

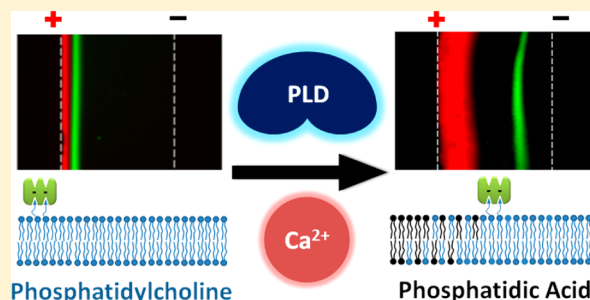
Monitoring Phosphatidic Acid Formation in Intact Phosphatidylcholine Bilayers upon Phospholipase D Catalysis

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S Supporting Information

ABSTRACT: We have monitored the production of the negatively charged lipid, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidic acid (POPA), in supported lipid bilayers via the enzymatic hydrolysis of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (PC), a zwitterionic lipid. Experiments were performed with phospholipase D (PLD) in a Ca^{2+} dependent fashion. The strategy for doing this involved using membrane-bound streptavidin as a biomarker for the charge on the membrane. The focusing position of streptavidin in electrophoretic-electroosmotic focusing (EEF) experiments was monitored via a fluorescent tag on this protein. The negative charge increased during these experiments due to the formation of POPA lipids. This caused the focusing position of streptavidin to migrate toward the negatively charged electrode. With the use of a calibration curve, the amount of POPA generated during this assay could be read out from the intact membrane, an objective that has been otherwise difficult to achieve because of the lack of unique chromophores on PA lipids. On the basis of these results, other enzymatic reactions involving the change in membrane charge could also be monitored in a similar way. This would include phosphorylation, dephosphorylation, lipid biosynthesis, and additional phospholipase reactions.



Phospholipids play important roles in numerous cell membrane activities, such as protein binding, intracellular signaling, apoptosis, and the synthesis of biomolecules.^{1–5} Different phospholipids and fatty acids can be interconverted through various enzymatic reactions on the surface of lipid membranes.⁵ This includes phosphatidic acid (PA), which typically accounts for 1–4% of the total lipid content of eukaryotic cells.⁶ PA is a central lipid in signaling reactions and serves as a precursor for the biosynthesis of many other lipids.^{5,7–9} It is also involved in regulating membrane curvature and the vesicle fusion process.^{1,9–12}

Activated by Ca^{2+} , phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylcholine (PC) to phosphatidic acid (PA) (Figure 1A).^{13,14} Such enzymatic activity is closely related to intracellular signal transduction, the presence of cancer cells, and other PA-related processes.⁹ A “ping-pong-like” catalytic mechanism has been proposed for PLD activity.¹⁵ Namely, PC binds covalently with PLD to form a PA-PLD complex with the release of choline. The P–O bond between PA and PLD is then cleaved by the nucleophilic attack of water on the distal phosphate ester.¹⁵ Real-time atomic force microscopy (AFM) measurements have demonstrated that PLD is released from the lipid bilayer surface after the reaction is completed.¹⁶

Typically, phospholipase reactions on phospholipids are monitored by measuring the concentration of the products which are evolved. For example, PLD activity has been monitored by measuring the concentration of free choline released from PC using a two-step chromogenic assay or using isotopically labeled choline.^{13,17–20} In the chromogenic assay, the released choline is first transformed into betaine and H_2O_2

by choline oxidase. With 4-aminoantipyrine and sodium 2-hydroxy-3,5-dichlorobenzenesulfonate, H_2O_2 then produces quinone dye in the presence of peroxidase. The concentration of the quinone dye can be determined by measuring its absorbance around 500 nm.^{13,18,19} In this case, successive experiments can result in compounded errors and makes the assay less accurate. Indeed, the concentration of the biologically relevant product, PA, has been particularly difficult to measure, simply because PA is a component of many other phospholipids and lacks unique absorption peaks that can readily be differentiated from other lipids. As a consequence, it has been difficult to directly monitor the PLD-catalyzed PC to PA conversion directly within intact lipid membranes. As such, there are current efforts directed toward developing new assays for the formation of PA in PLD-catalyzed PC hydrolysis. This includes very recent work exploiting the use of ion current passing through a nanopore in a black lipid membrane to follow PA production.^{21,22}

Herein, we report a simple and straightforward method that can directly determine the percentage of POPA in supported lipid bilayers (SLBs) by monitoring the electrophoretic-electroosmotic focusing (EEF) position of a fluorescently tagged streptavidin molecule that is bound to the membrane via a biotinylated lipid.²³ Negatively charged membrane-bound proteins like streptavidin can be focused on SLBs containing

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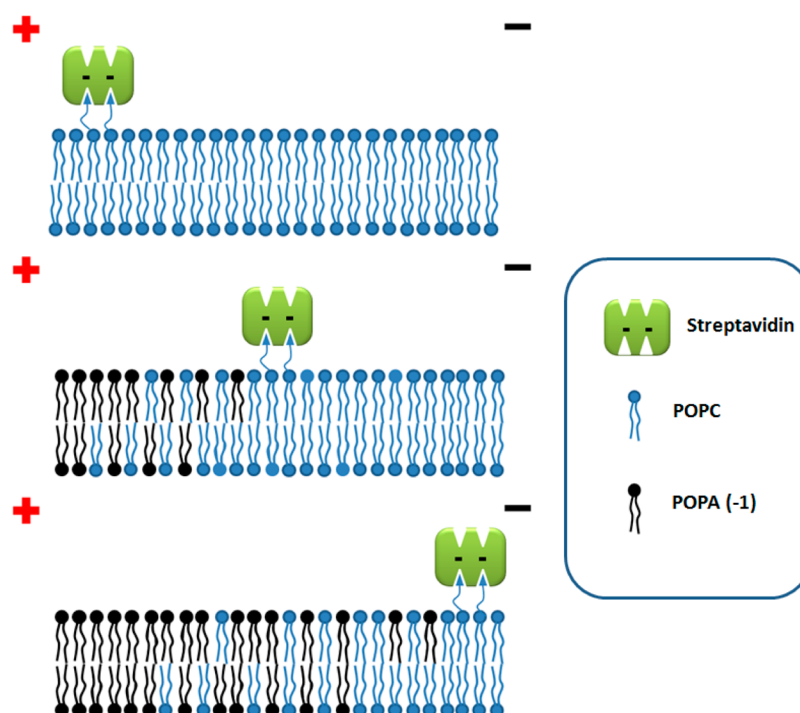


Figure 1. (A) Illustration of PLD catalyzed POPC to POPA conversion. (B) Illustration of the effect of negatively charged POPA lipids on the focusing position of a streptavidin biomarker in an EEF experiment.

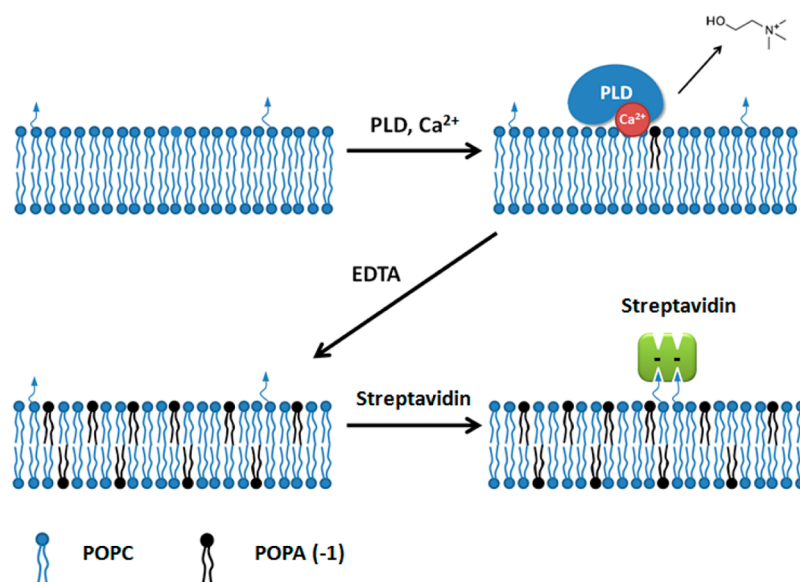


Figure 2. Schematic diagram of the experimental procedure for an EEF assay. In a first step, PLD is introduced above the POPC bilayer in the presence of Ca²⁺. This converts neutral POPC lipids to POPA. After the reaction, PLD and Ca²⁺ are removed from the SLB surface by rinsing in buffers containing EDTA. Finally, streptavidin biomarkers are introduced to the SLB and the EEF assay is performed.

negatively charged lipids by counteracting electrophoretic and electroosmotic forces (Figure 1B).²¹ These forces exactly balance each other at the focusing position. Moreover, the zeta potential of a focused protein should be equal to the local surface zeta potential at the focusing position, which is directly related to the local surface charge density. As such, streptavidin or other membrane bound proteins can be exploited as indicators of the local surface charge density. When an external electric field is applied laterally across an SLB, negatively charged lipids spontaneously build up a concentration gradient, generating higher negative surface charge density near the

positive electrode.^{21,24,25} The concentration of negatively charged lipids in the membrane strongly affects the focusing position of the protein because the shape and magnitude of the gradient that can be formed are directly involved.²³

This assay exploits the fact that POPC is uncharged (zwitterionic), while POPA is negatively charged at neutral pH (Figure 1A). Thus, the PLD enzymatic reaction can convert an initially neutral bilayer into one that contains negatively charged lipids. After the reaction is completed and the enzyme is washed away, the concentration of the negatively charged components can be monitored via an EEF assay, when

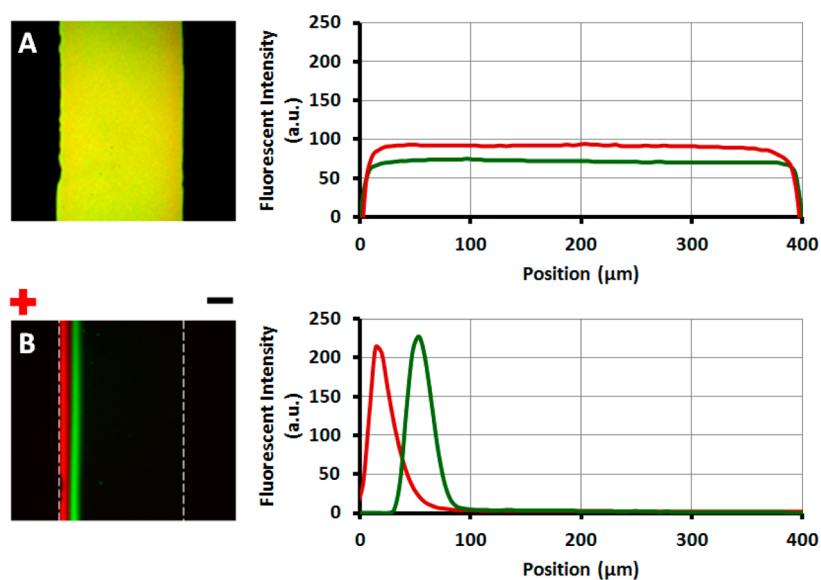


Figure 3. Fluorescence images of streptavidin (green) on an SLB containing 0.1% Texas Red-DHPE (red), 0.5% biotin-cap-DOPE and 99.4% POPC (A) before and (B) after commencing the EEF experiment in a 50 V/cm electric field.

streptavidin is subsequently attached to the surface (Figure 2). Specifically, the focusing position of the streptavidin marker will migrate ever closer to the negative electrode as the concentration of POPA is increased (Figure 1B).

EXPERIMENTAL SECTION

Materials. Fibrinogen was purchased from Sigma (St. Louis, MO), while phospholipase D was purchased from Enzo Life Sciences (Farmingdale, NY). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(cap biotinyl) (biotin-cap-DOPE), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidic acid (POPA) were purchased from Avanti Polar Lipids (Alabaster, AL). 1,2-Dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine-*N*-Texas Red (Texas Red-DHPE) and streptavidin were purchased from Invitrogen (Grand Island, NY). Polydimethylsiloxane (PDMS) was obtained from Dow Corning (Sylgard, silicone elastomer-184). The specific dye-labeled lipid and biotin-conjugated lipid/streptavidin pair were chosen for these experiments, as they represent standard lipid membrane chemistries that are readily compatible with the PA sensing assay developed herein.

SLB Formation. SLBs were formed by the vesicle fusion method on clean glass coverslips (Corning, NY, 22 × 22 mm, no. 2).^{26,27} The coverslips were cleaned in a boiling 1:4 solution of 7× detergent (MP Biomedicals, Solon, OH) and purified water. Purified water came from an Ultrapure Water System (Thermo Scientific Barnstead Nanopure Life Science, Marietta, OH). The coverslips were rinsed with copious amounts of purified water, dried with nitrogen gas, and annealed in a kiln at 500 °C for 5 h before use. Small unilamellar vesicles (SUVs) were prepared by vesicle extrusion. To do this, the lipids were mixed in chloroform. The chloroform was subsequently evaporated under a stream of nitrogen followed by vacuum desiccation for 4 h. Next, the lipids were rehydrated in a phosphate-buffered saline (PBS) solution which consisted of 10 mM sodium phosphate, 150 mM NaCl, and 0.2 mM sodium azide. The pH of the PBS solution was tuned to 7.4 with a small amount of 1 M HCl. The concentration of the lipids in solution was 1.0 mg/mL. After several freeze–thaw cycles, the solutions

were extruded through a polycarbonate filter (Whatman) with 100 nm pores.

To form SLB patches, fibrinogen monolayers were patterned on glass coverslips using a standard PDMS stamping method in a first step.²³ Next, SLB patterns were formed by vesicle fusion onto the bare glass areas on the patterned glass slide.^{21–23} Each SLB patch was about 2 mm long and 400 μm wide.

Fluorescence Imaging and Flow Cell Operation.

Epifluorescence images were obtained through a 10× objective using a Nikon E800 fluorescence microscope with a Roper Scientific MicroMAX 1024B charge-coupled device (CCD) camera (Princeton Instruments). A flow cell electrophoresis device was used to hold the pH and ionic strength conditions constant.^{23,28} All experiments were performed with a 10 mM Tris buffer solution at pH 7.5. The buffer was flowed through the channels at a rate of 25 mL/h per channel.

RESULTS AND DISCUSSION

In a first assay, SLBs were composed of 99.4% POPC, 0.5% biotin-cap-DOPE, and 0.1% Texas Red-DHPE. The biotin-cap-DOPE provided binding sites for streptavidin. Texas Red-DHPE was added to monitor the formation of the SLB, and also could be used as a secondary biomarker for obtaining information about the concentration gradient of POPA after performing electrophoresis. The movement of Texas Red-DHPE and streptavidin within the SLBs was investigated during electrophoresis without exposing the bilayer to any PLD. To do this, 5 nM Alexa-488-labeled streptavidin in 10 mM Tris buffer at pH 7.5 was introduced above the SLBs and incubated for 30 min. The streptavidin should bind tightly to the biotin-cap-DOPE lipids under these conditions.^{23,25,28} Next, unbound proteins were rinsed away using 10 mM Tris buffer. A fluorescence image of this system was obtained (Figure 3A). As can be seen, both the streptavidin and Texas Red-DHPE were uniformly distributed across the SLB. After the application of a 50 V/cm electric field for 45 min (Figure 3B), Texas Red-DHPE accumulated on the side of the SLB closest to the positive electrode, simply due to the electrophoretic force on this negatively charged dye-labeled lipid. Streptavidin also formed a narrow band very close to the positive electrode edge.

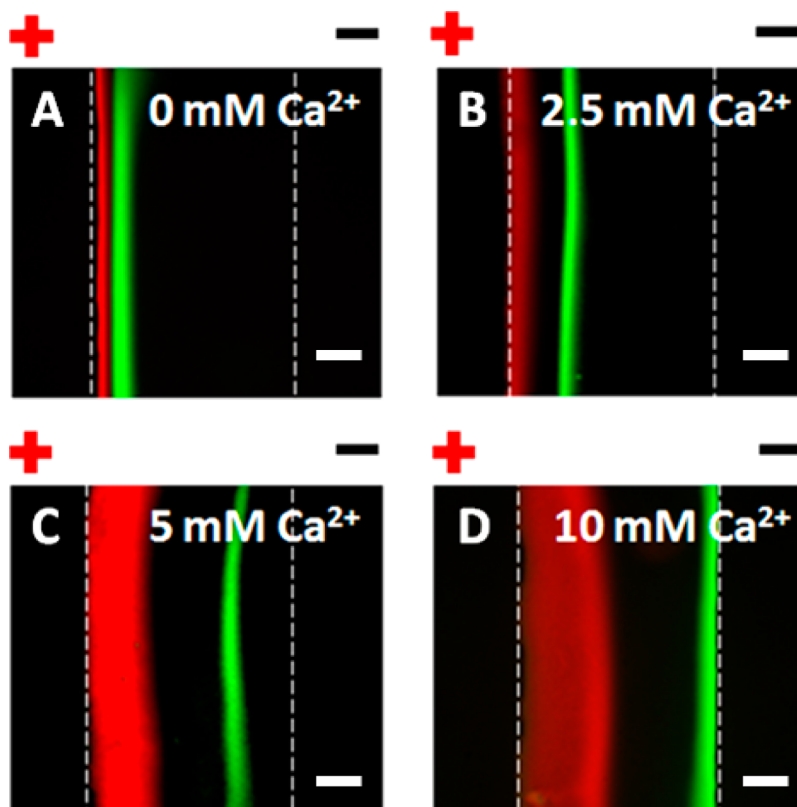


Figure 4. Electrophoretic-electroosmotic focusing (EEF) of Texas Red-DHPE and streptavidin biomarkers on SLBs after PLD-catalyzed POPA formation with (A) 0, (B) 2.5, (C) 5, and (D) 10 mM Ca^{2+} concentrations. The scale bars are 100 μm .

The focusing of streptavidin occurred because Texas Red-DHPE and biotin-cap-DOPE were negatively charged and established a shallow surface charge gradient.^{23,24} The line of focus for streptavidin was not quite as close to the positive electrode edge of the bilayer as the Texas Red-DHPE and biotin-cap-DOPE gradient. Moreover, the system shown in Figure 3B was at a steady state and the continued application of the 50 V/cm potential did not alter the profiles of either streptavidin or Texas Red-DHPE.

In a next set of experiments, we examined the effect of PLD activity on the same initial SLBs, as described in Figure 3. Because Ca^{2+} is a key activator of PLD,^{14,19} the PA conversion rates were measured at four different Ca^{2+} concentrations: 0, 2.5, 5.0, and 10 mM CaCl_2 (Figure 4). To perform an experiment, 10 μL of a 4.6 nM PLD solution were incubated over the SLB for 10 min. This was done in a 10 mM Tris buffer solution at pH 7.5 and 20 $^{\circ}\text{C}$. Next, the PLD solutions were rinsed away with Tris buffer that also included 30 mM EDTA to ensure removal of the Ca^{2+} and, hence, dissociation of the protein molecules.¹⁶ After this, 5 nM Alexa-488-labeled streptavidin was incubated over the SLBs for 30 min and rinsed away with buffer. EEF experiments were done in buffer with a 50 V/cm electric field. As can be seen in Figure 4A (no Ca^{2+}), streptavidin was focused to the same position as found in Figure 3B. Indeed, without Ca^{2+} , PLD should not have any significant catalytic activity.¹⁹ Therefore, no POPA was formed and the focusing position of streptavidin was unchanged, as expected. By contrast, the experiments with 2.5, 5, and 10 mM CaCl_2 all led to a change in the streptavidin focusing position. For example, with 2.5 mM Ca^{2+} , the streptavidin band focused to a position about 125 μm away from the positive electrode edge of the SLB (Figure 4B). Fluorescent line profiles for all

four streptavidin bands are provided in Figure 5. It should be noted that the formation of POPA was independently verified

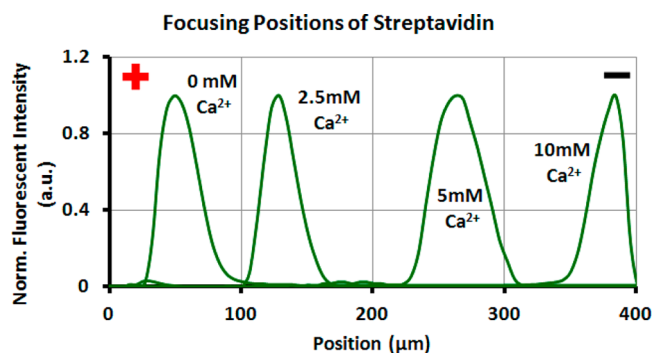


Figure 5. Line-scan fluorescence profiles of the streptavidin focusing positions on SLBs after the PLD-catalyzed reactions in Figure 4. All SLBs were incubated with PLD and Ca^{2+} for 10 min.

by performing matrix-assisted laser desorption/ionization (MALDI) experiments, and the expected masses were found. These data are provided in Figure S1 of the Supporting Information.

The zeta potential and structure of bilayer-bound streptavidin molecules should be essentially the same under all our experimental conditions. Moreover, the zeta potential of the SLB and the bound streptavidin should be equal to each other at the focusing line of the protein.²³ Therefore, the various focusing positions of the streptavidin bands in Figure 4 are indicative of differences in the negatively charged lipid gradients that are formed upon application of the electric field under the

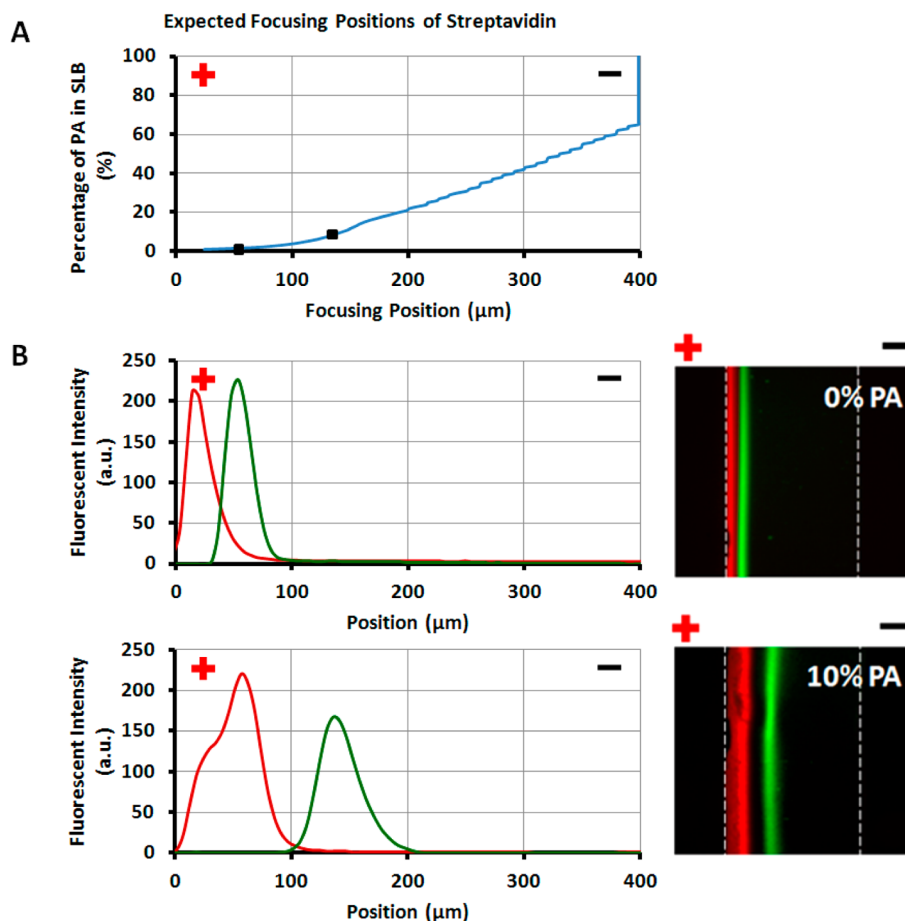


Figure 6. Effect of POPA concentration on the focusing position of streptavidin. (A) The expected focusing position calculated as a function of POPA concentration in the SLB.²³ (B) Focusing of Texas Red-DHPE and streptavidin with 0 and 10 mol % PA percentages. The ■ along the blue line in (A) represent the actual focusing positions found from the corresponding maxima in fluorescent intensity from the streptavidin in (B).

different experimental conditions. Specifically, as more negatively charged PA lipids are produced, the magnitude of the zeta potential adjacent to the positively charge electrode will be higher.²³ The gradient will also significantly broaden as the PA concentration increases. Thus, the focusing position of streptavidin will move ever further from the positive electrode upon the increase in PA concentration. Plots of the zeta potential as a function of position across the bilayer after the application of a 50 V/cm field with varying concentrations of negatively charged lipids are provided in Figure S2 of the Supporting Information.

The amount of POPA formed under the conditions employed herein should vary linearly with the concentration of Ca^{2+} .¹⁹ It should be noted, however, that with 10 mM Ca^{2+} , the streptavidin band was pushed all the way to the negative electrode edge of the SLB (Figure 4D), which set an upper limit to the amount of POPA that could be monitored with the current bilayer patch geometry and electric field conditions. The streptavidin focusing position, x , varied continuously as a function of POPA concentration up to this concentration limit, according to the following equation (Figure 6A):²³

$$\text{percentage}_{\text{POPA}} = K\{\exp[-v_d(x - r_f)/D] + 1\} \quad (1)$$

where $\text{percentage}_{\text{POPA}}$ is the mole percentage POPA in the SLB, K is a constant related to the experimental conditions, v_d (0.08 $\mu\text{m/s}$) is the drift velocity of POPA in the electric field, D (3.5 $\mu\text{m}^2/\text{s}$) is the diffusion constant of POPA, and r_f is a constant

with units of length and is related to the boundary conditions of the bilayer. See the Supporting Information for more details on the derivation of this equation.

The validity of this model was verified by running control experiments with known amounts of PA in the membrane. As can be seen, the plot of the expected focusing positions (Figure 6A) correspond very well with data points from 0 and 10 mol % POPA (Figure 6B). It should be noted that controls with higher PA concentrations are not provided, as vesicle fusion under these conditions required substantially altered buffer conditions which would ultimately effect subsequent electrophoresis experiments. On the basis of this calibration curve, the concentrations of PA produced with PLA under the various Ca^{2+} concentrations were estimated from the locations of the streptavidin bands. Specifically, the concentrations of POPA generated from the 0, 2.5, 5, and 10 mM Ca^{2+} experiments should be to be 0%, 7%, 33%, and at least 61 mol %, respectively. Plots with the zeta potential value as a function of position with each of these POPA concentrations is provided in Figure S3 of the Supporting Information. It should be noted that concentration values for POPA reported under each set of Ca^{2+} concentrations was corrected for the fact that the membranes also contained 0.6 mol % of negatively charged biotin-cap-DOPE and Texas Red-DHPE.

As can be seen from the data in Figures 4 and 6, the width of the Texas Red-DHPE band significantly broadened as the POPA concentration increased. Quantitative fluorescence line

scans for the Texas Red-DHPE data from Figure 4 are provided in Figure 7. As can be seen, the higher the Ca^{2+} concentration

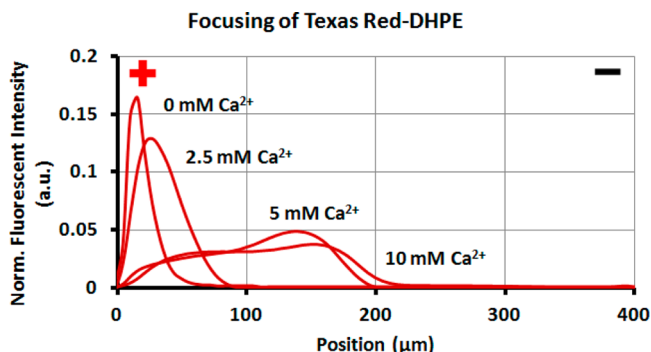


Figure 7. Fluorescence line-scan profiles of Texas Red-DHPE biomarker focusing positions on SLBs after PLD catalyzed reactions with 0, 2.5, 5, and 10 mM of Ca^{2+} .

(i.e., POPA mol %), the broader the Texas-DHPE band became. In addition, the position of this dye-labeled lipid band shifted away from the positive electrode. A maximum intensity peak could be observed on the negative electrode sides of these bands at the highest POPA concentrations. The reason for this should have two underlying causes. First, Texas Red-DHPE has a large headgroup, which is subject to moderate electroosmotic forces.²⁹ As such, this band will reach steady state closer to the positively charged electrode compared to POPA. Moreover, POPA's smaller headgroup should allow it to pack more readily on the side of the bilayer closest to the positive electrode. Since both POPA and Texas Red-DHPE are negatively charged, the high concentration of the former lipid should actually help exclude the latter from the most negatively charged portions of the membrane on the positive edge of the bilayer upon electrophoresis. These facts account for the fluorescence profiles observed with 5 and 10 mM Ca^{2+} as the Texas Red-DHPE builds up on electrophoretic grounds as close as it can to the region of highest POPA concentration from which it is depleted.

CONCLUSIONS

In conclusion, we have developed a label-free detection method that can characterize enzymatic membrane reactions by monitoring the focusing position in electrophoretic-electroosmotic focusing experiments. With comparison to conventional methods, the extent of the lipid headgroup hydrolysis reaction can be determined directly within the intact membrane because of the negative charge on the product lipid. This PLD enzyme reaction assay on SLBs requires far less materials than bulk assays. After detection, the SLBs can be restored to a well-mixed state by turning off the applied electric field.

In the future, other enzymatic reactions that involve changing the charges on lipids could be monitored in a similar fashion. There are a large group of such reactions, including phosphorylation, dephosphorylation, lipid biosynthesis, and related phospholipase reactions.^{3,5,7,8} As such, information on reaction rates as a function of membrane structure, cholesterol content, the presence of double bonds on the lipid tails, metal ions in solution, or related variables could all be tested. Because this assay is run in a supported lipid bilayer environment, it should be completely compatible with simultaneously running many experiments both as a function of solution conditions as

well as by tuning the chemical content of the bilayer. Such multiplexed assays could be performed by employing the appropriate microfluidic setup.^{30–32} As such, this assay has excellent potential for high-throughput/low sample volume analysis of enzymatic reactions on lipids.

ASSOCIATED CONTENT

Supporting Information

Mass spectrum of PC to PA conversion and the focusing positions of the biomarker as a function of PA mole percentages as well as extended calculations. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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