Structural Studies of Polymer-Cushioned Lipid Bilayers

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ABSTRACT The structure of softly supported polymer-cushioned lipid bilayers, prepared in two different ways at the quartz-solution interface, were determined using neutron reflectometry. The polymer cushion consisted of a thin layer of branched, cationic polyethyleneimine (PEI), and the bilayers were formed by adsorption of small unilamellar dimyristoylphosphatidylcholine (DMPC) vesicles. When vesicles were first allowed to adsorb to a bare quartz substrate, an almost perfect bilayer formed. When the polymer was then added to the aqueous solution, it appeared to diffuse beneath this bilayer, effectively lifting it from the substrate. In contrast, if the polymer layer is adsorbed first to the bare quartz substrate followed by addition of vesicles to the solution, there is very little interaction of the vesicles with the polymer layer, and the result is a complex structure most likely consisting of patchy multilayers or adsorbed vesicles.

INTRODUCTION

Supported model membranes have been used extensively to study the structure and function of biomembranes (Tamm and McConnell, 1985; Sackmann, 1996). For supported membranes to maintain the structural and dynamic properties of free biomembranes, the interaction between the membrane and the substrate should be minimized. Methods of accomplishing this include separating the membranes from their substrates by either a few monolayers of water or by soft polymer cushions (Sackmann, 1996). In the case of a thin water layer, the separation distance between the substrate and the biomembrane is dictated by the nature of the substrate-membrane forces and is typically 10–20 Å. Whereas lipid molecules are able to diffuse freely within the membrane, this presents a problem for transmembrane proteins, which can become immobilized because of direct interaction with the solid support (McConnell et al., 1986; Kalb and Tamm, 1992; Salafsky et al., 1996). Some proteins still maintain their function if their active site is far from the solid substrate. However, one cannot easily study phenomena such as lateral diffusion, clustering, or domain formation of proteins (common features of many cellular processes) due to the altered interactions of the proteins with the substrate surface.

One strategy for decoupling the biomembrane from the underlying surface is to rest the biomembrane on a soft hydrated polymer or polyelectrolyte film. The polymer film acts as a support for the biomembrane, not unlike the cytoskeletal support found in actual mammalian cell membranes (Jacobson et al., 1995). Various approaches to creating such polymer-lipid composite films have been reviewed by Sackmann (1996). To explore this idea, our work focused on the formation of a lipid bilayer physisorbed to branched polyethyleneimine (PEI), a water-soluble polymer, which is weakly positively charged in neutral or acidic aqueous environments. In addition, PEI is highly swollen in aqueous environments and thus acts as a deformable and mobile substrate for a biomembrane (Fig. 1).

The most common methods used to assemble biomembranes on surfaces are the Langmuir-Blodgett (LB) technique and vesicle fusion. Here we choose to focus on vesicle fusion for two reasons. First, incorporation of transmembrane proteins is possible with proteoliposomes. Second, the ease of vesicle preparation is an advantage for potential applications. Moreover, the fusion of vesicles to solid substrates has been established as a method for formation of solid supported bilayers on various substrates including glass, silicon, and mica (Tamm and McConnell, 1985; Bayerl and Bloom, 1990; Horn, 1984). Spinke et al. (1992) investigated the adsorption of phosphatidylcholine vesicles to a copolymer containing side chains resembling phospholipid tails and found an increase in thickness that corresponded to the thickness of a single bilayer. Neutron reflectivity has been used to investigate the structure of solid supported bilayers (Johnson et al., 1991; Koenig et al., 1996), but we were interested in determining the structure of polymer-cushioned biomembranes. Here we present structural data obtained from adsorbing PEI and phosphatidylcholine vesicles to quartz substrates by neutron reflectometry. We will show that the nature of the structure depends on the order in which the components are added to the system.

EXPERIMENTAL SECTION

Materials

Polyethyleneimine (MW = 1800 g/mol) was obtained from Polysciences (Warrington, PA). Dimyristoylphosphatidylcholine (DMPC) (MW = 678 g/mol) was purchased from Avanti Polar Lipids (Alabaster, AL). D_2O (99.9%) was from Sigma (St. Louis, MO), and potassium nitrate (Puratronic grade) was purchased from Alfa (Ward Hill, MA).

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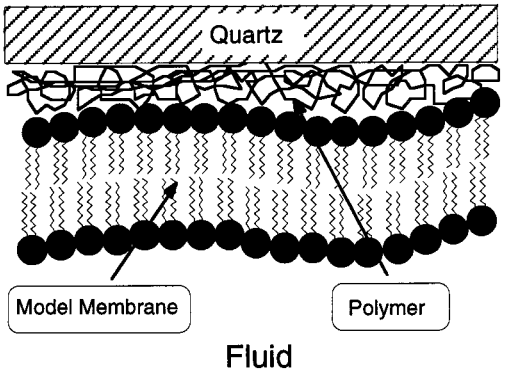

FIGURE 1 Conceptual diagram of polymer-cushioned bilayer at the solid-solution interface. In our case the solid substrate was optically polished monocrystalline quartz, which gives very low absorption of neutrons.

Vesicle preparation

Small unilamellar vesicles (SUVs) of DMPC were prepared by the method of Bangham et al. (1974). Briefly, MLVs (multilamellar vesicles) were prepared by hydrating a dried lipid film of DMPC with Millipore water (37°C for several hours) and subsequently sonicating with a probe sonicator (Fisher Sonic Dismembrator 300) and filtering through a 0.22-μm Millipore filter. The resulting SUVs have an average diameter of 400 Å, as found by particle sizing optical turbidity measurements (Microtrac UPA 150; Brookhaven Instruments Corp.).

Neutron reflectometry of PEI/vesicle adsorption in liquid-solid interface cell

The neutron measurements were made on the time-of-flight SPEAR reflectometer at the Manuel Lujan, Jr. Neutron Scattering Center at the Los Alamos National Laboratory, using a liquid-solid interface cell (for details see the accompanying paper, Kuhl et al., 1998). The range of neutron wavelengths was 1–16 Å. Typical counting times were 3–4.5 h. The reflectivity data were reduced by using the incident neutron intensity spectrum and plotted as $R^*Q_z^4$ versus the perpendicular scattering vector, Q_z (this accounts for a sharp Q_z^{-4} decrease of the reflectivity due to Fresnel's law). The error bars on the data represent the statistical errors in the measurements where the uncertainty in the Q_z resolution, σ_{Qz}/Q_z, was nearly constant over the measured scattering vector range, with a value of ~3%.

The monocrystalline quartz substrates were purchased from Atomergic Chemetals Corp. (Farmingdale, NY) and were polished to a flatness better than $\lambda/10$, and scratch/dig (S/D) = 80/60. The quartz substrates were cleaned in aqua regia, rinsed in Milli-Q water, and cleaned for at least 30 min in a Jelight model 342 ozone cleaner. PEI was added to the system as a 100 ppm solution in 0.5 mM KNO_3/D_2O, and DMPC vesicles were added to a final concentration of 0.14 mg/ml. All solutions were allowed to adsorb for at least 15 min before data collection. All preparations and measurements were made above the chain melting temperature of DMPC, 24°C.

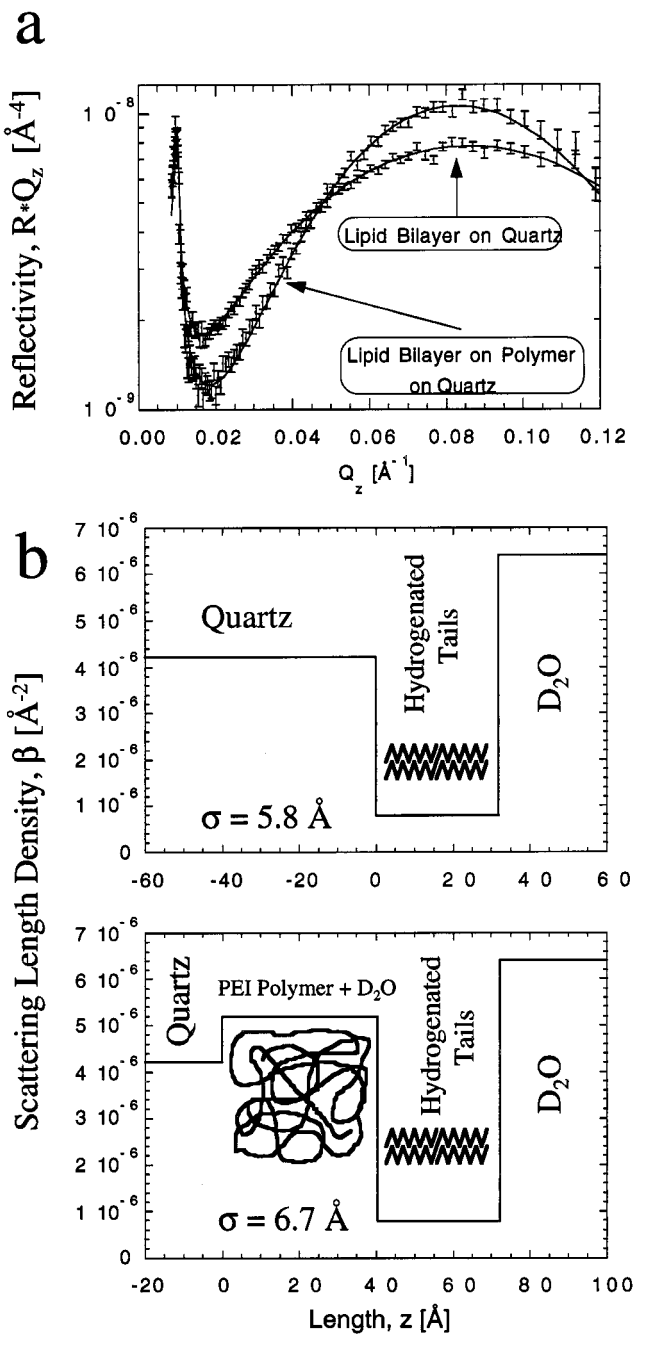

FIGURE 2 (*a*) Neutron reflectivity profile at the D_2O solution interface of vesicles adsorbed to quartz and after addition of PEI. The solid curves through the data are the fits made with the box models. (*b*) Corresponding scattering length density profile of the box model.

RESULTS

Method I: DMPC vesicle adsorption followed by PEI adsorption

DMPC vesicles adsorbed to form an almost perfect bilayer on the quartz substrate (Fig. 2). The data were fitted using a one-box model (Fig. 2 *b*) considering only the hydrocarbon tails. The reflectivities were calculated using the iterative, dynamical method (Russell, 1990), and one overall root mean square roughness factor, σ (Als-Nielsen, 1986) was used to smear out all of the interfaces. The fits included an additional parameter to normalize the calculated reflectivity to the data. The length scales and scattering length densities, β, used for the calculations are shown in Table 1. All scattering length densities presented in this paper are given with air as a reference medium. The calculated scattering length density of quartz was $4.2 \pm 0.1 \times 10^{-6}$ Å^{-2},

TABLE 1 Parameters from fits to the models discussed in the text

Composition	σ (Å)	β lipid tails (10^{-6} Å^2)	T lipid tails (Å)	β polymer (10^{-6} Å^2)	T polymer (Å)	χ^2
Step a	5.8 ± 0.4	0.77 ± 0.08	31.9 ± 0.6	—	—	1.9
Step b	6.7 ± 0.4	0.77 ± 0.08	31.9 ± 0.6	5.19 ± 0.03	40.4 ± 0.9	3.0

Step a. DMPC vesicles were adsorbed on pure quartz: a bilayer is formed (see Fig. 2).
Step b. DMPC vesicles were adsorbed on pure quartz, and after that PEI polymer was added (see Fig. 2).
The parameters correspond to the box models shown in Fig. 2 *b*. T refers to the thickness of the specified box in Ångstroms, and β to the scattering length density in Å^{-2} (air as a reference medium). σ is the Gaussian roughness of the interface. We used the reduced χ^2. For a good fit the χ^2 should be approximately equal to 1.

which was kept constant in the fitting procedure. The value of β for the hydrogenated phosphatidylcholine (PC) hydrocarbon tails is dependent on their packing density, e.g., 60 Å^2/molecule gives $\beta = -0.1 \times 10^{-6}$ Å^{-2}. We found the thickness of the DMPC box on quartz to be 31.9 ± 0.6 Å, with $\beta = 0.77 \pm 0.08 \times 10^{-6}$ Å^{-2}, which might be explained by 86% of the quartz surface being occupied by the bilayer and the rest by D_2O. We tried to fit the data to more complex models: 1) a three-box model with two more boxes for the headgroups, and 2) a four-box model in which each headgroup and tail was considered separately. These multiple-box models gave similar χ^2 values, and the results were consistent with the one-box model but were not sufficiently accurate to give additional insight into the structure. It is of interest, however, that a more complex (three-box) model indicates the existence of a headgroup/water layer ~11.7 ± 0.7 Å thick at the quartz interface, which is qualitatively in agreement with findings of Johnson et al. (1991).

After adding PEI to the system, we saw significant changes in the neutron scattering profile (Fig. 2 *a*) compared to DMPC on bare quartz. To account for this change, we introduced a box into our model to represent the swollen polymer, PEI/D_2O. We tried to accommodate the PEI/D_2O box at the following physically allowable positions: 1) above the outer lipid layer, 2) below the inner lipid layer, and 3) both above and below the lipid bilayer. This procedure gave a reasonable fit to the data at position 2), which was with the PEI/D_2O located between the quartz substrate and the inner phospholipid monolayer (Fig. 2 *b*). Note that this addition did not significantly alter the parameters of the bilayer alone, although we did allow the values to vary during the fitting procedure (Table 1).

Method II: PEI adsorption followed by addition of DMPC vesicles

The adsorption of PEI onto the bare quartz substrate did not show a substantial change in the reflectivity as compared to the bare quartz substrate (data not shown). However, the profile could be fit using a box model of either 1) 10 ± 1 Å width with a scattering length density of 3.44 ± 0.1 × 10^{-6} Å^{-2}, assuming 0.49:0.51 mixing by volume of D_2O to PEI; or 2) 20 ± 1 Å width with a scattering length density of 3.63 ± 0.1 × 10^{-6} Å^{-2}, assuming 0.53:0.47 mixing (the calculated scattering length density of dry PEI is $\beta = 0.56 \times 10^{-6}$ Å^{-2}). Subsequent injection of DMPC vesicles onto the PEI-coated surface gave a reflectivity profile (Fig. 3 *a*) that indicates that the resulting structure is complex. The multiple peaks at low Q_z values indicate that structures of a length scale ranging from 500 to 600 Å are present. Because of the complex nature of this profile, it is difficult to unambiguously determine the exact structure at the interface. However, we are certain that the resulting scattering length density is not due to a single bilayer above the PEI

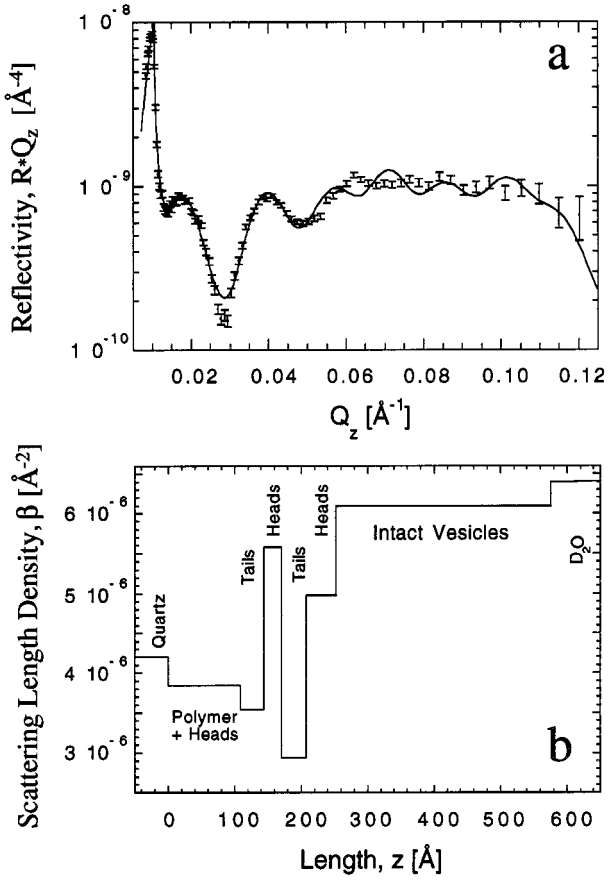

FIGURE 3 (*a*) Neutron reflectivity data at the D_2O solution interface of PEI adsorbed to a quartz substrate followed by the addition of vesicles. The solid curve through the data is the fit by the use of a multiple box model. (*b*) Corresponding scattering length density profile of the multiple box model.

cushion. Starting from simple box models, we were able to fit the data with reasonable confidence by using the model shown in Fig. 3 *b*. Although we do not present this model as a unique structure for these layers, this is a simple model that illustrates the complexity of the resulting structure.

DISCUSSION

Method I: DMPC vesicle adsorption followed by PEI adsorption

DMPC vesicles fused to the quartz substrate to form an almost perfect bilayer, as previously observed (Johnson et al., 1991). This is due to the strong van der Waals interaction between lecithin bilayers and quartz or mica (Horn, 1984). The neutron reflectivity profile enables us to probe the structure of the supported layer. The thickness of the box was 31.9 ± 0.6 Å, which is slightly lower than the value of 35 Å for fully extended hydrocarbon tails. This result, however, is consistent with a fluid bilayer.

To think that PEI could crawl beneath the lipid bilayer and lift it up may sound implausible, but after some consideration it is not unexpected or unprecedented. First, purely thermodynamic or energetic considerations suggest that, because PEI is positively charged, it should bind more strongly to the negatively charged quartz surface than the uncharged PC bilayer. Thus PEI is energetically favored to displace the bilayer. Second, it has been found by surface force measurements (Giasson et al., unpublished data) that a cationic polymer can displace a monolayer of a negatively charged surfactant adsorbed to a mica substrate with the surfactant layer remaining associated with the polymer. In addition, the lipid layer closest to the quartz substrate may not be a perfect layer, but may contain a small number of holes or defects. These may allow the PEI to access the underlying substrate. Finally, we see changes in the overall roughness factor σ (Table 1) when PEI is added to the system: $\sigma = 5.8 \pm 0.4$ Å for the DMPC bilayer formed on quartz, but upon the addition of PEI, σ increased to 6.7 ± 0.4 Å. Thus the soft PEI layer adds roughness to the bilayer, as expected, probably in the form of additional undulations and protrusions.

The thickness of the PEI layer beneath the DMPC bilayer was found to be ~40 Å. Moreover, the PEI is highly hydrated, and we estimate the adsorbed PEI to be ~80% water by volume. As discussed briefly earlier, one possible explanation for the apparent difference in the PEI thickness when measured against D_2O (~10 Å) versus against a bilayer and D_2O (~40 Å) is that the bilayer may give added contrast such that the true polymer thickness can be measured. When only the polymer is adsorbed there may be a diffuse layer that does not have sufficient contrast to allow for accurate thickness determination. It is also possible that because of the different interactions between PEI and the inner leaflet of the bilayer, the adsorbed PEI thickness is indeed greater than in the case in which no bilayer is present.

Method II: PEI adsorption followed by addition of DMPC vesicles

Although we do not see PEI directly in the neutron scattering profile, we have indirect evidence that PEI is adsorbing to the quartz substrate. The scattering profile for vesicles added to PEI-coated quartz (Fig. 3 *a*) is clearly different from when vesicles are added to a pure quartz substrate (Fig. 2 *a*). This has also been observed by Sohling et al. (1996) where PEI adsorption to silicon did not result in substantial changes in the x-ray scattering profile, but where negatively charged vesicles were able to adsorb to PEI-coated silicon but not to bare silicon. Moreover, our box model can accommodate a thin layer (~10 Å) of PEI on the surface. The thickness of PEI adsorbed to mica measured by surface force measurements is ~10 Å under compression (Wong et al., unpublished data). It is possible that PEI adsorbs in a different configuration on quartz, but as discussed below, the lower thickness observed from fitting the reflectivity data could be due to the weak contrast between D_2O and PEI. By enhancing the contrast, i.e., when a bilayer is next to PEI, we find a larger thickness for the PEI layer.

DMPC vesicles do not appear to form a uniform bilayer when adsorbed to PEI, because the reflectivity profile indicates a complex structure that most likely consists of multilayer complexes of PEI with bilayers as well as intact vesicles adsorbed to the surface. When the surface is rinsed with D_2O, some of the multiple peaks at lower Q_z values disappear, but this still appears to be a heterogeneous structure (data not shown). The reason for the lack of adsorption and rearrangement into an extended bilayer is probably because the absence of a strong interaction between PEI and DMPC bilayers.

Comparison of both methods and implications of results

The differences observed in the structures depended on the method of preparation, namely the order in which the components were added to the system. Because PEI and DMPC vesicles were present in the solution for both preparation methods, the observed differences in the reflectivity profiles must be due to the initial surface adsorption behavior. In the case where the vesicles are added to the polymer, multilayered aggregates form that may be kinetically trapped from their equilibrium structure. In the other case, where a uniform lipid bilayer forms first, the reflectivity profile is consistent with a polymer cushion beneath a uniform bilayer.

Our results showing the formation of stable bilayers on polyethyleneimine illustrates the concept of the balance of the interaction forces between the components of the lipid-polymer composite film and the underlying substrate (Elender and Sackmann, 1994). Polyethyleneimine is positively charged and electrostatically binds to the negatively charged quartz substrate. The electrostatic interaction is stronger than the van der Waals attraction between the lipid

and the quartz substrate, which leads to the displacement of the preformed DMPC bilayer and thus gives our desired structure. Recent work by Kühner and Sackmann (1996) has examined another case where the van der Waals attraction between the bilayer and the solid substrate dominates over the polymer-substrate interaction. There covalent attachment of a neutral dextran polymer layer to the substrate was required to prevent its desorption in the presence of the bilayer. It can be concluded that both systems tend to reach an equilibrium structure that is governed by the relative strengths of the underlying interactions in the lipid-polymer composite film. Thus the final structure and stability of polymer-supported membranes is highly dependent on the chemical nature of the individual components of the system.

CONCLUSIONS

Neutron reflectivity has allowed us to investigate complex and biologically relevant bilayer structures at the solid-liquid interface. We found dramatic differences in the structures resulting from two methods of preparation in which we only varied the order in which the components were added to the system. DMPC vesicles added to PEI-coated quartz substrates led to a nonhomogeneous structure consisting of a mixture of vesicles and multilayers of lipids. In contrast, PEI appears to diffuse beneath a preformed DMPC bilayer, cushioning the bilayer from the underlying quartz substrate. This may be a simple way to prepare other softly supported biomembranes.

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