI MS as well as reduce the complexity of the obtained mass spectra. Implementation of this approach is straightforward and should have application to a number of tissue profiling and imaging experiments.

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