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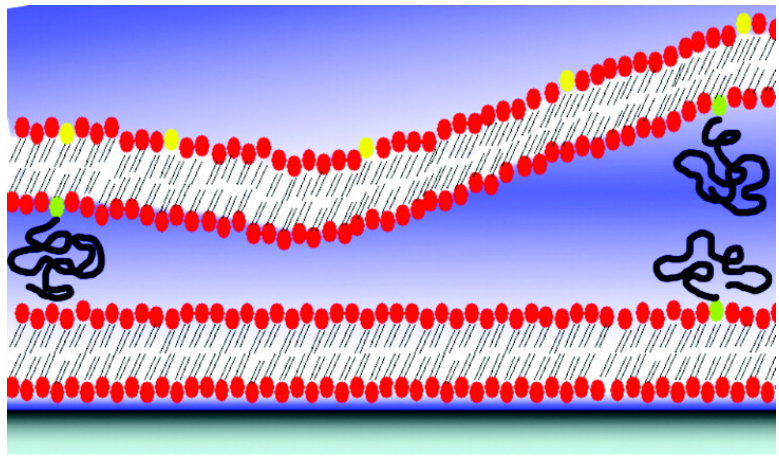

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Interaction of Cationic Lipoplexes with Floating Bilayers at the Solid−Liquid Interface†

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Neutron reflection has been used to study the interaction of cationic lipoplexes with different model membrane systems. The model membranes used are prepared as "floating" phospholipid bilayers deposited at a silicon/water interface and separated from the solid substrate either by an adsorbed phospholipid bilayer, polymer cushions composed of polyethylene glycol lipids, or a lipid monolayer adsorbed onto a chemically grafted hydrocarbon layer. The cationic lipoplexes studied are those formed by the complexation of calf thymus DNA with dimethyl-dioctadecylammonium bromide (DDAB), with either cholesterol or dioleoyl-L-α-phosphatidylethanolamine (DOPE) incorporated as "helper" lipid. The cationic lipoplexes are found to destroy three of the four types of (negatively charged) floating bilayers, with the rate of destruction dependent on the nature of the layer separating the floating bilayer from the silicon substrate. The only bilayers to remain intact after exposure to the lipoplexes were those fabricated above the chemically grafted (octadecyl) hydrocarbon layer. This supports the hypothesis that the high negative charge density of the SiO_2 layer on the silicon surface may influence, by way of electrostatic interaction with the cationic lipid, the interaction of the lipoplexes with the model bilayer. It is concluded that the floating bilayer supported on a chemically grafted hydrocarbon layer lends itself perfectly to the study of lipoplex−membrane interactions and, with sufficient exposure time, would allow a detailed characterization of the structures formed at the membrane interface during the interaction.

Introduction

The recent increase in biophysical studies of membranes, in particular, studies employing surface-specific detection techniques such as scanning probe, spectroscopic, or scattering methods, has spawned a revival of interest in the use of simple lipid bilayers as membrane models.[1−5]

Since Tamm and McConnell first reported model membranes prepared by the deposition of bilayers directly onto solid supports in 1985,[6] there have been a variety of other systems developed, including stacks of bilayers spin coated onto solid supports[7,8] or formed by solvent evaporation,[9] and single bilayers formed by a combination of Langmuir−Blodgett[10] and Langmuir−Schaefer[6] deposition, by the adsorption and fusion of vesicles from an aqueous solution,[11,12] or by a combination of Langmuir−Blodgett deposition and vesicle fusion.[13] Most recently, phospholipid bilayers have been deposited on silicon supports by coadsorption with a soluble surfactant.[14,15]

In the study of interactions taking place at the surface of such model membranes, the technique of neutron specular reflection[16−18] offers particular advantages: (1) the neutrons can penetrate condensed matter to probe buried interfaces, (2) they are scattered by atomic nuclei (rather than electrons) and so interact more strongly (than X-rays) with the elements most common in biological samples (viz., C, H, N, and O), which in turn means that the intensity of the incident radiation required is much lower so that sample degradation is less of a concern. The technique is particularly sensitive to the different scattering properties of hydrogen and deuterium, and this allows an experimental system to be designed specifically to enhance the site of investigation through deuterium labeling and contrast variation. The initial structure of the bilayer can be probed in detail using solvent contrast variation,[19,20] and then changes that result from alterations in temperature, pH, or ionic strength can be measured. With a similar strategy, it is also possible to observe the penetration and location of protein molecules in the bilayer and/or measure the modifications to the bilayer structure.[19,21] In the area of gene delivery, lipid exchange has previously been monitored during the reaction of hydrogenous cationic lipid

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vesicles[22] and hydrogenous lipoplexes[23] with supported deuterated phospholipid bilayers.

In all of the studies noted above, the model membranes that were used comprised bilayers directly adsorbed to a solid support, and the problem with these (single) bilayer systems is that they are localized close to the surface of a substrate and this constraint affects their hydration and restrains their flexibility. Given that the dynamic fluidity of cell membranes is critical to their biological function, this loss of freedom in an adsorbed bilayer significantly reduces its suitability as a model for probing membrane interactions. In the search for more appropriate model systems, therefore, researchers have looked to the use of bilayer stacks in which the adjacent bilayers are separated by a layer of water, giving a supported but unconstrained system that provides a better approximation of a cell membrane. The simplest characterizable stacked system is one of two bilayers, the first being adsorbed to the solid support and the second "floating" above the first. Charitat et al.[24] produced the first double bilayer system of this type using 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) or 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) transferred onto a hydrophilic silicon substrate using three Langmuir−Blodgett depositions followed by a Langmuir−Schaefer deposition. The phase behavior[20] and lipid packing[25] of the upper bilayer in such systems have been investigated using neutron reflection, with results suggesting that the behavior of the upper bilayer is consistent with that of a "free" system. The packing parameters of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) in the upper bilayer of a supported double bilayer were determined to be similar to those of vesicle systems.[25] In this case, an octadecyl-trichloro-silane (OTS)-coated substrate had to be used to allow for the formation of the floating DMPC bilayer because the previously described method[24] is not very efficient with short-chain phospholipids.

Alternative approaches to solving the problem of the constrained adsorbed bilayer have seen the incorporation of polymer cushions to separate the bilayer from the solid support. To decouple the bilayer and membrane proteins from their solid support, there have been numerous proposed spacers, including chemically modified lipids covalently bound to the surface,[26] polyethyleneimine (PEI),[27] the polysaccharide dextran, cellulose,[28] polyacrylamide,[29] agarose, chitosan,[30] and polyethylene glycol (PEG).[31] These various systems have seen only limited success, however, and it is often found that the bilayers are patchy and exhibit numerous defects.

In the work reported here, the aim has been to investigate new model bilayers suitable for studying the interaction of cationic gene-delivery vectors with membranes using neutron reflection. Callow et al.[23] investigated similar systems but used a single model bilayer directly adsorbed onto a hydrophilic silicon surface where a native oxide layer was present. The negative charge density of this silicon oxide layer is a concern when investigating cationic lipid complexes because of possible electrostatic interactions. Callow et al. used the model bilayer to investigate the role of neutral helper lipids included in the vectors.[22,23]

The first two model systems investigated here were double bilayers of the type prepared by Charitat et al.,[24] made from deuterated phosphocholine lipids. These two systems were prepared from d_{62}-DPPC or d_{83}-DSPC, with 10% alkyl chain deuterated 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylserine (d_{62}-DPPS) incorporated in the outermost bilayer leaflet (to provide a negatively charged outer surface to mimic a biological membrane). Two novel model systems were also investigated in which the distance between the bilayer and the solid substrate surface was increased by using a polymer cushion as a spacer. The first of this type of model was fabricated with a single bilayer separated from the substrate by a polymer cushion, and the second was fabricated with a double bilayer, with the polymer cushion separating the two bilayers. PEG polymer cushions appeared to present a suitable system to be used in the present study.[31] An added incentive to using a polymer spacer was to mimic the intracellular compartment of cells. Any lipoplexes crossing the outer bilayer should be detected by the change in scattering-length density to the polymer layer. PEG lipids of 2000 and 750 molecular weight were used with volume fractions of PEG of around 4%.

The final model system investigated here, again with the aim of minimizing the effect of the negative surface charge on the silicon substrate while maintaining the classical double bilayer model, incorporated a covalently bound hydrocarbon layer on the silicon block chemically grafted to the block using octadecyltrichlorosilane (OTS). The trichlorosilyl head groups of OTS react with the surface hydroxyl groups forming siloxy bonds, producing a hydrocarbon film with densely packed, extended chains. Once formed, the OTS film is very hydrophobic, and a monolayer of lipids can be deposited by lowering the substrate down through a surface monolayer (Langmuir−Blodgett deposition). Hughes et al.[25] reported the first use of an OTS self-assembled monolayer (SAM) in a double-bilayer system, prompted by the identification of some limitations in the fabrication procedure of double bilayers. Upon this hydrophobic base, three bilayer leaflets were deposited to form a hybrid double bilayer.

The intended architectures of the four model membrane systems described above are depicted in Figure 1.

Experimental Section

Materials. All chemicals used were obtained commercially and were used as received. Dimethyl-dioctadecyl-ammonium bromide (DDAB, purity >98%), cholesterol (Chol, purity >99%), dioleoyl-L-α-phosphatidylethanolamine (DOPE, purity >99%), solvents (chloroform, acetone, and ethanol; purity >99%), and octadecyl trichlorosilane (OTS, purity >+90%) were supplied by Sigma (Dorset, U.K.). 1,2-Dipalmitoyl-d_{62}-*sn*-glycerol-3-phosphocholine (d_{62}-DPPC, >98% deuterium purity), 1,2-dipalmitoyl-d_{62}-*sn*-glycero-3-[phospho-L-serine] (d_{62}-DPPS, >98% deuterium purity), 1,2-distearoyl-d_{70}-*sn*-glycero-3-phosphocholine-1,1,2,2-d_4-*N*,*N*,*N*-trimethyl-d_9 (d_{83}-DSPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (*h*-DSPC, purity >99%), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-750] (PEG750-DSPE, purity >99%), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (PEG2000-DSPE, purity >99%) were purchased from Avanti Polar Lipids (Alabaster, AL). Dimethyl-d_{74}-octadecylammonium bromide (d_{74}-DDAB) was purchased from QMX Chemicals. DNA sodium salt from calf thymus (the molecular weight is reported to be between 10 and 15 million Da) verified to be protein-free (by UV spectroscopy) was purchased from Sigma-Aldrich. D_2O (>99%) was provided by the Institut Laue-Langevin.

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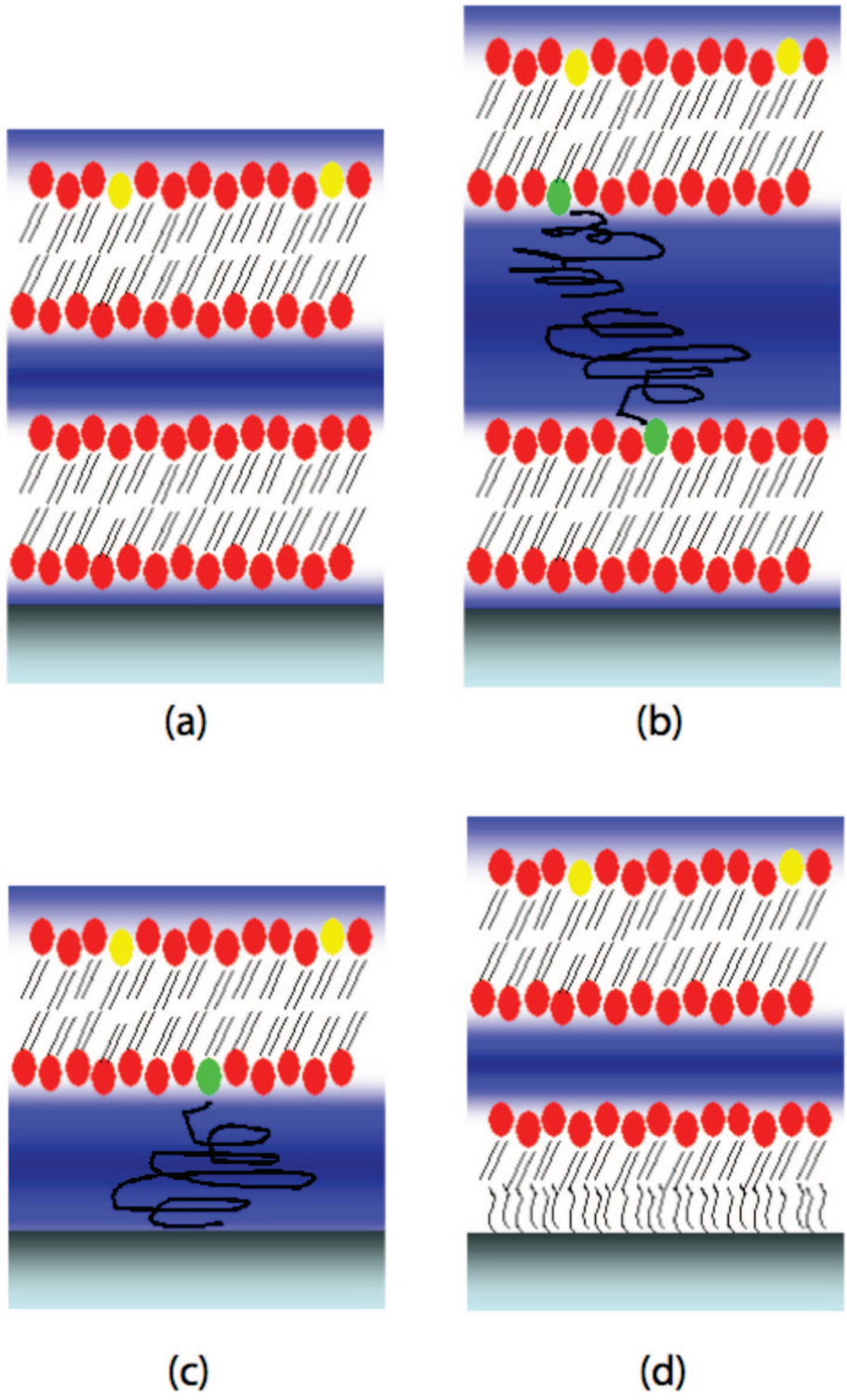

Figure 1. Graphical depiction of model bilayers: (a) Double bilayer, (b) PEG-cushion double bilayer, (c) PEG-cushion single bilayer, and (d) OTS-hybrid double bilayer. Solid circles are the phospholipid head groups PC (red), PS (yellow), and PE (green). Thin lines are alkyl chains; thick lines are PEG.

Contrast-match solutions were prepared by mixing D_2O and H_2O in the appropriate ratios to match the scattering-length densities of Si (CMSi: 38.1 vol % D_2O) and SiO_2 (CMSiO_2: 58.3 vol% D_2O) and the intermediate value of 4.13×10^{-6} Å^{-2} (CM4.13: 67.9 vol % D_2O).

Preparation of Solid Substrates. Silicon blocks with dimensions of $80 \times 50 \times 10$ mm^3, highly polished on one side by Siltronix Ltd. (Annemasse, France), were thoroughly cleaned before use by sequential bath sonication in the following solvents: chloroform, acetone, and ethanol. The smooth face of silicon has a native oxide layer of ~15 Å. A proven protocol for rendering the silicon surface hydrophilic was used immediately prior to lipid deposition. It included exposure to ozone for 30 min, produced by flowing oxygen in a UV chamber.[32]

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A SAM from OTS was directly chemisorbed to the silicon oxide surface. To prepare the OTS SAM layer, first the silicon block was prepared as described above. After UV/ozone exposure, the silicon substrate was sonicated for 15 min in H_2O and then thoroughly dried in an oven set at 50 °C for several hours. It was imperative to keep all glassware dry to prevent unspecific polymerization. In a nitrogen-filled glovebox, the substrates were submerged in a solution of 10 μL OTS in 100 mL of dodecane. The reaction was left to proceed for 12 h, after which time the substrates were ultrasonically cleaned in ethanol and then H_2O.[25] The layer grafted to the surface of the block was subsequently used in preparation of a double bilayer as described below, with the lipid deposition commencing from the point of the first downward Langmuir−Blodgett dip.

Deposition of Phospholipid Layers. A Langmuir trough was thoroughly cleaned before use with chloroform, followed by copious rinsing with Millipore water. The trough was filled with UHQ water, and the barriers were closed completely to verify the absence of any surface-active molecules. The first leaflet of a bilayer to be transferred to a hydrophilic silicon substrate requires an upward Langmuir−Blodgett deposition. The substrate was therefore lowered into the subphase of the Langmuir trough before a monolayer of the desired lipid composition was formed at the air/liquid interface. The lipid, which had been dissolved in chloroform, was added dropwise to the subphase surface using a Hamilton microsyringe. The solvent was allowed to evaporate before the monolayer was compressed and maintained, throughout the entire deposition process, at 40 mN/m. The silicon substrate was then raised, lowered, and raised again using a computer-controlled Nima dipper at a rate of 5 mm/min to complete the three Langmuir−Blodgett depositions (for the model shown in Figure 1a). The fourth and final (Schaefer) deposition was performed using a manual in-house dipper, with speeds of ∼0.2 μm/s and horizontal-level adjusters. The block was lowered through the lipid monolayer until it reached the Teflon sample cell, where it was clamped in the sample holder.[20]

Two samples were fabricated with different numbers of ethylene oxide (EO) subunits in the PEG chains grafted to the DSPE. PEG2000 (MW = 2000 g mol^{-1}) has 45 EO subunits, and PEG750 (MW = 750 g mol^{-1}) has 17 EO subunits. The samples were fabricated either by depositing a layer of *h*-DSPC using the Langmuir−Blodgett technique followed by two layers of 24:1 *h*-DSPC/*h*-DSPE-PEG and a fourth layer of 9:1 *h*-DSPC/*h*-DSPS using the Langmuir−Schaefer technique (for the model shown in Figure 1b) or by depositing a layer of 49:1 d_{83}-DSPC/*h*-DSPE-PEG (2000MW) using the Langmuir−Blodgett technique followed by a layer of 9:1 d_{83}-DSPC/d_{70}-DSPS using the Langmuir−Schaefer technique (for the model shown in Figure 1c). For the preparation of the final model bilayer (Figure 1d), an OTS layer was prepared (as described above), followed by two Langmuir−Blodgett depositions and a final Langmuir−Schaefer deposition.

Fabrication of Gene-Delivery Complexes. Vesicles were first prepared by hydrating thin films of DDAB/cholesterol (1:1) and DDAB/DOPE (1:1) in the required solvent (H_2O or D_2O) and were subsequently reduced in size by extrusion 49 times through polycarbonate filters (100 nm pore size) at 50 °C. Lipoplexes were prepared by admixing equal volumes of lipid and calf thymus DNA stock solutions such that the final concentration of DDAB was 0.1 or 0.01 mg/mL, with a cationic lipid/DNA (±) charge ratio of 1:1.

Neutron Reflectivity Measurements. Specular neutron reflectivity is widely used for the investigation of buried interfaces, allowing the determination of the structure of matter perpendicular to a surface or an interface. Measurements can be performed in situ, and only very small amounts of material are required. The technique is very sensitive to the thickness of the layer, the composition, and the roughness of the interfaces. Specular reflectivity, defined as the ratio between the reflected and the incoming intensities of a neutron beam, is measured as a function of the wave vector transfer, $Q = (4\pi/\lambda)$ sin θ (where λ is the wavelength and θ is the angle of the incoming beam with respect to the surface), and is proportional to the Fourier transform of the first derivative of the scattering-length density (SLD). Using H/D-substituted molecules can increase or decrease the contrast—the difference in scattering-length density—between layers to highlight specific layers. Further details on the technique, data analysis, and applications can be found in refs 16 and 18 and references therein.

Measurements were performed on the high-flux D17 reflectometer at the Institut Laue-Langevin (Grenoble, France) in time-of-flight mode using a spread of wavelengths between 2 and 20 Å with two angles of incidence of 0.7 and 3° to provide a Q range of 0.0077 to 0.3 Å^{-1}.[33]

The model bilayers were initially characterized in three or four solvent contrasts, each of different scattering-length density determined by the D_2O/H_2O ratio: (i) D_2O ($\rho = 6.35 \times 10^{-6}$ Å^{-2}) offered maximum contrast with the hydrogenated lipids, (ii) $CMSiO_2$ ($\rho = 3.41 \times 10^{-6}$ Å^{-2}) contrast matched the silicon oxide layer at the surface of the silicon substrate, and (iii) CMSi ($\rho = 2.07 \times 10^{-6}$ Å^{-2}) and (iv) H_2O ($\rho = -0.56 \times 10^{-6}$ Å^{-2}) offered maximum contrast with the deuterated lipids. When changing the contrast, the subphase was washed through with a 3-fold excess in volume of the new solvent, using a peristaltic pump with a flow rate of 2 mL/min. The gene-delivery complexes were added to and removed from the subphase using the same technique.

Analysis of Reflectivity Data. Lipid molecules can normally be split into two regions of rather different scattering-length density: the alkyl chains and the head group. When considering the lipid arrangement within a bilayer, splitting the molecules into two regions of different scattering density results in three layers: heads, chains, and heads. A model of each bilayer was constructed that was parsed into a series of parallel layers of homogeneous material. The reflectivity profiles were then model fitted following an optical matrix formalism, with each layer characterized by a scattering-length density (SLD), thickness, and interfacial roughness.[34] The scattering-length densities were fixed using theoretical values (Table I in Supporting Information) whereas the thickness and roughness of the layers were varied within physically realistic limits until the calculated reflectivity profile matched the measured profile. The data furnished through multiple contrast measurements were analyzed using the simultaneous fitting utilities of the program Motofit.[35]

Results and Discussion

Bilayers from d_{62}-DPPC: Characterization. Double bilayers prepared using chain-deuterated d_{62}-DPPC were successfully fabricated (model depicted in Figure 1a). The first three Langmuir−Blodgett depositions were made using only d_{62}-DPPC, and only the final Langmuir−Schaefer deposition was made using 9:1 d_{62}-DPPC/d_{62}-DPPS.

The reflectivity profile from a d_{62}-DPPC double bilayer was initially measured in H_2O, the solvent in which it was fabricated at 25 °C, to establish whether the depositions had been successful. The measured reflectivity profile was fitted using a nine-layer model, varying parameters over fixed, physically plausible values to minimize the χ^2 value. The fitted reflectivity data with the corresponding scattering-length density profile for the fits are shown in Figure I of the Supporting Information. The model at 25 °C can be interpreted as two bilayers that are 53 ± 2 and 59 ± 3 Å thick, with roughness values of 4 ± 1 and 6 ± 1 Å, respectively, separated by a 15 ± 1 Å thick solvent layer and the lower bilayer separated from the silicon block by a 5 ± 1 Å solvent layer. The model parameters agree with literature values,[21,25,37] except for the thickness of the upper bilayer, which is 3−5 Å thicker than generally reported. However, the presence of 10% DPPS in the upper bilayer may be responsible for this increase in thickness, either directly by influencing packing within the membrane or indirectly via repulsive forces from the negative charge density on the substrate surface. Alternatively, the

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unusually high surface coverage, approaching 100%, of the upper bilayer, as indicated by the transfer ratio value obtained during the deposition may maintain the lipid packing configuration from the deposition at 40 mN/m, more than a bilayer with only 70−80% surface coverage because there is less lateral free space for layer expansion. The less than 80% surface coverage of the lower bilayer, compared to a more normal coverage of 95%, may explain the unusually high surface coverage of the upper bilayer because there is less lipid in the lower bilayer to shield the attractive forces between the substrate and upper bilayer.

The temperature of the sample was subsequently raised to 45 °C, above the $L_\beta-L_\alpha$ phase-transition temperature for d_{62}-DPPC to mimic the L_α phase of a biological membrane.

In the fluid phase, the melted alkyl chains have a reduced thickness. The position of the second minimum in the measured reflectivity profile is more sensitive to the thickness of the alkyl chains of the first bilayer than to other components whereas the amplitudes and sharpness of the minima depend upon bilayer roughness. The shift of the second minimum to higher Q after increasing the temperature and the reduction in the sharpness of the minimum reflect a decrease in the alkyl chain thickness and an increase in roughness, as would be expected for an $L_\beta-L_\alpha$ phase transition. The shift to lower Q of the first reflectivity minimum reflects the swelling of the water layer thickness. The alkyl chain thicknesses decrease to 30 ± 1 and 32 ± 1 Å for the upper and lower bilayers, respectively, and the solvent layers swelled to 9 ± 1 and 25 ± 1 Å. A similar reduction in the alkyl chain thickness and swelling of the solvent layers has been previously observed for $L_\beta-L_\alpha$ lipid-phase transitions in planar double bilayers.[38] Interestingly, the $L_\beta-L_\alpha$ phase transition does not affect the head-group layers. The statistical accuracy in the region of the second minimum is poor for the sample at 45 °C, and no additional measurements were made in different solvent contrasts. As a consequence, the model used to fit the reflectivity profile cannot be considered to be a unique solution. Double bilayers composed of *h*-DPPC are stable to at least 80 °C.[38] However, deuterated lipids behave differently and have lower T_m value,[39] and in light of the deposition difficulties with d_{62}-DPPC, in marked contrast to *h*-DPPC, it was deemed preferable to continue the experiment in the gel phase. A temperature of 37 °C was therefore chosen to enable the comparison with physiological temperature. The reflectivity was measured in two solvent contrasts at 37 °C, namely, H_2O and D_2O. The data were simultaneously fitted with the same model as used for the data at 25 °C, showing that cycling the bilayer through the phase transition had no effect on the integrity of the bilayer.

Bilayers from d_{62}-DPPC: Exposure to Lipoplexes. The model bilayer was exposed to lipoplexes formed by the complexation of calf thymus DNA with a 0.1 mg/mL *h*-DDAB-Chol vesicle solution to mimic the conditions used by Callow et al.[22,23] It was predicted that there would be an increase in amplitude of the first minimum in the reflectivity profile resulting from an increase in contrast between the upper bilayer and the solvent due to hydrogenous lipid from the lipoplexes exchanging with the deuterated lipid in the model bilayer. On the basis of experiments performed by Callow et al.[22,23] reporting lipid exchange between lipoplexes and a single lipid bilayer, the experimental conditions were chosen to reproduce similar observations in the double-bilayer system. However, the actual measured changes have been modeled as the removal of *d*-lipid from the upper bilayer, which was replaced by solvent. In light of the poor contrast in scattering-length density between the solvent and bilayer, a large change in composition was required before a significant difference in the scattering curve was observed. After 8 h of exposure to the lipoplexes, the difference between the time slice profile and the original profile was sufficiently different to justify modeling. Figure 2 shows the reflectivity profiles measured after 8 and 18 h of exposure and the scattering-length density profiles corresponding to the line fits (no additional measurements were made in between these time slices). After 8 h, the upper bilayer was successfully modeled to include an additional 60% solvent whereas after 18 h only ∼5% of the lipid in the upper bilayer remained. In the studies reported here, unlike the results of Callow et al.,[22] the lipoplexes had a destructive effect on the upper bilayer of this model system. Unfortunately, the low scattering contrast between the lipid bilayer and solvent did not permit the kinetics of this interaction to be characterized accurately.

After 18 h of exposure, the lipoplex solution was washed out of the subphase, and the reflectivity profiles for the model system were measured under four different solvent contrasts (H_2O, CMSi, $CMSiO_2$, and D_2O) to enable the remaining membrane structure to be characterized with a high level of confidence (see Figure II in Supporting Information). A four-layer model was found to be sufficient to provide a simultaneous fit to the reflectivity profiles measured in the four solvent contrasts, after the removal of the lipoplexes following the 18 h exposure period. A single bilayer of 59 ± 2 Å thickness was obtained, supporting the notion that the lipoplexes had destroyed the upper bilayer. The remaining lower bilayer was modeled in a way that supports the trends observed by Callow et al.[22,23] in that a reduction in the scattering-length density of the alkyl-chain layer was seen to represent a 15% exchange of DPPC for *h*-DDAB-Chol, assuming an equimolar transfer of *h*-DDAB and cholesterol from the lipoplexes to the supported bilayer. However, the scattering-length-density profiles suggest an unequal distribution of *h*-DDAB between the upper and lower leaflets of the supported bilayer with *h*-DDAB localizing in the lower leaflet where the positively charged head groups are electrostatically attracted to the SiO_2 layer. This results in the loss of the distinct solvent layer between SiO_2 and the bilayer.

Bilayers from d_{83}-DSPC: Characterization. The concept of the first model system was carried forward to create a second model, but this time with greater scattering contrast between the model bilayers and the solvent, providing conditions more conducive to elucidating the time course of the interaction.

When designing the novel bilayer models, bilayer stability was considered to be critical, and because fully deuterated d_{75}-DPPC double bilayers have often been found (unpublished data) to be unstable even in the absence of lipoplexes and with chain-deuterated d_{62}-DPPC double bilayers shown here to be destroyed in the presence of lipoplexes, our subsequent double-bilayer models were fabricated using d_{83}-DSPC rather than d_{62}-DPPC. This provided for more stable double bilayers and gave higher transfer ratios in their preparation.

The second model bilayer was fabricated by depositing three layers of d_{83}-DSPC using the Langmuir−Blodgett technique and then adding a fourth layer of 9:1 d_{83}-DSPC/d_{70}-DSPS using the Langmuir−Schaefer technique. The d_{83}-DSPC double bilayer was first characterized using the three solvent contrasts provided by the subphases of H_2O, CMSi, and D_2O. The data collected were simultaneously fitted using a nine-layer model, varying parameters over fixed, physically plausible values to minimize the χ^2 value. The fitted reflectivity data with the corresponding scattering-length-density profiles for the fits are shown in Figure III of the

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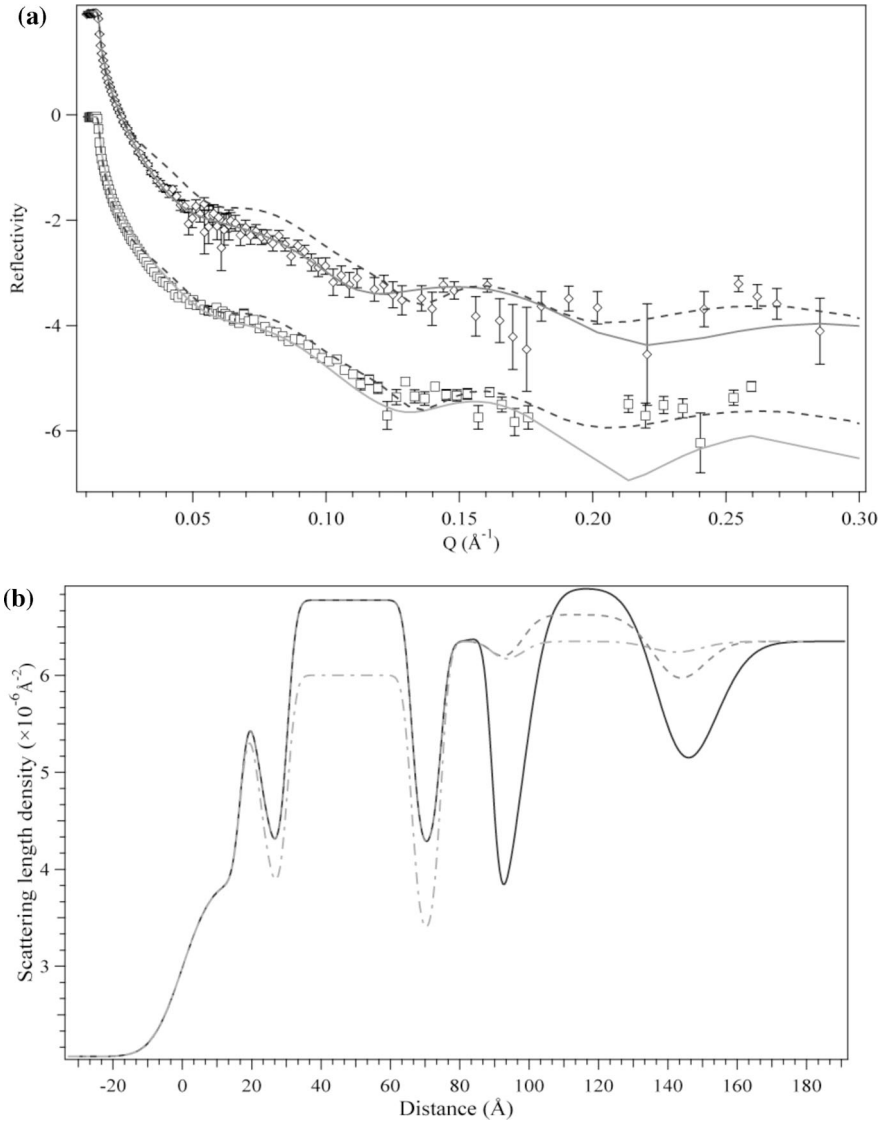

Figure 2. (a) Neutron reflectivity profiles and line fits from a d_{62}-DPPC double bilayer at 37 °C after 8 h (□) and 18 h (◊) of exposure to 0.1 mg/mL lipoplexes formed from DDAB/cholesterol. The dashed line is fit before exposure (data offset for clarity). (b) Scattering-length-density profiles corresponding to line fits before exposure (−), after 8 h (---), and after 18 h (-•-).

Supporting Information. This model can be interpreted as two bilayers 58 ± 2 and 56 ± 2 Å thick, separated by a 15 ± 1 Å thick solvent layer with the lower bilayer separated from the silicon block by a 3 ± 1 Å solvent layer. The scattering-length-density profiles show a high surface coverage following deposition, correlating with the measured transfer ratios of between 0.95 and 1.0.

Bilayers from d_{83}-DSPC: Exposure to Lipoplexes. The model bilayer was exposed to lipoplexes formed from a solution of 0.01 mg/mL *h*-DDAB-DOPE vesicles in H_2O. In contrast to the first model bilayer, here the concentration of lipoplex solution was reduced by a factor of 10 to slow the reaction. Furthermore, the reflectivity was measured in H_2O rather than D_2O to highlight *d*-lipid removal, and the time slices were measured in 1 h 45 min periods to obtain reasonable statistical accuracy, over a total exposure time of approximately 12 h. Measurements were performed at 25 °C.

The interaction of the lipoplexes with the double bilayer was modeled by sequentially adjusting the parameters for the different layers. It was not necessary to alter any parameter in the first five layers pertaining to the substrate, lower bilayer, and solvent gap. The changes to the reflectivity profile could not be modeled by the addition of layers to the model to represent the deposition of material from the lipoplexes forming new structures at the membrane interface. It was instead sufficient to model the data by a reduction in the scattering-length density of the upper bilayer. However, as Figure 3 shows, the reduction in the scattering-length density of the bilayer was not symmetrical between the upper and lower bilayer leaflets. The data at distances of between 80 and 150 Å on the *x* axis of Figure 3b represents the variation in the scattering-length density of the upper bilayer with exposure time. The asymmetrical nature of the reduction in the scattering-length-density profile over time seems to relate to the reduction in the amplitude of the second reflectivity minimum (Figure 3a). This reduction in amplitude could not be modeled adequately without treating the two leaflets of the upper bilayer independently. It is unlikely that the process of bilayer destruction is unsymmetrical because this would expose the hydrophobic alkyl chains to water, inducing lipid rearrangement. Rather, this suggests that some hydrogenous lipid from the lipoplexes could be mixing with the upper leaflet of the bilayer. Most of the reduction in the scattering-length density of

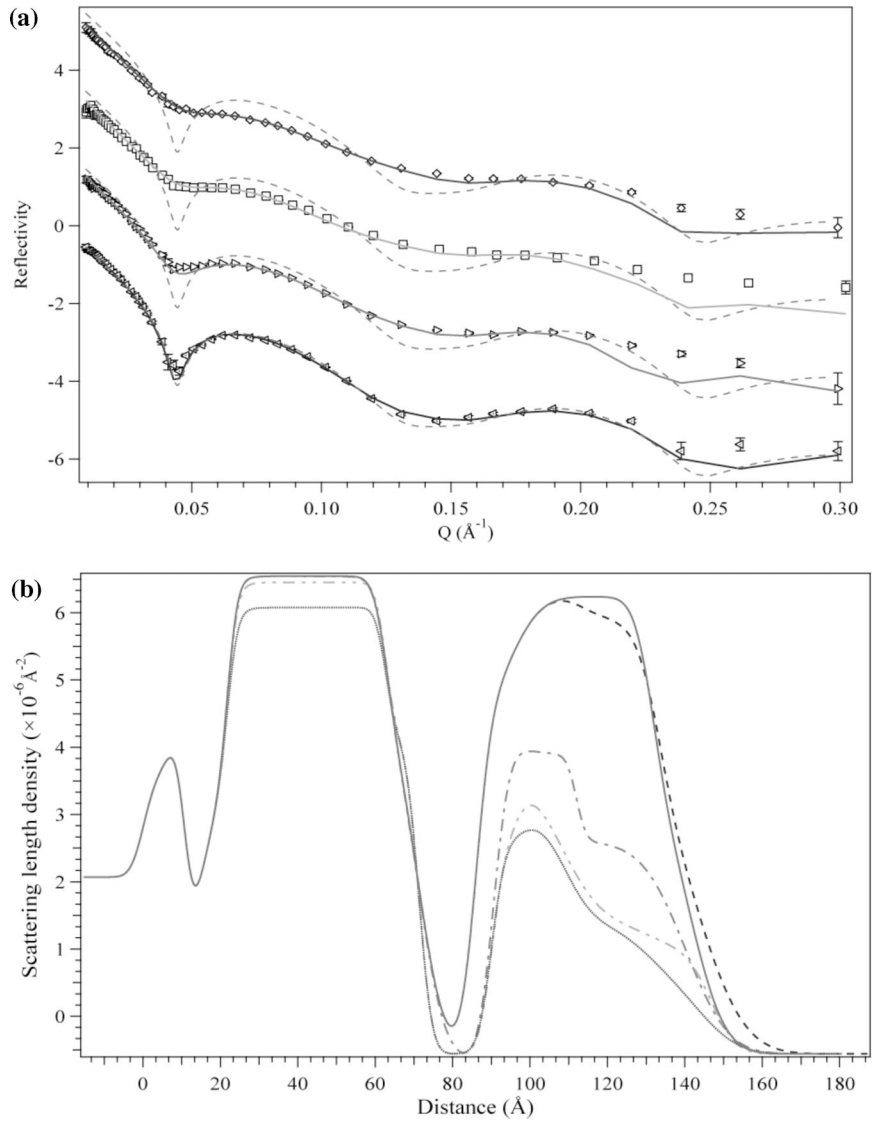

Figure 3. (a) Neutron reflectivity profiles from d_{83}-DSPC double bilayer in H_2O exposed to lipoplexes fabricated from 0.01 mg/mL DDAB−DOPE after 1 h (left-facing △), 5 h (right-facing △), 8 h 40 min (□), and 12 h 20 min (◇). The data are offset for clarity. The overlayed dashed line is the fit before exposure to the lipoplexes. (b) Scattering-length-density profiles corresponding to the line fits before lipoplex exposure (solid) and after 1 h (---), 5 h (-•-), 8 h 40 min (-••-), and 12 h 20 min (•••).

the model bilayer is attributable to the removal of lipid from the bilayer, replaced by solvent. Significant lipid mixing between the lipoplexes and the model bilayer could not be modeled to fit the data because the reduction in the scattering-length density of the head-group layers would be expected to decrease proportionally much less than that of the alkyl chains. This could not be modeled to fit the data, so significant bilayer destruction is likely to have occurred before the lipoplexes were washed out.

After 12 h of exposure of the membrane to the lipoplexes, the lipoplexes were washed out and replaced with D_2O, and the reflectivity of the final state of the double bilayer was measured. The use of this solvent contrast would have enabled the asymmetrical nature of the outer bilayer to be characterized in greater detail, given that any *h*-lipid from the lipoplexes located in the outer bilayer would give a high contrast with the solvent. However, it appears that the solvent exchange process removed the remains of the outer bilayer along with the lipoplexes. This observation is taken as evidence that the lipoplexes have a detrimental effect on the bilayer because the solvent in the sample was changed several times before the lipoplexes were added without any effect on the structure of the bilayer. The scattering-length-density profile (Figure IV of Supporting Information) corresponding to the line fit for the reflectivity profiles measured in D_2O shows that the reduction in the scattering-length density of the lower bilayer can be attributed to *d*-lipid for *h*-lipid exchange, consistent with the findings above.

Bilayers from PEG−Lipids. The PEG-cushion *h*-DSPC double bilayers (model 1b) were first characterized using three solvent contrasts (D_2O, CM4.13, and CMSi). The data collected were initially fitted using the same nine-layer model as used above except that the second solvent layer became the PEG cushion. The volume of an EO monomer is 61.4 Å^3 (ref 40) and therefore has a scattering-length density of 0.6×10^{-6} Å^{-2}. Each EO monomer has 2.5 water molecules associated with it,[41] meaning that the minimum solvent fraction in the PEG layers needed to be constrained to 50% in the model. However, after it was found necessary to use large roughness values to smear out the amplitude of the minima in the modeled profile to fit the data, the use of three layers to model the upper bilayer became unjustified because the partition of the thicknesses of the three layers within the bilayer could not be resolved from the

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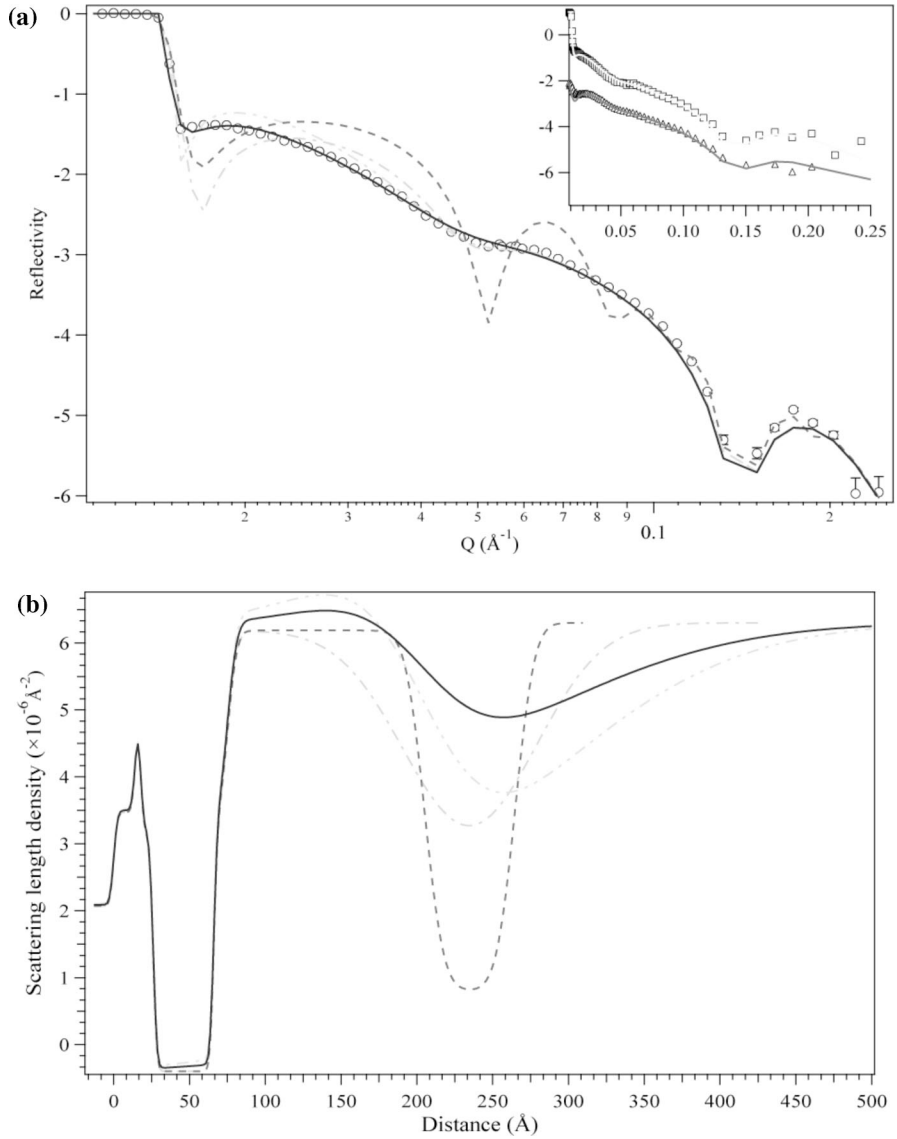

Figure 4. (a) Neutron reflectivity profile and line fits from PEG2000-cushion h-DSPC double bilayer in D_2O (circles). Solid line calculated from parameters in Table II in Supporting Information. Dotted and dashed lines represent variations, listed in Table 1, in the roughness and solvation values of the upper bilayer. The inset shows the measured reflectivity profiles in CM4.13 (□) and CMSi (△) and line fits again calculated from parameters in Table II in Supporting Information. Data offset for clarity. (b) Scattering-length-density profiles corresponding to the line fits in plot a.

reflectivity profile. Instead, we used a scattering-length density averaged across the totality of the bilayer of 0.2×10^{-6} Å^{-2} and a thickness of 60 ± 10 Å. The large error in this thickness results from inferior resolution from the abnormally large roughness of the sample.

PEG-2000 Cushion Double Bilayer: Characterization. The measured and calculated reflectivity profiles and the corresponding fitted scattering-length-density profiles for the PEG2000 *h*-DSPC double bilayer in D_2O are shown in Figure 4. The model describes two $\sim 60 \pm 10$ Å thick bilayers separated by 140 ± 5 Å. Despite PEG2000 having a radius of gyration of ~35 Å at a grafting density of 4 mol %,[42] the range of measurable repulsive force between layers containing PEG2000 at 4.5 mol % has been found to extend to 100 ± 10 Å.[43−46] The larger separation between the double bilayers modeled here is not significant because of the large roughness in the sample.

Table 1. Modified Upper Bilayer Roughness and Solvent Values from the PEG2000-Cushion *h*-DSPC Bilayer[a]

line fit	inner roughness (Å)	outer roughness (Å)	solvent (%)
---	10	10	10
-•-	40	40	10
-••-	40	40	50
—	40	110	50

[a] The line fits correspond to Figure 4 and indicate how the amplitudes of the reflectivity profile oscillations are largely influenced by these parameters.

In Figure 4, the various dashed lines indicate models with exactly the same parameters except that the roughness parameters describing the inner and outer interfaces of the upper bilayer and the percentage of solvent in this bilayer are varied (Table 1). The dashed-line model describes an upper bilayer with good surface coverage and acceptable roughness parameters. It is clear that

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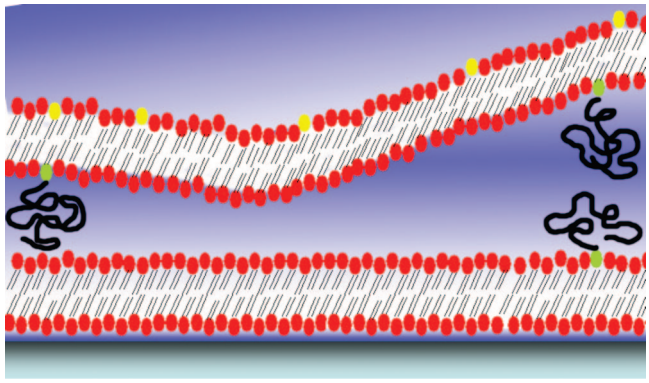

Figure 5. Graphical depiction of the PEG-cushion double-bilayer model. Solid circles are the phospholipid head groups PC (red), PS (yellow) and PE (green). Thin lines are alkyl chains, and thick lines are PEG. (See the text for more details).

the reflectivity profile is accurately described in terms of the positions of the minima, indicating that the models' thickness parameters are correct. To smooth out the amplitude of the interference fringes in the line fit for $Q > 0.05$ Å^{-1}, the roughness of the interface between the PEG cushion and the upper bilayer needed to be increased to 40 Å. The increase in roughness smears out the scattering contrast between the bilayer and solvent and seems to imply from the scattering-length-density profiles (Figure 4.b) that there is a low surface coverage of the upper bilayer, whereas the transfer ratios were, in fact, between 0.95 and 1.0. To obtain a model profile in good agreement with the data for $Q < 0.05$ Å^{-1}, the roughness of the bilayer−subphase interface needed to be greatly increased. It is not possible to determine whether this roughness is static or dynamic in origin, but in any event it seems probable that there are large undulations in the outer bilayer so that the use of a simple box model here is thus questionable; therefore, the conclusions based on its use (as described above) must be regarded with some caution.

Figure 5 shows a cartoon of a possible interpretation of the model used to fit the data.

PEG-2000-Cushion Double Bilayer: Exposure to Lipoplexes. The model PEG-cushion *h*-DSPC double bilayers were exposed to lipoplexes formed from 0.01 mg/mL d_{74}-DDAB−DOPE in D_2O for a total of ~55 h. Measurements were performed at 25 °C.

The interaction of the lipoplexes with the PEG-2000-cushioned double bilayers was modeled with a seven-layer model, assuming that the parameters of the first five layers pertaining to the lower bilayer remained unchanged. The modeling of the reflectivity profiles over time shows a progressive increase in the scattering-length density of the upper bilayer and a progressive decrease in the scattering-

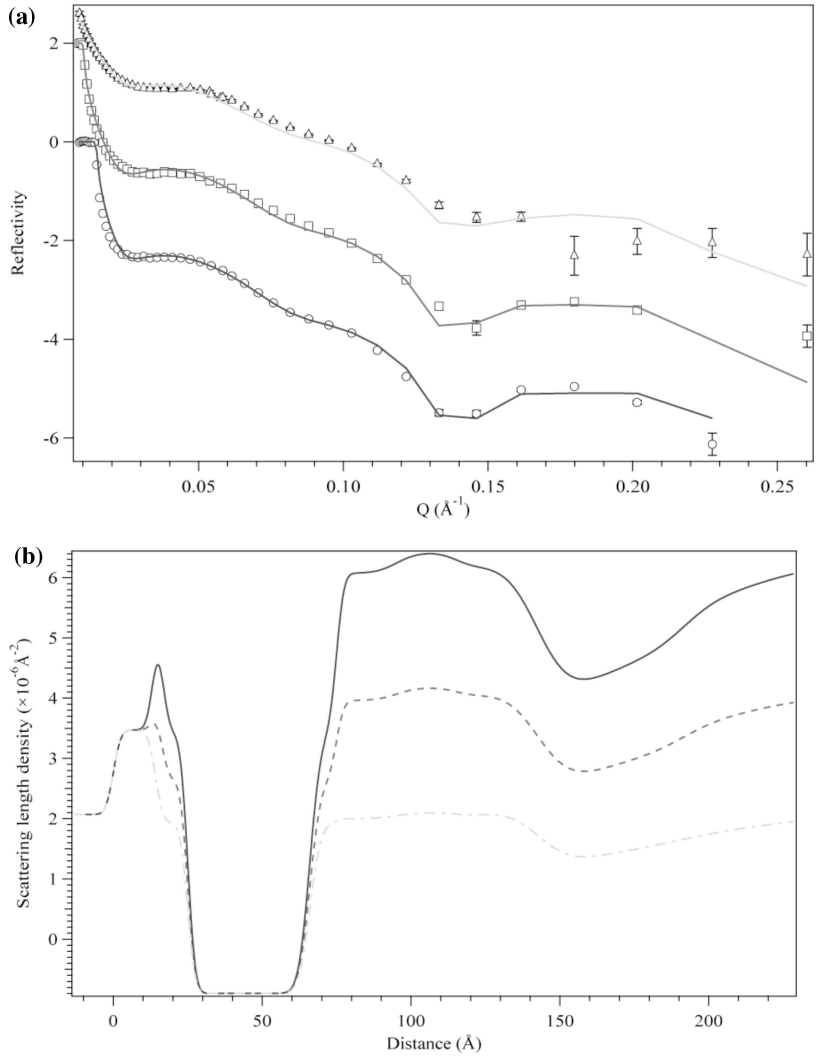

Figure 6. (a) Neutron reflectivity profiles and line fits from PEG750-cushion *h*-DSPC double bilayer in D_2O (○), CM4.13 (□), and CMSi (△). Data are offset for clarity. (b) Scattering-length-density profiles corresponding to the line fits in D_2O (−), CM4.13 (---), and CMSi (-•-). Model parameters are listed in Table III in Supporting Information.

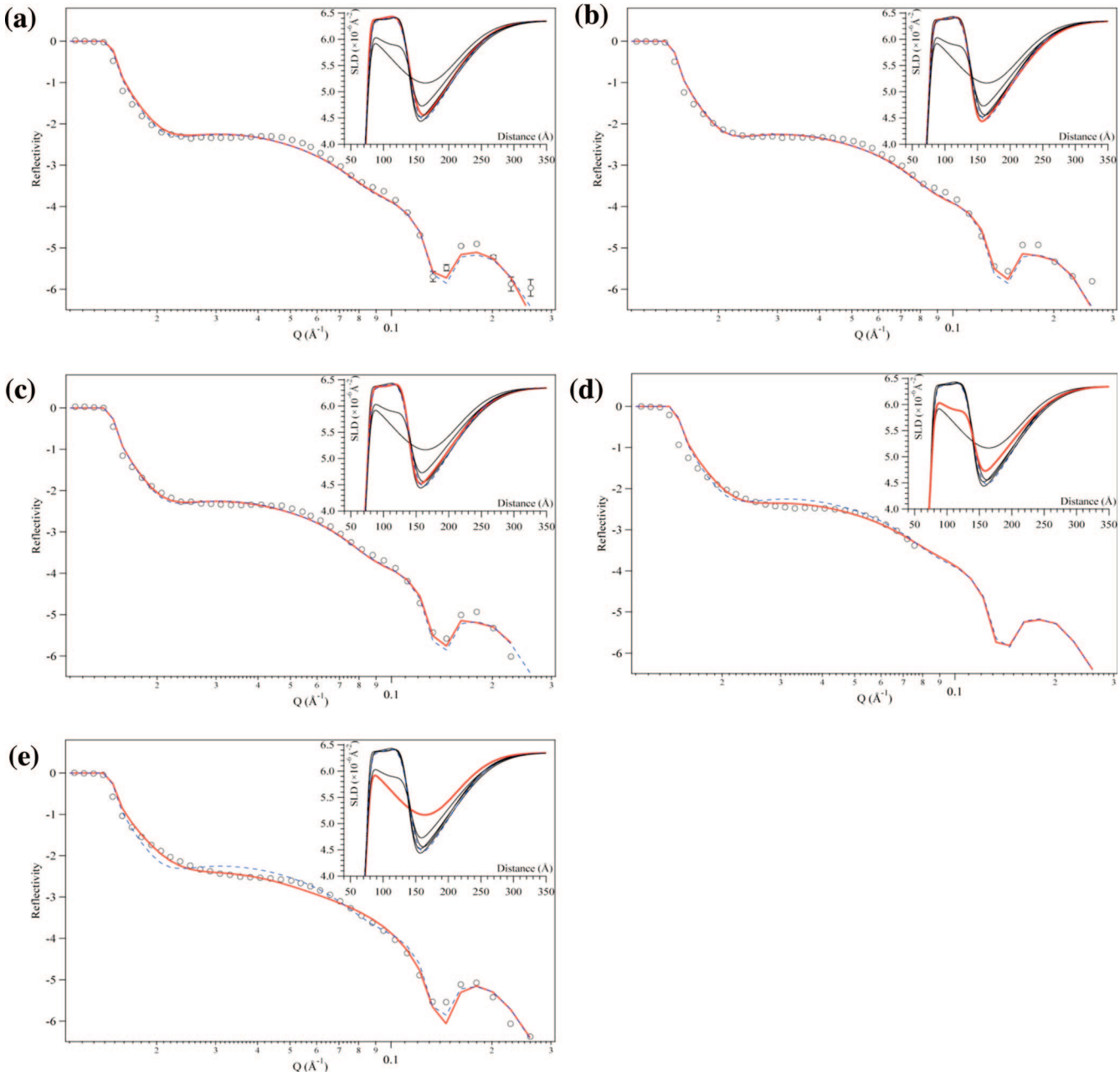

Figure 7. Neutron reflectivity profiles measured in D_2O with fitted lines (solid) from the PEG750-cushion *h*-DSPC double bilayer during exposure to lipoplexes fabricated from 0.01 mg/mL d_{74}-DDAB−DOPE after (a) 3, (b) 5.25, (c) 7.5, (d) 32.5, and (e) 56 h. The dashed line indicates the fit before lipoplex exposure. (Inset) Scattering-length-density profiles corresponding to the time-slice line fit (thick) showing only the distance range spanning the PEG and upper bilayer.

length density of the PEG-cushion layer (Figure V in Supporting Information). The interaction appears to progress in two phases. During the first 11 h of exposure, a major increase is seen in the scattering-length density of the upper bilayer. This is consistent with lipid from the bilayer being removed and replaced by subphase solvent. A second phase occurred over a much longer time period and involved a reduction in the scattering-length density of the PEG cushion. This reduction suggests that lipoplexes are accumulating in the PEG-cushion layer (Figure V in Supporting Information).

PEG-750-Cushion Double Bilayer: Characterization. The reflectivity profiles and scattering-length density profiles corresponding to the line fits for the PEG-750 *h*-DSPC double bilayer are shown in Figure 6. The model describes two ∼60 Å. thick bilayers separated by 60 ± 5 Å. According to the Alexander−de Gennes theory,[47] PEG750 is clearly in the mushroom state at 0.04 mol %, giving a thickness corresponding to the Flory radius of ∼20 Å. As with PEG-2000 *h*-DSPC, the modeled separation is greater than twice the expected extension length, and again the fitted values of the layer roughnesses are very high so that the use of a simple box model here is inappropriate and any conclusions based on the use of such a model must be regarded with caution.

PEG-750 Cushion Double Bilayer: Exposure to Lipoplexes. The model PEG-cushion *h*-DSPC double bilayers were exposed to lipoplexes formed from 0.01 mg/mL d_{74}-DDAB-DOPE in D_2O for a total of ∼55 h. Measurements were performed at 25 °C.

The interaction of the lipoplexes with the PEG-750-cushioned double bilayers was modeled as above, with a seven-layer model.

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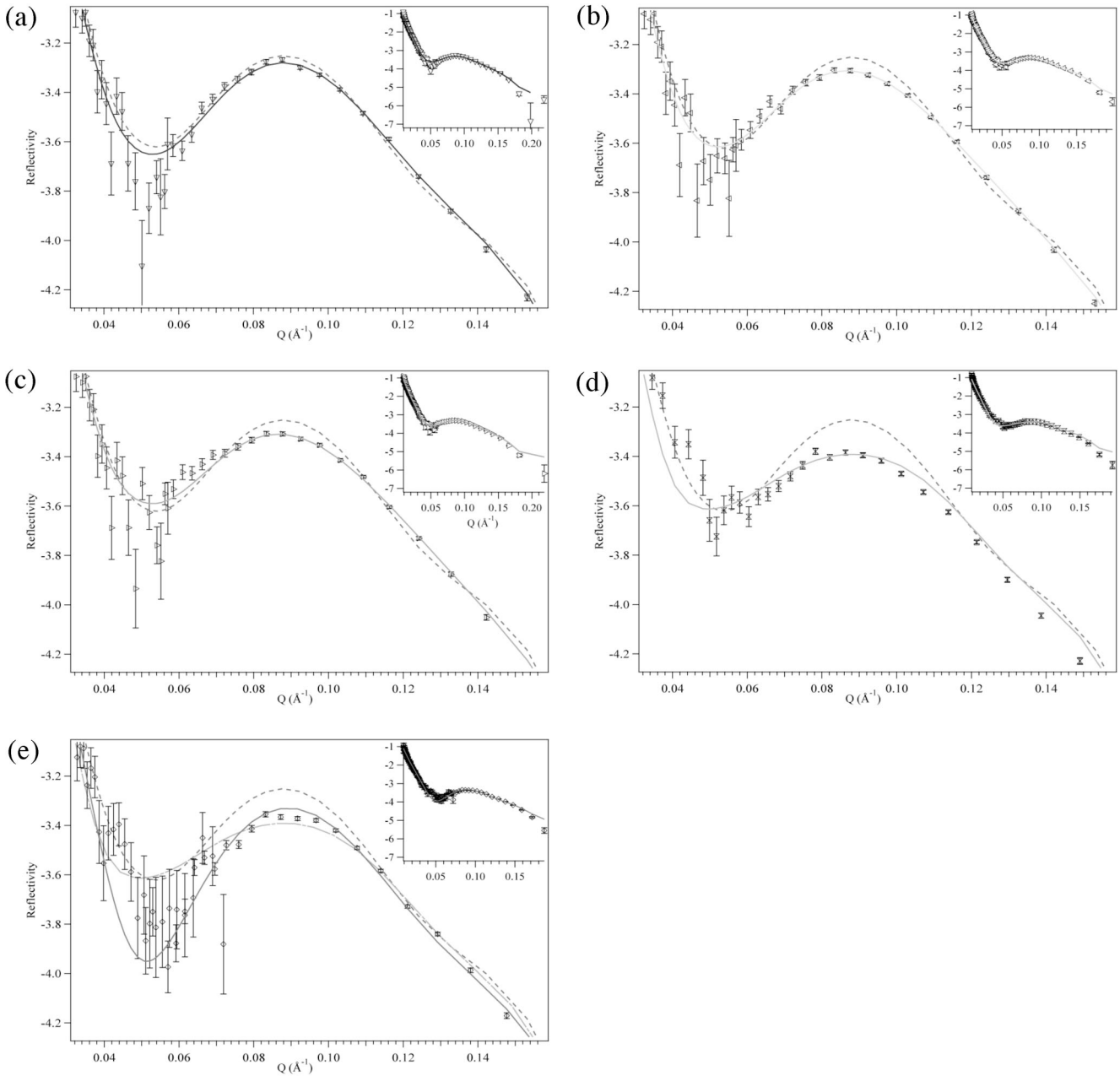

Figure 8. Neutron reflectivity profiles measured in H_2O with fitted lines (solid) from the OTS hybrid d_{83}-DSPC double bilayer during exposure to lipoplexes fabricated from 0.01 mg/mL DDAB−DOPE after (a) 3.5, (b) 7, (c) 10.5, and (d) 14 h and (e) after removal of the lipoplexes. The inset is the full measured Q range. The overlay in each plot is the fit before exposure to the lipoplexes (dashed line).

The reflectivity profiles measured over time during the 55 h exposure period, along with line fits and corresponding scattering-length-density profiles, are shown in Figure 7.

There appears to be little or no change in the reflectivity profile during the first 7.5 h of exposure of the lipoplexes to the PEG750-cushion double bilayer. The next time-slice measurement was made after 32.5 h, and the scattering-length-density profile obtained for the data shows only a small increase in the scattering-length density of the upper bilayer. In addition, there is a significant decrease in the scattering-length density of the PEG-cushion layer. After 56 h of exposure to the lipoplexes, the upper bilayer has lost 8% of its original constituent lipid, and further accumulation of lipoplexes in the PEG-cushion layer has occurred.

Single Bilayer with PEG-2000 Cushion: Characterization. The bilayer schematized in Figure 1c and prepared by depositing 49:1 d_{83}-DSPC/*h*-DSPE-PEG (MW = 2000) using the Langmuir−Blodgett technique followed by a layer of 9:1 d_{83}-DSPC/d_{70}-DSPS using the Langmuir−Schaefer technique was first characterized using three solvent contrasts (H_2O, CMSi, and D_2O). The data collected were simultaneously fitted using a five-layer model (data shown in Figure VI of Supporting Information). The predicted bilayer structure for this sample (Figure 1c) was a PEG cushion separating the bilayer by ∼35 Å from the SiO_2 surface. This 35 Å distance is the Flory radius and the predicted extension length of PEG (MW = 2000) at a grafting density of 2 mol %. However, a satisfactory fit for all three contrast data sets was obtained by excluding DSPE−PEG from the model, just leaving a single supported bilayer. With such a low volume fraction of DSPE in the DSPC bilayer, the change in the overall head-group-layer scattering-length density is negligible. The *h*-alkyl chains lower the scattering-length density of the hydrocarbon layer from 6.9×10^{-6} to 6.75×10^{-6} Å^{-2}, which is still too small to confirm either the absence or presence of DSPE. This left the separation distance of the bilayer from the

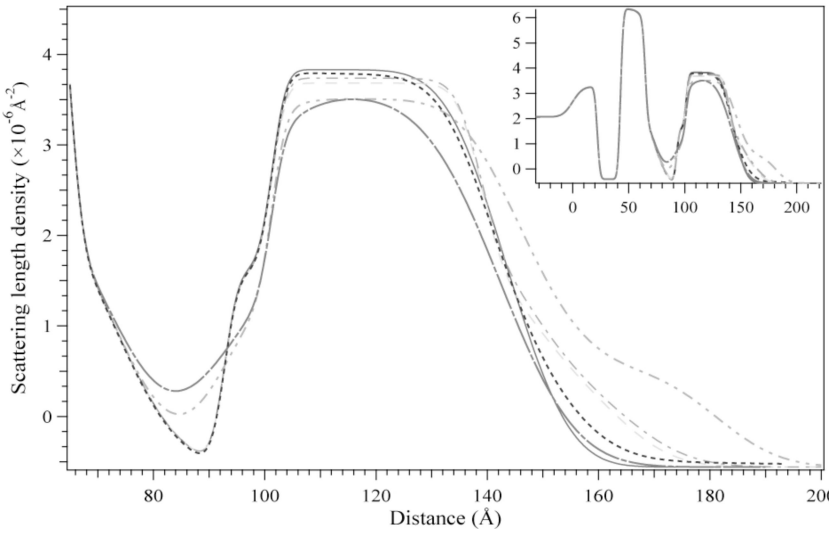

Figure 9. Scattering-length-density profile over the distance range of the upper bilayer only, corresponding to the line fits in Figure 8 before exposure (−); after 3.5 (•••), 7 (---), 10.5 (-•-), and 14 h (-•••); and after lipoplex removal (− −). The inset shows the full distance range of the SLD profile.

substrate as the only indication of the presence of PEG. Because the best-fit model was a bilayer 59 ± 2 Å thick separated from the solid substrate by a 3 ± 1 Å solvent layer, it appeared that little or no DSPE−PEG had been transferred.

Single Bilayer with PEG-2000 Cushion: Exposure to Lipoplexes. The sample was exposed to lipoplexes fabricated from a 0.01 mg/mL vesicle solution of *h*-DDAB−DOPE in H_2O over 12 h, during which three reflectivity measurements were recorded in H_2O for periods of 1.75 h (data shown in Figure VII of the Supporting Information). Measurements were performed at 25 °C. After 12 h, the lipoplex solution was removed and replaced with D_2O, and the final state of the bilayer was characterized. The reflectivity profiles of the three time slices and the final measurement in D_2O are indistinguishable, within experimental error, from the profile recorded for the bilayer before exposure (data shown in Figure VII of the Supporting Information), indicating that there is no interaction or formation of new structures at the bilayer surface. This is difficult to comprehend considering the nature of the changes that occurred in the double bilayer sample and in the work by Callow et al.[22] that showed that 70% of the lipid in a single-bilayer model had been exchanged for lipid from lipoplexes within the first hour of exposure. The most plausible conclusion is that DSPE−PEG is still present somewhere in the bilayer and by some undeterminable mechanism either slows down or prevents the interaction. The PEG may have prevented the lipoplexes from interacting with the bilayer just as PEGylated lipids have shown promising results as constituents of drug-delivery vehicles and biologically passivating coatings,[48−50] increasing the circulation time by preventing interactions with serum proteins because of steric stabilization. Whereas the resolution of the reflectivity profiles is insufficient to confirm or deny the presence of DSPE−PEG, the absence of an interaction here suggests that DSPE−PEG is still present in the bilayer.

Bilayers from d_{83}-DSPC on SAM: Characterization. Double bilayers from d_{83}-DSPC were successfully deposited on the grafted SAM (modeled as in Figure 1d). The double bilayer was first characterized using the three solvent contrasts provided by the subphases H_2O, CMSi, and D_2O (data shown in Figure VIII of the Supporting Information). The data collected were simultaneously fitted using an eight-layer model. This model can be interpreted as a 56 ± 3 Å bilayer separated from the lower 51 ± 3 Å thick OTS−composite bilayer by a 20 ± 1 Å solvent gap. The lower bilayer had ∼90% coverage, whereas the upper bilayer had a surface coverage of only ∼60%, which is lower than the normally obtainable 80% as a consequence of the total surface coverage of the OTS layer. The grafting procedure was developed to produce a less than perfect OTS layer that appears to be necessary for the successful deposition of the upper leaflets.[25]

Bilayers from d_{83}-DSPC on SAM: Exposure to Lipoplexes. The model bilayer was exposed to lipoplexes formed from the complexation of calf thymus DNA with a solution of 0.01 mg/mL *h*-DDAB-DOPE vesicles in H_2O. Measurements were performed at 25 °C. As above, with the d_{83}-DSPC double bilayer, the time slices were measured in 1 h 45 min periods over a total exposure time of approximately 12 h (Figure 8). The interaction of the lipoplexes with the double bilayer was modeled by assuming that the parameters of the first four layers pertaining to the lower bilayer remain unchanged. The speed and extent to which the reflectivity profile changed upon exposure of the bilayer to the lipoplexes were significantly less than for the d_{83}-DSPC double bilayer, where a pronounced, progressive removal of *d*-lipid from the upper bilayer was observed (Figure 3). The changes in the reflectivity profiles of the OTS-hybrid d_{83}-DSPC double bilayer could not be acceptably modeled, within plausible limits, according to the previously observed trends of bilayer destruction. Instead, an additional layer was required to model the presence of lipoplexes at the surface of the upper bilayer.

The changes in the reflectivity profiles were characterized by a decrease in the amplitude of the first maximum, indicative of a reduction in contrast between the upper bilayer and solvent that can result from *d*-lipid being removed and solvent (H_2O) taking its place. It can also be attributed to the formation of a layer at the interface of the upper bilayer with a scattering-length density greater than that of the solvent.

The changes in the reflectivity profiles are too small to elucidate the exact structural nature of the layer forming at the bilayer surface. However, a qualitative interpretation of the scattering-length-density profiles (Figure 9) is possible and indicates the accumulation of a larger amount of lipoplex material with increasing exposure time.

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The solid line fit in Figure 8e was obtained by assuming that the lipoplexes are completely removed after the exposure period. Therefore, the line fit has the increased amplitude in the region of the first minimum as described above. This fit falls within the error bars, however, as does the fit (-•••-) to the reflectivity profile measured during the exposure period after 14 h. Therefore, it is unclear whether the structures that form at the interface of the upper bilayer remain after the lipoplexes in the subphase have been removed. The scattering-length-density profile in Figure 9 shows the modeled changes to the upper bilayer corresponding to line fits to the time-slice profiles.

Conclusions

Biophysical studies of membrane−membrane interactions require well-defined model systems, and the work presented here represents part of our ongoing effort focused on characterizing the membrane interactions of gene-delivery lipoplexes in an attempt to correlate their interaction mechanism(s) and transfection efficiency. In our first studies of these systems,[22,23] we used single lipid bilayers supported on silicon and demonstrated a pronounced exchange of lipid between the lipoplexes and the membrane. As was noted earlier, however, these bilayers are not ideal as models of biological cell membranes: they have limited hydration and are rather constrained, and these deficiencies are further confounded by the fact that the electrostatic field due to the SiO_2 on the silicon surface might exert some influence on events taking place in the subphase above the membrane. In the studies reported here, therefore, we were interested to explore more suitable model membrane systems in which the bilayers encountered by the lipoplexes have properties closer to those of biological membranes and where the influence of the substrate surface charge was minimized. A range of different model membranes were explored (Figure 1), and neutron reflectometry, combined with extensive use of contrast variation, permitted an in-depth characterization of the systems not only in terms of their structure and composition but also in terms of their suitability for use in the study of lipoplex−membrane interactions.

The first of the model membrane systems investigated proved wholly unsatisfactory for use in studying the membrane interactions of gene-delivery systems. These model membranes involved two phospholipid bilayers fabricated from either DPPC or DSPC, with one bilayer adsorbed onto the silicon surface and the second bilayer floating above the first (Figure 1a). Given the earlier observation of lipoplex−membrane lipid exchange,[22,23] the experimental conditions for the reflectivity studies of these systems were chosen so as to be able to highlight the replacement of the deuterated lipid in the membrane with hydrogenous lipid from the lipoplexes. Even small amounts of hydrogenous lipid located in the upper floating bilayer would have resulted in a significant change in the reflectivity profiles. Such changes were not observed, however, and the modeling instead suggested that the upper bilayer was destroyed by the lipoplexes. Over an extended exposure time, it then seems that the destruction of the upper bilayer allowed the lipoplexes access to the lower bilayer and for a time period long enough that 15% of the bilayer was infiltrated by hydrogenous lipid. This secondary interaction of the lipoplexes with the lower bilayer is consistent with the results of Callow et al.[22] From these experiments, therefore, we conclude that the lipoplexes first cause lipid to be stripped away from the floating bilayer and then electrostatic forces between the negatively charged SiO_2 and the positively charged DDAB head groups result in the addition of DDAB molecules to the SiO_2 surface. To test this hypothesis and, if proven, to determine over what range the electrostatic forces are active, three different novel model membranes were designed, fabricated, and exposed to lipoplexes.

Two of these novel membrane systems involved a polymer cushion provided by the polyoxyethylene glycol head groups of PEG lipids, either sandwiched between the silicon block and a single lipid bilayer (Figure 1c) or else sandwiched between a supported and a floating lipid bilayer (Figure 1b). Although the former systems were fabricated seemingly successfully using PEG-2000 lipids, their reflectivity profiles proved difficult to model, and the results obtained following their exposure to lipoplexes were equally difficult to explain. The two systems involving PEG cushions sandwiched between two lipid bilayers, however (Figure 1b), − one involving PEG-750 and the other involving PEG-2000 − proved to be stable throughout three solvent changes, indicating that whichever long-range attractive forces, be it electrostatic or otherwise, act to retain the upper floating bilayer, they are effective over a greater range than the repulsive osmotic forces that operate between the polymer chains. Sadly, however, although it is reported that the roughness of the bilayers in such systems is unaffected even at high grafting densities of PEG,[51] the reflectivity data obtained here could be modeled using only a wholly unrealistic roughness for the outer floating bilayer. It seems probable, therefore, that there are large undulations in the outer bilayer, which makes it all but impossible to extract reliable structural information pertaining to the upper bilayer from the model. Given then that the main goal in this work was to be able to measure the structural changes to the (floating) bilayer that result from its interaction with lipoplexes, this is a major limitation of this type of model membrane and precludes the possibility of further studies with this model without employing significant changes to reduce the roughness.

The final model membrane system investigated involved a double bilayer supported above an octadecyl hydrocarbon layer chemically grafted to the SiO_2 surface (Figure 1d). This system was shown to be stable not only to solvents but also on exposure to lipoplexes. By some means, therefore, the destruction of the upper floating bilayer by the lipoplexes was avoided in this system. This supports the hypothesis that the high negative charge density of the SiO_2 layer may influence, by way of electrostatic interaction with the cationic lipid, the interaction of the lipoplexes with the model bilayer. It is concluded that this model membrane system lends itself perfectly to the study of lipoplex−membrane interactions and, with sufficient exposure time, would allow a detailed characterization of the structures formed at the membrane interface during the interaction.

Supporting Information Available: Tables and figures referred to in the text with Roman numerals. This material is available free of charge via the Internet at http://pubs.acs.org.

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