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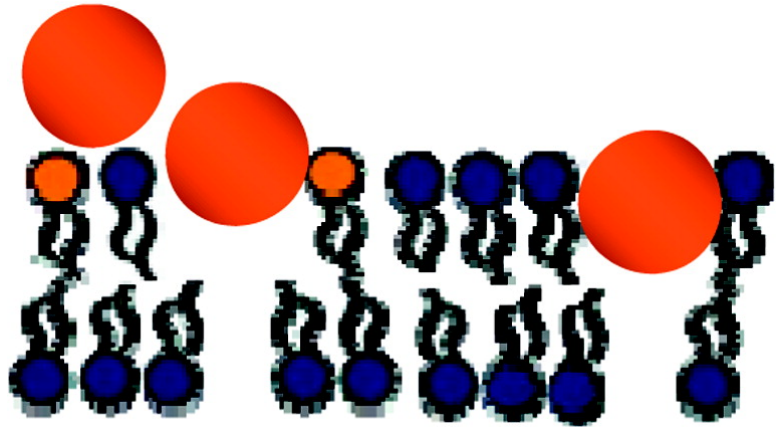

Specular Neutron Reflectivity Studies of the Interaction of Cytochrome c with Supported Phosphatidylcholine Bilayers Doped with Phosphotidylserine†

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Specular neutron reflectivity was used to study the time course and nature of the interaction of the positively charged, peripheral membrane protein cytochrome c with supported bilayers of zwitterionic 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC) containing the anionic lipid 1-palmitoyl-2-oleoyl-glycero-3-phosphatidylserine (POPS). The supported bilayers were prepared by deposition on silicon blocks of two monolayers of DOPC, the second of which contained either 10 or 20 mol % POPS at surface pressures of either 15 or 20 mN/m using a combination of Langmuir−Blodgett and Schaefer deposition techniques. Each supported bilayer was initially characterized by specular neutron reflectivity using subphases of 10 mM NaCl aqueous solutions. Regardless of POPS content and bilayer deposition pressure, the molecular architecture of the bilayers was similar. The addition of cytochrome c resulted in an almost immediate change in reflectivity, which was well modeled by assuming that an additional layer was present next to the outer leaflet of the bilayer. The thickness of this layer, which contained the volume fraction of ∼15% protein, was ∼30 Å (consistent with the cross-section of a single cytochrome c molecule). The addition of cytochrome c to the subphase also resulted in a change in the structure of the phospholipid bilayer, suggesting some penetration of cytochrome c into the bilayer. Specular neutron reflectivity studies after careful washing with solvent showed that although most of the protein was washed off by flushing 10 mM NaCl D_2O through the cell a small amount remained both within the bilayer and bound to the membrane surface.

Introduction

The interaction of specific membrane lipids with peripheral membrane proteins is key to understanding how cellular membranes become the scaffold for signaling in such important processes as apoptosis. Apoptotic cells are selectively marked for degradation by the appearance on the exterior surface of the plasma membrane of the oxidized form of negatively charged membrane lipid phosphatidylserine (PS), the so-called "eat-me" signal.[1,2] Normal, healthy cells, in contrast, do not express oxidized PS on their exterior and are therefore untouched by macrophages.[3] In normal mammalian cells, PS comprises ∼10% of the total amount of membrane phospholipid and is predominantly located on the inner leaflet of the plasma membrane. (For a review, see ref 4.) Although it is known that the oxidation and subsequent externalization of PS are mediated by its electrostatic interaction with the protein cytochrome c (cyt c),[5] surprisingly little is known about the molecular mechanisms governing this interaction despite the fact that it is key to understanding the role of cyt c in apoptosis.

What is known, however, is that early during apoptosis cyt c is released from the mitrochondria into the cytosol of the cell where it is converted to its pro-oxidant form by interaction with reactive oxygen species, also generated within the mitochondria. Cyt c is a heme-containing, peripheral membrane protein composed of 104 amino acids that is an electron carrier in the respiratory chain of eukaryotes. Having eight positive charges at physiological pH, it is a basic protein. Detailed structural studies revealed that cyt c has a stable prolate spheroid structure with a radius of 31 Å.[6] The pro-oxidant form of cyt c selectively catalyzes the peroxidation of unsaturated PS. Shortly after its oxidation, PS is found to accumulate on the exterior surface of the cell, presumably because its chemical modification serves to depolarize the molecule, leading to perturbation of the membrane structure.[5]

The present study aims to investigate the interaction of the oxidized form of cyt c with unsaturated phosphatidylserine using specular neutron reflectivity (SNR) in supported bilayers prepared from 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) bilayers and either 10 or 20 mol % palmitoyl-oleoyl-glycero-3-phosphoserine (POPS). Unsaturated lipids were selected for the preparation of the supported bilayers in order to mimic the fluid nature of the cell membrane. Because the supported bilayer was prepared by using two separate depositions of monolayer, it was possible to produce an asymmetric bilayer (in terms of lipid content), thereby mimicking the in vivo situation. In the present study, the layer closest to the substrate was composed entirely of DOPC whereas the outer layer comprised DOPC mixed with

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either 10% or 20% POPS. SNR studies were used to provide information about the way in which cyt c interacts with PS-containing membranes.

Materials and Methods

Materials. DOPC and POPS were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification after checking their surface pressure−area isotherms against those recorded for the same material in the literature.[7] Spectroscopic-grade chloroform and ethanol were used (AnalaR, BDH Chemical Ltd., Poole, Dorset, U.K.). An aqueous solution containing 10 mM NaCl (AnalaR, BDH Chemical Ltd., Poole, Dorset, U.K.) was used as a subphase for all experiments. Prior to its use, NaCl was heated to 400 °C for 2 h in a muffle furnace (model JP-DIN, Gallencamp, U.K.) to remove any organic impurities. Ultrapure water was obtained by either double distillation or filtration through a Millipore Milli-Q system to a resistivity of 18 $M\Omega^{-1}$. The ultapure water was confirmed to be spectroscopically pure (Chirascan, Applied Photophysics Laboratory, U.K.), and prior to use it was degassed by sonication for 15 min. Cytochrome c (type IV, horse heart) was purchased from Sigma Chemical Co. (Gillingham, U.K.). UV spectroscopy (Chirascan, Applied Photophysics Laboratory, U.K.) was used to determine that the cyt c used in these studies was ∼90% oxidized by comparison with the UV spectra for oxidized and reduced forms of cyt c (prepared using the method in ref 8). Prior to its use in the SNR studies, cyt c was dissolved in D_2O for sufficient time to ensure complete labile proton exchange. The bilayers were supported on single crystals of silicon with dimensions of 5 × 5 × 1 cm^3, polished on one face (111) to ∼5 Å roughness (Crystran Ltd., Dorset, U.K.).

Preparation of Monolayers. A Nima 611 Langmuir trough (volume 300 mL) fitted with a dipping attachment (Coventry, U.K.) was thoroughly cleaned, first using chloroform and then ethanol and finally copious amounts of ultrapure water. After cleaning, the trough was filled with an aqueous solution containing 10 mM NaCl, and the absence of any surface-active impurities was confirmed by closing the barriers of the trough and monitoring surface pressure by means of a strip of filter paper acting as a Wilhelmy plate dipping through the surface of the subphase and attached to a pressure sensor. Once the subphase was deemed to be clean, a 20 μL aliquot (Hamilton syringe, Bonaduz, Switzerland) of a 2 mg/mL solution of DOPC or DOPC/POPS (9:1 or 8:2 mol/mol) in chloroform was added to the surface of the subphase (unless otherwise stated, an aqueous 10 mM NaCl solution). After the evaporation of the chloroform (approximately 10 min), the lipid monolayer was slowly compressed (30 cm^2/min), and the surface pressure (π)−area (A) isotherm was recorded. The lipid monolayers were checked for the absence of hysteresis by repeated compression and decompression to surface pressures of just less than their collapse point and for stability by maintaining the surface pressure at a predetermined value over at least 1 h. (A film was deemed to be stable if the area changed by less than 2% over this time.) The experiments were performed at ambient temperature, ∼22 °C, considerably above the phase-transition temperature of the lipids comprising the bilayer.

The effect of cyt c on monolayers prepared from DOPC and DOPC/POPS was studied as follows. First, the lipid monolayer was compressed to ∼2 mN/m above the target pressure of 10 mN/m, and then the monolayer was held at a constant area per molecule. Once the film was deemed to be stable, 5 mL of a 72 μg/mL solution of cyt c was injected under the monolayer by means of a long-needle syringe in order to ensure the distribution of cyt c under all parts of the monolayer. The change, if any, in surface pressure of the monolayer in the presence of cyt c was recorded over time.

Preparation of Bilayers. Bilayers of phospholipid mixtures were prepared by the consecutive deposition of two monolayers on silicon blocks. The first bilayer was deposited using the Langmuir−Blodgett methodology and then by the Langmuir−Schaefer technique. Prior to deposition of the phospholipid bilayers, the silicon blocks were thoroughly cleaned by first rinsing and sonicating in chloroform, then acetone, and finally ethanol. The silicon blocks were finally rendered hydrophilic by treatment with UV/ozone for 30 min as described previously.[9] The first half of the bilayer was deposited on the silicon block at ∼25 °C using the Langmuir−Blodgett technique. This methodology involved first totally immersing the silicon block in the subphase of the Langmuir trough (namely, an aqueous solution of 10 mM NaCl) oriented such that the polished face of the block was at a right angle to the surface of the subphase. A 20 μL aliquot of 2 mg/mL DOPC in chloroform was then deposited on the surface of the trough. After the evaporation of the chloroform, the monolayer was compressed at a speed of 25 cm^2/min to the desired surface pressure (either 15 or 20 mN/m) and allowed to equilibrate for 5 min before the silicon block was withdrawn from the subphase at a speed of 7 mm/min while still maintaining the monolayer at the desired surface pressure using an automatic feedback control mechanism.

To deposit the second half of the bilayer, the Langmuir−Schaeffer technique was used. First, the Langmuir trough was emptied and cleaned as described above. A similarly cleaned Teflon cell was then placed in the bottom of the trough, and the trough was filled with an aqueous solution of 10 mM NaCl. A 20 μL aliquot of a 2 mg/mL lipid mixture composed of DOPC with either 10 or 20 mol % POPS in chloroform was deposited on the surface of subphase, and after evaporation of the chloroform, the monolayer was compressed to the appropriate surface pressure, namely, 15 or 20 mN/m. Prior to deposition of the second monolayer, the silicon block was oriented in such a way that the polished face with the first deposited monolayer faced downward toward the subphase.[9] The silicon block was very slowly lowered through the monolayer to allow the deposition of the second layer while maintaining the required pressure constant. The block was then pushed completely through the surface and into the Teflon cell, and the silicon block was secured in the Teflon cell under water as described elsewhere.[9] The bilayer-coated silicon block was stored immersed in the aqueous 10 mM NaCl solution in the sealed Teflon cell until required for the SNR experiments, at which point the aqueous 10 mM NaCl solution was slowly replaced with D_2O also containing 10 mM NaCl by carefully flushing three volumes of the deuterated solution through the cell using a peristaltic pump. This technique has been proven to leave the lipid film intact on the surface of the substrate.[9] Extreme care was taken at all times to avoid the introduction of air bubbles into the cell. The transfer ratios for the depositions of the first monolayer were all greater than 80%.

Specular Neutron Reflectivity. The monolayer experiments were carried out on the SURF beamline at the ISIS Facility, Rutherford Appleton Laboratory (Chilton, Oxfordshire, U.K.). The DOPC/POPS monolayers were prepared as described above and compressed to 10 mN/m on a D_2O subphase containing 10 mM NaCl (6.35 × 10^6 $Å^{-2}$ scattering length density (SLD) of the NaCl solution). It was determined that because of the low concentration of the salt its presence does not noticeably alter the SLD of the subphase solution. SNR measurements were recorded first for the monolayer alone using an incident angle of 1.5°. Following this, 5 mL of a 4.8 μg/mL solution of cyt c in D_2O was injected under the monolayer by means of a long-needle syringe to ensure, as far as possible, the even distribution of the cyt c solution under the monolayer. To ensure complete interaction between cyt c and the monolayer, the system was left to equilibrate for 1 h before the monolayer was characterized again using SNR. This equilibration time was determined from surface pressure measurements performed on a Langmuir trough. Throughout all experiments, the temperature of the film in the trough was maintained at 25 °C.

The bilayer experiments were carried out on the D17 beamline at the Institut Laue-Langevin (Grenoble, France).[10] For these studies, the contrast was provided by the various subphases used, namely, D_2O (SLD of 6.35 × 10^6 $Å^{-2}$), silicon-matched water (SiMW; composed of $0.62H_2O/0.38D_2O$ to give an SLD of 2.07 × 10^6 $Å^{-2}$),

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and silicon oxide-matched water (SiO_2MW; composed of 0.43H_2O/0.57D_2O to give an SLD of 3.41×10^6 Å^{-2}), all containing 10 mM NaCl. For the bilayer studies, the Teflon cells containing the bilayer-coated silicon blocks immersed in 10 mM NaCl in D_2O were placed in the sample rack on D17 at 25 °C, and the samples were accurately aligned in the beam. SNR measurements were then performed at three incident angles (0.8, 1.6, and 4°) to give a Q (momentum transfer) range of 0.0098−0.396 Å^{-1} using the instrument in time-of-flight mode in the 2−20 Å wavelength range. After measurements at all three angles had been recorded, 10 mM NaCl in D_2O was carefully exchanged using a peristaltic pump, first for 10 mM NaCl in SiMW and then for 10 mM NaCl in SiO_2MW, ensuring that three times the total cell volume of the new solvent was flushed through the sample cell prior to measurement with neutrons. After characterization of the various bilayers, the bilayers were exposed to a solution of cyt c in D_2O containing 10 mM NaCl so as to give a ratio of 1 cyt c molecule for every 12.5 molecules of POPS in the bilayer. The time course of the interaction of cyt c with the monolayer was monitored by recording reflectivity at one angle (0.8°) in 20 min time slices over a period of 80 min. After exposure of the bilayer to cyt c for 80 min, the subphase containing cyt c was replaced first with D_2O containing 10 mM NaCl and then with SiMW containing 10 mM NaCl, and the bilayer was recharacterized using the same three incident angles as originally used.

A bare silicon block, having no lipid deposited on it, was also characterized using subphases of 10 mM NaCl in D_2O and SiMW. Then, a solution of cyt c in H_2O containing 10 mM NaCl was added as described above, and the block was incubated in this protein solution for 60 min, after which time it was characterized using NR. Finally, the block was rinsed with 10 mM NaCl D_2O and recharacterized using NR.

Method of Analysis. In an SNR experiment, the reflected intensity is measured as a function of the incident angle. For a single interface, the specularly reflected intensity decays as a function of the fourth power of Q.[11] For an interface consisting of several layers, reflection will occur from each of the interfaces depending upon the refractive indices of each layer. A specular reflectivity curve therefore essentially reflects the neutron refractive index profile normal to the surface. The most common approach to analyzing reflectivity data is the optical matrix method, which can be used to analyze both monolayer and bilayer data. In this approach, a physically reasonable model of the distribution of components of the interface is constructed by subdividing the adsorbed layer into a number of parallel layers of uniform (or homogeneous) SLD. In the formalism used, although each layer is assumed to be of uniform SLD, the interfaces are broadened by roughness. Each layer in the model is therefore described by an SLD, thickness, and roughness. The parameters of the model are then adjusted until the best possible agreement is achieved between the theoretically calculated reflectivity curve and the measured data, while still obtaining a physically reasonable model.

In the present study, the lipid monolayer and bilayer were subdivided into two/three regions (depending on whether a monolayer or bilayer was being modeled). One region, representing the alkyl chain region of the lipid, was taken to comprise CH_3, CH_2, and CH=CH groups, and the other was taken to contain the lipid headgroups, including the acyl carbonyls, the glycerol backbone, and the phosphate and choline moieties, together with any water of hydration. When a bilayer was being modeled, two headgroup regions were required.

The scattering length density (SLD) for each sublayer was calculated from

$$\frac{\sum_{i=1}^{n} b_i}{v_{\text{particle}}}$$

where b_i is the scattering length for each moiety comprising the sublayer and V_{particle} is the volume of each moiety comprising the sublayer.[10] The SLDs of the various parts of the lipid molecules and silicon and its oxide are given in Table 1. The SLD of cytochrome c was calculated by assuming that all of the labile, accessible H^+ were exchanged for D^+.[12]

Table 1. Scattering Lengths, Molecular Volumes, and Scattering Length Densities of Materials Used in This Study

material	molecular formula	volume (Å^3)	scattering length density ($\times 10^{-6}$ Å^{-2})
palmitoyl chains	$C_{30}H_{62}$	800[a]	−0.22
oleoyl chains	$C_{34}H_{66}$	934[b]	−0.41
PC head	$C_{10}H_{18}NO_8P$	344[a]	1.75
PS head	$C_8H_{11}NO_{10}P$	321[a]	2.63
silicon	Si	20[a]	2.07
silicon oxide	SiO_2	47[a]	3.41
cytochrome c			3.54[c]

[a] Obtained from ref 21. [b] Calculated as described in ref 25. [c] In D_2O, it was assumed that most of all the labile protons were exchanged for deuterium.[26] The number of exchangeable protons was calculated as described in the Materials and Methods section.

Because the deposited bilayer was asymmetric in terms of its lipid content, it was necessary to calculate different SLDs for the outer and inner layers of the deposited bilayer. That is, the calculated SLD was based upon the mol % of POPS present. It was assumed that the negatively charged POPS remained in the outer leaflet because it was unlikely to migrate toward the negatively charged silicon block. Although the alkyl region of the bilayer was asymmetric, an average SLD was calculated for this region to reduce the number of variables in the modeling. It was found that dividing the region into two separate chain regions does not improve the fit. In the case of the supported bilayer, besides assigning physically reasonable values of sublayer thickness, SLD, and an estimate of interfacial roughness, a coverage parameter was also introduced to account for the fact that the bilayers may not completely cover the substrate.

The Motofit software, recently developed in ref 13, was used to analyze the neutron scattering data in the present study. When data sets of more than one contrast were collected for a sample, the SNR curves were analyzed simultaneously using Motofit. The software utilizes least-squares fitting to calculate a reflectivity profile from the input parameters and genetic optimization followed by Levenberg−Marquardt fitting to optimize the fit. In addition, the quality of the fit was assessed visually.

Results

Cytochrome c Binding to Monolayers Containing Phosphatidylserine. Figure 1 shows the surface pressure−area isotherms of DOPC and POPS monolayers and mixtures thereof spread on a subphase of 10 mM NaCl. Regardless of their compression state, the individual lipids and their mixture were in their liquid expanded state. As anticipated from a consideration of molecular structure, POPS exhibited the smallest limiting area (75 Å^2) and DOPC exhibited the largest (103 Å^2). Interestingly, the limiting areas for DOPC monolayers containing 10 and 20 mol % POPS lay closest to that obtained for POPS, suggesting nonideal mixing of the negatively charged and neutral constituents of the monolayer. In support of this, structural studies using Brewster angle microscopy on monolayers of saturated, symmetric dipalmitoyl phosphatidylcholine and dipalmitoyl phosphatidylserine reveal that at comparable surface pressure irregular domains form.[14]

When cyt c was injected into the D_2O (10 mM NaCl) subphase underneath a monolayer prepared from DOPC and containing 20 mol % POPS at a surface pressure of 10 mN/m, the surface

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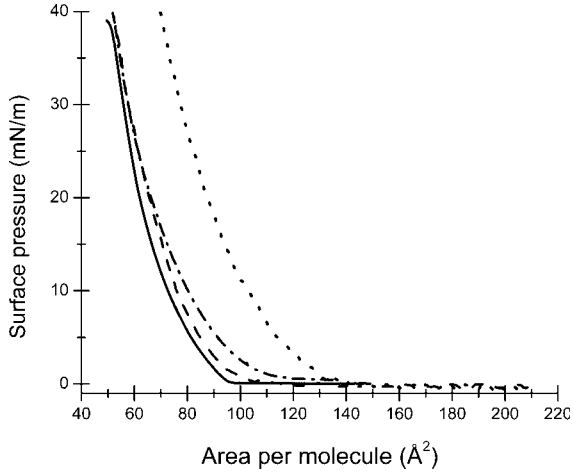

Figure 1. Surface pressure−area isotherms of pure DOPC (•••), 10% POPS (•-•-•), 20% POPS (---), and pure POPS (—) on a 10 mM NaCl subphase at 25 °C ($n = 3$).

pressure was seen gradually to increase by 7.8 ± 1.1 mN/m ($n = 3$) over 1 h, with the precise time course of the reaction being dependent on the rate of mixing of the subphase, an effect previously reported by Quinn and Dawson.[15] This observation suggests that any interaction between cyt c and a DOPC monolayer containing 20 mol % POPS was complete within 2 h under the experimental conditions used in the present study. When an equivalent volume of cyt c free water was added to the subphase, no change in surface pressure was observed over a time period of 1 h. Interestingly, compression to a higher surface pressure of 20 mN/m and injecting cyt c under this layer resulted in an increase in surface pressure of only 2.2 ± 0.5 mN/m ($n = 3$), suggesting that the interaction of cyt c with the monolayer is either less at the higher pressure or of a different nature. Note that when the same concentration of cyt c was injected into a subphase of 10 mM NaCl in water no effect on surface pressure was observed, which suggests that cyt c does not sit at the air−water surface.

An SNR study was performed to determine the extent of the binding of cyt c to a monolayer comprising 20 mol % POPS in DOPC spread on a subphase of D_2O containing 10 mN/m NaCl and compressed on a Langmuir trough to a surface pressure of 10 mN/m (SURF beamline, ISIS, Oxfordshire, U.K.). The NR of the air−water interface was characterized before and after the addition to the subphase of cyt c. After the interaction between the monolayer of DOPC containing 20 mol % POPS at 10 mN/m and cyt c was complete (as assessed by a tailing off of the increase in surface pressure), the monolayer was recharacterized using SNR. The parameters obtained from modeling the reflectivity curves thus obtained are summarized in Table 2.

The relatively thin hydrophobic chain region of the DOPC/POPS monolayer before the addition of cyt c is consistent with the unsaturated nature of the majority of the lipid chains present. Significantly, when cyt c was added, it was not possible to model the reflectivity curve obtained via the two-layer model used for modeling the reflectivity profile obtained in its absence because a third layer was required directly below the lipid monolayer, supporting the observation from the isotherm studies that cyt c interacts with the DOPC/POPS monolayer. Although the thickness of the lipid chain region was unchanged in the presence of cyt c, a slight increase in the SLD of the lipid headgroup region was observed, most probably because of the insertion of some of the cyt c into that region. The "extra"third layer required was 15 Å thick and contained 20 vol % cyt c and 80% solvent. Attempts to model a third (cyt c-containing) layer with a thickness of 31 Å, because this is the reported cross section of the protein,[6] were unsuccessful, suggesting that the protein either must have unfolded or else had become partially inserted into the lipid headgroup region of the monolayer; although it must be remembered that only one contrast was used for this study, the SNR study clearly shows that cyt c interacts with the DOPC/POPS monolayer.

Table 2. Model Parameters Used to Fit the Neutron Reflectivity Data from for Monolayer Composed of 20% POPS/80% DOPC at 10 mN/m without and with Cytochrome c, Assuming That the Protein Penetrates into the Headgroup

layer[b]	thickness (Å)	SLD (10^{-6} Å^{-2})	% water	roughness (Å)
(A) Monolayer Alone				
chains	15.5	−0.24	0	2.3
heads	9.1	1.91	40	2.1
(B) Monolayer with Cytochrome c Injected into Subphase				
chains	15.3	−0.24	0	2.4
heads	9.0	2.35[a]	28	2.3
protein	15.2	2.94	80	2.5

[a] The head SLD is a weighted average of protein and lipids to reflect the penetration of cyt c into the head region. [b] Because only one contrast was used, no error was calculated on the fit, and the χ^2 value was used to determine the goodness of fit.

Cytochrome c Binding to Bilayers Containing Phosphatidylserine. Figures 2 and 3 show the reflectivity profiles obtained using the three contrasts for the single supported DOPC bilayers containing 10 and 20 mol % POPS, respectively, deposited at 20 mN/m before the addition of cyt c. Also shown as an inset is the SLD profile obtained from fitting the data. The parameters obtained from fitting the SNR data are summarized in Table 3. In the analysis, the roughness parameters were linked for all layers because they were assumed to be dependent on the roughness of the silicon block. The structure of the bilayers modeled was very similar for both molar ratios of POPS when deposited at 20 mN/m (and 15 mN/m; data not shown). In all cases, a four-layer model was found to fit the data best, where the first layer was silicon oxide, which is known to vary slightly in thickness from block to block but was found to be ∼7 Å for the blocks used in this study.

The next three layers arise from the phospholipid bilayer, which was subdivided into the inner headgroup, the hydrophobic chains, and the outer headgroups. The bilayer coverage of the silicon substrate was found to be 60−70% by NR compared with >80% coverage calculated from the transfer ratio obtained from the Langmuir−Blodgett deposition, suggesting that some lipid may have been lost during the Schaefer deposition. Although the final coverage is lower than has been reported for some bilayer systems prepared from saturated lipids using the same methodology, it is undoubtedly a consequence of the unsaturated (rather than saturated) nature of the lipids were used although better coverage may have been attainable using a different method of bilayer formation.[16] Whereas others have reported a very thin layer of water between the silicon block surface and the phospholipid bilayer,[17,18] in our case it was not possible to distinguish between the inner head groups and this water layer. Thus, in the modeling process, the inner headgroup layer was allowed to be thicker to encompass the adjacent water layer.

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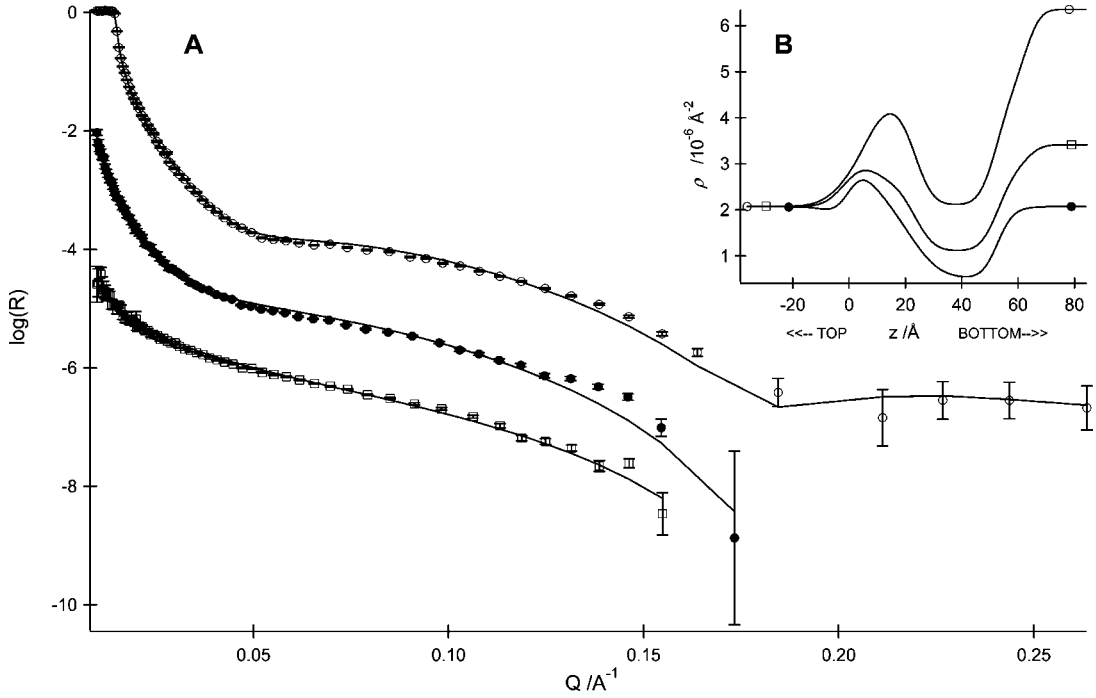

Figure 2. DOPC bilayer containing 10% POPS deposited at 20 mN/m. (A) Reflectivity data with D_2O (○), SiMW (♦), and SiO_2MW (□) solvents. For clarity, the reflectivity curves are offset by −1 on the *y* axis.

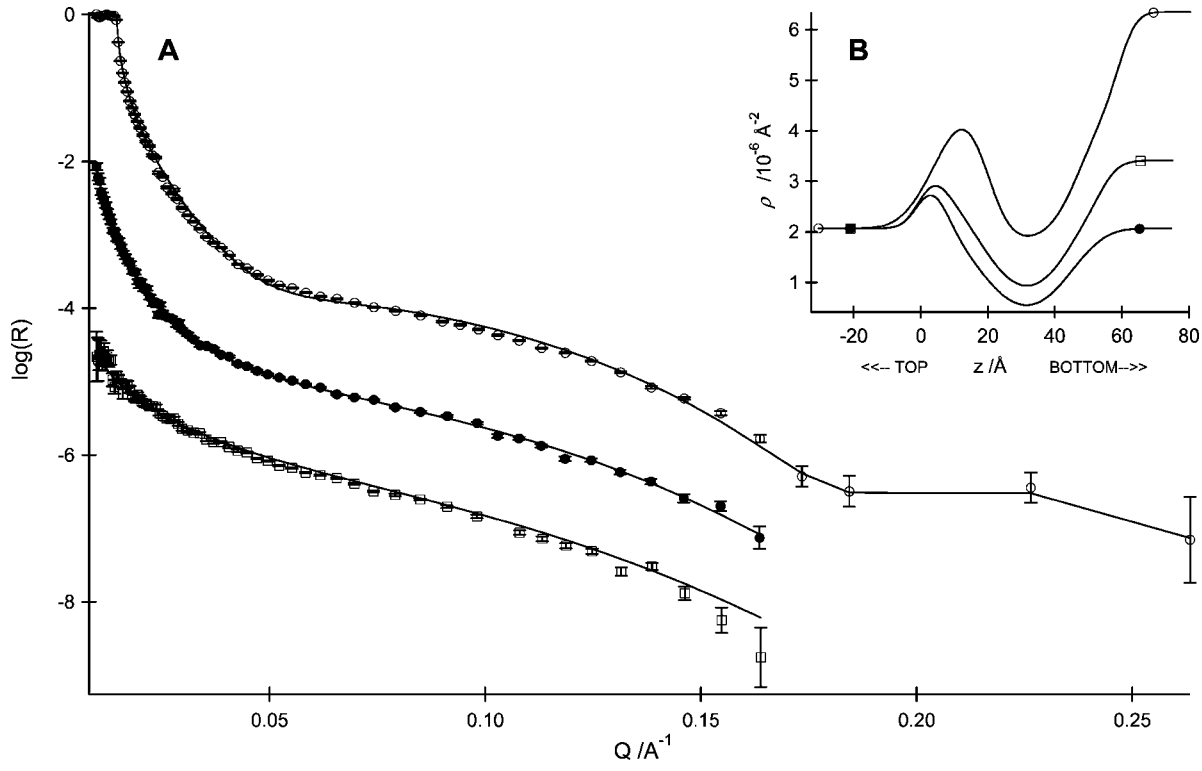

Figure 3. DOPC bilayer containing 20% POPS deposited at 20 mN/m. (A) Reflectivity data with D_2O (○), SiMW (●), and SiO_2MW (□) solvents. For clarity, the reflectivity curves are offset by −1 on the *y* axis. (B) SLD profile used to fit the data.

Furthermore, because the curves do not have any sharply defined features, it is prudent to fit the data with the least number of layers possible.

As expected, the solvent content of the chains is lower than that of the headgroup regions because it is determined by the percent coverage of the block by the bilayer. In the case of the two blocks, coverages were found to be 63% and 73% for the 10% PS and 20% PS blocks, respectively.

After full characterization of the bilayers using SNR, cyt c (dissolved in a 10 mM NaCl solution in D_2O) was injected into the sample cell, and the time course of the interaction was monitored by taking measurements at only one angle, namely,

Table 3. Parameters Derived from Fitting of the Bilayers Composed of Different Molar Mixtures of POPS with DOPC in the Presence of 10 mM NaCl

layer	oxide	heads[c]	chains	heads
(A) 10% POPS/90%DOPC Deposited at 20 mN/m				
thickness (Å)	7.4 ± 0.7	16.0 ± 1.1	29.3 ± 0.7	10.5 ± 0.7
SLD (10^{-6} Å^{-2})	3.41	1.75	−0.24	1.91
% water	0	50 ± 3	37 ± 1	60 ± 5
roughness (Å)	5.0 ± 1.2[b]			
(B) 20% POPS/80% DOPC Deposited at 20 mN/m				
thickness (Å)	7.5 ± 1.2	14.2 ± 2.8	26.0 ± 1.1	9.0 ± 1.2
SLD (10^{-6} Å^{-2})	3.41[a]	1.75[a]	−0.23[a]	1.83[a]
% water	0[a]	58 ± 12	27 ± 3	60 ± 4
roughness (Å)	7.5 ± 1.2[b]			

[a] These parameters were calculated and fixed during the fitting. [b] It was assumed that the roughness of each layer was the same. The roughnesses of all layers were linked during the fitting procedure in order to reduce the number of fitted parameters and decrease the variability of the fit. [c] The inner headgroup region includes the water layer between the lipid bilayer and the silicon substrate.

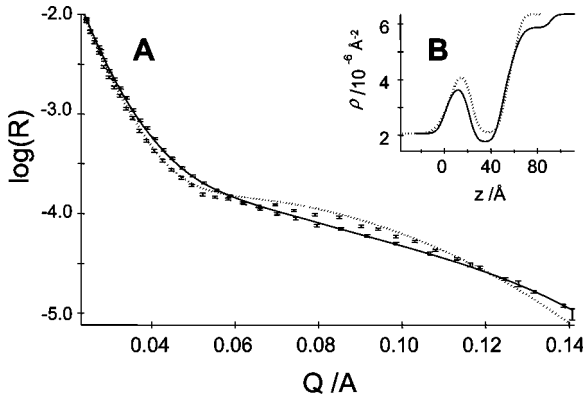

Figure 4. DOPC bilayer containing 10% POPS deposited at 20 mN/m. (A) Reflectivity data and (B) SLD profiles for the bilayer before (•••) and after (—) cyt c addition. The solvent used was D_2O.

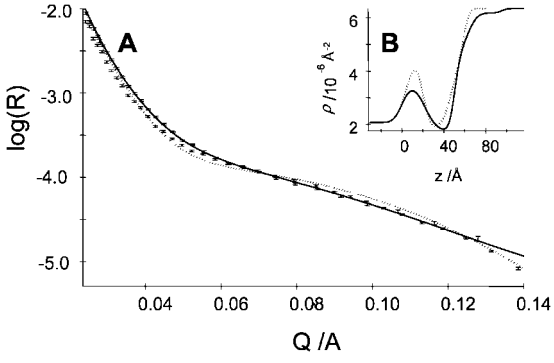

Figure 5. DOPC bilayer containing 20% POPS deposited at 20 mN/m. (A) Reflectivity data and (B) SLD profiles for the bilayer before (•••) and after (—) cyt c addition. The solvent used was D_2O.

0.8° every 20 min. The addition of cyt c resulted in an almost immediate change (i.e., within the first 20 min) in the reflectivity curves (Figures 4 and 5 for the bilayers containing 10% POPS and 20% POPS, respectively). Further measurements over 80 min, and in one case after 3 h, did not show any further alteration of the reflectivity curve, suggesting that the interaction was complete upon first contact.

The reflectivity profiles in the presence of cyt c for both bilayers containing 10% POPS and 20% POPS at 20 mN/m could not be satisfactorily modeled using the four-layer model that fit the bilayers in its absence; instead, a five-layer model was found to provide the best fit for the NR curves of bilayers with cyt c (Table 4). The 15 mN/m bilayer can be fit with a very similar model. The parameters obtained from modeling the bilayer containing 10 and 20 mol % POPS when deposited at 20 mN/m are shown in Table 5. In this model, the fifth layer had a thickness that closely matched the thickness of a single cyt c protein,[6] namely, 30 Å. The characterization of a bare substrate after incubation with cyt c (Table 5) also revealed that a protein layer of 30 Å was formed adjacent to the silicon oxide. Interestingly, the majority of the protein remained on the bare surface of the silicon block after rinsing.

In addition to the extra layer adsorbed onto of the bilayer, it was possible to model the bilayer only by decreasing the SLD of the hydrophobic chain and the headgroup regions and in particular the outer headgroups, suggesting a displacement of some of the water in the bilayer due to the penetration of cyt c into the bilayer. However, regardless of the molar ratio of POPS present, the amount of cyt c contained within the bilayer was greatest toward the outer leaflet of the bilayer. This observation is consistent with the fact that the bilayer is asymmetric and the outer leaflet contains the anionic lipid, which will interact with the positively charged cyt c. It is worth commenting that for all bilayers the inner headgroup region contains ∼40% solvent whereas the outer headgroup region contains ∼30% solvent.

Table 4. Parameters Derived from Fitting Bilayers Composed of Different Molar Mixtures of POPS with DOPC in the Presence of 10 mM NaCl after Incubation with Cytochrome c for at Least 20 Minutes

layer	oxide	heads[c]	chains	heads	protein
(A) 10% POPS/90% DOPC Deposited at 20 mN/m					
thickness (Å)	7.4[a]	13.8	27.4	10.4	32.3
SLD (10^{-6} Å^{-2})	3.41[a]	1.75[a]	−0.24[a]	1.91[a]	3.54[a]
% water[b]	0[a]	40	21	29	83
% cyt c[b]	0[a]	10	16	30	13
roughness (Å)	5.0[a]	5.0[a]	5.0[a]	5.0[a]	7.1
(B) 20% POPS/80% DOPC Deposited at 20 mN/m					
thickness (Å)	7.5[a]	14.0	25.1	9.7	36.6
SLD (10^{-6} Å^{-2})	3.41[a]	1.75[a]	−0.23[a]	1.83[a]	3.54[a]
% water[b]	0[a]	47	9	35	85
% cyt c[b]	0[a]	17	18	25	15
roughness (Å)	7.5[a]	7.5[a]	7.5[a]	7.5[a]	10.6

[a] These parameters were fixed during the fitting. [b] Calculated from the fitted SLD of the layer. [c] The inner headgroup region includes the water layer between the lipid bilayer and the silicon substrate. [d] Because only one contrast was used, no error was calculated for the fit, and the χ^2 value was used to determine the goodness of fit.

Table 5. Model Parameters Derived from Fitting the Bare Block without Any Lipid before and after Incubation with Cytochrome c (10 mM NaCl) for at Least 20 Minutes

layer	oxide
Bare Silicon Substrate	
thickness (Å)	16.6±0.5
SLD (10^{-6} Å^{-2})	3.41[a]
% water [b]	0[a]
roughness (Å)	7.1±0.4

layer	oxide	protein
Bare Silicon Substrate in the Presence of cyt c		
thickness (Å)	16.6[a]	29.4 ± 0.6
SLD (10^{-6} Å^{-2})	3.41[a]	2.10
% water[b]	0[a]	63 ± 3
% cyt c [b]	0[a]	37 ± 3
roughness (Å)	7.1[a]	8.7 ± 0.5

[a] These parameters were fixed during the fitting. [b] Calculated from the fitted SLD of the layer.

This difference is likely due to the fact that the model used assumes that the inner headgroup region includes the layer of water between the bilayer and the silicon substrate.

After measurement of the bilayer with cyt c, the cell was flushed with 10 mM NaCl in D_2O using three times the total cell volume, and the SNR measurements were repeated. D_2O solvent was then replaced with SiO_2MW, also containing 10 mM NaCl. Both of these measurements gave reflectivity profiles that indicated that whereas most of the cyt c had been washed off a small amount (~6% by volume) remains bound to the surface of the bilayer and about one-third remains in the bilayer, with the majority of this close to the silicon surface. Significantly, however, the structure of the bilayer remained intact throughout the solvent-exchange process.

Discussion

The time course and nature of the interaction of cyt c with monolayers and bilayers containing the anionic lipid POPS were studied using Langmuir trough and neutron reflectivity studies. In the Langmuir trough experiments, it was obvious that whereas cyt c interacts with monolayers containing 20 mol % POPS it appears to do so preferentially at low pressures. However, monitoring interactions using changes in surface pressure can be misleading, as is made clear in the present study. From the combined use of Langmuir trough experiments and SNR studies performed on monolayers and bilayers prepared at low surface pressures, it is clear that cyt c interacts with the membranes differently at different pressures, although at all pressures it appears to attach to the exterior of the negatively charged membrane, a phenomena that is not unreasonable for a positively charged peripheral membrane protein. The differences in the location of cyt c at the different surface pressures are probably a consequence of the differences in density of the negatively charged POPS headgroups. The conclusions of the present study are in agreement with those of Demel et al.,[19] who used radiolabeled cyt c to prove its presence below a monolayer of POPS at higher surface pressures despite no change in surface pressure being observed by Langmuir trough experiments.

Maierhofer et al.[20] used SNR of monolayers containing the anionic lipid dimyristoylphosphatidylglycerol at surface pressures of 30 mN/m and above to show that cyt c bound to the negatively charged lipid. In this study, the thickness of the additional cyt c-containing layer was 27 ± 2 Å, which is similar in thickness to the diameter reported for cyt c.[6] In the monolayer study reported here, the thickness of the cyt c-containing layer was found to be ~15 Å. Although only one contrast was used in the present study, it is pertinent to note that in the studies reported by Maierhofer et al.[20] the experiments were also performed at higher surface pressures (i.e., 30 mN/m as opposed to the 10 mN/m used in the present study). Because of the lower surface pressure (and the consequent lower density of negatively charged POPS head groups) in the present study, it is more likely that cyt c changes conformation, unfolds, or partially penetrates into the headgroup region of the monolayer. At higher surface pressures, the higher density of the negatively charged headgroups means that it is more likely that cyt c adsorbs onto the exterior surface of the monolayer.

The structures of deposited single bilayers of mixed DOPC/POPS were found by SNR, and the effect of the adsorption of cyt c to these bilayers was determined. It was found that bilayers containing either 10 or 20 mol % POPS are similar in structure. As expected, the thickness of the outer headgroup was larger compared to that of a pure zwitterionic phosphorylcholine head alone as a result of the presence of the negatively charged phosphorylserine groups,[21] but it was not possible to resolve the structure of the inner headgroup from the layer of water between the bilayer and the silicon substrate. The thickness of the hydrocarbon chain region was relatively small as a result of the unsaturated nature of the hydrophobic chains.

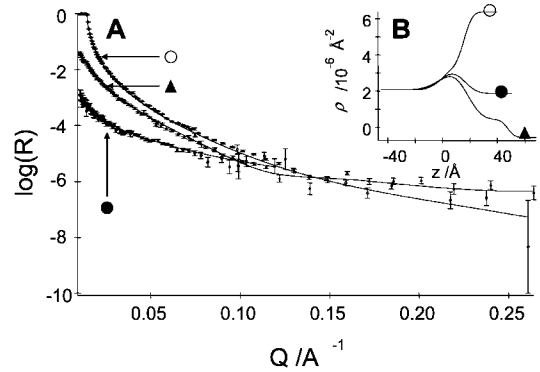

Figure 6. Bare silicon substrate. (A) Reflectivity data with D_2O (○) and SiMW (●) and after cyt c addition in H_2O (▲). (B) Corresponding SLD profiles.

The thickness of the mixed palmitoyl/oleoyl chain region was found to be <30 Å, which is consistent with a published study where the thickness of the palmitoyl chain region of bilayers was reported to be 32 ± 2 Å.[17] The effective length of a saturated hydrocarbon tail in its fluid phase can be approximated as 80% of its extended length (D_c)

$$D_c = 1.5 + n \times 1.265 \text{ Å}$$

where n is the number of carbon atoms.[22] From this equation, for palmitoyl the expected D_c was calculated to be 18.4 Å, and for oleoyl it was 17.3 Å,[22] giving ~37 Å as the maximum length of the hydrocarbon region of the bilayer. The model-fitted length of the hydrocarbon region in the POPS/DOPC bilayers was found to be <30 Å, indicating tilt or overlap of the chains.

The addition of cyt c resulted in changes in the reflectivity profile of the bilayers. The results reported here suggest that cyt c forms a heavily hydrated layer of protein attached to the outer surface of the bilayer and penetrates into the bilayer, most probably penetrating into water-filled "gaps" in the bilayer formed by the relatively low surface coverage. Furthermore, there was little difference found in the amount of adsorbed cyt c and the thickness of the bilayers with different amounts of POPS present. The pressure at deposition also had very little effect on the structure of the bilayers (data not shown).

For the two POPS-containing bilayers deposited at 20 mN/m, the thicknesses of the layers containing cyt c were found to be 32 and 36 Å, slightly larger than the diameter of cyt c (31 Å) as found by X-ray crystallography.[6] In addition, in the presence of cyt c there was a decrease in the SLD of all layers of the bilayer, which may be interpreted as solvent displacement by cyt c. It is possible that this incorporated cyt c is binding specifically to POPS but, as seems likely from the calculated coverage, it may also be filling in the water gaps in the bilayer caused by incomplete coverage. Note here that most of the bound cyt c is confined to the outer leaflet of the bilayer, which would suggest

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that cyc c is binding specifically to POPS because the model bilayer was produced in such a way as to contain all of the anionic lipids in its outer leaflet.

The use of supported bilayers allowed us to examine the tightness of the association of cyt c with POPS, something not readily possible with monolayer studies. The flow of the 10 mM solvent through the cell was seen to cause the partial detachment of cyt c from the bilayers, suggesting that the binding of cyt c to the model bilayers is partially electrostatic. Most of the cyt c that remained after rinsing was found to be close to the substrate. Interestingly, the layer of cyt c formed on a bare block was also difficult to wash off, with only about 30% being removed.

Although the location of cyt c in membranes containing cardiolipin has been studied using a variety of techniques,[23,24] to date there have been no studies reported on the interaction of cyt c with membranes containing POPS. Because of the emerging role of cyt c/POPS in the clearance of apoptotic cells, it is important to determine how cyt c interacts with POPS. Using this bilayer system, it will be possible to study the conditions under which the signaling pathway for cell clearance takes place.

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