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Fluorescent Lipo-Beads for the Sensitive Detection of Phospholipase A_2 and Its Inhibitors

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Abstract

Phospholipase A_2 (PLA_2) is a membrane lytic enzyme that is present in many organisms. Human PLA_2 has emerged as a potential biomarker as well as a therapeutic target for several diseases including cancer, cardiovascular diseases, and some inflammatory diseases. The current study focuses on the development of lipo-beads that are very reactive and highly sensitive to PLA_2. To develop the best supported lipid bilayer formulation, several lipid combinations were investigated using 10 μm porous silica beads. The reactivity of PLA_2 was monitored via the decrease in particle fluorescence because of the release of entrapped fluorescent dye from the particle pores or the disintegration of a fluorescent lipid constituted on the bilayer upon lipid hydrolysis using flow cytometry. The enzyme binding studies indicate that lipo-beads with bulky fluorescent tags in the lipid head group and anionic lipids produce a more pronounced response. The kinetic studies suggest that these lipo-beads are very reactive with PLA_2 and can generate a detectable signal in less than 5 min. The enzyme inhibition studies were also conducted with two known PLA_2 inhibitors, varespladib and quercetin. We find that quercetin can hydrolyze the supported membrane, and thus inhibition of PLA_2 is not observed; however, varespladib has shown significant PLA_2 inhibition on lipo-beads. We have demonstrated that our lipo-bead-based

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Supporting Information

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Chemical structures of RPE, DMPC, DMPG, varespladib, and quercetin; long-term storage stability of different lipo-beads at 5 °C; stability of different lipo-beads after exposure to synthetic urine for 30 min; effect of Ca^{2+} on the stability of the SLM; PLA_2 activity on 20:80 DMPG/DMPC-coated fluorescein-encapsulated beads at 18 °C (gel phase) and 30 °C (liquid phase); effect of quercetin on DMPC lipo-beads; PLA_2 inhibition by ANXA3 for 20:80 DMPG/DMPC lipo-beads; and EC_{50} and PLA_2 concentrations needed for a 25% decrease of the fluorescence intensity (PDF)

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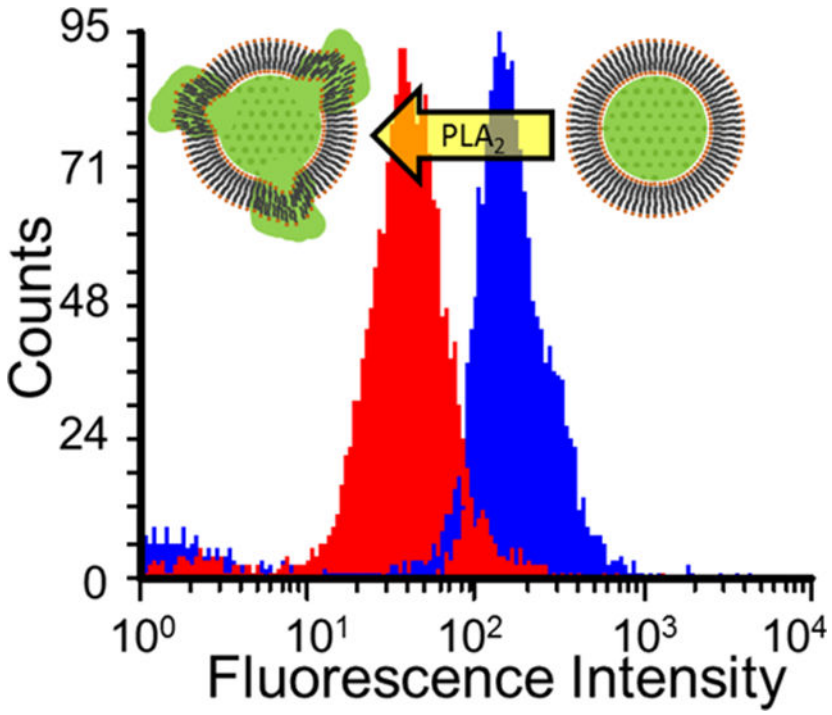
approach can detect annexin-3, a known disease biomarker, as low as 10 nM within 5 min after incubation.

Graphical Abstract



Keywords

phospholipase A_2; annexin A-3; lipo-beads; supported lipid bilayers; flow cytometry

INTRODUCTION

The human phospholipase A_2 (PLA_2) enzyme has emerged as a potential diagnostic biomarker for several diseases including breast, colon, pancreatic, lung, and prostate cancer and cardiovascular diseases and as a therapeutic target for pancreatitis, sepsis, atherosclerosis, and several other inflammatory diseases.[1–10] Although overexpressed PLA_2 in biological fluids and tissues under diseased conditions enables the enzyme to be targeted as a disease marker, under pathogenic conditions, PLA_2 is directly or indirectly responsible for the progression of several diseases. PLA_2 catalyzes the hydrolysis of phospholipid membranes; thus, the investigation of PLA_2–membrane interaction will provide opportunities to develop new methods of disease diagnostics and in vitro analysis in drug development.

There are six main variants of PLA_2, and their functions in the human body are different;[11] yet, all six variants of PLA_2 are capable of hydrolyzing the sn-2 position of the fatty acid chain of phospholipids present in cell membranes and produce arachidonic acids and lysophospholipids.[11–13] Besides, the specificity of PLA_2 from different sources for a given

phospholipid is similar.[5] Thus, a common approach that exploits the PLA_2–membrane interaction can be utilized to detect any variant of PLA_2.

Biological cells can be used as substrates to study the enzyme–membrane interaction; however, the presence of proteins, carbohydrates, and other extracellular components can interfere with PLA_2 reactivity, thus hindering the sensitivity and selectivity of the detection. Further, culturing and handling the cells can be tedious and complex, and long-term storage of cells is not convenient. On the other hand, model lipid membrane-based approaches are simple and easy to prepare and handle and thus provide simple and effective ways to investigate specific membrane hydrolysis. Also, PLA_2 is more reactive with organized phospholipid bilayers,[14] and model membrane systems can be customized with specific lipid compositions, thereby enhancing the enzyme's response significantly.

The PLA_2–lipid membrane interaction has been investigated using model lipid systems comprised of lipid vesicles or supported lipid membranes (SLMs). In lipid vesicle-based studies, colorimetric,[3,15] radiometric,[16] and steady-state fluorescence techniques[17–19] have been implemented to monitor the enzyme–membrane interactions. Furthermore, lipid vesicles have been utilized in PLA_2-mediated drug delivery for several cancer types.[20,21] Though lipid vesicles are the most commonly used substrates to explore PLA_2–membrane interactions[3,15–17,19] and may be more suitable for drug-delivery applications, they possess some inherited limitations that are not conducive for disease diagnosis purposes. Lipid vesicles have a short half-life and undefined size and shape; they tend to fuse and are prone to leak the content. In this regard, the use of SLMs is more favorable as the surface of the solid support renders the extra stability to the lipid membranes owing to the membrane–surface interaction.[22]

Lipid bilayers assembled on microspheres (lipo-beads) are advantageous as they enable the utilization of a sensitive high-throughput method such as flow cytometry.[23] The process of forming a lipid bilayer on microspheres is simple and efficient and well characterized. When small unilamellar lipid vesicles (SULVs) mix with microspheres, a single lipid bilayer of about 5 nm thickness will be formed around the microspheres spontaneously because of electrostatic interactions between the vesicles and microspheres. A water layer of about 2 nm trapped between the particle surface and the bilayer helps to retain the fluidity of the lipid bilayer.[24,25] The defined size, spherical shape, and bilayer fluidity provide a more cell-like structure, a high surface-to-volume ratio (thus increased reactive surfaces), and ease of handling and storage for an extended period without compromising the reactivity and sensitivity.

A microsphere-based approach has been developed to study the PLA_2–lipid bilayer interaction by Chemburu et al. using fluorescence superquenching of silica microspheres derivatized with a fluorescent polyelectrolyte.[26] However, the use of a preassembled cationic polyelectrolyte adds an extra synthesis step; an additional reagent (quencher) is needed to detect the enzymatic response, making the approach more complex, and relying on two consecutive binding events can cause an undesirable signal loss. Nevertheless, this work has laid the foundation for a more thorough study to develop more sensitive yet simple fluorescence microsphere-based detection for PLA_2. A more detailed kinetic and binding

study of PLA_2 on lipid monolayer-coated styrene-divinylbenzene beads was reported by Kim and his co-workers.[16] In their work, the use of radiolabeled lipids was unconvincing, and the necessity of separating hydrolyzed fatty acids followed by their radiometric analysis was tedious and time-consuming. Further, the hydrophobicity of styrene beads allows only the formation of a lipid monolayer, which is a less accurate representation of the cell membrane. None of the work has studied the use of lipo-beads for PLA_2 inhibition, which will be advantageous in analyzing new PLA_2 inhibitors as therapeutic drugs as well as disease markers such as annexins that inhibit PLA_2.

In this work, we demonstrate the use of fluorescent lipo-beads comprised of various lipid formulations for fast, sensitive, and specific detection of the PLA_2–membrane interaction. We utilized two types of fluorescent lipo-beads, one, which has a fluorescent dye encapsulated in porous silica particles using a nonfluorescent lipid coating and another with plain porous silica particles coated with a fluorescent lipid bilayer. In both types, the bilayer will be disintegrated from beads upon reacting with PLA_2, resulting in a decrease in the bead fluorescence.[27] Most PLA_2 inhibition studies have been performed with either lipid micelles or vesicles,[3,15,28–31] and to the best of our knowledge, this is the first time that lipo-beads are used for enzyme inhibition studies. We envision that these inhibition studies will initiate the use of lipo-beads in analyzing therapeutic candidates for several diseases. Here, we demonstrate the proof of concept for inhibition studies using two known inhibitors of PLA_2, varespladib and quercetin. Then, we explore the feasibility of using lipo-beads to detect annexin A3 (ANXA-3), a potential biomarker for prostate and several other cancer types.[32,33]

EXPERIMENTAL SECTION

Materials.

1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dipentadecanoyl-*sn*-glycero-3-phosphocholine (15:0 PC), 1,2-dimyristoyl-*sn*-glycero-3-phosphorylglycerol (DMPG), and rhodamine phosphoethanolamine (14:0 Liss Rhod PE, RPE) were purchased from Avanti Polar Lipids (Alabaster, AL). Porous silica microspheres of 10 μm diameter with 10 nm pores (Nucleosil 100–10) were purchased from Macherey-Nagel Inc. (Duren, Germany). PLA_2 from porcine pancreas, varespladib (LY315920), quercetin dihydrate, phosphate-buffered saline (PBS) (0.01 M phosphate buffer, 0.0027 M KCl, 0.137 M NaCl, pH 7.4 at 25 °C), fluorescein sodium salt, hydrochloric acid (36.5–38%), ammonium hydroxide solution (28–30%), calcium chloride, and chloroform were purchased from Sigma-Aldrich (St. Louis, MO). Hydrogen peroxide (30%) was purchased from VWR (Radnor, PA). Recombinant human annexin A3 (ANXA-3) was purchased from Creative BioMart (Shirley, NY) and Abcam (Cambridge, MA).

Preparation of Fluorescent Lipo-Beads.

The lipo-beads with encapsulated fluorescent dye were prepared by coating a nonfluorescent lipid bilayer on porous silica particles soaked in fluorescein dye, and the lipo-beads with a fluorescent lipid bilayer were prepared by coating the plain porous silica particles with a lipid bilayer comprised of DMPC and a fluorescent derivative of PE (RPE). Additionally, for

demonstration purposes, lipo-beads with fluorescein encapsulated and coated with DMPC and RPE were also prepared. Beads with encapsulated fluorescein dye were composed of various nonfluorescent phospholipid bilayers with varying molar composition of lipids (100% DMPC, 100% PC, and 20:80 DMPG/DMPC). For all lipo-bead preparations, first, a known amount of porous silica microspheres were cleaned in a solution of 4% NH_4OH and 4% H_2O_2 (w/v) at 80 °C, followed by a rinse in a solution of 4% H_2O_2 and 4% HCl (w/v) at 80 °C. SULVs prepared by bath sonication were used to form lipid bilayers on particles, as reported elsewhere.[27] Briefly, a lipid solution prepared in chloroform was kept under a stream of N_2 to evaporate the solvent and to form a lipid film at the bottom of a glass vial. For lipid mixtures, lipids in chloroform were mixed at the desired molar composition before forming the lipid film. The lipid film was further dried in a vacuum desiccator for an hour and hydrated by mixing with PBS (pH 7.4, 0.01 M). The hydrated lipid was sonicated using a bath sonicator (VWR, Radnor, PA) until the solution became clear, indicating the formation of SULVs. For fluorescein-entrapped microspheres, 100 mg of surface cleaned particles were soaked in 10.0 mL of 1.0 mM fluorescein sodium salt in PBS for 48 h while gently shaking. SLMs were formed by vortexing particles in 500.0 μL of SULV solution (1 mM of total lipid) for 5 min, followed by 20 min shaking using a vortex mixer (VWR, Radnor, PA). The excess lipid and fluorescein dye were removed by rinsing the prepared particles in PBS several times. To prepare lipo-beads coated with fluorescent lipids; 100.0 mg of cleaned particles were used with 500.0 μL of SULV solution (1 mM of total lipid) comprising 100:1 M ratio of DMPC and RPE. The excess lipid was removed as before. Each lipo-bead sample was suspended in 500.0 μL of PBS and stored at 5 °C for extended use. The loss of lipo-bead fluorescence was used to assess the stability of the particles. Thus, the fluorescence intensity of the beads was measured prior to each experiment using a FACSCalibur flow cytometer (BD Bioscience, San Jose, CA). Bead suspensions that were below a predetermined threshold intensity were deemed unstable and discarded.

Confocal Fluorescence Imaging of Lipo-Beads.—To demonstrate the encapsulation of fluorescein and the formation and stability of lipid bilayers on particles, fluorescence imaging was performed using an Olympus Fluoview FV10i confocal laser scanning microscope. Three types of lipo-beads, (a) fluorescein-entrapped porous beads with 100% DMPC lipid bilayer, (b) plain porous beads with a 100:1 (molar ratio) DMPC/RPE lipid bilayer, and (c) fluorescein-entrapped porous beads with 100:1 DMPC/RPE lipid bilayers were imaged after 21 days of preparation. Excitation and emission wavelengths of 473 and 510 nm, respectively, were used for fluorescein, wherein the excitation and emission wavelengths of 559 and 600 nm, respectively, were used for RPE.

Stability Studies of Lipo-Beads.—The stability of DMPC, 100:1 DMPC/RPE, and 20:80 DMPG/DMPC lipo-beads stored at 5 °C was monitored for 3 weeks after preparation. A 10 μL portion from 10 mg/mL stock lipo-beads was diluted to 100 μL with PBS for the fluorescence analysis. All measurements were done in three replicates.

Interaction of PLA_2 with Lipid Bilayers on Beads.

The interaction of PLA_2 with the lipid bilayer on beads was explored via the release of fluorescein dye (Figure 1d) and the disintegration of the fluorescent lipid, RPE, (Figure

1e) from the bead surface. The activity of the enzyme was evaluated for four different lipo-bead formulations at two different temperatures, below and above the phase transition temperature (T_m) of the major lipid constituent of the bilayer. In each test, 50.0 μL of 1 mg/mL lipo-beads suspended in PBS was taken into a microcentrifuge tube and incubated with 45.0 μL of 4.1 μM PLA_2 and 15.0 μL of 0.01 mM Ca^{2+} for 30 min. Lipo-beads in enzyme-free PBS with and without 0.01 mM Ca^{2+} were used as controls, and each test was performed in three replicates. A temperature-controlled digital water bath was used to maintain the desired temperature. The lytic activity of the enzyme was measured via the decrease in the fluorescence intensity of the particles. The fluorescence intensities of the particles before and after reacting with the enzyme were measured at 525 nm for fluorescein-entrapped microspheres and at 585 nm for fluorescent lipid-coated microspheres using a BD FACSCalibur flow cytometer. The percentage decrease was calculated as shown in eq 1

$$\text{Percent decrease} = \left(\frac{I_0 - I_t}{I_0}\right) \times 100 \quad (1)$$

Here, I_0 is the initial fluorescence intensity of the particles and I_t is the intensity of the particles after reacting with the enzyme. All data collection and analyses were performed using BD CellQuestTM Pro (BD Bioscience, San Jose, CA) and FCS Express 6 (De Novo Software, Glendale, CA) software, respectively.

PLA_2 Binding and Kinetic Studies with Lipo-Beads.

Enzyme binding and kinetic assays were performed on different lipo-beads to determine the most reactive lipid composition, optimal PLA_2 concentration, and incubation time. During the binding studies, 45.0 μL of PLA_2 with various concentrations and 15.0 μL of 0.01 mM Ca^{2+} for DMPC, PC, and 100:1 DMPC/RPE and 0.3 mM of Ca^{2+} for 20:80 DMPG/DMPC were mixed with 50.0 μL of 1 mg/mL lipo-beads in microcentrifuge tubes. Lipo-beads incubated in enzyme-free PBS with and without Ca^{2+} were used as controls, as mentioned previously. All assays were performed at temperatures where the major lipid of the bilayer was in its liquid phase. The fluorescence intensity of the particles in each sample was measured as before. The optimal PLA_2 concentration for each lipid composition obtained from the binding studies was used for the respective kinetic experiments. For each kinetic assay, 1 mg/mL suspension of lipo-beads was used with 0.01 mM Ca^{2+} for DMPC, PC, and 100:1 DMPC/RPE lipo-beads and 0.3 mM Ca^{2+} for 20:80 DMPG/DMPC lipo-beads. A constant volume of bead sample was withdrawn from the microcentrifuge tube at known time intervals for flow cytometry measurements.

PLA_2 Inhibition with Model Inhibitors.

The efficacy of lipo-beads for PLA_2 inhibition was evaluated with two model inhibitors, varespladib and quercetin. For varespladib, 35.0 μL of the inhibitor solutions in PBS with various concentrations ranging from 0 to 75.0 μM were mixed first with 50.0 μL of 1.0 μM PLA_2 for 30 min at 25 °C. Then, 50.0 μL solution of 1 mg/mL lipo-beads was incubated with the mixture for another 30 min while maintaining the liquid phase of the primary constituent lipid. A calcium concentration of 0.01 mM was maintained for

100% DMPC, 100% PC, and 100:1 DMPC/RPE and 0.3 mM for 20:80 DMPG/DMPC lipo-beads. A positive control comprised of all reagents, but the inhibitor and a negative control comprised of the PBS buffer were also used. For quercetin, 45.0 μL of 4.1 μM PLA_2 was preincubated with 45.0 μL of 35.0 μM quercetin before mixing with 50.0 μL of 1 mg/mL 100% DMPC lipo-beads. This mixture was incubated at 30 °C for 30 min before measuring the fluorescence intensity of the particles. The positive control and the negative control were used as before.

Determination of Annexin A-3.

ANXA-3 was determined via PLA_2 inhibition using 20:80 DMPG/DMPC and 80:15:5 DMPC/DMPG/RPE lipo-beads. For 20:80 DMPG/DMPC lipo-beads, 10.0 μL of 100.0 nM PLA_2 was mixed with 10.0 μL of 10.0 μM Ca^{2+} and 10.0 μL of 55.0 nM ANXA-3 in PBS. A sample of 20.0 μL of 1 mg/mL 20:80 DMPG/DMPC lipo-beads was added to the preincubated enzyme–ANXA-3 mixture. For 80:15:5 DMPC/DMPG/RPE lipo-beads, 10.0 μL of 20.0 nM PLA_2 was preincubated with 10.0 μL of different concentrations of ANXA-3 and 10.0 μL of 10.0 μM Ca^{2+} before adding to the 20.0 μL of 0.5 mg/mL 80:15:5 DMPC/DMPG/RPE beads. The reaction mixture was incubated at 30 °C for 30 min before measuring the fluorescence intensity of the particles.

The percent inhibition and percent PLA_2 activity for two model inhibitors and ANXA-3 were calculated using eqs 2 and 3

$$\text{Percent inhibition} = \left(\frac{I_{\text{D, PLA}_2} - I_{\text{D,Inh}}}{I_{\text{D, PLA}_2}}\right) \times 100 \tag{2}$$

$$\text{Percent PLA}_2\text{ activity} = (\,100 - \text{percent inhibition}\,) \tag{3}$$

where $I_{\text{D, PLA}_2}$ is the decrease in the signal for PLA_2 and $I_{\text{D,Inh}}$ is the decrease in the signal in the presence of the PLA_2–inhibitor mixture. I_{D} was calculated by $\frac{I_0 - I_t}{I_0}$ (see eq 1).

Statistical Analysis.

All experiments were conducted in triplicate with average measurements reported. Statistical analysis (unpaired *t*-test) was conducted using GraphPad Prism software to report the variations in different measurements at a 95% confidence interval, where it was necessary. Data were considered statistically significant when p-value < 0.05.

RESULTS AND DISCUSSION

Preparation of Fluorescent Lipo-Beads.

PLA_2 is more reactive with phospholipid bilayers containing choline, glycerol, and ethanolamine in the lipid-phosphate head group.[34,35] Therefore, we utilized several combinations of choline, glycerol, and ethanolamine containing phospholipids as constituents of the supported bilayer. When selecting them, lipids with phase transition temperatures closer to room temperature (around 25 °C) were considered for convenience

during the preparation and experimentation. Among the three phospholipids tested, glycerol containing DMPG has a net negative charge at physiological pH, and PLA_2 is more reactive with the anionic lipid.[3,18,36–38] The silica surface is also negatively charged at pH 7.4;[39–41] thus a stable SLM containing 100% DMPG could not be obtained because of strong repulsive electrostatic interactions. Consequently, DMPG was mixed with zwitterionic DMPC to form stable bilayers. Fluorescence spectroscopy-based analysis is simpler and more sensitive than other techniques that have been used to determine the membrane–enzyme interaction in previous studies. Further, a high throughput, fluorescence-based method such as flow cytometry can easily be implemented to monitor the loss of fluorescence in lipo-beads as a consequence of membrane hydrolysis. The presence of a bulky fluorophore in the fluorescently tagged lipid may restrict the enzyme's access to the membrane. To circumvent this, we used dye-encapsulated microspheres with a nonfluorescent lipid coating (Figure 1a). Yet, to find the efficacy of using lipo-beads with fluorescently tagged lipids, silica microspheres coated with fluorescent lipids were also investigated (Figure 1b). In this study, two choline-containing zwitterionic lipids, DMPC (14:0) or PC (15:0), were used as the primary lipid constituent of the bilayer because most mammalian cell membranes are enriched with phosphatidylcholines.[42] Even though the structure of these two lipids differs only by one carbon atom in the fatty acid tail (Figure S1), their phase transition temperatures are 24 and 35 °C. We used 20 mol % DMPG to explore the effect of bilayer charge on PLA_2 reactivity. We found that stable lipo-beads with anionic lipids can be formed only when the molar ratio of DMPG is 20% or less under our experimental conditions; thus we kept 20 mol % as the upper limit for the anionic lipid. When the fluorescent tagged lipid RPE is incorporated into the bilayer, we found that 1–5 mol % of RPE would produce lipo-beads with the maximum fluorescence intensity. This can be due to the fluorescence self-quenching at a high fluorophore concentration. During the bead preparation, lipid solutions were maintained above the phase transition temperature of the major lipid, and the prepared lipo-beads were kept at 5 °C during storage. The stability studies conducted for 3 weeks shows that there is no significant loss in particle fluorescence, confirming that the lipid coating on beads is intact even after 3 weeks if kept below the phase transition temperature of the major lipid (Figure S2). Further, these lipo-beads were also stable in artificial urine (Figure S3). Additionally, confocal fluorescence imaging was performed on these beads after 3 weeks from preparation to further confirm the stability and integrity of the bilayers (Figure 1a–c). The obtained images clearly prove that the bilayers are intact and stable even after 3 weeks from preparation. In Figure 1a, fluorescein is retained by the nonfluorescent lipid coating. The sharp fluorescent edge in the sphere (Figure 1b) suggests the existence of a lipid bilayer comprising the fluorescent lipid, RPE, whereas Figure 1c demonstrates the presence of fluorescein in pores and RPE in the coating.

Interaction of PLA_2 with Lipo-Beads.

PLA_2 requires Ca^{2+} as a cofactor for the enzymatic hydrolysis of the lipid sn-2 ester bond.[11–13,42] In the vesicle-based studies, it has been shown that a high calcium concentration can disrupt vesicles because of membrane fusion.[43,44] However, a systematic study on the effect of Ca^{2+} concentration on solid SLMs has not been reported. To develop a stable, highly sensitive, and reproducible bead-based substrate for the PLA_2 assay, the determination of the optimum Ca^{2+} concentration is essential. From a series of optimizing

experiments, we confirmed that a high calcium content could disrupt the supported membrane to some degree (Figure S4), and we limited the maximum concentration to be used with minimum disruption effect to 0.3 mM Ca^{2+}. In Figure 2, the reaction of PLA_2 on three different lipo-beads, while keeping the enzyme and calcium concentrations constant, is summarized. Upon PLA_2 reaction with lipo-beads, the fluorescence intensity of particles is decreased because of the release of the trapped dye (Figure 1d) or the loss of fluorescent lipid (Figure 1e) as a consequence of bilayer disintegration from the particle surface. Our data suggest that dye-encapsulated DMPC lipo-beads are more reactive (Figure 2a) than the dye-encapsulated PC lipo-beads (Figure 2b). When the incubation temperature is below the phase transition temperature of each lipid, the decrease in the fluorescence intensity is 38 and 33% for DMPC and PC, respectively; however, when the incubation temperature is above the phase transition temperature, PLA_2 is more reactive with both lipo-bead types, and the decrease in the fluorescence intensity is now 59 and 47%, respectively. The increased enzymatic activity could be due to the thermal induction or due to the increased fluidity of the membrane, which favors membrane hydrolysis.[18,45] Another possible cause is that the lipids in the liquid phase are more structurally disordered than those in the gel phase, and it has been reported that PLA_2 is more active on sites with structural defects.[46] Our observation with solid-supported bilayers is in good agreement with the PLA_2 interaction with lipid vesicles.[18,37,45] The difference in activity between PC and DMPC lipo-beads is due to the change in the fatty acid chain lengths as reported by Hoyrup et al. in a lipid vesicle study.[47] The statistical analysis of the data suggests that the average fluorescence decreases in the two lipo-bead types are not statistically significant ($p = 0.36$) when the lipids are in their gel phase; however, the decrease is statistically significant ($p = 0.04$) when the lipids are in the liquid phase. This observation indicates that the effect of phase change has become more pronounced than the carbon chain length of the fatty acid in the liquid phase. Though the data from lipo-beads with fluorescent lipids (RPE/DMPC) cannot be directly compared with the dye leakage data owing to the two modes of fluorescence loss, the RPE/DMPC beads show greater sensitivity for PLA_2. The percent fluorescence decrease in these particles is higher for comparable PLA_2 concentrations, and there is no significant difference in responses between the gel and liquid phases (72 and 74%, respectively, Figure 2c). When RPE is present, it is not necessary to hydrolyze the whole bilayer to generate a marked decrease in the fluorescence signal. It has been shown that PLA_2 first hydrolyzes the outer monolayer before reaching to inner layer.[35] With the bulky group of rhodamine present, we hypothesize that most RPEs are assembled into the outer monolayer, which results in a significant fluorescence decrease upon reacting with the enzyme, a hypothesis worth investigating further. The slow diffusion of the trapped dye can contribute to the low release of the fluorescein in encapsulated particles, resulting in a lower percent decrease in the particle fluorescence. One can anticipate that phase mismatching due to the presence of two lipids might enhance the PLA_2 reactivity.[48] The phase transition temperatures for DMPC and RPE are 24 and 42 °C, respectively, and by incubating the reaction mixture at 30 °C, only DMPC is in the liquid phase, whereas RPE is in the gel phase. However, the low abundance of RPE (1%) might not generate a phase mismatched bilayer; thus, we conclude the phase mismatch effect is negligible. Even though PLA_2 is more reactive with bilayers containing anionic lipids, there was no activity for 20% DMPG at 0.01 mM Ca^{2+} (Figure S5). However, when the Ca^{2+} concentration is increased from 0.01 to 0.30 mM, the

reactivity of PLA_2 was significantly enhanced (Figure 3c). In the presence of an anionic lipid in the bilayer, Ca^{2+} binds first to the anionic lipid before activating PLA_2,[49,50] and thus it is necessary to have an extra amount of the divalent cation. Therefore, a higher concentration of Ca^{2+} was used in tests with DMPG to follow.

PLA_2 Binding and Kinetic Studies with Lipo-Beads.

EC_{50} is the 50% effective concentration of the enzyme for the hydrolysis of the phospholipid bilayer, and here it is used to compare the reactivity of the enzyme on different lipo-beads. The EC_{50} obtained from the enzyme dose–response curves in Figure 3 confirms that PLA_2 has enhanced reactivity with lipo-beads containing anionic lipids (Figure 3c). The EC_{50} for the bilayers containing 20% anionic lipid was 112 nM, whereas for beads with pure zwitterionic lipids, PC and DMPC, the values were 197 and 278 nM, respectively (Figure 3b,a). Figure 3 further suggests that the potency of the enzyme is higher for anionic lipo-beads, though the efficacy is greater for zwitterionic lipo-beads. We anticipate that by further increasing the amount of anionic lipids in the bilayer, more sensitive lipo-beads can be obtained. The EC_{50} for the lipo-beads with RPE is 89 nM, and its dose response curve suggests that PLA_2 is more effective and potent with these lipo-beads than other tested lipo-beads (Figure 3d). As explained earlier, the presence of RPE in the outer layer and less effect from the diffusion-limited release can be the factors for the enhanced reactivity. If we consider a 25% decrease as the minimum detectable response, 100:1 DMPC/RPE lipo-beads have the minimum detection limit of about 48 nM (Figure 3d), whereas 100% DMPC lipo-beads have the minimum detection limit of 92 nM (Figure 3a). The EC_{50} and PLA_2 concentrations needed for a 25% decrease in the fluorescence intensity are summarized in the table of Supporting Information (Table S1).

The kinetic data suggest that PLA_2 generates a distinguishable signal in less than 5 min of initiating the reaction (Figure 4). For 100% DMPC and 100% PC lipo-beads, the percent decrease of the mean fluorescence intensity is rapid and it reaches the maximum decrease within 15 min (Figure 4a,b), whereas for the anionic lipo-beads, the maximum decrease reaches within 5 min (Figure 4c), further confirming the enzyme's enhanced reactivity with negatively charged lipids. On the other hand, lipo-beads with the fluorescent lipid, RPE, reaches the maximum decrease in 10 min (Figure 4d), and the extent of decrease is also greater in this time period, confirming the data in Figure 2c. The kinetic data suggest that both anionic lipo-beads and RPE lipo-beads are fast reactive; yet, RPE lipo-beads are the most sensitive.

Inhibition Assays with Model Inhibitors.

Under pathological conditions, PLA_2 behaves as a membrane disruptor that triggers several disease conditions, including Alzheimer, multiple sclerosis, and Parkinson disease.[51,52] Thus, the development of PLA_2 inhibitors as therapeutic agents will be important, and we envision that these lipo-beads can be utilized in in-vitro studies to investigate the efficacy and potency of new PLA_2 inhibitory drugs. To demonstrate the proof of concept for utilizing lipo-beads in inhibition studies, known PLA_2 inhibitors, varespladib and quercetin, were analyzed. The percent inhibition and the percent PLA_2 activity were calculated using eqs 2 and 3 as described before. The varespladib data indicates that the extent of inhibition

varies with different lipid substrates (Figure 5). Though DMPC and PC beads show the inhibition (Figure 5a,b), it is not significant as in the anionic DMPG beads (Figure 5c). The statistical analysis shows that there is no significant difference in inhibition in all three lipo-beads (thus no linear correlation) above 5 μM of varespladib (p = >0.05), which indicates that the inhibition is saturated at micromolar concentrations. The lipo-beads comprised of RPE (Figure 5d) show a linear correlation, though the inhibition is less pronounced. As mentioned before, when RPE is present, it is not necessary to hydrolyze the whole bilayer to generate a marked decrease in the fluorescence signal; thus, the direct correlation of enzyme–inhibitor interaction is suggested, whereas in DMPC, PC, and DMPG beads, the direct correlation of enzyme–inhibitor interaction is not observed. Jain et al. have reported that PLA_2 has a membrane binding site and a catalytic site, and inhibitors can have three different modes of action. In the first mode, the inhibitor competes with the substrate to bind to the catalytic site of the enzyme, whereas in the second mode, the inhibitor changes the organization of the bilayer, thus affecting the enzyme–substrate binding, and in the third mode, the inhibitor binds directly to the active site or to the interfacial binding site of the enzyme. Therefore, the extent of inhibition depends on the mode of inhibition. Furthermore, the type of lipid substrate can also influence the extent of inhibition.[53] Thus, a combined effect from the mode of inhibitory action and the type of lipid substrate is anticipated for the overall PLA_2 inhibition on different lipo-beads. The more pronounced inhibition in DMPG beads and the linearity in RPE beads suggest that a combination of DMPG and RPE lipids might produce more sensitive and appropriate lipo-beads. A thorough investigation of the inhibitory effects and mechanisms on different lipo-beads is worth investigating, though it is beyond the scope of the current work. However, our data suggest that these lipo-beads can be utilized to explore the PLA_2 inhibition by target molecules, and it is vital to choose the correct type of lipid substrate for PLA_2 inhibitory studies.

The bioflavonoid quercetin, which is normally used for the treatment of inflammation and arteriosclerosis, did not show any inhibition of PLA_2 activity when tested with lipo-beads. Instead, it showed a significant disruption of the lipid bilayer (Figure S6). The lytic activity of quercetin could be due to the more structural polarity of quercetin, which helps to interact with the polar head group of a lipid molecule via hydrogen bonding, thus permeating the lipid membrane (see Supporting Information for structures, Figure S1). A previous study of quercetin on 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine vesicles has confirmed this type of interaction.[54] In another study, quercetin on human erythrocyte membranes showed that quercetin could affect the fluidity of the membrane, thus destabilizing the membrane.[55]

Determination of Annexin A3.

Annexins have emerged as disease biomarkers for cancer, diabetes, and inflammatory diseases.[56–59] They are a family of proteins that binds to phospholipids and are also known to inhibit PLA_2 activity.[1,31,60] In this work, we performed preliminary studies on annexin detection as another proof of concept for the study using lipo-beads for PLA_2 inhibition. Our initial ANXA-3 test with anionic lipo-beads generated a moderate result (Figure S7). The test showed that lipo-beads can be implemented to detect the protein, however, the sensitivity was not high enough for a good detection. Our PLA_2 binding data so far have suggested that DMPG and RPE lipo-beads are the most effective in PLA_2 detection. Thus, to

enhance the sensitivity of lipo-beads further, from a quick inquisitive experiment, we found that lipo-beads with 80:15:5 DMPC/DMPG/RPE would perform better. The dose–response curve for this new lipid formulation gave an EC_{50} of 15 nM for PLA_2 (Figure 6). We chose 20 nM PLA_2 for the inhibition assays with varying ANXA-3 concentrations. Figure 7 shows that ANXA-3 as low as 10 nM can easily be detected with 35% PLA_2 inhibition. However, to establish the detection limit of our method, a more comprehensive study has to be performed with a wider range of ANXA-3 concentrations. ANXA-3 is currently detected via enzyme-linked immunosorbent assay (ELISA) and has a detection limit of about 0.05 nM (assaypro.com). In comparison to ELISA, the minimum concentration we could detect during this study is 100 times higher. However, our on-going work suggests that the current limit can be lowered further by enhancing the reactivity of lipo-beads by changing the lipid composition and the size and porosity of silica particles. The reported data in Figures 6 and 7 are the average of a minimum of three replicates, and the small standard deviations indicate that the method has good reproducibility. Further, our approach is simple and rapid, and reagents are comparatively inexpensive.

Comparison of the Sensitivity of Lipo-Beads.

The sensitivity of lipo-beads was evaluated by comparing the PLA_2 concentrations needed for a 25% decrease of fluorescence intensity of lipo-beads obtained from the dose response curves (Figures 3 and 6). The average % decrease for the most reactive beads at the highest PLA_2 concentration we tested in this study was about 75%, and we chose one-third of this maximum to be the criteria for the minimum detectable signal. The data suggest that 85:15:5 DMPC/DMPG/RPE lipo-beads are the most sensitive for PLA_2 detection, with a concentration needed for a 25% signal decrease as low as 6.20 nM (see Table S1 in the Supporting Information).

CONCLUSIONS

In this work, we explored several formulations of lipids as bilayers supported on porous silica microspheres to develop a rapid and sensitive particle-based detection method for PLA_2–membrane interaction. We have also demonstrated the feasibility of using these lipo-beads for indirect determination of biomarkers that are capable of inhibiting PLA_2. We envision that these lipo-beads will be beneficial for disease diagnosis and drug discovery studies. These lipo-beads are very reactive and can generate a detectable signal in less than 5 min. Further, they are stable for several weeks in storage at 5 °C. We have shown that even though PLA_2 can hydrolyze most phospholipid membranes, it is essential to have a correct combination of phospholipids for the enhanced reactivity. The current study found that lipo-beads comprised of phosphatidyl ethanolamine tagged with a bulky fluorescent molecule at the hydrophilic head and anionic lipid DMPG provide fast and sensitive detection of PLA_2-mediated lipid hydrolysis. Finally, we have demonstrated that ANXA-3, a known disease biomarker, can be detected as low as 10 nM using these lipo-beads. The sensitivity of these lipo-beads can be further improved by optimizing the lipid composition in the supported bilayer. Further, it is our aim to extend the current study to analyze ANXA-3 and other annexins in biological fluids. These sensitive lipo-beads will be useful as a simple, fast,

and inexpensive method for the detection of PLA_2 and its inhibitors at clinically relevant concentrations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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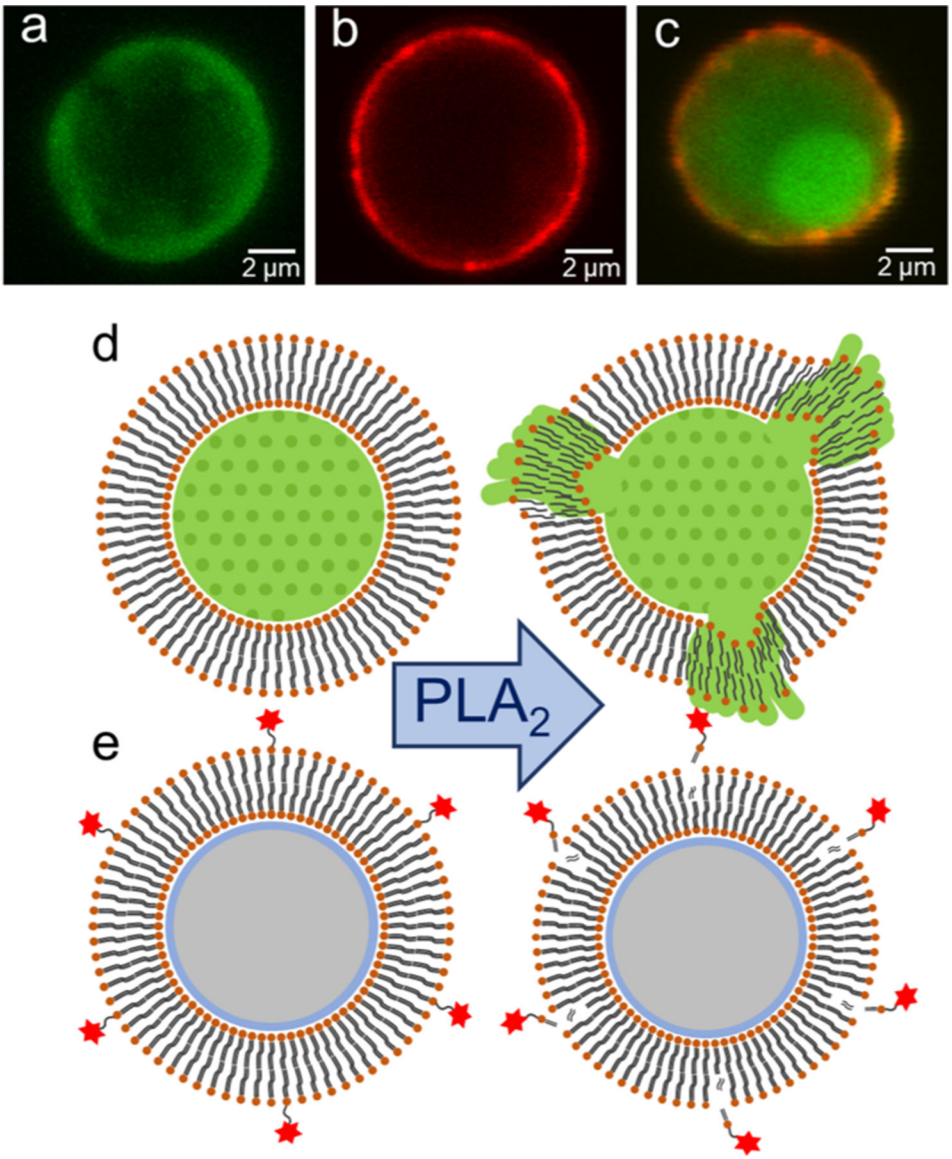

Figure 1.

Formation of fluorescent lipo-beads and the two approaches for detecting membrane–PLA_2 interaction. Lipo-beads (a) with a nonfluorescent lipid bilayer with encapsulated fluorescein, (b) with a lipid bilayer comprised of fluorescent lipid, RPE, and (c) with a lipid bilayer comprised of RPE and encapsulated fluorescein. The concept of release of (d) fluorescein dye and (e) fluorescent lipid because of the PLA_2-mediated membrane hydrolysis.

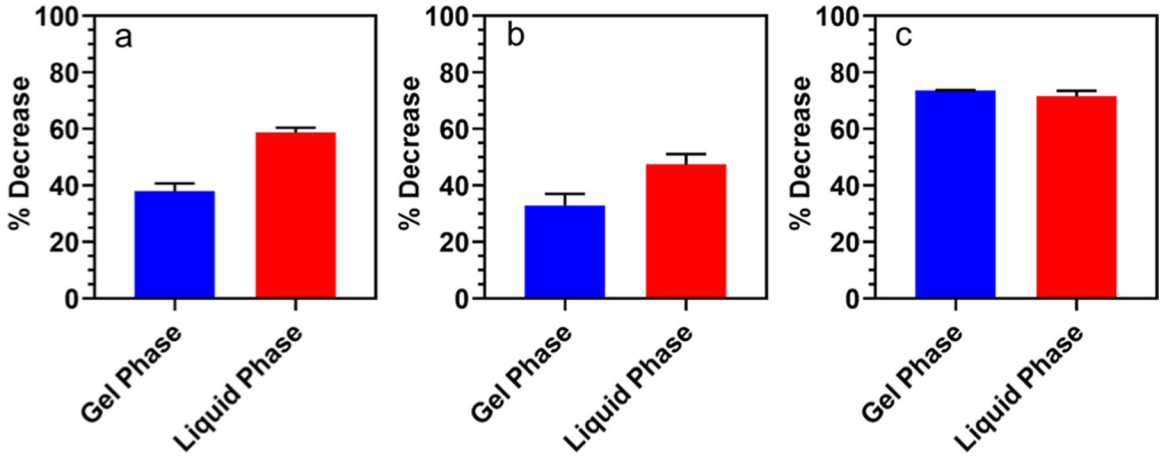

Figure 2.

PLA_2 activity as the percent decrease of fluorescence intensity for three different lipo-beads in gel and liquid phases of the primary lipid. (a) DMPC at 18 °C (gel phase) and 30 °C (liquid phase), (b) PC (15:0) at 30 °C (gel phase) and 41 °C (liquid phase), and (c) 100:1 DMPC/RPE at 18 °C (gel phase) and 30 °C (liquid phase).

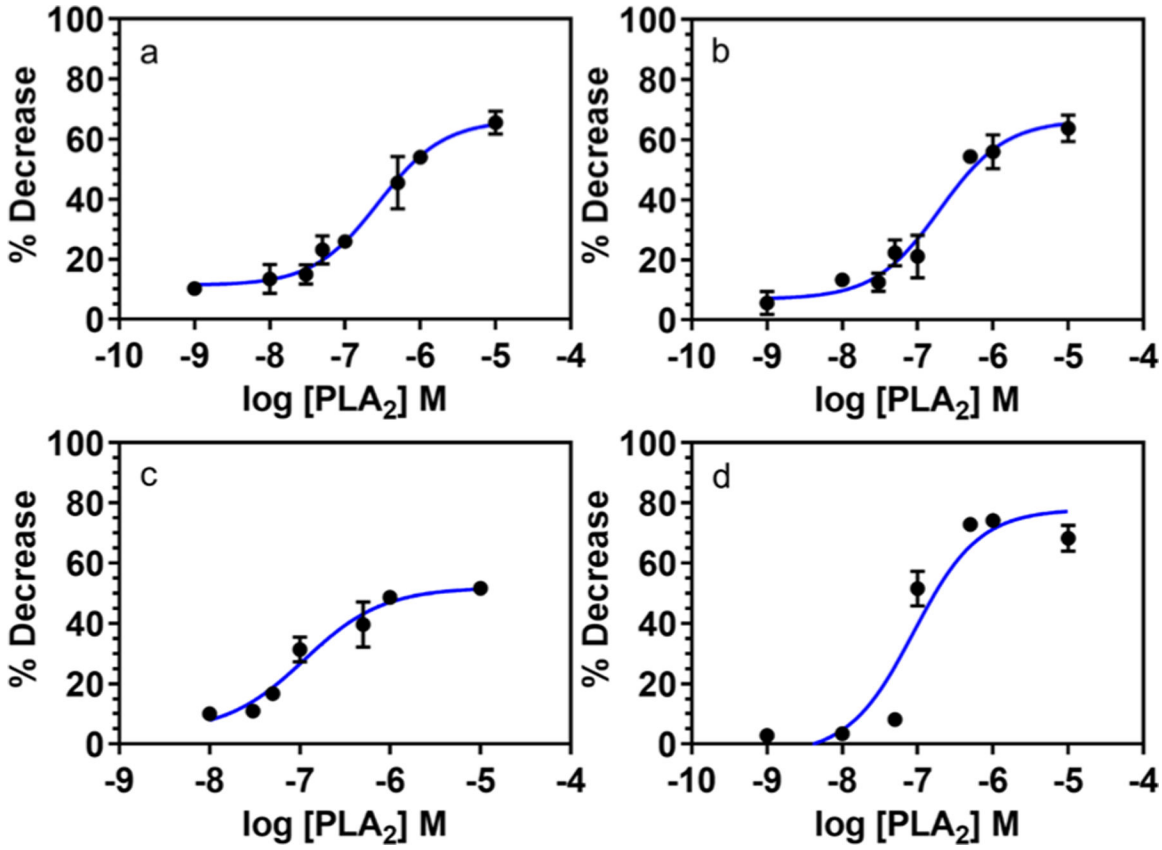

Figure 3.

Dose–response curves for PLA_2 interaction on different lipo-beads at the liquid phase of the primary constituent lipid. (a) DMPC, (b) PC, (c) 20:80 DMPG/DMPC, and (d) 100:1 DMPC/RPE. The PLA_2 activity was measured as the percent decrease of fluorescence intensity of lipo-beads after reacting with PLA_2 at different concentrations ranging from 1 nM to 10 μM, except for (c) where the range is from 10 nM to 10 μM.

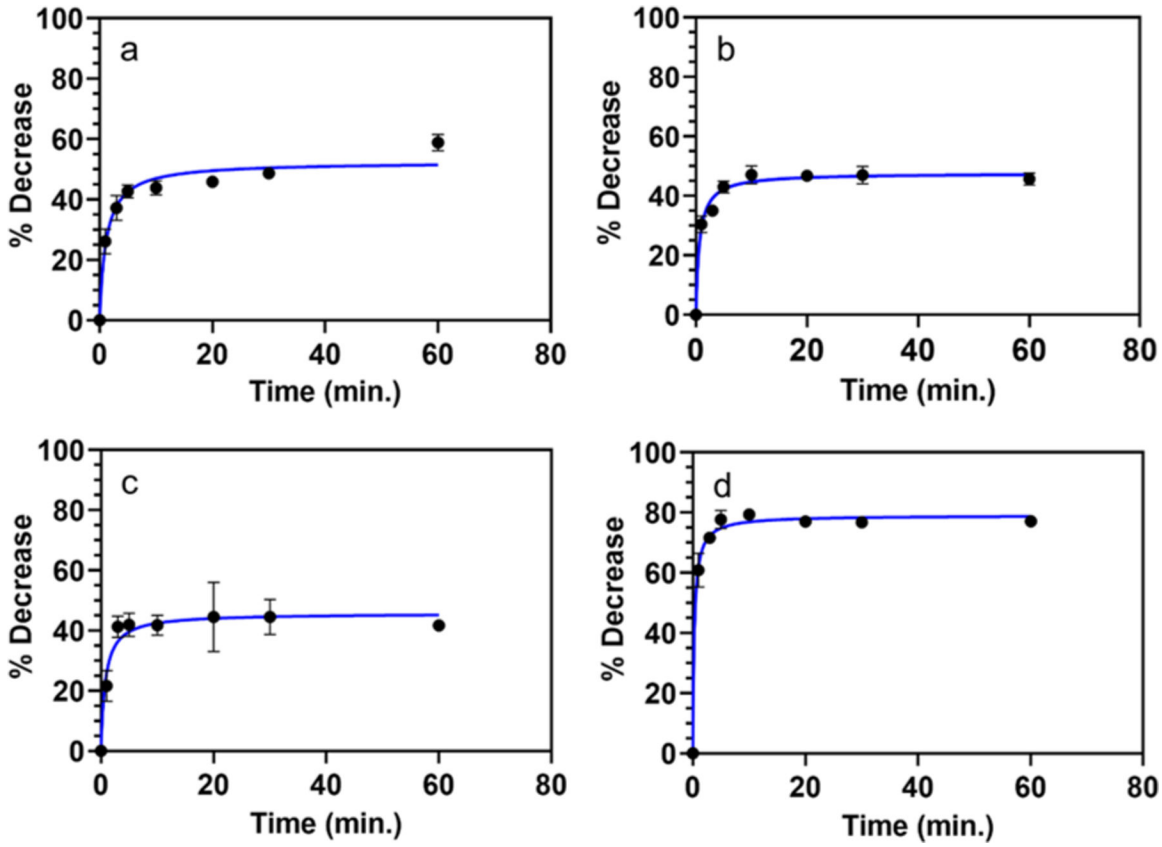

Figure 4.

PLA_2 kinetic graphs for different lipo-beads at the liquid phase of the primary constituent lipid. (a) DMPC, (b) PC, (c) 20:80 DMPG/DMPC, and (d) 100:1 DMPC/RPE. The kinetic studies were performed by measuring the decrease in the lipo-bead fluorescence over time at a PLA_2 concentration.

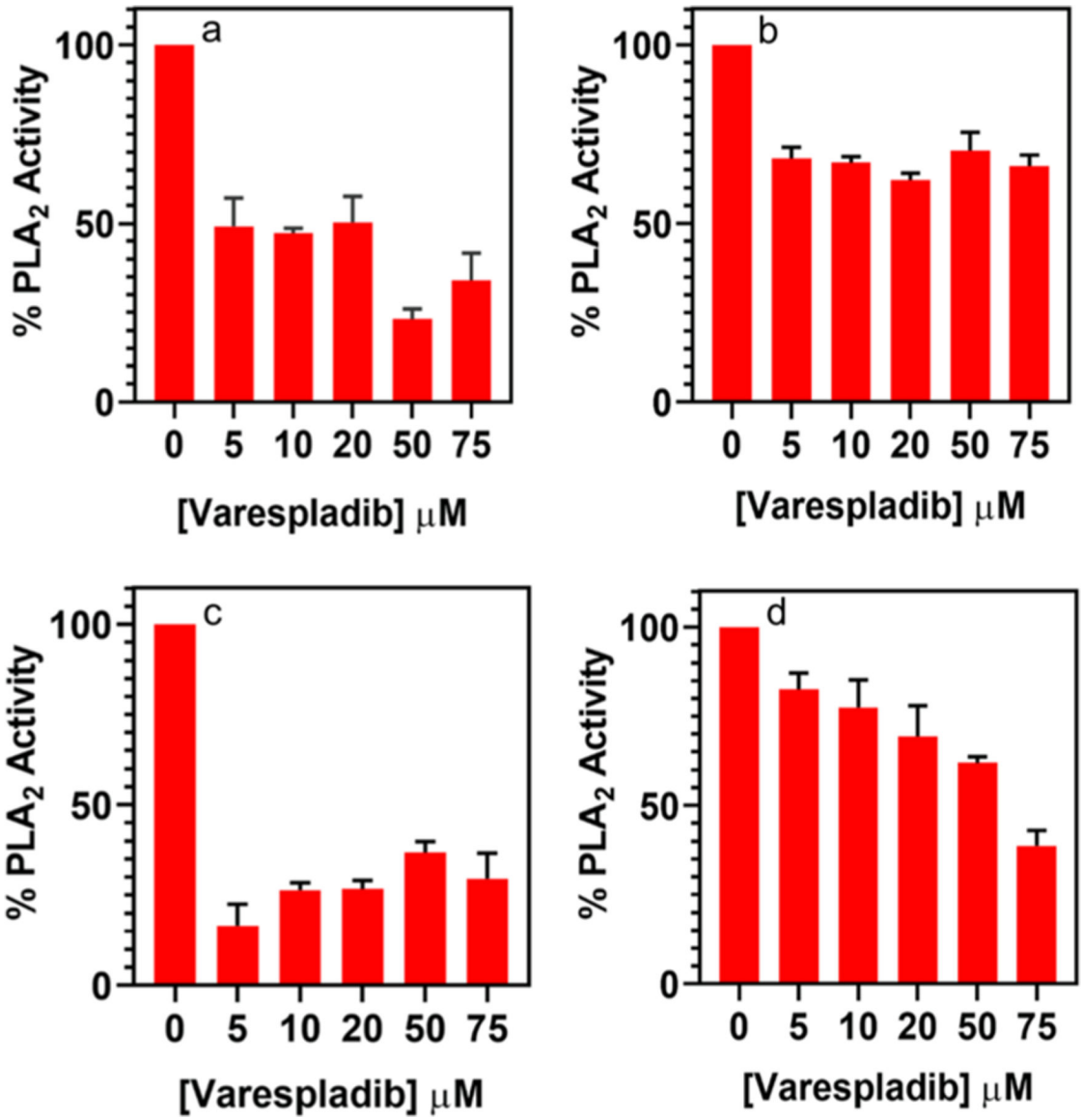

Figure 5.

% PLA_2 activity in the presence of varespladib for different lipo-beads at the liquid phase of the primary constituent lipid. (a) DMPC, (b) PC, (c) 20:80 DMPG/DMPC, and (d) 100:1 DMPC/RPE. The extent of PLA_2 inhibition was converted to the % PLA_2 activity using eqs 2 and 3.

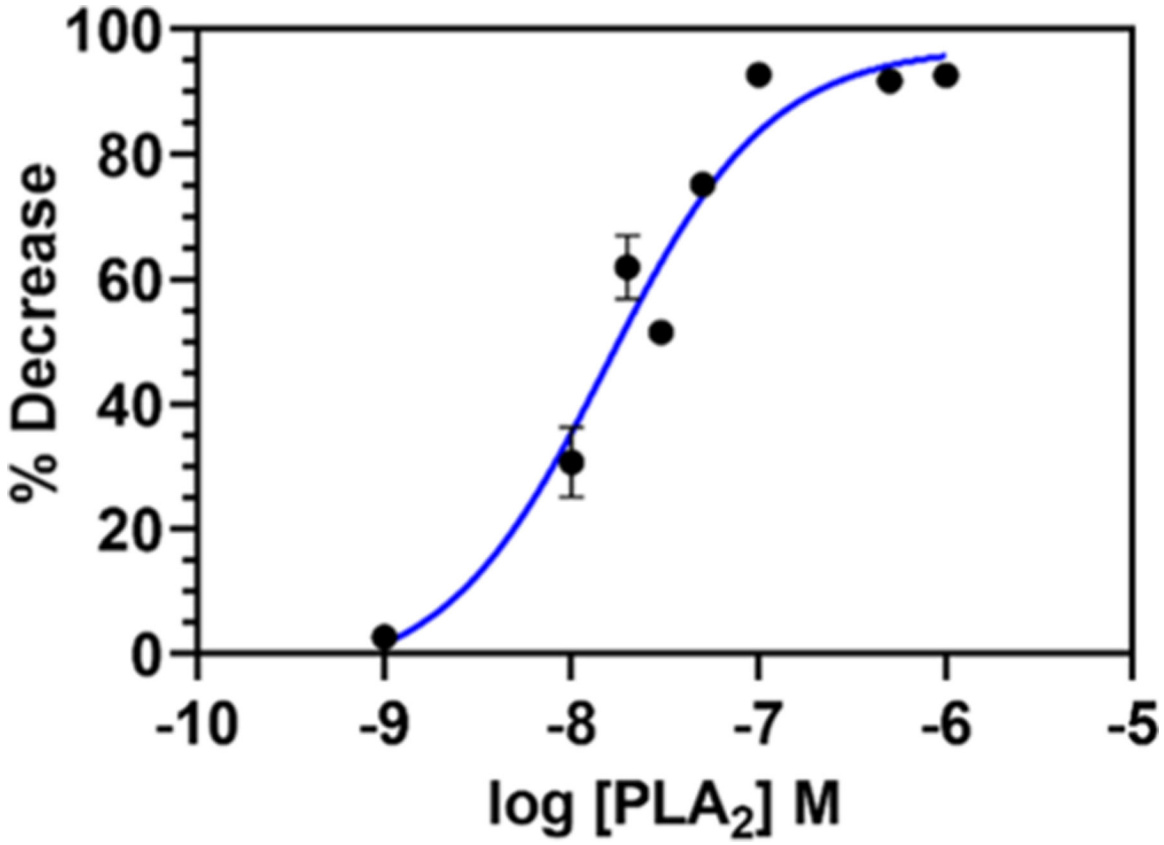

Figure 6.

Dose–response curve for PLA_2 hydrolysis of the lipid bilayer with 80:15:5 DMPC/DMPG/RPE lipo-beads at 30 °C. The enzyme activity at different concentrations of PLA_2, ranging from 1 nM to 1 μM, was calculated as the percent decrease of the fluorescence intensity of lipo-beads using eq 1.

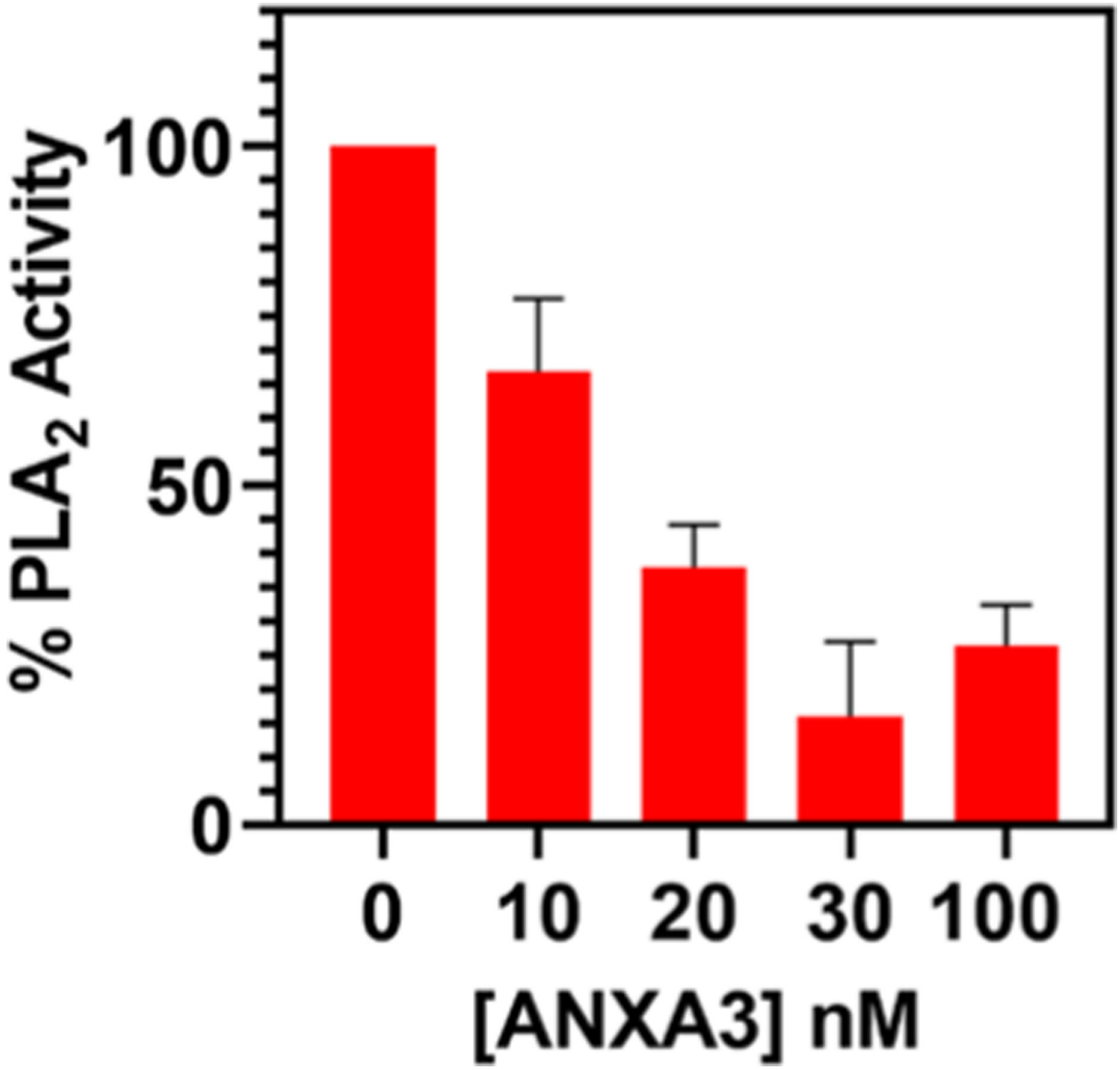

Figure 7.

Detection of ANXA-3 via the inhibition of PLA_2 activity using lipo-beads comprised of the 80:15:5 DMPC/DMPG/RPE phospholipid bilayer. The PLA_2 concentration was maintained at 20 nM while varying the inhibitor ANXA-3 concentration from 10 to 100 nM.