Preparing Biological Samples for Analysis by High Vacuum Techniques

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Abstract

Time of flight secondary ion mass spectrometry (ToF-SIMS) and scanning electron microscopy (SEM) provide valuable complementary information about the molecular composition and morphology of biological samples, but both techniques are performed under high vacuum, which is not compatible with hydrated samples. Designing a suitable method to prepare biological (hydrated) samples for high vacuum conditions is important to obtain reliable and scientifically meaningful results from ToF-SIMS and SEM and to enable the routine use of these techniques for characterization. This article will compare freeze-drying and critical point drying for preparing adherent and nonadherent cells for ToF-SIMS and SEM analyses.

Introduction

Imaging mass spectrometry (IMS) is gaining popularity for the analysis of biological samples because it directly measures the chemical distribution across tissues and cells without the need for labels.[1] In fact, several major pharmaceutical companies are using IMS to screen the distribution, retention, and metabolism of new compounds in tissue sections and entire body sections. One type of IMS is time of flight secondary ion mass spectrometry (ToF-SIMS), which offers label-free, high spatial resolution (<100 nm) chemical imaging of molecules (native and exogenous) on the surface (top few Angstroms) of biological samples.[2] For instance, ToF-SIMS can be used to monitor the specificity of MRI contrast agents in tissue.[3] The technique can detect all of the elements and small molecules, typically encompassing a mass range of 1 amu to ~2000 amu, although masses up to 10,000 amu have been detected.[4] This mass range makes ToF-SIMS an attractive characterization method for projects involving small molecule binders, metallic-based nanoparticles, lipids, peptides, and small proteins.[5-7] Imaging ToF-SIMS can interrogate areas ranging from $2\times2\ \mu m^2$ to $7\times7\ cm^2$, which spans the size range from subcellular analysis to whole organism analysis.[5-7]

Scanning electron microscopy (SEM) is another critical technique in the biological analyst's tool kit and it provides complementary morphological information with superior lateral resolution (~ 3 nm). SEM offers a large depth of field, which enables a large area of the image to remain in focus at once. In addition, it produces high lateral resolution images that enable finely spaced details to be easily observed. Sample preparation on non-hydrated samples is typically straightforward and involves making the samples electrically grounded. Hydrated samples are challenging to image because the vacuum conditions cause water sublimation and sample shrinkage, which alters the morphology of interest. Also, hydrated samples are generally insulating materials, which are difficult to ground electrically, and therefore charge under the electron beam.

Proper sample preparation is critical to achieve meaningful results for ToF-SIMS and SEM analyses of biological (hydrated) samples because both techniques operate under high vacuum. The low pressure and room temperature conditions cause sample water to rapidly sublime, which drastically alters the native morphology (sample shrinkage) and chemical distribution of the biological sample.[8] Therefore, it is important to identify a sample preparation method for ToF-SIMS that will maintain the native chemical distribution and for SEM that will maintain the native sample morphology. The optimal method will retain both the native chemical distribution and morphology on a nanometer scale. Fast-freeze, freeze-fracture has been used successfully by several labs to prepare biological samples for high vacuum, but the necessary infrastructure is not commonly found on typical laboratory instrumentation.[8,9] Freeze-drying and critical point drying are alternative sample preparation methods that are commonly used in the microscopy community to preserve biological samples.[10-11] Freeze-drying involves rapidly freezing samples in a cryogen, such as nitrogen slush or liquid ethane, and then gently subliming the water by controllably increasing sample temperature. Critical point drying is a multi-step process that enables a phase transition from liquid to gas without the effects of surface tension that occur during air drying and freeze-drying. During critical point drying, samples are treated with a fixative, such as gluteraldehyde, and then a series of dehydration baths of increasing dehydrant concentration. Ethanol is an example of a common dehydration fluid. When the sample is fully dehydrated, the dehydration fluid is exchanged for liquid CO_2 (LCO_2) in the critical point dryer chamber and the chamber is brought to critical point temperature and pressure conditions (-31 °C and 1072 psi for LCO_2) to remove all fluid.

This article compares freeze-drying with critical point drying for preparing adherent and non-adherent cells for SEM and ToF-SIMS analyses. After drying, the samples were analyzed by SEM to verify that the cell morphology was retained (the cells did not shrink) and by ToF-SIMS to confirm that the native chemistry of the cells was not altered. The best preparation method will maintain both the morphology and chemistry of biological samples because that will enable a single sample to be examined by the complementary characterization techniques.

Experimental Method

Instrumentation

ToF-SIMS experiments were conducted on an ION-TOF model ToF-SIMS IV instrument that was upgraded to a three-lens Bi_n^+ polyatomic primary ion source.[12] The instrument also features a dual source column (DSC) with Cs and electron impact (Ar, SF_5^+) sources for depth profiling measurements and a low electron flood gun for charge compensation. For the experiments described here, 25 kV Bi_1^+ or Bi_3^+ were used to collect spectra and images. Sample position was manipulated using an ECSOSY (Raith, Dortmund, Germany) stage controller, which allows sample movement in the *X, Y*, and *Z* directions as well as sample rotation (*R*) and angling (*T*). The desired position was aligned using *in-situ* optical microscopes. Samples were mounted on the holder using aluminum clamps.

SEM images were captured on a Zeiss model Supra-55 Field Emission SEM using a 3 keV beam energy and the SE2 detector. The samples for SEM were mounted with conductive double-stick tape on Al stubs and then coated with platinum. The instrument has the variable pressure option (enables imaging beam sensitive and non-conducting samples), however it was not used for this study.

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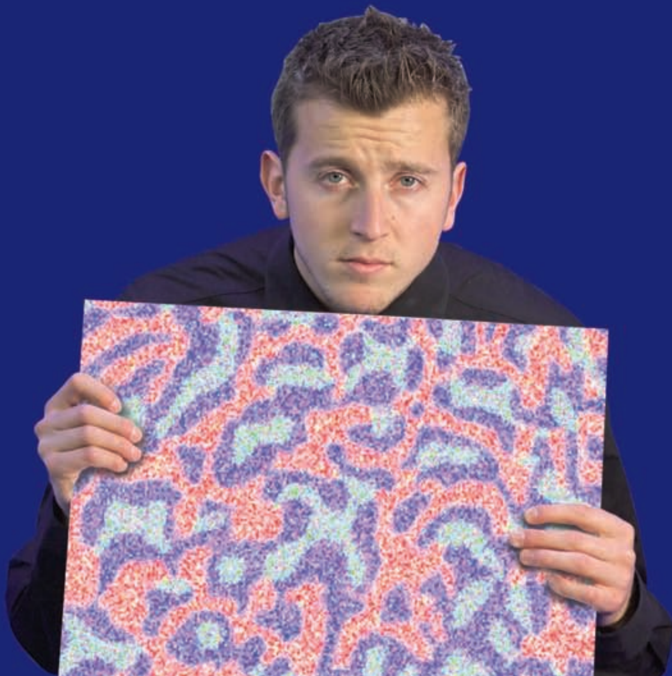

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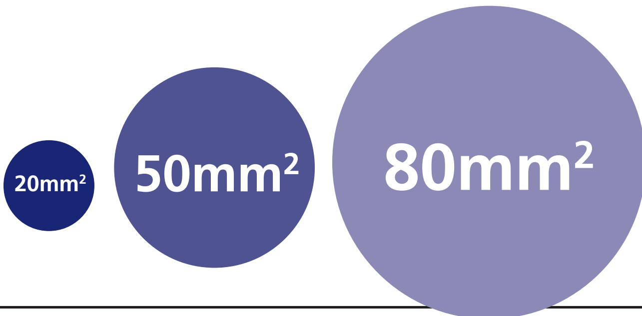

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Sample Preparation

Cell Culture

LS174T human colorectal carcinoma cells (CL188, American Type Culture Collection (ATCC)) were cultured in media composed of Eagle's minimum essential medium (ATCC) with Earle's BSS and 2 mM L-glutamin (EMEM)/1.0 mM sodium pyruvate, 0.1mM nonessential amino acids, 1.5 g/L sodium bicarbonate, and 10% fetal bovine serum. Silicon substrates (8 × 8 mm^2) were placed in the cell culture flasks so that the LS174T cells would grow onto the silicon surfaces. The substrates were cut to this size so they could fit into the sample holders of the dryer chambers. Before drying treatments, the samples were washed several times with phosphate buffered saline. The LS174T samples were used as an example of an adherent cell line.

Killed *Escheria coli* bacteria O157:H7 (KPL) were received and rehydrated per manufacturer instructions. Sterne spores were grown using new sporulation medium, collected by flooding the culture plate, dislodged with a cell spreader, purified by centrifugation in 15mL tubes, and re-suspended in water. The *E. coli* bacteria were used as an example of a killed nonadherent cell while the Sterne spores were used as an example of a living nonadherent cell. In certain experiments, *E.coli* cells or Sterne spores were captured using superparamagnetic beads (Invitrogen) and labeled using gold nanoparticles for spectroscopic analysis. The spectroscopic data is beyond the scope of this article; however the images collected with SEM will be discussed.

Freeze-Drying

The LS174T-containing silicon substrates that were destined for freeze-drying were removed from solution with a pair of reverse action tweezers. The excess fluid was wicked away by gently touching a chem-wipe to the edge of the silicon. The silicon was then rapidly plunged into the cryogen and stored under liquid nitrogen until freeze-drying. In the experiments described here, the cryogen was liquid nitrogen, and a Millrock (Kingston, NY) Tech 24L freeze-drier was used. Nitrogen slush was also explored as a cryogen, but maintaining a critical depth of slush proved to be very difficult, therefore, those results will not be presented here. After freezing, the samples were transferred under liquid nitrogen to a shelf in the freeze-drier. Experimental drying conditions are discussed in detail below.

The *E. coli* bacteria and Sterne spores were prepared for freeze-drying experiments after binding to beads and nanoparticles in solution. Before being placed onto filters, they were concentrated using a magnetic field. The bead-cell samples were then placed onto 0.2µm filters, and most of the liquid was removed using vacuum filtration. Samples containing some residual moisture were placed onto a shelf in the Millrock 24L Tech freeze-drier and an appropriate freeze-dry cycle was engaged. It should be noted that several other methods of sample preparation were investigated including plunge freezing in liquid nitrogen and plunge freezing in nitrogen slush on copper substrates and silicon wafers; however, the best results for these nonadherent samples were obtained using a track etch filter as the substrate and a shelf-drying method.

The shelf drying method used a commercial grade laboratory freeze dryer. This unit was a tray freeze dryer, which offered the primary advantages of allowing intimate or physical contact via the shelf with samples. Due to the improved thermal exchange from enhanced conductivity, gentler transitions, more extensive control over the drying process was achieved. Slow controlled process changes were advantageous to secondary, or "bound" moisture removal. The sublimation of bound or secondary water was difficult to accomplish without careful control of process conditions, often-excessive heat or energy would have resulted in disruptive damage to structural features of samples. Tray freeze dryers are used commercially to produce optimal or highest degree drying in materials due to the precise control of the thermal process.

A tray based freeze drier is essentially a two-chamber system; the chambers are stacked vertically relative to each other. The upper chamber contains the shelves, used to hold or support samples, and fluid is pumped through the shelves to chill or heat the shelves. The lower chamber is used to capture the water from the drying process, and leads to a vacuum pump used to depressurize the chambers. Vacuum is used to increase the volatility of the water bound in the samples, and to assist in the drying process. This type of unit differs from traditional freeze-drying in that a standard freeze drier is usually a condenser and vacuum system connected to a manifold.

The process conditions used to freeze dry these samples were designed to slow the rate at which temperature changes occurred to help protect the structural integrity of the parts. In order to assist with damping the thermal changes or compensate for the sample size relative to the mass of the freeze dryer, we packed several liters of free water in the system. The process starting point was chosen as -60^0C, so that the samples could be safely pre-frozen in a separate cryogenic process. The freeze dryer was then engaged under vacuum (100 mTorr). The process was run in 5^0C steps with 3 hour holds at each temperature up to 35^0C. The ramp rate or temperature change for each process step was 1 hour. A final hold at 35^0C was performed for 4 hours to complete sublimation. The unit was then brought to room temperature, and up to atmospheric pressure.

Critical Point Drying

Critial point drying (CPD) was performed using a Tousimis (Rockville, MD) Samdri 795 drier. The LS174T-containing silicon

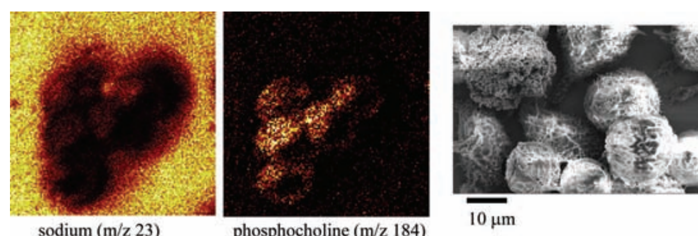

Fig. 1. ToF-SIMS images (71x71 µm2 field of view) of Na (left) and phosphocholine (middle) and SEM image (right) for LS174T cells frozen in liquid nitrogen and freeze-dried.

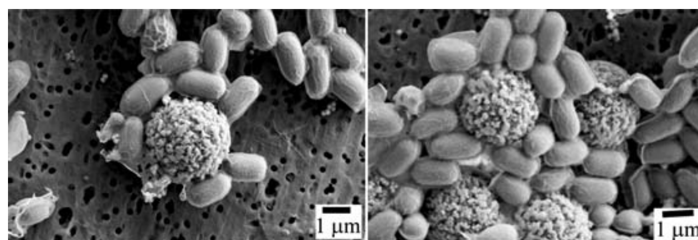

Fig. 2. SEM images of bead-Sterne-nanoparticle samples on a membrane substrate that were shelf freeze-dried in the Millrock Tech 24L freeze-drier. Beads are ~2.8 µm in diameter and appear rough. Sterne are ~1 µm x ~0.5 µm and appear slightly wrinkled. Nanoparticles are not evident in these images.

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samples were placed in a dish containing 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). The cells were allowed to undergo fixation for five minutes. The fixative solution was replaced with fresh 2% gulteraldehyde/0.1 M sodium cacodylate buffer solution and the samples were treated for an additional 20 minutes. Next, the fixative solution was removed from the dishes and 25% by volume ethanol/deionized water was added for five minutes. The samples were then processed in a series of additional dehydration solutions (50%, 70%, 90%, 100%, 100%, 100% ethanol) for five minutes each. After complete dehydration, the samples were critical point dried in the Tousimis drier. The drier chamber was partially filled with the dehydrant, 99.5% pure ethanol, and was suited with bone-dry, 99.8% purity LCO_2. The instrument was operated through the standard cycles of cooling, solvent exchange (purging for 10 minutes), heating and pressurizing to the critical point (31°C, 1072 psi), and venting, as described in the instrument instruction manual.

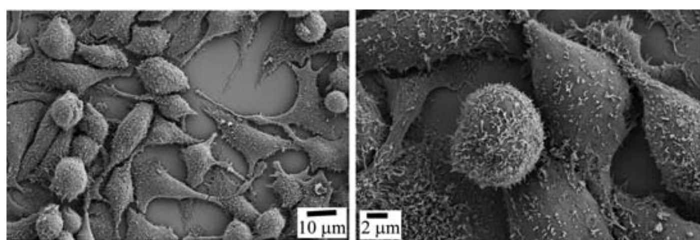

Fig. 3. SEM images of critical point dried LS174T cells.

The *E. coli* bacteria and Sterne spores were prepared for CPD on track etch filters with a pore size of 0.4 µm or smaller. A filter apparatus was assembled, and 1mL of dilute cell suspension or bead-bound cell suspension and 1mL of 2% gluteraldehyde in 0.1M sodium cacodylate buffer pH 7.4 were mixed together in the filter apparatus, and allowed to fix for 5 minutes. A vacuum was used to pull most of the fixative solution through the filter, leaving only fixed cells on the filter surface. Care was taken not to allow the filter to become dry during the sample preparation process. Fresh 2% gluteraldehyde in 0.1M sodium cacodylate buffer replaced the initial aliquot of fixative, and was allowed to stand for 20-30 minutes. Again, vacuum was used to remove fixative, and the cells were rinsed 3 times with 0.2M sodium cacodylate buffer. Once samples were fixed, dehydration was performed. The dehydration procedure involved pulling vacuum to remove most of the final buffer solution, and adding 25% ethanol solution to the sample for 5-10 minutes. The samples were then processed in a series of additional dehydration solutions (50%, 70%, 90%, 100%, 100%, 100% ethanol). After complete dehydration, the samples were critical point dried in the Tousimis drier, as described above.

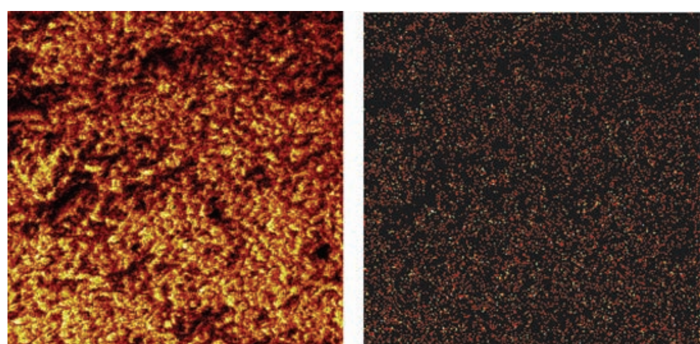

Fig. 4. ToF-SIMS images (500x500 μm^2 field of view) of Na (left) and phosphocholine (right) for critical point dried LS174T cells.

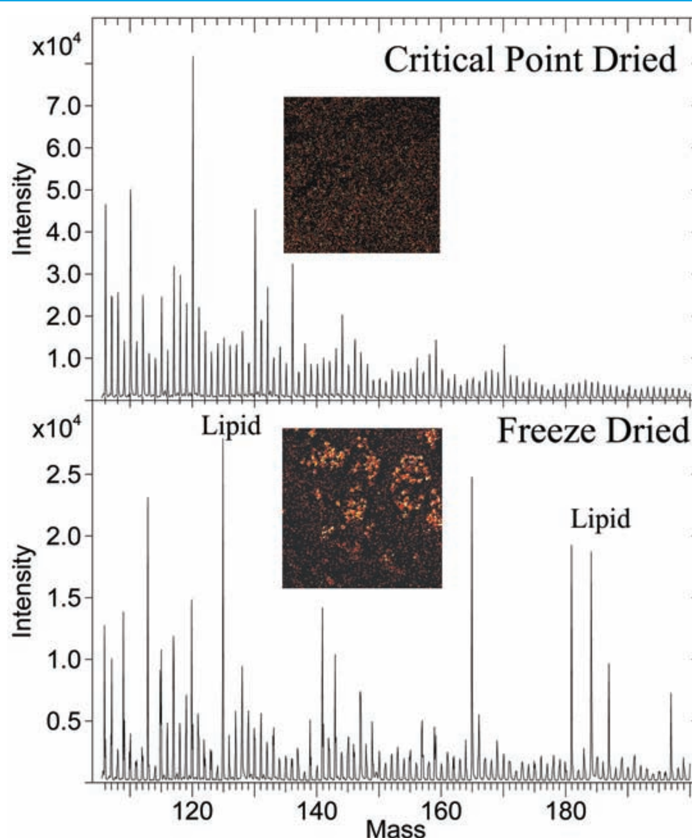

Fig. 5. Comparison of mass spectra for freeze-dried and critical point dried cells. Lipids were not detected after treatment with critical point drying. The insets show the corresponding SIMS images for m/z 184 phosphocholine.

Results and Discussion

The chemical distribution and sample morphology of freeze-dried and critical point dried LS174T cells were assessed using ToF-SIMS and SEM. LS174T cells were plunge frozen in liquid nitrogen and placed into a Millrock Tech 24L shelf freeze-drier. Although liquid nitrogen does not freeze as rapidly as nitrogen slush or liquid ethane, it easily can be maintained at deep volumes, and is less dangerous to work with. Therefore, samples prepared in this experiment could be frozen with a high velocity and a deep plunge in a typical chemical safety hood. ToF-SIMS images showed that the chemical distribution of the LS174T cells was maintained on the micrometer-scale (Figure 1). Phosphatidylcholine is a lipid that is highly concentrated in cell membranes and that fragments during the ToF-SIMS process to yield phosphocholine (m/z 184).[13] A cluster of five cells was evident from a lack of sodium signal and the corresponding phosphocholine image showed that lipid co-located with the cells. Because the chemical distribution was maintained, this sample preparation method is appropriate for ToF-SIMS. SEM images (see the right panel of Figure 1) showed that cell morphology remained largely unperturbed, although the cells experienced mild shrinkage. Therefore, this sample preparation may not be the optimal choice for morphological studies using SEM.

Additional freeze-drying studies were performed using non-adherent samples that were prepared on track etch filters. In these experiments, prior to drying, the cells were captured with super-paramagnetic beads and labeled with spectroscopic nanoparticle tags. The bead-cell-tag complex was captured on the membrane and rinsed before being placed onto a shelf in the freeze-drier. A

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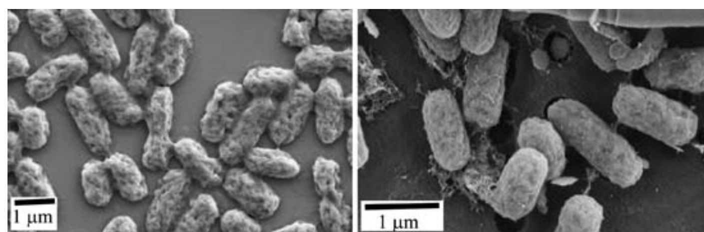

Fig. 6. SEM images of killed E. coli O157:H7 bacteria prepared by air-drying (left) and critical point drying (right).

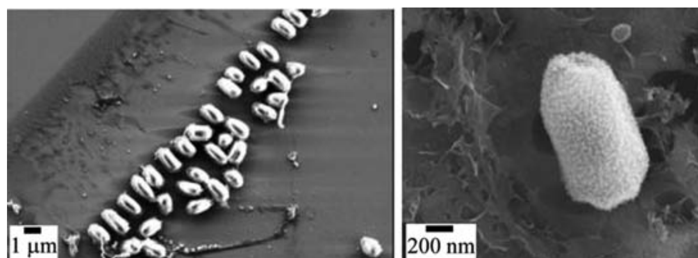

Figure 7. SEM images of Sterne spores prepared by air-drying (left) and critical point drying (right).

high number of bead-cell complexes were captured; Figure 2 shows representative SEM images. Due to the non-violent nature of shelf-drying, the bead-cell bonds appear to have been preserved; however, some wrinkling of the Sterne membrane is noticed and can be attributed to mild shrinkage of the sample during the freeze-dry process. Additionally, the spectroscopic nanoparticle tags are not present in the sample, although each sample was examined spectroscopically before freeze-drying and did exhibit a spectroscopic signal. The absence of the nanoparticle tag indicates some disruption in the binding mechanism caused by the freeze-dry process. Overall, for imaging cellular material, this type of shelf-drying remains a viable option however it may require further optimization to prevent cell shrinkage and disruption of moieties bound to the cell surface. The bacteria samples were not analyzed by ToF-SIMS because of the small size of the cells.

The other sample preparation technique that was evaluated was critical point drying. SEM images of the critical point dried LS174T cell samples verified the presence of a large population of cells across the entire substrate. The cells exhibited excellent morphology, including easily discernable microvilli (Figure 3). The observation of microvilli was especially significant because the small size (diameter = 100 nm; length = 100 -2000 nm) of the structures exemplified that critical point drying was effective at maintaining morphology down to the nanometer-scale. Interestingly, phosphocholine was not detected by ToF-SIMS analysis of the critical point dried cells. Figure 4 shows ToF-SIMS images and Figure 5 shows ToF-SIMS spectra. It is likely that the ethanol-based dehydration treatment extracted the lipids from the cells, which is disadvantageous for many fundamental biological investigations. However, removal of lipids may be a novel sample treatment for ToF-SIMS experiments in which lipids are not of interest. The presence of lipids, such as phosphatidylcholine, has been an obstacle to diversifying the biological applications of ToF-SIMS because it suppresses signals from other analytes. By extracting lipids and maintaining most of the cellular structure (as evidenced by the SEM images), it is probable that other species (ex. metabolites, pharmaceuticals) will be detectable with ToF-SIMS.

E. Coli and Sterne bacteria were used to compare air-drying and critical point drying for the preparation of non-adherent biological samples. SEM images of air dried *E. Coli* showed severe shrinkage, a rough looking texture, and appeared to be relatively flat compared to the *E. Coli* that were prepared by critical point drying and imaged under the same conditions (Figure 6). critical point drying *E. Coli* SEM images showed three-dimensional bacterial structures, and the absence of shrinkage or morphological disruption. Similarly, air dried Sterne appeared to shrink and did not retain their 3-dimensional structure. Conversely, critical point drying Sterne retained their 3-dimensional structure down to the fine-details of the spore-coat as seen in an SEM image of a single bacterium (Figure 7). The bacteria samples were not analyzed by ToF-SIMS.

Summary

In summary, deep plunge freezing in liquid nitrogen and freeze-drying was a viable preparation of biological samples for ToF-SIMS experiments because the chemical distribution was unchanged during the treatment. Freeze-drying was not an optimal sample preparation method for SEM experiments because this treatment caused mild shrinkage to both the adherent and non-adherent biological samples. Critical point drying was a better choice for preparing samples for SEM experiments because the sample morphology was maintained down to the nanometer-scale. Phosphatidylcholine was not detected by ToF-SIMS for adherent cells that were critical point dried, probably because the dehydration process extracted lipids. Although critical point drying was not suitable to prepare cells for ToF-SIMS experiments that require the analysis of lipids, it is possible that the method will be appropriate for other biological applications where lipid suppression is beneficial. Future experiments are planned to investigate the utility of fixation and dehydration as a preparation method for nonlipid ToF-SIMS experiments of biological samples. ■

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