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ARTICLE *in* LANGMUIR · AUGUST 2006

Impact Factor: 4.46 · DOI: 10.1021/la060853q · Source: PubMed

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Fluorophore-Encapsulated Solid-Supported Bilayer Vesicles: A Method for Studying Membrane Permeation Processes

Thomas. L. Williams, Margarida M. L. M. Vareiro, and A. Toby A. Jenkins*

Department of Chemistry, University of Bath, Bath BA2 7AY, United Kingdom

Received March 30, 2006. In Final Form: June 1, 2006

This letter describes a new method for studying the interaction of the membrane-lysing enzyme phospholipase A_2 (PLA_2) with phospholipid bilayers by simultaneous measurements of enzyme binding and vesicle lysis using surface plasmon resonance (SPR) and permeabilization using surface plasmon field-enhanced fluorescence spectroscopy (SPFS). The PLA_2 inhibitor dimethyl-eicosadienoic acid was incorporated into the surface-bound vesicles and support bilayer in order to study its role in preventing PLA_2-mediated vesicle lysis. This methodology has a generic applicability for the study of a range of membrane-disrupting agents.

Introduction

A range of cell membrane mimics based on solid-supported lipid bilayers have been created by various groups to enable the study of membrane-bound proteins, peptides such as ion channels, pore-forming toxins, and membrane-lysing agents.[1] They have been used in a number of applications, including the development of new biosensors [2] and studies of ion channels and pumps.[3,4] Methods for creating biomimetic lipid bilayers, including black lipid membranes, glass-supported lipid bilayers, and thiol−lipid-supported bilayers, have been devised, with a review being provided in ref 1. A good biomimetic model lipid membrane should possess some or all of the properties of cellular lipid membranes: fluidity, both in and out of the lipid plane; space for the insertion of membrane proteins above and below the bilayer; and blocking to the transport of small molecules and ions except via ion channels/pumps or as a consequence of permeation processes.[5] The design of such systems is nontrivial. For example, although it is possible to create a simple blocking membrane using a thiol−lipid support on gold substrates, such membranes often have limited space between the bottom leaflet and the substrate for protein incorporation and may show limited fluidity.[6] A number of investigators have attempted to create lipid bilayers across small apertures on micromachined systems. These systems have many advantages but do not always lend themselves to studies of protein binding with the membrane and can be complex and difficult to fabricate.[7] One potential approach for improving the properties of biomimetic bilayers is to tether whole vesicles to a solid support. The advantage of such a system is that vesicles combine the relatively high electrical resistance of a lipid bilayer with the fluidity of a cellular biomembrane. However, the problem of measuring the interaction of proteins/peptides with the membrane then presents itself. A number of attached vesicle systems have been investigated in recent years, using a range of supports to attach vesicles to the surface, including coupling of vesicles to surface-adsorbed biotin-tagged bovine serum albumin via streptavidin[8] and confinement of single fluorophores in vesicles using a physisorbed lipid bilayer−streptavidin on glass.[9] Jung et al. investigated the binding of PLA_2 to surface-immobilized vesicles using SPR only. The vesicles were supported on the surface using biotin−streptavidin coupling. They determined the equilibrium dissociation constant of 6×10^{-7} mol dm^{-3}.[10] Recently, separate studies by Boxer and Höök have extended the tethered vesicles concept by attaching oligonucleotides to vesicles and measuring hybridization with complementary surface-immobilized oligonucleotides attached to a solid-supported lipid bilayer.[11,12]

The tethered vesicle system detailed here is a development of the work of Boukobza et al,[9] but with a more stable vesicle system using a thiol−lipid bound-support lipid bilayer. A schematic of the tethered vesicle system is shown in Figure 1. In our system, the gold film has two key functions: it stabilizes the support bilayer by covalently binding thiol lipids to the surface, and it has a surface plasmon wave that can be excited to resonance by incident light via the surface plasmon resonance effect. In the work detailed here, combined SPR/SPFS allows both binding of PLA_2 and membrane permeation via the diffusion of fluorophores through the membrane away from the evanescent SPR wave into bulk solution to be measured.

The final part of this study illustrates the effect of the PLA_2 inhibitor dimethyl-eicosadienoic acid, which prevents reduced lysis of vesicles. The action of the inhibitor allowed the quantification of the binding of PLA_2 with the membrane by fitting the change in SPR reflectivity versus titrated concentration to the Langmuir equation.

PLA_2 release in biological systems is a critical part of fatty acid biosynthesis but if introduced in excess can cause tissue damage (for example, in some snake bites) and inflammatory disease such as arthritis.[13] Compounds that inhibit PLA_2 action

* Corresponding author. E-mail: a.t.a.jenkins@bath.ac.uk.

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10.1021/la060853q CCC: $33.50
Published on Web 06/20/2006

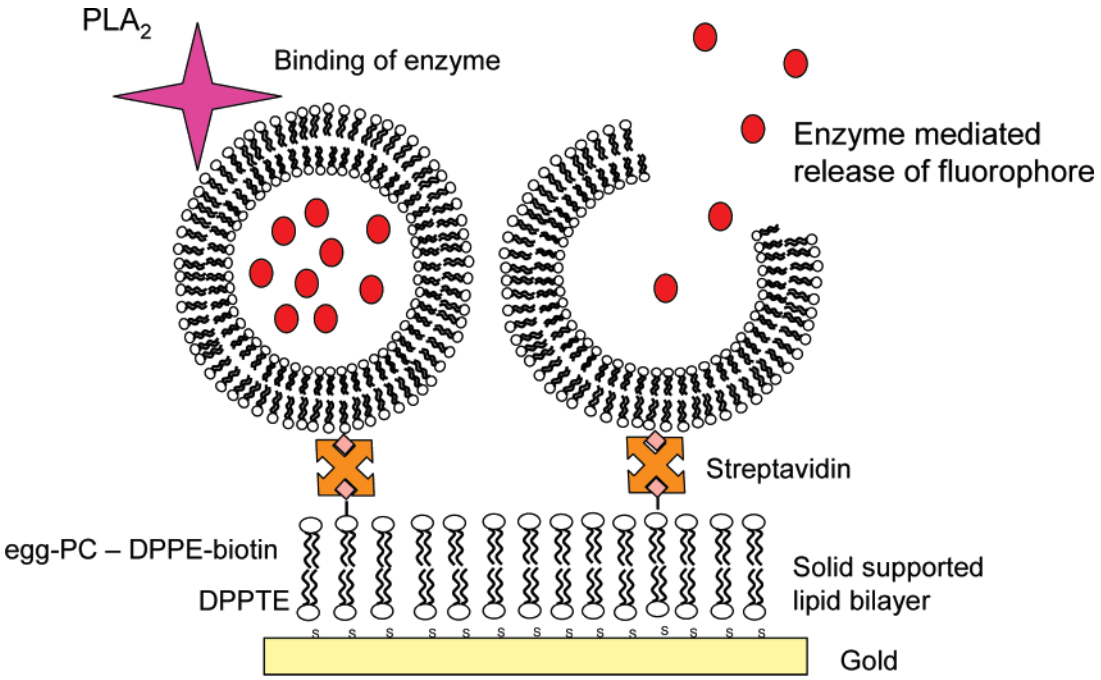

Figure 1. Schematic of the solid-supported bilayer vesicle illustrating the lysis action of PLA_2 on the membrane causing the release of the encapsulated fluorophore.

could therefore have important clinical applications. In this study, dimethyl-eicosadienoic acid was used both as a control measurement and as a proof of principle for potential applications of this technique for screening potential PLA_2 inhibitors.

Materials and Methods

Gold was thermally evaporated onto high refractive index LaSFN9 glass (Hellma Optik, Hellma, Germany) to a thickness of 50 nm in an Emitech K975X (Emitech U.K.) thermal evaporator. After evaporation, the slides were annealed at 450° C for 90 s and then cleaned under ozone for 10 min before being immersed in a 1 mmol dm^{-3} ethanolic solution of thiol−lipid, dipalmitoyl-phosphtidylthiolethanol (DPPTE) (Avanti, Alababster, AL), for 16 h to form a self-assembled monolayer (SAM). Large unilamellar vesicles (LUVs), with a diameter ca. 100 nm, consisting of egg-phosphatidylcholine (egg-PC) (Lipid Products, Nutfield Nurseries, U.K.) mixed with 1 mol % dipalmitoyl-phosphoethanolamine-*N*-biotinyl (DPPE-biotin) (Avanti), were formed by extrusion. A liposome suspension was prepared by mixing of lipids in chloroform, drying under vacuum for 12 h, and dispersing the dry lipids in 10 mmol dm^{-3} HEPES, 0.15 mol dm^{-3} NaCl, and 2 mmol dm^{-3} $CaCl_2$ buffer, pH 7.4 (all from Sigma, U.K) to give a final lipid concentration of 0.4 mg mL^{-1}. The vesicles were then fused onto the DPPTE surface to form a biotinylated lipid bilayer. Streptavidin (500 nmol dm^{-3}, (Sigma U.K.) was subsequently added to the bilayer. Finally, 100-nm-diameter vesicles consisting of 99 mol % 1,2-dimyristoyl-phosphotidyl-choline (DMPC) (Sigma U.K.) and 1 mol % DPPE-biotin encapsulating a free (nonconjugated) fluorescent probe, Alexa-fluor 647 carboxylic acid at a concentration of approximately 100 nmol dm^{-3} (Invitrogen, U.K.), were tethered to the solid-supported lipid bilayer using streptavidin (Figure 1). In the experiments used to follow the effect of the PLA_2 inhibitor, dimethyl-eicosadienoic acid, the inhibitor was added in chloroform to the dry lipid prior to hydration in buffer and extrusion. A final concentration of 0.1 mg mL^{-1} inhibitor was present in the vesicle suspension, which equates to approximately 10 mol % of the total.

Following the attachment of vesicles to the support bilayer−streptavidin matrix, the system was rinsed with fresh buffer to remove nonencapsulated fluorophores. SPR was used to follow the construction of the modified surface. Full details of the SPR/SPFS system

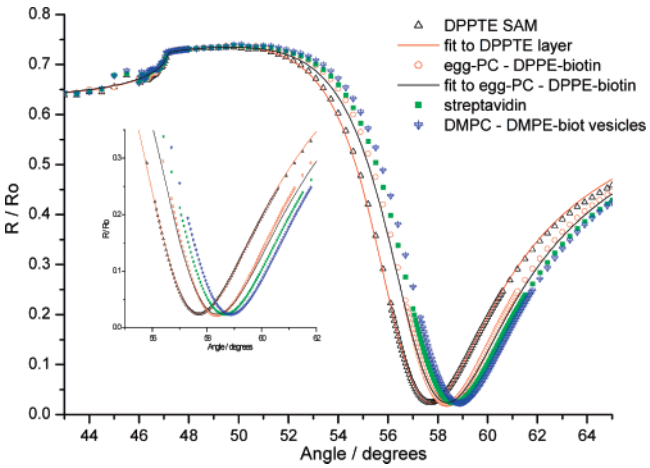

Figure 2. Angle-resolved SPR reflectivity curves showing the buildup of components of the tethered vesicle system and the Fresnel fit to the DPPTE SAM layer and egg−PC−DPPE−biotin adlayers. The angular minima are shown enlarged in the inset.

are provided in refs 14 and 15. SPR measures the angle of resonance when light is coupled into a thin metal film, normally via a prism. The angle at which surface plasmon resonance takes place, observed as a reflection minimum, is highly sensitive to changes in the dielectric constant. The adsorption or loss of a surface film on a metal surface within the evanescent field can thus be measured in situ and in real time. The home-built instrument used in these experiments followed the changes in reflection intensity at an angle of around 1.5° lower than the resonance angle. Hence, changes in reflected light intensity at the fixed angle allowed real-time measurement of enzyme binding and vesicle lysis.

Results and Discussion

Figure 2 shows the angle-resolved SPR measurements as the support matrix is built up and vesicles are immobilized. The final addition of fluorophore encapsulated in biotinylated DMPC

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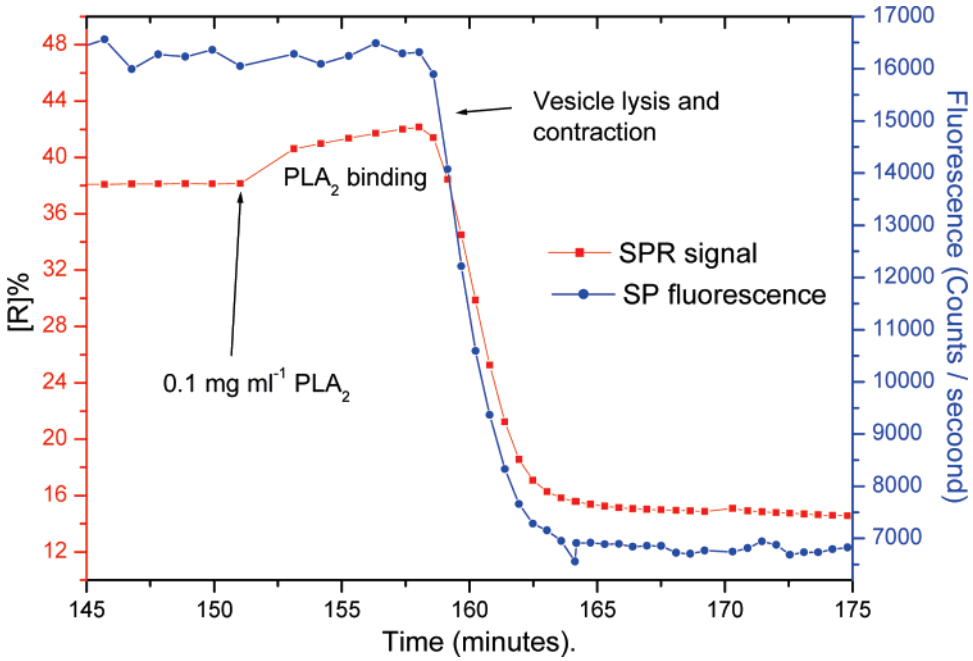

Figure 3. SPR measurements (left *y* axis) and SPFS measurements (right *y* axis) showing the initial attachment of PLA_2 followed by the subsequent lysis of vesicles.

LUVs was measured under noncontinuous illumination to prevent photobleaching of the fluorophores by the laser.

The egg−PC−DPPE−biotin layer SPR resonance curve was fitted to Fresnel equations using software from the Knoll group (Max-Planck-Institute for Polymer Research, Mainz, Germany). A thickness of 34 ± 3 Å was obtained, assuming a lipid film refractive index of 1.49.[16] The degree of streptavidin and DMPC−DPPE−biotin vesicle binding was estimated by noting that for biological adlayers a shift in the plasmon resonance angle of 0.10° equates approximately to a mass change of 0.5 ng mm^{-2} on the surface.[14] A surface density of 1.5×10^{10} streptavidin mm^{-2} was calculated from an SPR minima shift of 0.3° (Figure 2) and 7.7×10^4 DMPC vesicles mm^{-2} (minimum shift of 0.2°). This suggests a relatively low density of vesicles on the surface (a complete vesicle monolayer would have a density of 1×10^8 vesicles mm^{-2}).[17] Following vesicle immobilization, SPR and SPFS measurements were carried out in order to study both the stability of the adsorbed vesicle and the effect of introduction of PLA_2 onto the tethered vesicles. This relatively low density of adsorbed vesicles, given the amount of adsorbed streptavidin, suggests that in the initial binding phase many vesicle are not stable and may undergo deformation or even rupture perhaps because of non-DPPE−biotin-mediated interaction with the surface-bound streptavidin. However, those vesicle that do successfully bind appear to retain their fluorescence (and hence presumably their structure) over 30 min or more, prior to PLA_2-induced lysis.

This secretory PLA_2 (Worthington Biochemical, U.K.) hydrolyses the ester linkage of phospholipids, causing membrane lysis.[18] The SPR instrument was modified for combined fluorescence measurements by the addition of a photomultiplier tube (PMT) (Hamamatsu, Japan) to detect light emitted perpendicular to the plane of the gold film, placed at 90° to the photodiode.[14] Prior to the addition of PLA_2, the baseline fluorescence of the adsorbed vesicles was measured. After the stability of the tethered vesicles had been established, in terms of a reasonable constant fluorescence and SPR reflectivity for 30 min, PLA_2 was added at a concentration of 0.1 mg mL^{-1} in vesicle buffer to the tethered vesicles. The SPR and SPFS responses are shown in Figure 3.

Figure 3 shows the initial attachment of PLA_2 to the tethered vesicles and support surface, observed as an increase in reflectivity, followed by a lysis event around 10 min later. The lysis is observed both as a decrease in reflectivity (measured by SPR) and a reduction in fluorescence due to the release of encapsulated fluorophore. After lysis and surface rinsing, a further angle-resolved SPR measurement was made. A decrease of 0.3° in the resonance angle was measured, corresponding to a mass loss of 1.5 ng mm^{-2}. This is greater than the mass of adsorbed vesicles (1 ng mm^{-2}) and suggests that the support bilayer is being lysed in addition to the adsorbed vesicles, possibly resulting in the loss of biotinylated lipid−streptavidin units from the surface. The question of whether the vesicles are actually being lysed, or simply being lost as intact vesicles was investigated in a separate experiment. A biotin−streptavidin support layer (resistant to PLA_2-catalyzed lysis), similar to that used by Jung et al., was used to support the vesicle−fluorophore system. The addition of PLA_2 caused a decrease in fluorescence but only a small loss of mass density from the surface (0.1°) decrease in the resonance angle, showing that attached vesicles were being directly lysed.[10] We conclude that PLA_2 lysed both the support bilayer and the attached vesicles.

The measurements appear to correlate roughly with a study by Sanchez et al, who used two-photon fluorescence measurements to study vesicle lysis by PLA_2.[18] This study, which imaged vesicles in real time, observed a delay of ca. 8 min between enzyme attachment and vesicle lysis. SPR measurements of the effect of PLA_2 on the bilayer support−streptavidin matrix, without attached vesicles, were also made to investigate the effect of PLA_2 on the support bilayer. A small decrease in reflectivity after initial PLA_2 adsorption was observed, but the effect was an order of magnitude smaller than when vesicles were attached.

The final part of this study investigated the effect of the proposed PLA_2 inhibitor, dimethyl-eicosadienoic acid (Sigma,

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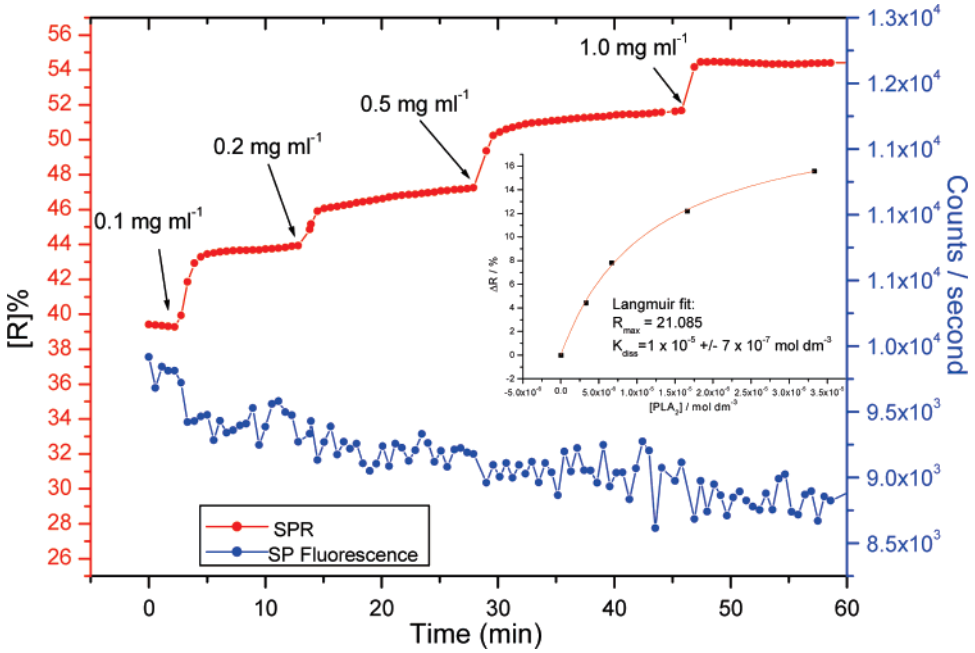

Figure 4. SPR and SPFS measurements of the titration of PLA_2 at concentrations between 0.1 mg mL^{-1} (3.33×10^{-6} mol dm^{-3}) and 1 mg mL^{-1} (3.33×10^{-5} mol dm^{-3}) onto tethered vesicles and the support bilayer surface containing the PLA_2 inhibitor dimethyl-eischaodecanoic acid. The Langmuir adsorption isotherm, plotting the change in SPR reflectivity vs PLA_2 concentration, is shown in the inset.

U.K.). Dimethyl eicosadienoic is a known PLA_2 inhibitor. To be effective, the inhibitor had to be introduced into the vesicle bilayer during preparation. Simply having the inhibitor in the buffer following vesicle immobilization was insufficient to prevent lysis probably because of its low solubility in aqueous buffer. Figure 4 shows the titration of PLA_2 onto the surface. The contrast with Figure 3 is clear; it can be seen that PLA_2 binds to the surface but does not induce any significant lysis of the adsorbed vesicles. The change in SPR reflectivity following titration and binding of PLA_2 was plotted versus the concentration of added PLA_2 and fitted to the Langmuir equation ($\Delta R = R_{max}\cdot[PLA_2]/([PLA_2] + K_D)$) in order to obtain a value for K_D, the dissociation constant for PLA_2 with the surface. A K_D of 1×10^{-5} mol dm^{-3} was calculated, which is somewhat higher than that determined by Jung, although they used a different methodology to determine their value and studied a different lipid system.[10] The inhibitory action of dimethyl-eicosadienoic acid can be seen. The eiscosadienoic acid does not effect the binding of the enzyme, hence it can be concluded that its PLA_2 inhibitory action must come from interfering with the actual lysis of the glycol unit in the lipid rather than preventing the attachment of the enzyme to the bilayer. This method of inhibition is in contrast to the well-studied PLA_2 inhibitor, manoalide. This natural marine compound, from the sea sponge *Luffariella variabilis*, irreversibly binds to PLA_2, causing its permanent inactivation.[19]

Conclusions

In this letter, the utility of using combined SPR/SPFS to study both the attachment of PLA_2 to surface-bound lipid vesicles and the subsequent lysis of the vesicle has been demonstrated. The PLA_2 system was chosen as a reasonably well understood model system to test and illustrate the potential of using this methodology to study membrane permeation. The action of the inhibitor, dimethyl-eicosadienoic acid, was studied, and provided it was incorporated into the vesicle, it exhibited an effective inhibitory action to PLA_2-mediated lysis. This result concurs with whole cell patch-clamp measurements made on the PLA_2 inhibitory action of dimethyl-eicosadienoic acid on gastric myocytes by Yang et al.[20] This study has attempted to illustrate the utility of surface-adsorbed vesicles as lipid bilayer mimics, with combined SPR/SPFS providing a synergistic methodology for studying toxin binding and membrane permeation events, and illustrates a potential screen for PLA_2-inhibiting drugs.

Acknowledgment. We thank the Natural Environment Research Council, the University of Bath, and The Royal Society for funding this work.

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