Quantification of Tight Binding to Surface-Immobilized Phospholipid Vesicles Using Surface Plasmon Resonance: Binding Constant of Phospholipase A_2

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Abstract: A new and sensitive method for quantifying high affinity binding of proteins (and potentially other ligands) to the surfaces of phospholipid bilayered vesicles is demonstrated. A planar, high-density streptavidin monolayer is first immobilized onto a surface plasmon resonance (SPR) sensor slide containing a mixed monolayer of biotin-terminated and hydroxyl-terminated poly(ethylene oxide) alkylthiolates tethered to the gold surface. Phospholipid vesicles containing 0.3% biotin-functionalized headgroups are then bound to this streptavidin monolayer to make a high-density, planar layer of *intact* vesicles. The absolute amount of protein binding to the vesicle layer can be monitored by SPR in real time to extract equilibrium and kinetic information under flowing solutions. Coupled with a catalytic assay to monitor the solution-phase concentration of enzyme, the value of the dissociation equilibrium constant for the complex of cobra venom phospholipase A_2 (PLA_2) bound to phosphatidylcholine vesicles was determined to be $6 \pm 2 \times 10^{-7}$ M. In principle, the method could be extended to determine dissociation constants as low as 10^{-10} M. The vesicles bind a maximum of 1 PLA_2 per 74 ± 16 outer leaflet phospholipids. The method provides a practical solution to a number of problems encountered with previous methods to quantify tight interfacial binding of proteins to vesicles. Spectral probes attached to enzymes or vesicles, which may perturb interfacial binding, are not required. Furthermore, surface-supported vesicles offer improved sensitivity over planar bilayers and are physiologically more relevant.

Introduction

Numerous species bind to the phospholipid interfaces of biological membranes and initiate a variety of physiological effects. Therefore, it is of great interest to have a sensitive method for measuring the equilibrium binding constants and kinetics for such binding. Here, we present a new approach for this and demonstrate it by measuring the equilibrium constant for the binding of a phospholipase to a phospholipid membrane.

Enzymes such as phospholipases that operate on highly water-insoluble components of biological membranes must be bound to the phospholipid interface to gain access to their substrates. Many of these enzymes are peripheral membrane proteins that can exist in both membrane-bound and water-soluble states. Such is the case for a large family of enzymes called phospholipase A_2 that hydrolyze the *sn*-2 ester of glycero-phospholipids.[1] The affinity of these enzymes for vesicles depends dramatically on the molecular composition of the vesicle bilayer.[2] The role of specific amino acid residues on the membrane binding surface of 14-kDa secreted phospholipases A_2 that control interfacial binding selectivity is being delineated by site-directed mutagenesis studies.[3−6] Such studies are important because they offer clues about the function of secreted phospholipases A_2. For example, the high affinity of human group IIA phospholipase A_2 for phosphatidylglycerol vesicles is consistent with the proposed role of this enzyme as an anti-bacterial agent since bacterial membranes, but not mammalian cell membranes, are highly enriched in phosphatidylglycerol.[7]

It is thus important to develop an efficient, accurate, and generally applicable method to quantify the binding of peripheral membrane proteins to phospholipid vesicles. Although several methods have been developed to obtain equilibrium dissociation constants for enzymes bound to phospholipid interfaces, they have limitations especially when the enzyme engages in high affinity interaction with vesicles. Resonance energy transfer has been used to detect the close proximity of an enzymic tryptophan

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with a fluorescent membrane probe.[2,8] This method suffers from the fact that the enzyme must contain a fluorescent residue such as tryptophan. Introduction of a tryptophan residue on the membrane binding surface of the protein can greatly influence interfacial binding.[5,9] Furthermore, the energy transfer efficiency depends dramatically on the lipid composition of the vesicle,[5] so that low fluorescent signals do not prove that there is little interfacial bonding. Many methods for measuring protein−vesicle dissociation constants involve separating vesicles from bulk solution by centrifugation followed by analysis of the amount of protein in the supernatant and pellet fractions. Vesicles composed only of lipids do not sediment well unless they are loaded with sucrose,[10] and sucrose-loaded phosphatidylcholine vesicles resist sedimentation probably due to sucrose leakage.[9] In addition, recovery of protein from the centrifuge tube is often low unless detergent is present,[10] and this problem is especially difficult when low concentrations of components must be used to measure dissociation constants in the submicromolar range. Vesicles containing a small amount of biotinylated phospholipid can be precipitated by cross-linking with solution-phase streptavidin,[11] but efficient precipitation occurs only in the presence of high micromolar to millimolar concentrations of lipids, so that dissociation constants below $\sim 10^{-6}$ M cannot be measured.

In this study, we have developed a simple method, based on intact vesicles immobilized on a surface plasmon resonance (SPR) sensor, to obtain the dissociation equilibrium constant, K_d, for the reaction Enzyme·Vesicle ⇌ Enzyme + Vesicle. It is applicable to lipids or lipid mixtures that form bilayered vesicles and to proteins that undergo reversible association to their surface. We have used SPR to directly measure the maximal binding and equilibrium fractional surface coverage as the concentration of enzyme in a recirculating solution is stepped up and down in a manner similar to that of the stepwise surface titration method previously described for the study of protein−protein and protein−DNA interactions.[12,13] The surface coverage is plotted versus concentration as an adsorption isotherm to determine the equilibrium dissociation constant, K_d, for the enzyme−vesicle interaction. The sensitivity of this method is limited only by the sensitivity of the assay for the aqueous enzyme, and not by the sensitivity of the SPR system. Because of this, K_d values $<10^{-10}$ M can be measured, in principle, which previously has not been possible. Furthermore, nonspecific binding of protein to non-vesicle surfaces in the flow system is of no consequence since such binding does not contribute to the SPR signal.

SPR has been used to determine the K_d of several toxins and enzymes on lipid surfaces.[14−17] However, most of those studies relied on the determination of the association and dissociation rate constants by fitting adsorption and desorption curves, which is not as accurate as measuring true equilibrium binding values and correlating the actual fractional coverage with the actual solution concentration. Also, the ratio of these rate constants gives the equilibrium dissociation constant only when a simple kinetic model applies: Enzyme·Vesicle ⇌ Enzyme + Vesicle, with only a single vesicle-bound enzyme species. Those fits often do not properly consider depletion of the solution near the surface due to adsorption and slow diffusion, which can lead to an inaccurate rate constant. A number of those studies were performed on biotinylated lipid vesicles immobilized using SA or avidin bound within the dextran coating of a BIACore SPR Chip,[14,17] which might introduce further complications to the kinetic analysis due to other mass transfer issues specific to these hydrogel coatings.[18,19] In addition, those studies did not consider the significant decrease in solution-phase protein concentration due to nonspecific adsorption to the system (i.e., walls of tubing, flow cell, etc.), unlike our method which takes any loss of enzyme into account by measuring the actual free enzyme concentration. Finally, none of those studies demonstrated that the probe vesicles were bound *intact* into the gel coatings. Here, we use a *planar* array of vesicles, which are proven here to be intact and are not complicated by any surrounding gel.

An alternative method which has been commonly used for immobilization of phospholipids results in hybrid bilayer membranes (HBMs). Many HBMs have been previously described and well characterized.[20−25] Phospholipid vesicles or micelles adsorb, rupture, and assemble onto a substrate with an alkylthiol monolayer, creating a single, planar monolayer of phospholipids with their hydrocarbon tails oriented toward the chains of the alkylthiol monolayer and their polar heads oriented up toward the buffer as shown in Figure 1a. Although these surfaces are well-defined,[20−24] there may be problems when using them as model membrane systems, primarily due to the decreased fluidity of the phospholipid monolayer caused by its constraint by the underlying solid-like alkyl monolayer. This and other issues with HBMs will be discussed in more detail below.

The more physiological alternative we use here is shown schematically in Figure 1b. The foundation of the substrate is a gold surface functionalized with a mixed monolayer of biotin-terminated alkylthiols (BAT) and short-chain poly(ethylene oxide)-terminated alkythiols (PEO). The PEO alkylthiolate, which has four ethylene oxide units and terminates in an OH group as shown in Figure 1b, resists nonspecific adsorption of solution components such as proteins.[26] The BAT immobilizes a streptavidin (SA) linker monolayer onto the surface. These mixed monolayers immobilize SA onto a Au surface with high coverage, specificity, and activity.[27−31]

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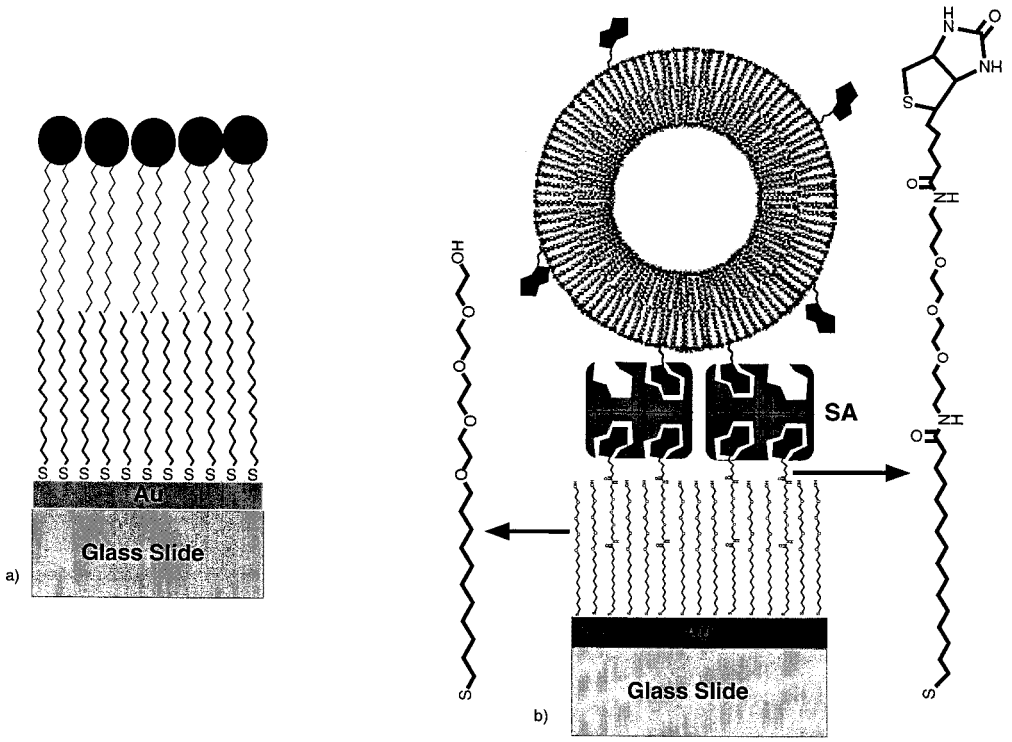

Figure 1. (a). Schematic of a hybrid bilayer membrane (HBM) (not to scale). The top layer represents the lipids with their polar heads (filled circles), while the lower layer represents the alkylthiols bound to the Au substrate. No attempt is made here or in Figure 1b to show the tilting of the alkyl chains from the surface normal (typically ~30°). (b). Schematic of the intact immobilized biotinylated vesicle monolayer (not to scale). The biotin in a mixed $DO_{et}PC$-biotinylated vesicle is bound to free binding sites on streptavidin which is itself bound to biotin in a mixed BAT/PEO monolayer adsorbed on the Au substrate.

The amount of SA that binds to these biotin-containing monolayers is dependent on the mole fraction of biotin in the film.[27,28] A monolayer with ~30% BAT (which results if the ethanol solution composition has a BAT/PEO ratio of 1/9) has been shown to lead to an optimal density of approximately 230 ng of SA/cm^2 (or 3800 Å^2/SA),[28,29] which is ~80% of the density of a two-dimensional *C*(2,2,2) crystalline SA monolayer.[32]

The streptavidin−biotin (SA−biotin) couple was chosen because of its high binding constant, ~10^{13} M^{-1},[33] and the fact that each streptavidin has four equivalent sites for biotin and is known to bind to these biotin-containing monolayers in such a way as to expose two of its sites away from the surface as shown in Figure 1b. For these reasons and because of the easy availability of biotinylated molecules, SA-functionalized surfaces have been utilized as linker monolayers for attachment of antibodies, DNA, proteins, and their binding partners to gold surfaces.[27,29−31] Since it has been demonstrated that phospholipid vesicles can be bound intact to aqueous-phase SA using the SA−biotin linkage without aggregation, stress, strain, fusion, or lysing and with their fundamental properties maintained,[14,34−36] it is expected that vesicles would make good physiological models if bound to a solid via SA. Here, we have used this SA linker monolayer to attach *intact* phospholipid vesicles containing a small amount of phospholipid with a biotinylated headgroup to gold surfaces.

These surface-bound vesicles form the surface sites with which the K_d values of membrane-binding proteins have been determined using SPR. Because these immobilized vesicles are quite fluid and can swell in response to protein or ligand binding, they offer a more physiologically relevant model of membranes than systems based on planar monolayers or bilayers attached directly to solid surfaces, as we show below.

In this paper, this technique for measuring K_d is demonstrated for phospholipase A_2 from cobra venom (PLA_2) binding to zwitterionic phosphatidylcholine vesicles. Since PLA_2 selectively hydrolyzes the phosphatidylcholine *sn*-2 ester linkage when bound to vesicles, we have used vesicles of 1,2-dioleyl-*sn*-glycero-3-phosphocholine ($DO_{et}PC$), where the fatty acyl ester linkages have been replaced with ether linkages to render them unreactive.

Materials and Methods

Materials. 1,2-Dioleyl-*sn*-glycero-3-phosphocholine ($DO_{et}PC$) was prepared as described.[37] 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine with a biotin$CONH(CH_2)_6CO$ group amide linked to the ethanolamine amino group (biotin-DOPE) was purchased from Avanti Polar Lipids Inc. PLA_2 from *Naja naja* venom was purchased from Sigma (cat. no. P6139). Recombinant wild-type core streptavidin was expressed and purified as previously described[38] and kindly provided by Professor P. S. Stayton (University of Washington). Biotinylated alkylthiol and the short-chain poly(ethylene oxide) alkylthiol were synthesized as described[39] and provided by Professors T. Sasaki and

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B. D. Ratner, respectively (University of Washington). Ellman's reagent (5,5′-dithio-bis-(2-nitrobenzoic acid)) was from Pierce. 1,2-Dithiodecanoyl-1,2-dideoxyl-*sn*-glycero-3-phosphocholine (dithio-$DC_{10}PM$) was a gift from H. S. Hendrickson (University of Washington). 1,2-Ditetradecyl-*sn*-glycero-3-phosphomethanol (DTPM) was prepared as described.[37] Bovine serum albumin (BSA) was from Sigma.

Substrate Preparation. Glass microscope slides (Fisher) were coated with 20 Å of chromium and then 500 Å of Au (Johnson Matthey, 99.9999% purity) by electron beam evaporation, as previously described.[28] The gold was functionalized with alkylthiols as previously described with a solution mixture of 10 mol % BAT and 90 mol % PEO in ethanol.[28]

Surface Plasmon Resonance (SPR) Measurements. The instrument used here is based on a planar prism (Kretschmann) configuration and has been described and characterized in more detail elsewhere.[40] White light is directed through a prism at a fixed angle (78° from normal) onto a gold-coated substrate and adsorption onto the gold sensor surface is observed by monitoring the shift of the SPR minimum. This shift is converted into a surface coverage (ng/cm^2 or molecules/cm^2) using a formalism for quantitative SPR described elsewhere.[40] Essentially, the volume of adsorbate per unit area was estimated from the SPR shift using the known refractive index of the solvent and the adsorbate and a calibration based on the sensor response to bulk refractive index changes. This volume of adsorbate was converted to mass using the specific volume of the molecule in the same solvent. The index of refraction for the proteins used was 1.57, with a specific volume of 0.77 mL/g.[40,41] For the $DO_{et}PC$, the index of refraction was estimated to be 1.49[24,42−44] with a specific volume of 0.92 mL/g lipids.[45] For the intact vesicles and for protein adsorption on them, the depth-related attenuation of the surface plasmon intensity (∼30%) was properly considered in applying the formalism previously described.[40]

The functionalized substrate is enclosed in an 80 μL flow cell attached to either a stop flow system or a continuous flow system. The stop flow system is driven by a syringe pump (Cavro) with two injection valves (VICI) to switch between buffer and sample loops and has been described previously.[28] After a steady baseline is observed in buffer, adsorption is initiated by injection of sample from the sample loop to the flow cell with a time constant of ∼1 s. After adsorption is complete, the surface is rinsed to remove physisorbed sample and to correct for any baseline shift due to a refractive index difference between the sample solution and the buffer.

The continuous flow system experimental procedure is similar to that described above. A Milligat pump (GlobalFIA), a positive displacement piston array pump, provides continuous and nearly pulseless flow (at a rate of 160 μL/min). As the solution exits the flow cell, the liquid can be directed out to waste or back to a reservoir for recirculation. The enzyme concentration is adjusted by addition of buffer or an aliquot of a more concentrated enzyme solution to the reservoir. Because of the nature of the recirculating flow, it takes a few minutes for such an addition to be fully mixed throughout the total system volume. In control experiments, we followed the time dependence of the concentration as a reservoir containing 200 μL of a higher refractive index solution was circulated into the system. The expected damped oscillations observed showed that complete mixing is achieved in less than 10 min. To ensure complete mixing during experiments, the solution in the reservoir was stirred and recirculated for at least 10 min after each addition of buffer or enzyme solution. For these experiments, the solutions were recirculated to ensure that binding of the enzyme to all surfaces had reached steady state. Thereafter, analysis of a sample of solution from the reservoir should provide the concentration of free enzyme at equilibrium.

Biotinylated Vesicles. Phospholipid vesicles were biotinylated by including during their preparation a small amount of phospholipid with biotin covalently attached to the headgroup as previously demonstrated.[11,34] Biotin-DOPE (in chloroform) was added to $DO_{et}PC$ (in chloroform) to yield 0.3 mol % of biotin-DOPE. The resulting solution was dried with N_2 and placed in a desiccator for ∼30 min in vacuo to remove traces of chloroform. Buffer (50 mM Tris-HCl, 50 mM KCl, 1 mM $CaCl_2$, pH 7.6) was added to the dried lipids to yield ∼1.0−0.1 mM of total lipid. After being subjected to six freeze−thaw/vortex cycles using a dry ice/acetone mixture and a 30 °C water bath, the lipid solution was passed through two polycarbonate membranes (100 nm pore, Avestin Industries, Ottawa, Ontario) at least 19 times using the Lipofast extruder (Avestin Industries), to produce the desired solution containing unilamellar vesicles with 0.3% biotin-DOPE lipids. As shown previously, the procedure produces vesicles of 80 ± 25 nm diameter.[46] This diameter corresponds to 4.7×10^4 lipids (total) per vesicle, or 2.9×10^4 outer lipid headgroups per vesicle, assuming that the packing density of lipids is the same as in Langmuir−Blodgett films (∼0.7 nm^2 per lipid[47] at the reported surface pressure of the vesicle of ∼30 dynes/cm[48]).

Preparation of Surface-Immobilized Vesicles. Samples with PEO/BAT monolayers were dosed with ∼0.05 mg/mL of SA in 50 mM Tris buffer as previously described[28] until the adsorption appeared to reach a saturation coverage of SA as observed with SPR. After being rinsed with pure buffer to remove any physisorbed SA, the above solution containing biotinylated vesicles was diluted ∼50% with buffer, introduced in the same buffer, and allowed to bind to the immobilized SA until equilibrated.

Measuring K_d for PLA_2 Binding. A solution of PLA_2 (in 50 mM Tris buffer) was circulated over the immobilized vesicles, and the adsorption was monitored with SPR. Additional PLA_2 was added to the solution to increase the concentration until the addition of more PLA_2 did not result in an increase in adsorption. This coverage was assumed to be saturation. The PLA_2 solution was then diluted by addition of buffer to the reservoir to remove a fraction of the bound PLA_2, and a new equilibrium was allowed to form. This was repeated multiple times until all of the bound PLA_2 was removed from the surface as observed by SPR. To determine the PLA_2 concentration at each step, samples of the PLA_2 solution were collected from the reservoir (after establishing equilibrium) into containers with Tris buffer (25 mM Tris-HCl, 100 mM KCl, 0.1 mM $CaCl_2$, pH = 8.5) containing 1 mg/mL bovine serum albumin to decrease protein loss to the walls of the container. These collected samples were assayed for enzyme concentration as described below.

PLA_2 Assays. A thiolester assay was used to determine the concentration of PLA_2 in samples from the SPR experiment.[49] The catalytic rate was determined by monitoring the change in absorbance at 412 nm as the free thiol formed on hydrolysis of the thiolester bond reacted with Ellman's reagent. The concentration of PLA_2 was found by calibration of the PLA_2 activity with standard solutions. The substrate for PLA_2 assays was prepared by drying a mixture of stock solutions of DTPM and dithio-$DC_{10}PM$ in chloroform with a stream of N_2. The remaining solvent was removed in a Speed-Vac (Savant Inc.) for 30 min. Buffer (1 mL of 25 mM Tris buffer) was added to make 90 mol % DTPM and 10 mol % dithio-$DC_{10}PM$, containing a total lipid concentration of 1.1 mM. Vesicles were formed by sonication.[50] Stock solutions of PLA_2 were diluted with 1 mg/mL BSA in order to prevent loss of enzyme to the walls of the tubes.

The final assay solution consisted of 0.8 mM Ellman's reagent, 100 μM DTPM, 10 μM dithio-$DC_{10}PM$, and 1 to 20 ng/mL PLA_2. First, the spectrophotometer thermostated at 30 °C was zeroed at 412 nm with just the substrate and buffer (25 mM Tris buffer stored in a 30 °C

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water bath) in a cuvette. Then, Ellman's reagent was added, and, after equilibration for two minutes, a stable baseline was recorded. Finally, the PLA_2 was added and its activity determined by measuring the increase in absorption at 412 nm. Between each assay, the cuvette was rinsed with a 5% solution of sodium hypochlorite (Clorox), 0.1 M HCl, and deionized water. The enzyme activity was linear with respect to the amount of enzyme within the concentration range used.

Dye Experiments. Extruded $DO_{et}PC$ vesicles were prepared in 50 mM Tris buffer containing 5 mM 5-carboxyfluorescein (Molecular Probes) as described above. The vesicles with entrapped dye were separated from the remaining dye using a size-exclusion column with G-25 Sephadex (Superfine, Pharmacia). The vesicles were allowed to adsorb on an immobilized SA layer as described previously. Rinses after adsorption were assayed for traces of dye to ensure that all nonbound vesicles and residual dye were removed from the flow cell. The surface-immobilized vesicles were exposed to a known volume of 1% Triton X-100 solution to burst the vesicles, and the resulting solution was assayed. The amount of dye in the detergent rinse was determined using a standard curve with known dye concentrations in detergent-containing buffer. Fluorescence intensity was monitored with a fluorimeter using excitation and emission wavelengths of 492 and 518 nm, respectively.

Hybrid Bilayer Membranes (HBMs). The method used to prepare HBMs on Au was based on previous literature.[22,25] A 0.1 mM solution of $DO_{et}PC$ in buffer as described above was sonicated, resulting in small unilamellar vesicles. The vesicles were injected into the SPR flow cell onto a Au substrate previously functionalized with hexadecanethiol as described above. The adsorption and assembly of the lipids were followed with SPR for 6−8 h until a stable HBM was formed. PLA_2 was injected onto the rinsed HBM and its binding monitored.

Results

The SPR response for a typical experimental sequence to study PLA_2 binding to immobilized, *intact* vesicles is shown in Figure 2. The experiment starts with the BAT/PEO monolayer already on the Au substrate in the SPR flow cell. At arrow a, SA was injected, resulting in a typical SPR wavelength shift of ∼10.0 nm due to SA immobilization. The SPR shift for six experiments averaged 9.6 ± 0.9 nm, which corresponds to the adsorption of $2.2 \pm 0.2 \times 10^{12}$ SA/cm^2 onto the surface (using the methods outlined above for converting SPR shifts to absolute surface coverages). This agrees with the saturation coverage of SA, 2.4×10^{12} SA/cm^2, on this particular composition of BAT/PEO previously observed.[28]

At arrow b in Figure 2, the biotinylated vesicles were injected into the flow cell. The SPR shift shown of ∼30 nm was typical of the average shift observed in six similar experiments (30.4 ± 1.9 nm). Doubling the concentration of vesicles did not lead to larger shifts, showing that saturation is reached (not shown). This saturation SPR shift corresponds to $8.9 \pm 0.6 \times 10^{14}$ lipids/cm^2 or 1.89×10^{10} vesicles/cm^2. This corresponds to about 1 vesicle for every 117 SA, or 5300 nm^2 per vesicle. This is close to the cross-sectional area of these ∼80 nm diameter vesicles injected (∼5030 nm^2), consistent with the formation of a densely packed monolayer of spherical vesicles on the chip surface. Assuming that none of the surface area of an immobilized vesicle is blocked by the biotinylated lipids present, by the SA-coated Au surface nearby, nor by adjacent vesicles, the total surface area of exposed lipid available for enzyme binding on one such vesicle is $\sim 2.01 \times 10^4$ nm^2 (based on their average diameter of ∼80 nm). Multiplying by the surface concentration of vesicles (1.89×10^{10} vesicles per cm^2 of Au), this gives a total exposed vesicle area of 3.8 cm^2 per cm^2 of Au sensor surface.

To explore if *intact* vesicles were binding to the SA, vesicles with entrapped dye were adsorbed to an immobilized SA layer in the same manner as described above. After adsorption, the surface was rinsed, and no dye was found to be present in the rinse, indicating the vesicles were not leaking or bursting. The SA bound vesicles were then rinsed with a 1% Triton X-100 solution, bursting the vesicles and releasing 2.0×10^{-11} moles of entrapped dye from the SPR flow cell, as measured by fluorescence analysis. On the basis of the concentration of dye loaded into vesicles (5 mM) and the Au surface area in the flow cell (1.6 cm^2), this corresponds to 4.0×10^{-6} cm^3 of trapped volume, or 1.8×10^{10} vesicles per cm^2 of Au area. The vesicle surface density measured by SPR is within 5% of this value, confirming that the vesicles are adsorbed intact. In a control experiment that was identical in all ways *except* that no vesicles were included in the original solution flowed across the SA layer (which nevertheless had the same dye concentration), only 2.0×10^{-12} mol of entrapped dye were rinsed from the SPR flow cell (i.e., 10% of the dye observed with vesicles present). This shows that at least 90% of the dye observed with vesicles present came from inside the adsorbed vesicles rather than from the walls of the flow system or other components of the surface film.

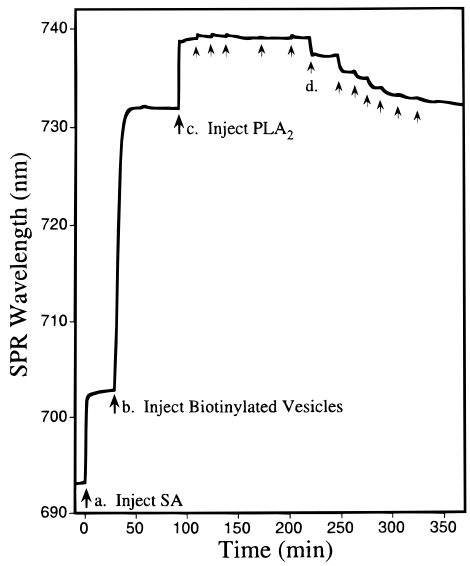

Figure 2. Construction of a biotinylated-$DO_{et}PC$ vesicle substrate (starting with the mixed BAT/PEO layer on Au) and determination of K_d for PLA_2 as observed with SPR. (a) A recirculating mixture of 0.05 mg/mL SA in 50 mM Tris buffer was introduced to the flow cell, resulting in an increase in the SPR wavelength due to binding of SA to the biotin in the mixed BAT/PEO layer. After ∼20 min, the sample was rinsed with pure buffer. (b) A 0.8 mM solution of biotinylated-$DO_{et}PC$ vesicles was introduced to the flow cell and an increase in SPR wavelength was seen due to binding of the vesicles to the SA layer. After ∼30 min the adsorption appeared to reach saturation, and the sample was rinsed with buffer. (c) A 2.5×10^{-5} M recirculating solution of PLA_2 was introduced to the flow cell, and the PLA_2 bound to the vesicles. Additional PLA_2 was then added (indicated by arrows) to the solution to increase its concentration until a saturation coverage was obtained. (d) The PLA_2 solution was diluted seven times (indicated by arrows), resulting in different $[PLA_2]_{bound}$ for each solution concentration and was eventually replaced with buffer to completely rinse the PLA_2 from the surface. Solution-phase aliquots were taken to assay for free PLA_2 concentration just before each arrow (i.e., before changing the PLA_2 concentration but after establishing equilibrium).

Arrow c in Figure 2 indicates the time when the solution of PLA_2 was first injected across the chip surface containing the immobilized vesicles. The concentration of PLA_2 flowing through the flow cell was increased (indicated by successive arrows) until a saturation coverage was reached at a PLA_2 concentration of $\sim 6 \times 10^{-5}$ M. Four such experiments gave a saturation SPR shift of 6.3 ± 1.4 nm for PLA_2, which

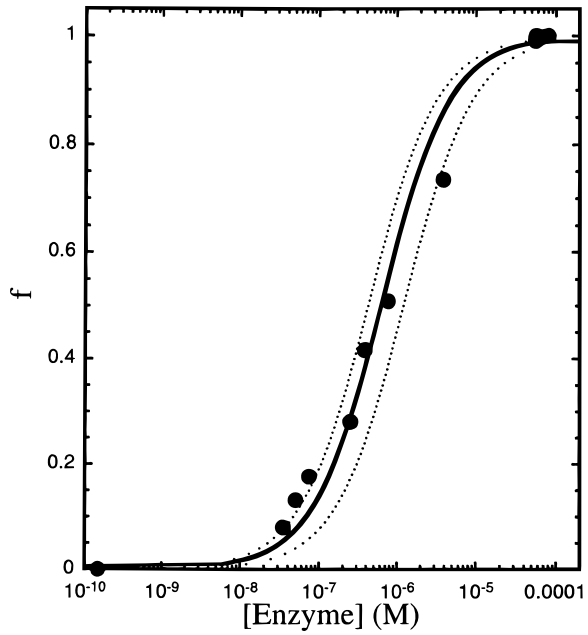

Figure 3. Plot of the fraction of PLA_2 binding sites on the vesicles that are filled (at equilibrium) by PLA_2 (f) versus the log of the solution-phase PLA_2 concentration. The solid curve represents the regression fit to eq 2 giving a K_d of $6 \pm 2 \times 10^{-7}$ M. (The dotted curves represent plots for K_d at these error-bar limits.)

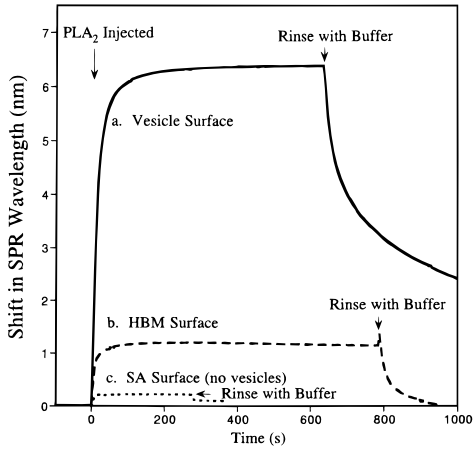

Figure 4. Stop-flow SPR experiments of PLA_2 adsorption on lipid surfaces. (a) Adsorption of PLA_2 from a 2×10^{-5} M solution onto a HBM of $DO_{et}PC$. (b) Adsorption of PLA_2 from a solution of the same PLA_2 concentration onto intact biotinylated $DO_{et}PC$ vesicles immobilized to the SPR chip surface through a SA monolayer. (c) Control experiment showing the adsorption of PLA_2 on the same surface as (b) but without vesicles (SA on the BAT/PEO monolayer).

corresponds to $6.9 \pm 1.5 \times 10^{12}$ PLA_2 per cm^2 of Au (or 1.9×10^{12} PLA_2 per cm^2 of outer lipid surface) or approximately 1 PLA_2 per 74 ± 16 outer lipid molecules (since each lipid occupies ~0.7 nm^2, see above). This packing density is lower than the value of 1 PLA_2 per 35−40 lipid headgroups reported for free vesicles,[2] but this difference is small considering error bars. The difference may be attributed to the proximity of the vesicles to the SA layer, the presence of biotin headgroups, and the close packing between vesicles, which probably renders some of their outer lipid surface inaccessible to protein. When no vesicles were immobilized on the surface (SA on the mixed BAT/PEO monolayer only), the PLA_2 adsorption was < 3% of that observed with vesicles present, as shown in Figure 4, curve c (dotted curve). This is consistent with PLA_2 binding being specific to the vesicles.

By subsequently lowering the concentration of the PLA_2 in the solution (arrow d in Figure 2), the equilibrium fractional amount of PLA_2 bound diminished. Further decreases in PLA_2 concentration (subsequent arrows) led to greater decreases in bound PLA_2. The fractional amounts of PLA_2 bound are plotted versus the log of concentrations (as determined by enzyme activity assay) at each of these steps in Figure 3. The dissociation constant is defined as:

$$K_d = \frac{[\text{Sites}]_{free} \cdot [\text{E}]_{free}}{[\text{Sites}]_{bound}} \quad (1)$$

where $[\text{Sites}]_{bound}$ is the number of PLA_2 binding sites occupied by bound enzymes, $[\text{Sites}]_{free}$ is the number of sites free for binding, and $[\text{E}]_{free}$ is the concentration of enzyme in solution, all at equilibrium. This can be rearranged and simplified to give:

$$f = \frac{[\text{E}]_{free}}{[\text{E}]_{free} + K_d} \quad (2)$$

where f is the fraction of PLA_2 binding sites on the vesicle surfaces that are filled (at equilibrium) at each specific PLA_2 concentration in the aqueous phase. We assume here that K_d is independent of f (i.e., no lateral interactions between bound enzymes). The amount of bound PLA_2 was determined by SPR and normalized to the amount of PLA_2 binding sites that are filled at saturation ($f = 1.0$). A binding site can be thought of as the number of lipids to which one PLA_2 binds (~40). The value of K_d for PLA_2 dissociating from $DO_{et}PC$ vesicles was determined by fitting the data of Figure 3 to eq 2, as indicated by the solid curve through the data. The best-fit value of $6 \pm 2 \times 10^{-7}$ M is similar to the K_d of 2×10^{-7} M reported for phospholipase A_2 from a different cobra species binding to 1,2-ditetradecyl-phosphatidylcholine vesicles.[50] The fractional coverage of the enzyme increases somewhat less steeply with concentration than the fit to eq 2, suggesting that K_d actually increases slightly with coverage. This is typical of weakly repulsive lateral interactions between adsorbed species.

Measurements of PLA_2 binding were also performed on HBMs of $DO_{et}PC$. In these experiments, small unilamellar vesicles of $DO_{et}PC$ were first allowed to fuse and assemble onto a pre-assembled hexadecanethiol monolayer on Au as described in Materials and Methods. Six such experiments produced an average of 9.9 ± 2.4 nm SPR shift which corresponds to $\sim 2.1 \pm 0.5 \times 10^{14}$ lipids/cm^2 or 0.48 ± 0.11 nm^2 per lipid, close to the value of ~0.7 nm^2 per lipid in a Langmuir−Blodgett monolayer (see above). In these initial experiments, a 2×10^{-5} M PLA_2 solution in 50 mM Tris buffer was injected into the flow cell in a stop-flow manner.

Curve a (dashed curve) of Figure 4 shows a 1.2 nm SPR shift as the PLA_2 binds to the HBM surface, typical of the average (1.2 ± 0.2 nm) observed in such experiments. This SPR signal corresponds to $1.0 \pm 0.2 \times 10^{12}$ PLA_2/cm^2 or about 1 PLA_2 for every 208 ± 35 lipid headgroups. Curve b (solid curve) of Figure 4 shows the same experiment performed with the intact vesicles immobilized on the chip surface instead. In this case, a SPR shift of ~6.4 nm results, corresponding to 7.0×10^{12} PLA_2/cm^2 (or 1.8×10^{12} PLA_2/cm^2 of outer lipid surface). This is a packing density of one PLA_2 for every ~73 lipid headgroups, 3-fold larger than on the HBM.

Discussion

There are a number advantages of using the intact immobilized vesicle surface as a model membrane for studying

peripheral protein−membrane interactions or the interactions of other species with membranes or with membrane-bound species. The experiments presented here have clearly demonstrated that the biotinylated vesicles can be bound *intact* onto the surface of an SPR sensor chip without significant changes in the properties of the vesicles. The vesicles bind PLA_2 with an equilibrium constant and packing density similar to those of aqueous-phase vesicles. Our results demonstrate that a dense layer of vesicles can be adsorbed onto the surface, so that signals (like those due to protein binding in SPR) are large for this geometry. In addition, nonspecific binding of the protein to the SPR chip was not a problem with this geometry, at least with the cobra venom PLA_2.

The HBMs offer an advantage over the intact vesicle surface in that their surface is entirely planar and orientational effects could be studied. However, the PLA_2 binding results clearly indicate there is a difference in the quality of the lipid layer in vesicles and HBMs which also has been observed by others.[51] Even though the vesicles have nearly 4 times more surface area, for the same concentration of enzyme we find that the vesicles still allow almost 3 times higher enzyme-to-lipid binding ratio than the HBM surface. The net effect leads to >6-fold signal enhancement and a more physiological environment. Recently, it has been suspected that HBMs are more constrained than true vesicle membranes.[23,52] This difference was attributed to the changes in the physical properties of the lipid layer in HBMs, such as density or fluidity, which may in turn restrict an enzyme's ability to adopt a favored conformation.[53] More importantly perhaps, when a peripheral membrane protein such as the PLA_2 studied here adsorbs to the surface, it partially inserts between the lipids[54] and thus squeezes into the lipid layer to some extent. With an HBM, this leads to very energetically unfavorable compression of the lipid monolayer or to an effectively higher surface pressure. (The surface pressure and energy of a lipid bilayer increases dramatically with packing density.) This destabilization may shut down enzyme adsorption at the unusually low saturation coverage observed here on HBMs (one PLA_2 per ∼208 lipid headgroups).

This is apparently not a problem with the immobilized, intact vesicles which bind a higher density of PLA_2 (one per ∼74 lipids). We note that free vesicles are considered in such respects as very good models of real biological cell membranes. Since water can easily pass through the bilayer of intact vesicles, their diameter will simply increase as the adsorbing protein squeezes into the bilayer, so that there will be no marked increase in surface pressure or bilayer energy upon protein adsorption to the intact vesicle surfaces. This allows supported vesicles to more closely mimic the behavior of free vesicles or real cells and, therefore, to provide more meaningful saturation coverages and binding constants for protein adsorption. However, HBMs are better from a nonspecific adsorption standpoint as there are no other surfaces besides exposed lipids, unlike the intact vesicle monolayer which potentially offers sites for nonspecific binding to the underlying SA or BAT/PEO. Much work has been done to prepare improved model membranes on planar solid surfaces that mimic biological membranes. For example, Heyse et al.[55] have recently used unique types of planar trilayer model membranes and obtained an equilibrium binding constant for transducin binding to the membrane that is only ∼50% below the value obtained for disk membranes.[56]

The surface-bound intact vesicles used here conveniently provide a direct way to measure protein-membrane affinities, whose values should be more physiologically relevant than those of such planar systems. The method avoids some previously described problems[12,13] that are inherent in measuring association and dissociation rate constants to obtain K_d.

The stepwise titration shown in Figure 2 allows adsorption measurement at a number of protein concentrations on the same surface instead of multiple measurements on different surfaces, which may result in error due to differences in the availability of binding sites for the different experiments. Also, when analyzing adsorption or dissociation kinetic curves, mass transfer must be considered, particularly in cases of low concentration or high affinity interactions. Since the present technique measures the amount of bound enzyme at equilibrium, such issues are avoided.

By adding the direct assay of free enzyme in solution to this SPR method, the accuracy of this method is improved. In previous studies, it was assumed that protein loss due to nonspecific binding to the flow cell or tubing was negligible compared to the initial concentration of protein in solution, and in some cases the free protein concentration was calculated by subtracting the amount of protein bound to the vesicles from the total protein in the system initially.[12,13] This assumption may be valid when the protein concentration in the bulk solution is high, but nonspecific binding is a common problem at the low concentrations needed ($<10^{-6}$ M) for strongly binding protein. With the present method, assaying the solution at equilibrium for each titration step avoids these issues.

In the method reported here, it is the sensitivity of the free-protein enzyme assay, not the SPR measurement, which limits the ability to measure low values of K_d in the case of tight protein−vesicle interaction. The thiolester phospholipid assay used in this paper can measure PLA_2 activities as low as 0.2 nmol/min, which allows the detection of $\sim 10^{-9}$ M of enzyme.[49] To measure higher affinity interactions, a more sensitive assay method could be employed. In the case of PLA_2, a radioactivity assay would allow for measurement of $K_d < 10^{-10}$ M.[57] For such strong binding, the off rates would be so slow that the waiting time for equilibrium to be established might be prohibitively long when decreasing the concentration of enzyme (i.e., amount bound). The drift rate of the SPR baseline might become comparable to that of the real signal, but this can be minimized by inclusion of an internal reference channel.[58,59] One could also determine the equilibrium-bound enzyme coverage after extremely long equilibration times (over which the baseline drifts too much for direct measurement) by a difference method, whereby the number of remaining free sites is measured from the rapid SPR response to a post-equilibrium, stepwise increase in the enzyme concentration to a large value, which quickly saturates the remaining free sites. To overcome long waiting times, one could do the experiment with increasing concentration increments, whereby the difference method also would not be necessary. Thus, the technique reported here has promise to complement measurements of K_d by fluorescence-

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and centrifugation-based techniques, and possibly to extend the limits of its measurement to much lower K_d values.

There are still some issues that need to be further examined, so it is not yet clear that this technique can be applied to all enzymes and lipids. Some anomalous behavior of anionic vesicles in the presence of Ca^{2+}, even when the vesicles are bound prior to exposure to Ca^{2+}, were observed (experiment not presented). Until the exact nature of these problems which are most likely due to vesicle aggregation facilitated by Ca^{2+}, is clarified, this method is not yet recommended for highly charged vesicles in the presence of Ca^{2+}. In addition, although nonspecific binding to the sensor surface was not a problem with cobra venom PLA_2, we did encounter enough nonspecific binding of the human group IIA PLA_2 to our SPR chip surface in the absence of adsorbed vesicles, such that control experiments were necessary to measure and correct for it. (This protein nonspecifically sticks strongly to many types of surfaces including various types of plastic and glass.[60]) Finally, we point out that the method also will allow the study of the kinetics of formation and dissociation of complexes formed by proteins (and other ligands) with the surfaces of vesicles. Note that Figure 4 (curve a), for example, also contains this kinetic information for PLA_2−vesicle interactions.

Conclusion

A new method for studying the equilibrium (and kinetic) constants for the binding of species to biological membranes has been demonstrated. We have bound ∼80 nm diameter phospholipid vesicles intact onto a gold SPR sensor surface using a streptavidin linker layer with biotinylated alkanethiols, and biotinylated lipids incorporated at low concentrations (∼0.3%) in the vesicles. An equilibrium titration approach was used to measure the dissociation constant for PLA_2 binding to such biotinylated-$DO_{et}PC$ vesicles, using SPR to monitor the amount of PLA_2 which bound to these vesicles. The concentration of free enzyme at equilibrium was directly determined by a catalytic assay to ensure accuracy and sensitivity. A value of $K_d = 6 \pm 2 \times 10^{-7}$ M was found, with a saturation coverage of one PLA_2 for every $\sim 74 \pm 16$ outer lipid headgroups, similar to the values expected based on studies of free vesicles. This may facilitate the study of the binding of a variety of mutant phospholipases and other species to membranes for which the determination of K_d has been difficult with other methods.

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