

Accelerator Mass Spectrometry-Based Signal Generation for Thin Layer Chromatography

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A highly sensitive accelerator mass spectrometry (AMS)-based assay for determination of the ^{14}C content in the scraped silica gel after thin layer chromatography (TLC) has been established. In the present study, ^{14}C -labeled inositol or its cellular derivatives was selected as a model analyte to evaluate the designed assay, since a series of phosphatidyl inositols (PIs) are known to be derived from inositol and to play pivotal roles as precursors or second messengers for several cell signaling processes. After incubating HeLa cells with ^{14}C -labeled inositol, allowing the dosed ^{14}C -labeled inositol to be converted to a series of ^{14}C -labeled PIs and phosphoinositides through biochemical processes, the phospholipids were extracted and separated by the conventional TLC. The resulting TLC plates were scraped and graphitized for AMS measurement. The obtained data demonstrates the intracellular distribution of the phosphatidyl inositols, implying that the developed assay is better and more convenient than the conventional ^{32}P -based methods.

Phosphatidyl inositol (PI), a major class of phospholipids, is an important lipid, both as a key membrane constituent and as a participant in essential metabolic processes in all plants and animals, either directly or *via* a number of metabolites.¹⁻⁶ In particular, PI and the phosphatidylinositol phosphates (phosphorylated forms of PI, also known as phosphoinositides) have a central position in cell signaling and regulation, including calcium homeostasis, membrane trafficking and cytoskeletal dynamics.¹⁻⁶ They are able to achieve signaling effects directly by binding to cytosolic proteins or cytosolic domains of membrane proteins *via* their head groups, and in this way, they can regulate the activity of a group of at least a dozen related enzymes, which in turn control many key cellular functions, including differentiation, proliferation, metabolism and apoptosis.¹⁻⁶ Indeed, the biological actions of the various components released have been the subject of intensive study over the last twenty years.

PIs and phosphoinositides can be intracellularly synthesized from *myo*-D-inositol, which have a six-membered ring with one axial hydroxyl in position 2 with the remainder equatorial, *via* a series of enzyme-mediated reactions in cells, and it is generally known that seven forms of phos-

phoinositides (Fig. 1) are associated with intracellular signal processes and enzymes.⁷⁻⁹ Of importance, the PI3-kinase pathway, which regulates many cellular functions, including

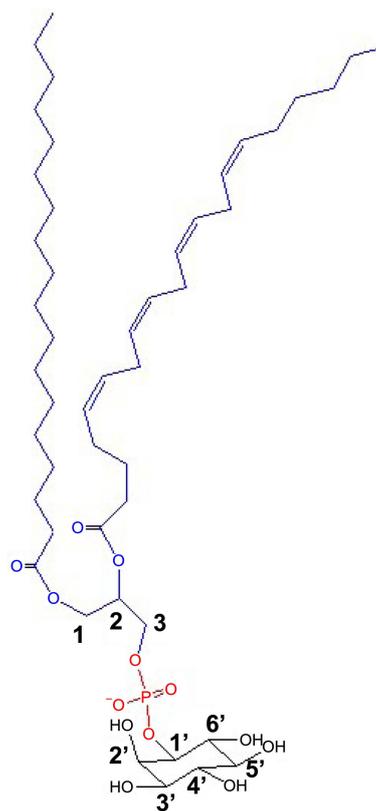


Figure 1. Structure of phosphatidylinositol (PI), which is originated from <http://en.wikipedia.org/wiki/File:Phosphatidylinositol.png>. The hydroxyl moieties at 3', 4', and 5' in the head group can be phosphorylated to produce seven PI derivatives: phosphatidylinositol 3-phosphate (PI3P), phosphatidylinositol 4-phosphate (PI4P), phosphatidylinositol 5-phosphate (PI5P), phosphatidylinositol (3,4)-bisphosphate (PI(3,4)P₂), phosphatidylinositol (3,5)-bisphosphate (PI(3,5)P₂), phosphatidylinositol (4,5)-bisphosphate (PI(4,5)P₂), and phosphatidylinositol (3,4,5)-trisphosphate (PI(3,4,5)P₃, or simply PIP₃). It should be noted that PI3P, PI4P, and PI5P are collectively known as PIP, and that PI(3,4)P₂, PI(3,5)P₂, and PI(4,5)P₂ are as PIP₂.

lipid metabolism, is frequently mutated or activated in several cancer cells and thus the expression patterns or mass distribution of the phosphoinositides have been reported to differ from cells to cells, being affected by several acyltransferases in the remodeling pathway (Land's pathway).^{3,5,6}

To date, however, it is still challenging to analyze and verify the exact mass distribution of the seven forms of phosphoinositides under physiologically relevant conditions, which may allow us to explain how the mass distribution of the phosphoinositides are correlated with the given conditions of the cells or cell organelles.^{3,5,6} In the present study, a novel thin layer chromatography (TLC)-based approach in combination with a highly sensitive detection method of accelerator mass spectrometry (AMS) is employed, in order to quantitatively analyze and verify the exact mass distribution of the seven forms of phosphoinositides in cells (Fig. 2). AMS is a technology for radiocarbon dating and is now in development for tracing ^{14}C -labeled compounds in biological systems.^{10,11} Differently from the conventional liquid scintillation counting method, AMS physically counts radioisotopes affording attomole to zeptomole ^{14}C per mg of total carbon sensitivity with a few percent precision, and importantly the ^{14}C content in the intact silica gel from the TLC plate can be directly and quantitatively measured by AMS.¹⁰ Even in case that a very low concentration (around 300 dpm) of ^{14}C labeled material is introduced directly into live cells, AMS can track down either the parent material or its metabolites under the given conditions, as is used in the present study.

To verify the proof-of-concept, HeLa human cervical cancer cells were incubated with MEM media for 4 days in the presence of ^{14}C -labeled *myo*-D-inositol to allow it to be converted to PI and its derivatives through biochemical process, followed by extraction of phospholipid from the cell lysates. After running a TLC plate using an eluting solution (4.6:1.7:1.5:1.4:0.8, CHCl_3 :acetone:MeOH:acetic acid: H_2O) with the extracted phospholipid, the TLC plate was stained by iodine and compared with the reference R_f values of phosphoinositides (0.5, 0.33, and 0.2 for PI, PIP, and PIP_2 , respectively).¹²

Very interestingly, our AMS data shown in Figure 2 clearly reveals that most of the phosphoinositides derived from the introduced ^{14}C -labeled *myo*-D-inositol are existent as PIP, although approximately 17-fold smaller amount of PI is also found. It should be noted that when the spotted volume onto the TLC plate is known, the measured $^{14}\text{C}/^{12}\text{C}$ ratio of the TLC fraction can be converted to the fraction of modern carbon, and thus to other quantitatively meaningful values, such as concentration, in order to compare the detected PI and PIP with the initial amount of the introduced ^{14}C -labeled *myo*-D-inositol. Our results indicate that the smaller fractionation of the TLC plate or use of HPLC for lipid separation may improve the resolution of PI-related lipidomics, which is under investigation.

In summary, we developed a novel detection system based both on TLC and AMS to study the mass distribution of the PI and its derivatives in cells under physiologically relevant

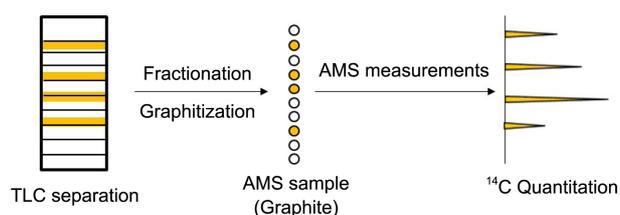


Figure 2. Schematic illustration of accelerator mass spectrometry (AMS)-based signal generation for thin layer chromatography (TLC), in order to verify the mass distribution of PI and phosphoinositides in cells. After scraping the TLC plate and adding 5 μL of tributyrin to each TLC fraction collected to increase ^{12}C content to the sample, $^{14}\text{C}/^{12}\text{C}$ ratios can be measured by AMS, allowing for the estimation of the intracellular mass distribution of the inositol derivatives.

conditions. This work can be extended in the near future to HPLC-based separation of cellular PI and phosphoinositides for the phosphoinositide-related lipidomics or mass distribution studies, which may contribute as well to drug development for pharmaceutical therapy of lipid related diseases through mostly presenting cellular phosphoinositide profile and phosphoinositide distribution in tissue.

Experimental Section

General Methods. Unless otherwise noted, reagents were obtained from commercial suppliers and were used without further purification. Doubly-deionized water was used whenever necessary. All experiments were performed in duplicate.

Cell Culture and Phospholipid Extraction. HeLa human cervical cells were obtained from Korean Cell Line Bank. HeLa cells were cultured at 37 $^{\circ}\text{C}$ with 5% CO_2 incubator in MEM supplemented with 2 mM L-Glutamine, 10% fetal bovine serum, 10 IU/mL penicillin and 0.01 mg/mL streptomycin. HeLa cells were incubated with 340 dpm of ^{14}C -

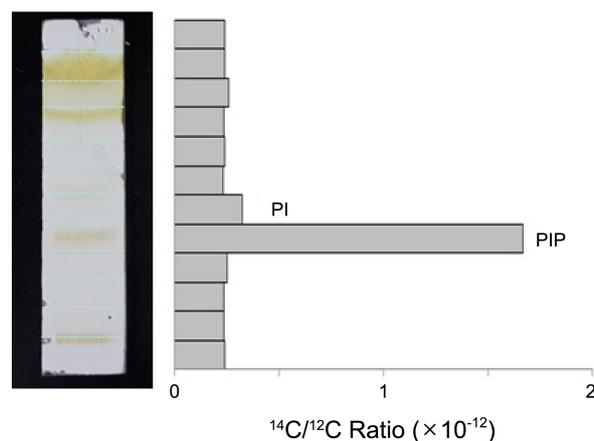


Figure 3. After separation of the extracted phospholipids from cells by TLC, the resulting TLC plate is stained with iodine (left). The R_f values of the stained signals can be compared with the reference data (for example, Ref. 12). $^{14}\text{C}/^{12}\text{C}$ ratios of the graphite samples from the scraped TLC fractions are measured by AMS. The R_f value of the highest $^{14}\text{C}/^{12}\text{C}$ ratio from the TLC fragment #5 corresponds to PIP.

American Radiolabeled Chemicals, Inc.) in 10 mL of MEM medium for 4 days. After incubation, phospholipids were extracted from cell lysates by using Folch procedure, commonly used for phospholipid extraction.^{12,13} In brief, collected cells were treated with 20 mL of CHCl₃:MeOH (2:1) and agitated for 20 min. After filtration, phospholipid solution was washed twice with 4 mL of 0.9% NaCl solution. The lower chloroform phase obtained from the above process was evaporated under vacuum until the volume of extracted phospholipid reached 2 mL.

TLC-based Separation of the Extracted Phospholipid.

To separate PI and phosphoinositides, we utilized the pre-coated TLC plates (1 cm × 5 cm) immersed for 5 seconds in a potassium oxalate and EGTA solution (containing 1% potassium oxalate (w/v) and 1 mM EGTA in methanol and water solution). After drying at 100 °C for 30 min, the extracted phospholipid solution was spotted several times on the prepared TLC plate, followed by running the TLC with an eluting solution (4.6:1.7:1.5:1.4:0.8, CHCl₃:acetone:MeOH:acetic acid:H₂O).¹² Then the TLC plate was removed from the chamber and dried under vacuum for 30 min to eliminate any remaining carbon source from organic solvents. After the TLC plate was stained by iodine, it was carefully scraped using cleaned wide-blade knife to obtain 10 fractions for AMS measurement.¹⁴⁻¹⁶

Pretreatment of the Scraped TLC Fractions and AMS Measurement. 5 μL of tributyrin was added to each TLC fraction collected, and the resulting TLC fractions were converted to solid carbon for AMS measurement using a two-step process according to the literature.¹⁰ Briefly, the tributyrin-added silica gels in Tin capsules were oxidized to CO₂ using the elemental analyzer and the evolved CO₂ was reduced to graphite by using hydrogen gas and iron powder as a catalyst at 520 °C for 3 h. Large C samples (> 500 mg)

were split and ¹²C:¹⁴C ratios were measured by AMS in KIST Accelerator Laboratory.

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