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Quantitative Detection of Purines in Biologically-Relevant Films with TOF-Secondary Ion Mass Spectrometry

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Abstract

In purine-depleted environments, the *de novo* purine biosynthetic pathway is catalyzed to ultimately produce inosine monophosphate (IMP), a purine invisible using current optical microscopy methodology. These enzymes form a complex, termed the “purinosome,” to replenish IMP levels. Before cellular chemical imaging may be applied to monitor the distributions and fluctuations in purine levels, it is necessary to develop a scheme to quantitatively detect purines. Here, IMP and other purines in biologically-relevant matrices have been detected quantitatively. These methods provide a TOF-SIMS protocol using C_{60}^+ primary ions to determine the concentration of biomolecules in a cell such as HeLa at the nanomolar level.

Keywords

Trehalose film; cellular imaging; depth profiling; TOF-SIMS; C_{60}

Introduction

In purine-depleted environments, the *de novo* purine biosynthetic pathway is catalyzed by six enzymes in ten biochemical steps to ultimately produce inosine monophosphate (IMP), a purine invisible to current optical microscopy methodology.^{1–3} It has been shown by fluorescent-labeling that these enzymes interact to form a cluster termed the “purinosome.”¹ Abnormal regulation of the *de novo* purine biosynthesis is related to various human diseases, including immunodeficiency, myopathies, and others.^{2,3} Therefore, the purinosome may be considered as a pharmacological target for therapeutic treatment.^{1,2}

Extensive chemical information concerning the purinosome is sparse and details about which subcellular compartment contains these enzymes is unknown.^{1,4} The most direct way to prove and trace dynamic action of the purinosome, chemically, is to determine the associative distributions of IMP, the sole product of the pathway. Ultimately, the distribution of IMP could be conveyed in a three-dimensional chemical representation to provide evidence of purinosome function. However, it is clearly a challenge to develop analytical methods to measure micromolar concentrations of IMP at the single cell level.

The main focus of this study is to develop a method of relative purine quantitation in biologically-relevant matrices by TOF-SIMS that can provide information about other metabolic processes. Two approaches are considered. In the first instance, IMP standard solutions were doped into a trehalose spin-cast thin film. Trehalose has been previously shown to be a successful matrix for depth profiling of peptides.⁶⁻⁷ Since IMP is readily converted into adenosine monophosphate (AMP), AMP-doped trehalose spin-cast standards were also created to demonstrate linearity, specificity, and simultaneous detection for purines related to the pathway. In a second instance, spin-cast films of a HeLa cell homogenate, spiked with known concentrations of IMP resuspended in trehalose were shown to yield quantitative concentration information. The results show the feasibility of simultaneously determining analyte concentration of biomolecules, a powerful approach for cellular analysis.

Experimental

Cell homogenate-containing sample preparations

HeLa cells, an immortal cervical cancer cell line, were grown in a T-75 flask using Dulbecco's Modification of Eagle's Medium (DMEM) (Cellgro), supplemented with 10% Fetal Bovine Serum (Cellgro), and a penicillin-streptomycin solution (10,000 I.U. penicillin, 10,000 µg/mL streptomycin) (Cellgro). Cells remained in an incubator maintained at 5% CO₂ and 37 °C, until 100% confluence.

The homogenization protocol was adapted from Core G Subcellular Fractionation of RAW264.7 Protocol PP00004301 to suit the HeLa cell line. To detach cells from the flask, 1X Trypsin-EDTA (Cellgro) was added. After centrifugation, the growth media was removed and cells were resuspended in isolation media (250 mM sucrose (Sigma, 99.5%), 10 mM HEPES (Cellgro), 1 mM EDTA (EMD, 99.0-101.0%), nanopure water, at pH of 7.4), and centrifuged at 1000 rpm for 5 minutes. Isolation media were removed, cells were resuspended in hypotonic media (100 mM sucrose (Sigma, 99.5%), 10 mM HEPES (Cellgro), 1 mM EDTA (EMD, 99.0-101.0%), at pH of 7.4), and centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded. The cellular pellet and 2 mL supernatant was homogenized in a glass homogenizer and centrifuged at 1000 rpm for 10 minutes.

Following this step, two different routes of sample preparation were performed. First, for the IMP-doped cell homogenate/sucrose solutions, the pellet was resuspended in the hypotonic media, 5 µL drops of this solution were dried on Si shards, and dried under a stream of nitrogen. For the second route, the homogenate solution was spin-cast into a trehalose film, as discussed below.

Solutions for spin-cast films

Spin-cast films were prepared with 0.125 M aqueous D-(+)-trehalose dehydrate (Sigma, 99%), solution. Pre-sliced 5 mm × 5 mm Si wafers (Ted Pella Inc.) were used as substrates for all films. All water for the preparations was purified by a Milli-Q System (Millipore, U.S.A.) with a resistivity of 18.2 MΩ cm and an organic content less than 5 ppm. The Si wafers were prepared by a procedure described elsewhere.⁷

The respective IMP- and AMP-doped trehalose solutions at varying concentrations were prepared *via* serial dilutions. Solid IMP (Sigma, 99.5%) and AMP (Spectrum, 99%), respectively, were dissolved in the trehalose solution to make a stock solution. Different aliquots of this solution were diluted with trehalose solution to make the concentration standards. The IMP- and AMP-doped trehalose solution was also made in this manner with IMP and AMP at equal concentrations.

For the IMP-doped cell homogenate/trehalose spin-cast films, the hypotonic media was removed and the homogenate pellet was re-suspended in 8 mL of trehalose solution. The resulting solution was separated into two aliquots. Solid IMP was dissolved in the first to create a stock solution. This stock solution was diluted serially with the remaining aliquot to create the concentration standards.

Concentration standards of IMP and AMP were confirmed with a NanoDrop 1000 spectrophotometer ($R^2=0.999$) at 223 and 227 nm, respectively.

Spin-casting procedure

Spin-cast films were created with 250 μL of the respective solutions. They were spin-cast onto the prepared Si wafers by a procedure described previously.⁷

TOF-SIMS analysis

The analysis was performed on a TOF-SIMS instrument previously described elsewhere.⁸ Briefly, the instrument is equipped with a C_{60}^+ primary ion source by Ionoptika Ltd. (Southampton, U.K.) at a 40° angle relative to sample normal.¹⁰ For depth profiling, a dc 100 pA beam of 40 keV C_{60}^+ was utilized to sputter through the spin-cast films with a raster area of $500\text{ }\mu\text{m} \times 500\text{ }\mu\text{m}$ in 4 s intervals. Between these erosion cycles, negative ion mode TOF-SIMS mass spectra were obtained at an ion fluence of $3 \times 10^{10}\text{ }\text{C}_{60}^+$ ions- cm^{-2} into a raster area of $200\text{ }\mu\text{m} \times 200\text{ }\mu\text{m}$ within the eroded area. The primary ion beam was pulsed for 100 ns at 3000 Hz.

For the first instance, the magnitude of the purine signal was determined by integrating the peak area at half-width and half-maximum at the corresponding mass value for each mass spectrum acquired during the complete depth profile – from the surface of the film to the Si interface. Because the spin-casting preparations are reproducible in terms of thickness and surface homogeneity⁷, this allowed the comparison of the integrated purine intensities of the depth profiles for the different concentration standards, respectively. For the second instance concerning the analyses with cell homogenate, the value of “total counts” was calculated by taking the integrated peak area at half-width and half-maximum of each sample, respectively, during a surface spectrum from a $150 \times 150\text{ }\mu\text{m}^2$ area with a primary ion dosage of $1.0 \times 10^{12}\text{ }\text{C}_{60}^+$ ions- cm^{-2} .

Results and Discussion

Spin-cast films of trehalose doped with varying concentrations of IMP were prepared and analyzed as a biologically-relevant model to determine if differences in IMP concentration could be detected in a defined volume. The concentration of IMP in a HeLa cell is

approximately 100 μM (6×10^{16} IMP molecules/ cm^3).¹ As demonstrated in Figure 1a, using the IMP-doped trehalose spin-cast film standards, IMP can be precisely determined well into the low nanomolar regime. This experiment has been reproduced successfully in triplicate with a coefficient of determination, R^2 , greater than 0.99 at each instance. This result implies that trehalose is an excellent matrix for reliable quantitation between spin-cast films containing nanomolar concentrations of IMP and, potentially, other purines. Similar to related work with biomolecules⁶⁻⁷, it appears trehalose offers enhanced stability to the purines during the primary ion bombardment. The combination of these features lends to the belief that IMP and other purines could be detected within a cell if matrix effects,^{9,11-12} which currently prevent this type of analysis, are overcome.

While IMP is the only purine whose presence after a stage of purine depletion would be the only molecular evidence of the *de novo* purine biosynthetic pathway¹, it is valuable to monitor the distributions of other purines during the process. As demonstrated in Figure 1b, AMP was examined using the same protocol as for IMP in the same nanomolar concentration range. With this capability it should be feasible to monitor the rate and distribution of the conversion from IMP to AMP during purine replenishment.

As shown in Figure 2, the mass signature peaks for $[\text{IMP-H}]^-$ and $[\text{AMP-H}]^-$, respectively, can be resolved within a mass spectrum of a trehalose spin-cast film doped with identical concentrations of IMP and AMP, despite an insignificant amount of isotopic overlap. When observing this spectrum, it should be noted that the trehalose peak, $[\text{trehalose-H}]^-$, is present at m/z 341, closely within the mass range of IMP and AMP. It has a similar intensity to the purine samples at nanomolar concentrations. This peak provides a convenient reference and internal standard. Tracking the intensity of this peak between depth profiles of different spin-cast films serves as an indicator of film homogeneity between samples and possible primary ion beam current fluctuations.

While trehalose is an accepted biologically-relevant model matrix, nanomolar concentration standards of IMP were created in a cell homogenate solution to serve as a matrix most similar to the cellular environment. As noticed from Figure 3a, the correlation reflects a relatively high amount of variability between concentrations. This result is most probably a result of the characteristic surface inhomogeneity of dried cellular homogenate. In addition, there is chemical noise associated with the homogenate that can generate isobaric interferences. While qualitative data may be obtained, there is excessive variability making obtaining a reproducible and relatively-quantitative result difficult. However, when the cellular homogenate is resuspended in trehalose solution and spin-cast, as seen in Figure 3b, the results are much improved. The slight variability is suspected to be introduced by matrix effects often caused by excessive salts in cellular growth media.^{9,13} Similar to previous spin-cast trehalose films, these spin-cast films have a glassy appearance, thus, lending to a more uniform surface which was key to the correlation and repeatability of the experiment. IMP-doped cell homogenate/trehalose spin-cast films could provide a standard technique to determine the concentration of a certain biomolecule in a cell or any other defined biological volume.

When trehalose is used as a matrix the number of intact purine molecules is enhanced relative to when neat IMP is bombarded directly. The trehalose matrix appears to be linked to the prevention of purine molecule fragmentation. Thus, both purine quasi-molecular ions are formed, presumably by the loss of a proton from the parent molecule. The purine molecules were completely consumed in the depth profile with little fragmentation indicated by the major fragment of IMP and AMP, hypoxanthine as $[M-H]^-$ present at m/z 135. The purine quasi-molecular ions appear to be protected from fragmentation largely by the trehalose matrix; perhaps, similar in theory as to how MALDI matrices prevent excessive fragmentation of high-mass molecules. In addition, despite damage accumulation characteristically incurred by depth profiling, the integrated purine signal is reproducible and gives a linear response to varying concentration.

Conclusions

These concentration studies provide promising evidence that IMP concentration can be seen at biological levels relevant to the dynamic actions of the *de novo* purine biosynthetic pathway. Biologically-relevant concentration standards can be analyzed quickly on the day of cellular analysis to obtain relative concentration data. Trehalose proves to be a matrix essential to observe quasi-molecular purine ions linearly, in regards to concentration. These results suggest the possibility of temporal studies for the distribution and concentration of purines in a biological sample.

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Figure 1.

The relative quantitation of the (a) IMP- and (b) AMP-doped trehalose spin-cast films is shown. The coefficient of determination is (a) $R^2 = 0.993$ and (b) $R^2 = 0.999$, respectively.

AMP- and IMP-doped trehalose spincast film

Figure 2.

The mass signature peaks for $[IMP-H]^-$ and $[AMP-H]^-$ are resolved here within a mass spectrum of a trehalose film doped with IMP and AMP, respectively.

Figure 3.

The relative quantitation of the (a) IMP- and (b) AMP-doped cell homogenate/trehalose spin-cast films is shown. The coefficient of determination is (a) $R^2 = 0.7974$ and (b) $R^2 = 0.9746$, respectively.