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Laser Desorption/Ionization Mass Spectrometric Assay for Phospholipase Activity Based on Graphene Oxide/Carbon Nanotube Double-Layer Films

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Abstract: A new method for quantitative phopholipase activity assays using mass spectrometry (MS) and a supported thin film consisting of a graphene oxide (GO)/carbon nanotube (CNT) double layer as a substrate for laser desorption ionization (LDI) has been developed. Phospholipids were very efficiently analyzed by LDI−time-of-flight (TOF) MS on the GO/CNT films, presumably because of the affinity of phospholipids for the GO/CNT surface. Therefore, the rate of lipid hydrolysis was conveniently measured using LDI−TOF mass spectra obtained from a GO/CNT surface on which the phospholipid hydrolysis reaction mixtures had been spotted by comparing the mass-peak intensities of reactants and products. The present platform for phospholipase assays based on MS and GO/CNT double-layer films enables quantitative measurements at low cost, allows assays to be performed in a short time, and is compatible with an array format, unlike conventional assay methods.

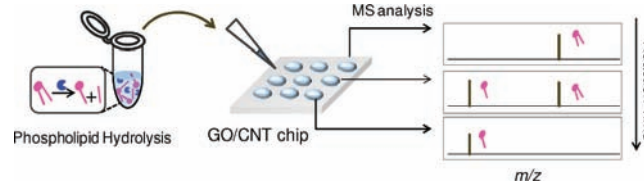
Phospholipases are a class of enzymes that play an important role in cell membrane systems by controlling the levels of free fatty acids through hydrolysis of phospholipids.[1] They are classified into five major classes, types A1, A2, B, C, and D, depending on their sites of hydrolysis on phospholipids, and have been reported to be involved in lipid metabolism, plant−pathogen interactions, inflammation, cellular signaling, and cancer development.[2] For example, phospholipase C (PLC) activates protein kinase C by generating diacylglycerol and increasing the calcium ion concentration.[3] Overexpression of phosphoinositide-specific PLC is involved in cancer development through participation in epidermal growth factor receptor (EGFR) signaling by activating the kinase Raf.[4] Therefore, it is important to establish a simple, quantitative, and efficient assay for the lipid hydrolysis activity of phospholipases.

Several phospholipase assay methods involving fluorogenic substrates,[5] HPLC,[6] ^{31}P NMR spectroscopy,[7] acid−base titration,[8] and so forth have been developed. In general, substrate labels should be avoided in enzyme activity assays if possible because they can affect the enzyme activity and preparing labeled substrates is not cost-effective. HPLC and acid−base titration are laborious methods that are not suitable for high-throughput screening.

Mass spectrometry (MS) is an attractive method for enzyme activity assays in which molecular weights of substrates change during enzyme reactions. MS-based assays do not require any labeling in most cases.[9] However, conventional MS analysis requires purification of analytes or pretreatment to remove other components of enzyme reaction mixtures (e.g., salts), and this hampers the wider application of MS in enzyme assays. The development of MS-compatible array methods available for rapid parallel screening and analysis would be beneficial.

Here we report a new platform for MS-based phospholipase activity assays that utilizes a supported graphene oxide (GO)/carbon nanotube (CNT) double-layer thin film as a substrate for laser desorption ionization (LDI). Conventional matrix-assisted laser desorption/ionization (MALDI) MS requires organic matrix molecules, which generate undesired interference peaks in the low-molecular-weight region. In fact, many alternative matrixes, including carbon-based materials such as graphite,[10] CNTs,[11] and pencil lead,[12] have been developed to eliminate complex matrixes. Recently, graphene was used as a new matrix for analysis of low-molecular-weight compounds[13] and single-stranded DNA.[14] The present strategy relies on efficient energy absorption of GO/CNT films from a laser, transfer of the energy to the analytes (phospholipids and their products cleaved by phospholipases, which have been deposited on the GO/CNT double-layer film), and subsequent desorption/ionization of the analytes. Previously, our group reported the fabrication of supported double-layer films of reduced GO and CNT for a large-area transparent electrode.[15] Recently, we discovered that small molecules can be analyzed using conventional LDI−time-of-flight (TOF) MS without an additional matrix when the molecules are spotted on a GO/CNT film.[16] Among these small molecules, phospholipids were very efficiently analyzed by LDI−TOF MS on the GO/CNT films, presumably because of their affinity of phospholipids for the GO/CNT surface.[17] Therefore, the rate of lipid hydrolysis could be followed using LDI−TOF mass spectra obtained from circular areas on the GO/CNT surface where the phospholipid hydrolysis reaction mixtures had been spotted at different time points by comparing mass-peak intensities of the reactants and products (Scheme 1).

Scheme 1. Strategy for Phospholipase Activity Assays Based on LDI MS and Supported Thin Films of GO and CNT



We first prepared GO-immobilized substrates by immersing aminated glass coverslips or silicon wafers in an aqueous suspension of GO, which was prepared according to a modified Hummer's method, to induce immobilization of negatively charged GO onto positively charged substrates by electrostatic interactions (Figure 1a).[15] Atomic force microscopy (AFM) images showed that GO sheets covered the entire surface, overlapping with adjacent GO sheets along the edges (Figure S1 in the Supporting Information). Subsequently, the GO-covered substrate was immersed in a solution of aminated multiwalled CNTs to form GO/CNT double-layer films. An even distribution of immobilized CNTs on the GO surface was observed by AFM and scanning electron microscopy (SEM) (Figure 1b,c).

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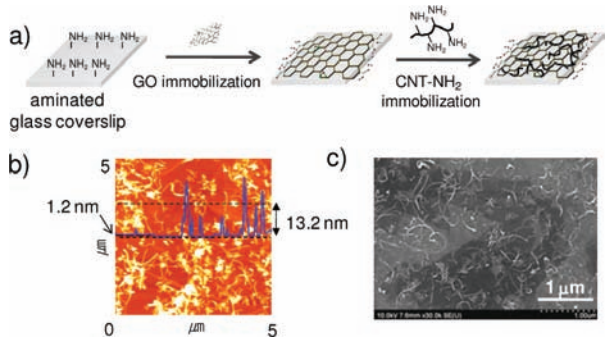

Figure 1. Preparation and characterization of GO/CNT double-layer films. (a) Supported GO/CNT films were fabricated by immersion of aminated glass coverslips or silicon wafers in an aqueous solution of GO followed by CNT immobilization. (b) AFM image and height profile and (c) SEM image of a GO/CNT film showing the homogeneous surface distribution of CNTs and good surface coverage of GO.

In the present study, we demonstrated our strategy using two different types of phospholipase, namely, phospholipase A2 (PLA2) and PLC, and dipalmitoylphosphatidylcholine (DPPC) as the substrate lipid (Figure 2). PLC catalyzes the hydrolysis of glycerophosphate ester bonds of phospholipids to diacylglycerol (DAG, M2) and the phosphorylated headgroup (M4). PLA2 hydrolyzes the sn-2 acyl ester linkage of phospholipids to free acids (M5) and 1-acyl-2-lysophospholipids−lysophosphatidylcholine (LysoPC, M3) when this specific substrate, DPPC, is used.[18]

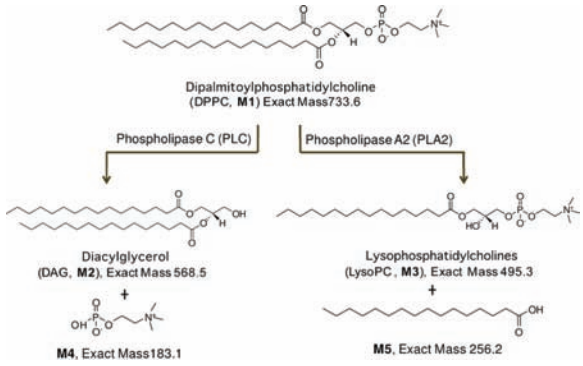

Figure 2. PLC- and PLA2-mediated cleavages of DPPC, which yield different products, DAG and LysoPC, whose mass peaks can be well-resolved in mass spectrometry. The exact masses of the species involved in each enzymatic reaction are given.

We next performed time-dependent hydrolysis reactions of DPPC catalyzed by PLC and PLA2 (Figure 3). To measure the PLC activity, an aqueous solution was prepared in buffer solution (pH 7.3) containing 14.8 units/L PLC, 50 mM Tris-HCl, and 20 mM $CaCl_2$. The enzyme solution was mixed with 2 mM DPPC dissolved in chloroform in a reaction vessel. Shaking the vessel formed an emulsion of aqueous and organic phases in which hydrolysis of DPPC occurred. In the time-dependence experiment, 1 μL aliquots of the chloroform phase were taken out and spotted onto a GO/CNT chip at intervals of 10 min. The prepared chip was then loaded directly into the LDI−TOF mass spectrometer, and mass spectra were taken from each spot without any further treatment or matrix application. The peaks corresponding to the DPPC hydrolysis product DAG ($[M2 + Na]^+$, m/z 591.4; $[M2 + K]^+$, m/z 607.4) increased gradually with time while those of DPPC ($[M1 + H]^+$, m/z 734.6; $[M1 + Na]^+$, m/z 756.5) decreased (Figure 3a). The relative amount of each component was calculated by measuring the relative intensity I [relative amount of MX = $I_{MX}/(I_{M1} + I_{M2})$], and this was plotted against reaction time (Figure 3c). In the case of PLA2, the enzyme was prepared in a reaction buffer (pH 8.9) containing 560 units/L PLA2, 160 mM Tris-HCl, 3 mM $CaCl_2$ and then reacted with 2 mM DPPC in chloroform (Figure 3b). The mass peaks corresponding to LysoPC, the DPPC hydrolysis product of PLA2, were observed at m/z 496.3 ($[M3 + H]^+$) and 518.2 ($[M3 + Na]^+$) and increased gradually, whereas the mass peaks of DPPC decreased as the reaction proceeded. Because a background mass peak from the LDI chip at m/z 496 overlapped with a peak corresponding to LysoPC, we modified the above equation for calculating the relative amount of each component to include subtraction of the intensities of the background peaks (which were almost constant in the GO/CNT chips used) from the intensities of the mass peaks in order to more accurately represent the relative peak intensities [relative amount of MX = $I_{MX}'/(I_{M1}' + I_{M3}')$, in which $I_{MX}' = I_{MX} - I_B$, where I_B represents the background peak intensity] (Figure 3d). In both PLC and PLA2, time-dependent substrate hydrolysis was observed, and the relative amounts of reactants and products could be conveniently calculated from the mass spectra (Figure 3). In addition to time-dependent assays, enzyme-concentration-dependent assays were also successfully performed by varying the concentrations of PLC and PLA2 (Figure S2).

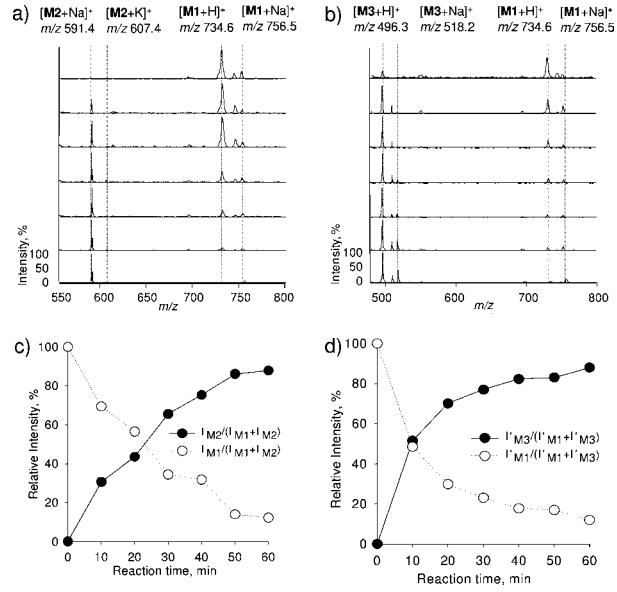

Figure 3. (a, b) Time-dependent LDI−TOF mass spectra for hydrolysis of the lipid substrate DPPC by (a) PLC and (b) PLA2. (c, d) Analysis of the relative intensities of the peaks corresponding to substrate and product obtained from (c) PLC and (d) PLA2 as functions of reaction time.

We next carried out inhibition assays of PLA2 and PLC to confirm that the present method is quantitative and applicable to inhibitor assays. Ethylenediaminetetraacetic acid (EDTA) and tricyclodecan-9-yl xanthogenate (D609) were chosen as PLC inhibitors.[19] PLC in a reaction buffer (14.84 units/L) was mixed with 2 mM DPPC in chloroform in the presence of various concentrations of each inhibitor and incubated with shaking for 2 h. Mass spectra were obtained after spotting reaction mixtures onto a GO/CNT chip. Dose-dependent inhibition curves based on relative peak intensities were plotted. The values of IC_{50} (the concentration that inhibits enzyme activity down to 50%) were 3.3 and 0.3 mM for EDTA and D609, respectively (Figure 4a,b). For inhibition assays of PLA2, EDTA and sodium cholate were chosen as model inhibitors.[20] A mixed solution of PLA2 in buffer (2240 units/L) and DPPC (2 mM) was incubated with various concentrations of

each inhibitor for 1 h. The IC_{50} values for EDTA and sodium cholate were 6.5 and 58.2 mM, respectively.

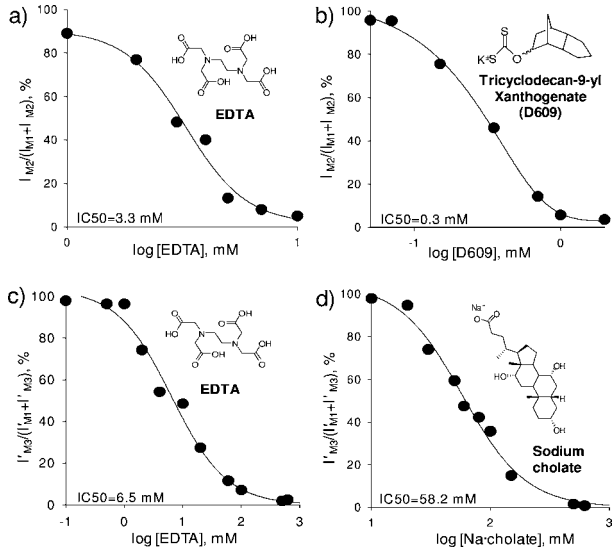

Figure 4. Dose-dependent inhibition of phospholipase reactions was performed with inhibitors. Inhibition of PLC by (a) EDTA and (b) D609 and inhibition of PLA2 by (c) EDTA and (d) sodium cholate were examined, and IC_{50} values were obtained.

One important aspect of the present method is that multiple enzymatic reactions occurring in a single reaction mixture can be conveniently analyzed because all of the species in the mixture would be mass-resolved. To corroborate this assumption, we performed hydrolysis of DPPC in the presence of both PLC and PLA2. As expected, both of the hydrolysis products M2 and M3 were observed from the mixed solution of DPPC, PLA2, and PLC after incubation for 2 h (Figure 5). The capability to perform multiple enzyme activity analyses from a single mixture in which multiple enzyme reactions occur is a great advantage of the present method, especially for investigating sequential enzyme reactions and discovering inhibitors of multiple enzymes by chemical screening.

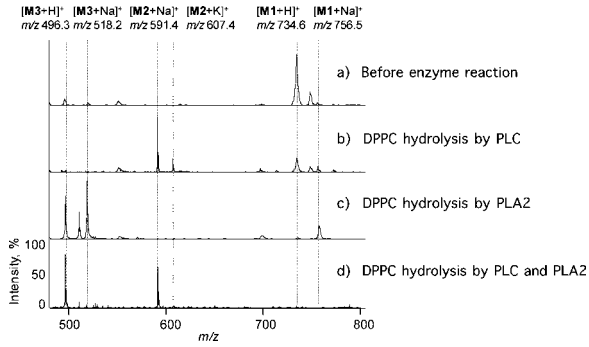

Figure 5. Characterization of two different phospholipase activities from a single reaction mixture using LDI−TOF MS. MS spectra of (a) DPPC, (b) DPPC hydrolysis by PLC, (c) DPPC hydrolysis by PLA2, and (d) DPPC hydrolysis by both PLC and PLA2 are shown. Peaks corresponding to products are clearly distinguished from each other in the mass spectra.

We next conducted an experiment to compare the uniformity of mass-peak intensities depending on areas exposed to the laser beam in the GO/CNT-based method to that in a typical MALDI−TOF MS experiment. The present method involves MS-based analysis that does not require mixing and cocrystallization with a matrix. Therefore, a sample-deposited GO/CNT surface should show few region-dependent differences in mass-peak intensities (unlike conventional MALDI−TOF MS, in which there are "sweet spots"[21]), provided that the analytes are evenly distributed within a confined area on the GO/CNT surface. To compare the uniformity of peak intensities between methods based on an organic matrix and a GO/CNT surface, LysoPC was used as an analyte for detection. The absolute intensities of the mass peaks varied significantly depending on the region when the organic matrix 2,5-dihydroxybenzoic acid (DHB) was used, but with the GO/CNT-chip LDI method, uniform intensities were obtained wherever the laser spot was applied (Figure 6a). Obtaining uniform peak intensities is especially important for the analysis of enzyme activities on biochips to obtain more reliable quantitative data. For parallel enzyme activity assays in high-throughput format, multiple samples should be screened quantitatively in a short time. The present assay method requires only spotting of samples on the GO/CNT surface for sample preparation without extra purification steps or expensive reagents (Figure 6b).

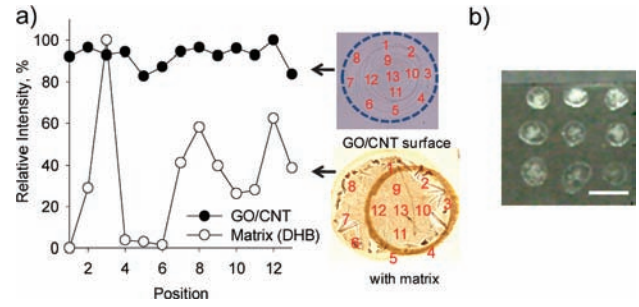

Figure 6. Advantages of the LDI−chip assay. (a) Comparison of uniformity of peak intensity between formats based on an organic matrix and on a GO/CNT chip. (b) Photo of a sample array prepared on a GO/CNT film fabricated on a glass coverslip (scale bar: 2 mm).

In conclusion, we have developed a new LDI−TOF MS-based platform for assays of phospholipid hydrolysis mediated by phospholipases that employs supported GO/CNT double-layer films. The films were used as the surface to absorb and transfer the energy of a laser to deposited analytes for desorption and ionization of the analytes. This is the first study to utilize GO/CNT hybrid films for mass spectrometric analysis of enzyme activities. The present platform for phospholipase activity assays has important advantages in comparison with the conventional method. First, the GO/CNT LDI chip makes the present method cost-effective, because expensive reagents, additional enzymes, isotopes, and fluorophores are not required. Second, the GO/CNT chips can be stored for more than several months under ambient conditions. Third, this method is technically simple and compatible with an array format. Thousands of samples can be spotted, and analysis of the array can be carried out at any time. Fourth, multienzymatic reactions can be conveniently characterized, as all of the analytes in a mixed solution should be mass-resolved in the MS-based assay. We believe that the present method will be widely applicable to enzymatic activity studies of phospholipases in cancer biology and chemical screening for discovering phospholipase inhibitors as drug candidates because of its simplicity, quantitativeness, reproducibility, cost-effectiveness, and compatibility with the array format.

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0062074), the Nano R&D Program (2008-2004457), and the National Honor Scientist Program.

Supporting Information Available: Experimental details, SEM and AFM images of GO-coated and CNT-coated substrates, and DPPC hydrolysis data for reactions with different concentrations of PLA2 and PLC. This material is available free of charge via the Internet at http://pubs.acs.org.

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(16) We found that the supported GO/CNT double-layer film can be harnessed for LDI MS of various small organic molecules. LDI MS is efficient on GO/CNT films, presumably because both GO and CNTs absorb UV light (337 nm) and transfer the energy to analytes on their surface. The effectiveness of the GO/CNT double-layer film as an LDI substrate was much higher than those of either GO or CNT alone (see Figures S5 and S6 for comparision of resolution and sensitivity). Detailed studies of small-molecule analysis by LDI−TOF MS on GO/CNT films will be published elsewhere.

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