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Fluorous Enzymatic Synthesis of Phosphatidylinositides

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Abstract

A fluorous tagging strategy coupled with enzymatic synthesis is introduced to efficiently synthesize multiple phosphatidylinositides, which are then directly immobilized on a fluorous polytetrafluoroethylene (PTFE) membrane to probe protein-lipid interactions.

Phosphatidylinositol (PtdIns) is a membrane-bound lipid that features a hydrophilic inositol head unit linked with a hydrophobic diacylglycerol (DAG) through a phosphate group.^[1] Three of the five hydroxyls in inositol undergo dynamic phosphorylation/dephosphorylation processes to generate the seven known phosphatidylinositides (PIs). Due to their interactions with a wide range of effector proteins,^[2] PIs are one family of the most versatile signaling molecules that regulate many cellular processes such as cell proliferation and vesicle transport.^[3] Abnormal levels of PIs have been associated with multiple diseases including cancer and neurodegenerative diseases.^[4] However, the detailed mechanisms by which PIs regulate different diseases are largely unknown, partly because of the difficulty in generating PI derivatives as cellular probes.

PIs and their derivatives are notorious for their structural complexity, with seven stereogenic centers and the hydroxyl groups around the inositol head unit having similar reactivity. Most of the synthetic strategies require selective protection and deprotection of the hydroxyl groups, and usually take more than 15 steps to synthesize one PI.^[5] The synthetic efforts are daunting when multiple PIs are targeted. In addition, PIs contain both the highly hydrophilic inositol phosphate head group and highly hydrophobic aliphatic side chains, making them difficult to purify from the reaction mixtures. Despite elegant work from several groups on developing novel methods and convergent strategies to prepare PIs and their derivatives,^[5] efficient synthesis of various PIs remains a technical challenge.

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Dedicated to Professor Dennis P. Curran on the occasion of his 60th birthday

Using enzymes as catalysts in organic synthesis has long been an alternative method to traditional organic synthesis.^[6] This approach has not been extended to PI synthesis although multiple enzymes that catalyze the formation of various PIs from PtdIns are well studied.^[7] The highly hydrophilic nature of the inositol phosphates head group further makes it difficult to separate the PIs from the enzymatic reaction mixtures containing inorganic salts. Utilizing highly fluorinated (fluorous) tags to assist separation of enzymatic products from mixtures over fluorous media^[8] has also been explored. For example, kinetic resolution of a fluorous ester has been carried out in a fluorous triphasic separative reaction to generate pure products without chromatography.^[9] Recently, fluorous tagged oligosaccharides have been used as enzymatic substrates in Nimzyme assays to detect enzymatic activities in cell lysates.^[10] However, these developments are focused on one-step enzymatic transformation and further applications of the products are not explored. We introduce here “fluorous enzymatic synthesis” (Fig. 1) where tandem enzymatic reactions are used to generate multiple probes after purification through fluorous solid phase extraction (FSPE)^[8a]. These probes can then be used as enzyme reporters, or be directly immobilized on a fluorous surface to form a microarray^[11] to investigate protein-small molecule interactions. PtdIns(4,5)P₂ is the most well-studied PI and functions as a substrate of multiple enzymes including phosphoinositide 3-kinase (PI3K) and phospholipase C (PLC).^[12] To validate “fluorous enzymatic synthesis”, we designed the fluorous PtdIns(4,5)P₂ derivative **1** with the fluorous tag at the *sn*-2 position. The long alkyl chain was used to ensure similar hydrophobicity and membrane localization as endogenous PtdIns(4,5)P₂ and to minimize the effect of the fluorous group on the head unit, where most metabolic reactions take place. The fluorophore was also added at the *sn*-1 position for sensitive monitoring of subsequent reactions. To synthesize **1** (Scheme 1), the fluorinated acid **2** was generated by the radical addition of the according perfluorinated iodide C₆F₁₃I with undec-10-enoic acid followed by reduction with lithium aluminum hydride.^[13] Coupling of **2** with the alcohol **3** and subsequent removal of the *p*-methoxybenzyl (PMB) protective group provided **4** in 90% yield. The alcohol in **4** was then phosphorylated and coupled with the inositol head group **5**,^[5a] and the resulting intermediate was oxidized with *t*-BuOOH to generate **6**. Next, both benzyloxycarbonyl (Cbz) and benzyl (Bn) groups were removed by hydrogenolysis while the methoxymethyl (MOM) group was removed by treatment with trimethylsilyl bromide (TMSBr) followed by methanolysis. The fully deprotected **7** was produced in 81% yield. Selective coupling of the terminal amine in **7** with *N*-hydroxysuccinimide (NHS) ester of fluorescein **8** provided the fluorous, fluorescent PtdIns(4,5)P₂ derivative **1**. The critical micelle concentration (CMC) of **1** was measured as 17 μM (Fig. S1), similar to that of the endogenous PtdIns(4,5)P₂ suggesting that the fluorous **1** is a good mimic as the endogenous PtdIns(4,5)P₂.^[14]

To investigate whether the tagged PtdIns(4,5)P₂ derivative worked as the enzyme substrate, the fluorous **1** was treated with purified PI3K, a kinase that phosphorylates endogenous PtdIns(4,5)P₂ to form the corresponding PtdIns(3,4,5)P₃ under standard PI3K reaction conditions.^[7a] The reactions were monitored by fluorescent detection of both PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ on TLC (Fig. 2). The starting material was cleanly converted to the product in 6 h under standard assay conditions. Likewise, when treated with PLC, another metabolic enzyme that utilizes PtdIns(4,5)P₂ as a substrate, fluorous **1** was completely

converted to the product DAG without any indication of formation of side products (Fig. 2). In cellular systems, PIs work as both starting materials for and products of multiple enzymatic reactions. To demonstrate that such complexity can also be recapitulated in the fluoros enzymatic synthesis, the tandem sequence of enzymatic reactions were investigated. The fluoros PtdIns(3,4,5)P₃ generated from the PI3K reaction followed by FSPE was directly used in a reaction catalyzed by phosphatidylinositol 3,4,5-triphosphate 5-phosphatase 2 (SHIP2)^[7c] to produce the corresponding PtdIns(3,4)P₂. As measured by TLC, the reaction generated a single product within 6 h. After FSPE, the fluoros PtdIns(3,4)P₂ was further subjected to dephosphorylation by phosphatase and tensin homolog (PTEN)^[7d] to yield the corresponding PtdIns(4)P. Again, only the desired product was produced which was further purified through FSPE (Fig. 2).

Both ³¹P NMR (Fig. 3) and MS (Fig. S2) were used to demonstrate the efficiency of FSPE purification. As described above, fluoros PtdIns(4,5)P₂ was first treated with PI3K and the resulting product was used as the substrate in SHIP2 reaction. To purify a product through FSPE, the reaction mixture was added to a column packed with fluoros silica. The column was first eluted with 20% and then 60% MeOH in water. As shown in Figure 3, the ³¹P NMR of the reaction mixture is dominated by ATP, the phosphate donor for the phosphorylation, and its hydrolyzed product ADP, with little signal for the PtdIns(3,4,5)P₃ product before FSPE. In contrast, after FSPE, the 60% MeOH fraction gave only resonances corresponding to PtdIns(3,4,5)P₃. These results suggest that FSPE efficiently enriches the desired fluoros product. Such enrichment is also critical for the MS analysis. No molecular ion was detected for PtdIns(3,4,5)P₃ before FSPE while the major signal in the MS spectrum after FSPE was the enzymatic product PtdIns(3,4,5)P₃ (Fig. S2). A similar phenomenon was observed for the SHIP2 reaction: in both ³¹P NMR or MS spectra, the desired reaction product was only detected after FSPE (Fig. 3 and Fig. S2).

Although cleavable linkers can be envisioned to avoid the trace of the fluoros tag on the enzymatic products, we chose to use non-cleavable fluoros tag for this work so that the enzymatic products could be directly immobilized on a fluoros surface for a small molecule array. We chose to use Teflon as the fluoros surface because the fluoros array used in the literature^[11] is no longer commercially available. Equal molar amounts of the three fluoros tagged PIs, PtdIns(4,5)P₂, PtdIns(3,4,5)P₃, and PtdIns(3,4)P₂, from the enzymatic reactions were spotted on a perfluorinated Teflon membrane for immobilization. Because each PI contains the same fluorophore fluorescein, the efficiency of loading was quantified by fluorescence scanning at the excitation/emission wavelengths of 488/520 nm (Fig. 4A). Indeed, the intensities of the three lipids were in the same range suggesting that they have similar capacity to immobilize on the Teflon membrane. When the quantity of the immobilized lipid doubles, so does the fluorescence intensity. The membrane was then incubated with biotin-conjugated antibody against PtdIns(3,4,5)P₃. After washing with buffer, the membrane was treated with Cy5-streptavidin and scanned for Cy5 signal ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 633/670$ nm) after washing with buffer (Fig. 4B). Only fluoros PtdIns(3,4,5)P₃ was detected by anti-PtdIns(3,4,5)P₃. Similarly, both fluoros PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ were detected by anti-PtdIns(4,5)P₂ (Fig. 4C) while anti-PtdIns(3,4)P₂ recognized fluoros PtdIns(3,4)P₂ and PtdIns(4,5)P₂ (Fig. 4D). These binding profiles are consistent with the intrinsic affinity of each antibody with different endogenous PIs,^[15]

suggesting that the fluorous tag does not significantly interfere with the antibody-lipid interactions.

Conclusions

In conclusion, a fluorous enzymatic synthesis strategy was developed to make multiple PIs which can be directly immobilized onto a fluorous microarray. Our experiments clearly demonstrate the advantage of the combination of fluorous tagging and enzymatic reactions in making multiple PIs at both the synthesis and the separation stages. Although cleavable linkers can be readily incorporated to remove the fluorous tag at the end of the enzymatic syntheses, this work purposely leaves the tag intact after enzymatic reactions so that the products could be directly immobilized onto a fluorous microarray. Indeed, the antibodies against PIs selectively recognize the fluorous-tagged lipids with the same binding profiles as their non-tagged parents, suggesting that the fluorous tag has little impact on their functions. This strategy should also be applicable to other complex endogenous small molecules whose biosynthetic enzymes are well characterized.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Schematic illustration of “Fluorous Enzymatic Synthesis”. The enzymatic products can be directly immobilized on a fluorous surface.

Fig. 2.

Enzymatic synthesis with PI3K, PLC, SHIP2, and PTEN. The reaction progression was monitored by TLC.

Fig. 3.

Efficiency of FSPE on product purification and characterization with ^{31}P NMR.

Fig. 4.

Selective binding profiles of fluorescent lipids with antibodies on a fluorescent membrane.

Scheme 1.
Synthesis of the fluororous substrate PtdIns(4,5)P₂.