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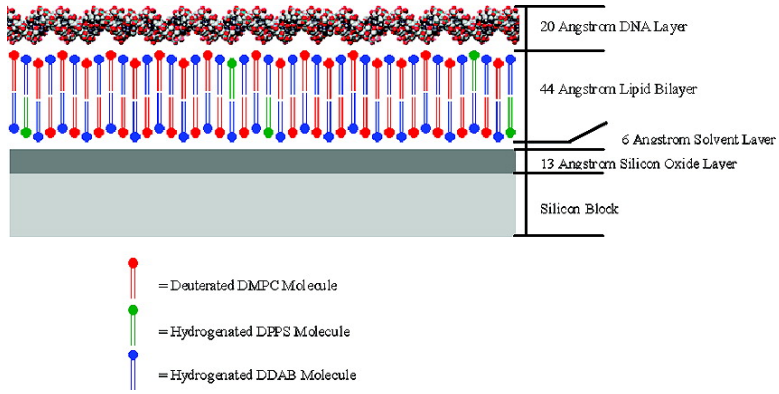
Langmuir, **2009**, 25 (7), 4181-4189• DOI: 10.1021/la802847h • Publication Date (Web): 23 December 2008



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Interaction of Cationic Lipid/DNA Complexes with Model Membranes As Determined by Neutron Reflectivity†

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Received August 31, 2008. Revised Manuscript Received November 13, 2008

Transfection of cells by DNA for the purposes of gene therapy can be effectively engineered through the use of cationic lipid/DNA "lipoplexes", although the transfection efficiency of these complexes is sensitive to the neutral "helper" lipid included. Here, neutron reflectivity has been used to investigate the role of the helper lipid present during the interaction of these lipoplexes with model membranes composed primarily of zwitterionic lipid 1,2-dimyristoylphosphatidylcholine (DMPC) together with 10 mol % 1,2-dipalmitoylphosphatidylserine (DPPS). Dimethyldioctadecylammonium bromide (DDAB) vesicles were formed with two different helper lipids, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE) and cholesterol, and complexed with a 1:1 charge ratio of DNA. The interaction of these complexes with the supported phospholipid bilayer was determined. DOPE-containing lipoplexes were found to interact faster with the model cell membrane than those containing cholesterol, and complexes containing either of the neutral helper lipids were found to interact faster than when DDAB alone was present. The interaction between the lipoplexes and the model membrane was characterized by an exchange of lipid between the membrane and the lipid/DNA aggregates in solution; the deposition of (additional) lipid on the surface of the model cell membrane was not apparent.

Introduction

The complexes formed between cationic liposomes and DNA ("lipoplexes") were the first nonviral gene-delivery vectors to be developed;[1] they present a promising alternative to viruses for the introduction of foreign genetic material into eukaryotic cells because they lack the biohazards associated with the use of viruses. Lipoplexes, in contrast to viral vectors, are easily prepared by simple admixing of an aqueous dispersion of cationic liposomes with (negatively charged) DNA. Complexation between the DNA and cationic liposomes then occurs spontaneously, driven predominately by electrostatic interactions, although counterion release[2] and hydrophobic interactions[3,4] are also thought to be important. It should be noted, however, that whereas the formation of lipoplexes is straightforward the structures of the resulting aggregates are frequently varied and very complex.[5−7]

One drawback to the clinical use of lipoplexes is their relatively low transfection efficiency in comparison with that achieved using viruses. To improve lipoplex design, it is essential to understand the structure−activity relationships governing transfection efficiency. However, this is a far from trivial task owing to the multitude of influencing factors. Of particular importance is the nature of the interaction of the lipoplex with the biological barriers it encounters before its DNA payload can enter the nucleus of the cell. Of these barriers, the cell membrane is considered to be especially important. Although it was initially proposed that lipoplex entry into cells necessitated direct fusion of the complex with the cell membrane,[1] it is now thought probable that the common route of entry of lipoplexes is via endocytosis.[8,9] However, because of the great variability in structure of the different types of lipoplexes coupled with the variable composition and surface properties of eukaryotic cell membranes, it is likely that the entry route exploited by lipoplexes may vary markedly from cell type to cell type.[10]

In this report, we have used neutron reflection measurements in combination with isotopic (H/D) substitution to study the interaction between various types of lipoplex and solid-supported model membranes. Neutron reflection is an ideal technique for such studies because it affords the opportunity to highlight specific molecules or molecular fragments in the multicomponent systems so that a detailed picture of the membranes and their interactions with the lipoplexes can be developed at a molecular level. Indeed, we have previously used the same technique successfully to investigate the interaction of lipid vesicles composed of the cationic lipid dioctadecyldimethylammonium bromide (DDAB) with and without the neutral "helper" lipids dioleylphosphatdiylethanolamine (DOPE) and cholesterol, with a model cell membrane.[11] The present report extends this earlier study by determining the extent and nature of the membrane interactions formed from these vesicles in the presence of added DNA. From these latter studies, we have been able to determine the relative rates of interaction of the lipoplexes with the model membrane and to compare these to the rates of interaction of the corresponding (DNA-less) cationic liposomes. The results of

† Part of the Neutron Reflectivity special issue.

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10.1021/la802847h CCC: $40.75
Published on Web 12/23/2008

these studies are discussed in the context of lipoplex design and in relation to transfection mechanisms and the efficiency of gene delivery.

Materials and Methods

Neutron Reflectivity. In a neutron experiment, the specular reflection perpendicular to the reflecting surface, R, is measured as a function of the wave vector transfer, $Q = (4\pi/\lambda)\sin\theta$, where θ is the glancing angle of incidence and λ is the wavelength of the incident neutron beam. $R(Q)$ is related to the scattering-length density (ρ) across the interface, $\rho(z)$,[12] by

$$R(Q) = (16\pi^2/Q^2)|\rho(Q)|^2 \quad (1)$$

where $\rho(Q)$ is the 1D Fourier transform of $\rho(z)$,

$$\rho(\mathrm{Q}) = \int \exp(-\mathrm{i}\kappa z)\, \rho(z)\, \mathrm{d}z \quad (2)$$

with $\rho(z)$ being a function of the distance perpendicular to the interface (z).

The scattering-length density $\rho(z)$ is related to the composition of the adsorbed species by

$$\rho(z) = \sum_j n_j(z) b_j \quad (3)$$

where $n_j(z)$ is the number of nuclei per unit volume and b_j is the scattering length of nucleus j.

In a typical analysis, the measured data are compared with reflectivity profiles calculated using the optical matrix method applied to different model density profiles.[13] Any model typically consists of a series of layers, each with a uniform scattering-length density ρ and a thickness t. An additional parameter σ, which measures the interfacial roughness between any two consecutive layers, may be incorporated if necessary. The calculated profile is compared with the measured profile, and ρ and t for each layer are varied until the optimum fit to the data is found. Although a given reflectivity profile may be satisfactorily accounted for by a variety of different structural models, the use of several profiles measured under different isotopic contrasts can usually ensure that the fitted model provides an unambiguous solution.

This technique, known as contrast variation, relies on the fact that different nuclei scatter neutrons with quite different amplitudes and, in the case of protons and deuterons, with opposite phase. Therefore, using a combination of hydrogenated and deuterated materials the reflectivity profile of a system can be substantially changed while keeping the same chemical structure at the interface. Moreover, it is possible (by adjusting the H/D ratio) to prepare solvents that are contrast matched to the silicon substrate used in the neutron reflectivity experiments, and the contrast between the surface and the solvent is then zero, giving a reflectivity profile arising only from the interfacial material.

The data described below were measured at the Institute Laue-Langevin using the D17 reflectometer and at ISIS using the SURF reflectometer. All measurements were made at 30 °C. Initial model fitting was performed using the programs AFIT[14] and WETDOC,[15] both allowing the simultaneous analysis of all data sets from the same sample under different H_2O/D_2O solvent contrasts. These initial model fits were then used as templates for simultaneous fitting of the experimental data using the MOTOFIT program.[16] Model parameters were constrained to impose symmetry on the lipid bilayers with each headgroup region having the same thickness, scattering-length density, and solvent volume present. The errors reported are those provided from the variance-covariance matrix calculated during the Levenberg−Marquardt optimization. Scattering-length density profiles were calculated using Parrat32.[17]

Materials. All chemicals used were obtained commercially and were used as received. Dimethyldioctadecylammonium bromide (*h*-DDAB, purity >98%), cholesterol (*h*-Chol, purity >99%), dioleoyl-L-a-phosphatidylethanolamine (*h*-DOPE, purity ≥99%), and DNA from calf thymus (DNA) were supplied by Sigma (Dorset, U.K.). Deuterated 1,2-dimyristoyl-d_{54}-*sn*-glycerol-3-phosphocholine-1,1,2,2-d_4,*N*,*N*,*N*-trimethyl-d_9 (*d*-DMPC, >98% deuterium purity) and hydrogenated 1,2-dipalmitoyl-*sn*-glycero-3-[phospho-L-serine] (*h*-DPPS), purity >99%) were purchased from Avanti Polar Lipids (Alabaser, AL). D_2O was provided by the Institut Laue-Langevin and was filtered through 0.22 μm pore-size filters (Millipore, Bedford, MA) before use.

Lipid/DNA Complex Preparation. Vesicles were prepared using the thin film method as described previously.[11] The required mass of lipid was placed in a round-bottomed flask and dissolved in excess chloroform. The chloroform was then removed by rotary evaporation at 50 °C, and the resulting lipid film was stored overnight at ~5 °C. An aliquot of D_2O (at 25 °C) was added to give a final concentration of 1 mg/mL lipid, and the solvent was allowed to hydrate the lipid under atmospheric pressure at 50 °C. When the lipid was fully hydrated, the multilamellar vesicles so formed were size reduced by probe sonication (Soniprobe, Lucas Dawe Ultrasonics) for 30 min over ice. Vesicle suspensions of lower concentration were prepared by the dilution of these 1 mg/mL preparations.

Throughout the following text, all lipoplex samples contain a final concentration of 0.1 mg/mL DDAB. Where there is a mixture of lipids present, the molar ratio of DDAB to helper lipid is 1:1. All vesicle preparations used in this study had mean vesicle radii of less than 40 nm (determined by dynamic light scattering using a Malvern 4700c light-scattering instrument; Malvern Instruments Ltd., Malvern, Worcester, U.K.) and can therefore be considered to be unilamellar.

Calf thymus DNA was then added to these vesicle preparations, giving a final charge ratio of 1:1 cationic lipid to DNA. The resulting complexes have been characterized by dynamic light scattering and small-angle neutron scattering and exhibit a d spacing of ~65 Å resulting from the formation of ordered lipid/DNA structures.[18]

Preparation of the Model Membrane. Lipid bilayers were deposited on the (111) surface of silicon single crystals by using a combination of the Langmuir−Blodgett (first layer) and Langmuir−Schaeffer (second layer) techniques.[19] Briefly, after immersing the highly hydrophilic block (a silicon crystal previously cleaned with organic solvents and UV/ozone treatment[20]) in the subphase, a 9:1 molar ratio of *d*-DMPC/*h*-DPPS was spread on the surface of the water at a pressure <0.1 mN/m, and after allowing the evaporation of the solvent, the monolayer was compressed to a surface pressure of 30 mN/m. By withdrawing the block from the water at a speed of 5 mm/min, a monolayer of lipid was deposited on its surface. The solid was then rotated by 90° and slowly reimmersed with the large face parallel to the water surface for the formation of the bilayer. The pressure was kept constant throughout the deposition process. Stable, reproducible bilayers (containing 9:1 *d*-DMPC/*h*-DPPS as confirmed by neutron reflectivity[11]) were obtained using this methodology.

Solvents. Four D_2O/H_2O mixtures were prepared for use in characterizing the layers found at the surface of the silicon block. Before use, all solvents were filtered through 0.22 μm pore-size membranes to remove any large contaminants. The four solvents each had different scattering-length densities (ρ), thus contrasting with or contrast matching different regions of the sample. D_2O ($\rho = 6.35 \times 10^{-6}$ Å^{-2}) offered maximum contrast with the hydrogenated lipid, $CMSiO_2$ solvent ($\rho = 3.41 \times 10^{-6}$ Å^{-2}) contrast matched the

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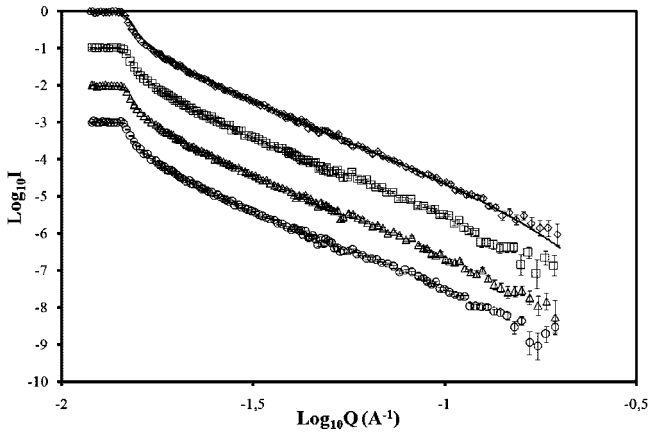

Figure 1. Clean silicon surface characterized with D_2O used as the solvent (◇) and then exposed to *h*-DDAB/DNA complexes for 2 (□), 4 (Δ), and 6 h (○). The solid line represents the fit to the surface of the bare silicon block. Data are offset for reasons of clarity.

silicon oxide layer at the surface of the silicon substrate, CMSi solvent ($\rho = 2.07 \times 10^{-6}$ Å^{-2}) contrast matched the silicon substrate, and H_2O ($\rho = -0.56 \times 10^{-6}$Å^{-2}) resulted in the maximum contrast with the deuterated lipid present at the silicon surface. To change the solvent contrast a 3-fold excess of the required solvent, pumped into the sample environment[21] (15 mL volume) at a rate of 150 mL/h was found to be sufficient.

Exposure of Model Membranes to Lipid/DNA Complexes. D_2O solutions of the various lipid/DNA complexes were introduced into the subphase above the model membranes following the same procedure as detailed above for the change in solvent. Where multiple H/D solvent contrasts were used to characterize the structures found at the interface at a certain time point, the lipid/DNA complexes were removed prior to data collection using the process described above.

Results and Discussion

Studies were first performed to determine the time course of interactions between various lipid/DNA complexes and the model membranes. A D_2O solution of each type of complex was injected into the subphase above a model membrane (characterized as previously[11]), and the neutron reflectivity of the system was then monitored as a function of time over a period of several hours. To provide a benchmark for the investigations involving the model membranes, a study was also performed to look at the time course of the interaction between *h*-DDAB/DNA complexes and a bare silicon block.

Time Course Studies. *Bare Silicon Block Exposed to h-DDAB/DNA Complexes.* The reflectivity data collected for the clean silicon block were modeled as described previously,[11] with a single uniform layer used to simulate the silicon oxide coating at the block surface. This layer was assigned a fixed scattering-length density (ρ) of 3.41×10^{-6} Å^{-2} (the value expected for SiO_2), and the reflectivity was fitted assuming a thickness of 13 ± 2 Å, with an interfacial roughness of 2 ± 1 Å.

The injection of *h*-DDAB/DNA complexes into the subphase above the silicon block gave no significant change in the reflectivity profile observed over a period of 6 h (Figure 1), and it is thus concluded that there are no lipid- and/or DNA-containing structures deposited on the silicon surface during this time.

We have previously shown that the exposure of a bare silicon surface to *h*-DDAB vesicles (in the absence of DNA) results in an immediate change in the interfacial reflectivity, corresponding to the rapid deposition of lipid on the surface of the hydrophilic, negatively charged silicon oxide layer.[11] We deduce, therefore, that this deposition onto the silicon block is governed by electrostatic interactions, with the cationic (DNA-less) DDAB vesicles being strongly attracted to the surface but the net neutral DDAB/DNA complexes experiencing no such an attraction.

Model Membrane Exposed to h-DDAB/DNA Complexes. In studying the time course of the interaction between *h*-DDAB/DNA complexes and a precharacterized model membrane, the neutron reflectivity of the system was monitored as a function of time over a period of 25 h (data not shown), after which the lipid/DNA complexes were removed and the absorbed layer was characterized in four solvent contrasts (D_2O, $CMSiO_2$, CMSi, and H_2O). Even after an exposure time in excess of 1 day, the neutron reflectivity of the membrane shows very little change, with a slightly increased level of solvent in the membrane but only minor changes in the ρ and thickness of the layers required to model the two ($t = 0$ and 25 h) data sets (Table 1).

After 25 h of exposure, the ρ of the membrane's headgroup layers have decreased by 0.9×10^{-6} Å^{-2}, and the ρ of its hydrocarbon core has decreased by 0.3×10^{-6} Å^{-2}. These changes are interpreted as the result of lipid exchange between the deuterated components of the membrane and the hydrogenous material of the DDAB/DNA complex, as was observed for membranes exposed to *h*-DDAB vesicles in the absence of DNA.[11]

The exchange of lipid, both in the presence and absence of DNA, is a slow process. Indeed, only minor changes in the structural parameters used to fit the reflectivity are required after many hours of exposure to *h*-DDAB vesicles or *h*-DDBA/DNA complexes. The increase in thickness of the headgroup layers, the reduction in thickness of the hydrocarbon layer, and the increase in the volume of solvent present within these layers (Table 1) may be caused by the disruption of the packing of the lipid molecules. During the preliminary stages of lipid exchange between the membrane and the *h*-DDAB molecules of the lipid/DNA complex, the bilayer will be composed of lipids with hydrocarbon chains of varying length (C14 for DMPC, C16 for DPPS, and C18 for DDAB). The presence of these different hydrocarbon chain lengths will cause lateral variations in packing density and thus will disorder the hydrocarbon layer of the membrane.

Model Membrane Exposed to h-DDAB/Chol/DNA Complexes. On exposing a model membrane to *h*-DDAB/Chol/DNA complexes, a significant change in reflectivity of the sample was observed immediately (data collected within the first hour of exposure) with the rate of change slowing after 2 h (Figure 2).

Therefore, not only do *h*-DDAB/Chol/DNA complexes interact more rapidly with the membrane than *h*-DDAB/Chol vesicles[11] but they also interact more rapidly than the *h*-DDAB/DNA complexes.

Whereas the increase in the rate of membrane interaction for *h*-DDAB/Chol vesicles versus *h*-DDAB vesicles is apparent only on a time scale of several days,[11] the increase in the rate of interaction for *h*-DDAB/Chol/DNA complexes versus *h*-DDAB/DNA complexes can be observed in a matter of hours. Modeling the interaction of the *h*-DDAB/Chol/DNA complexes in a similar manner to that used for *h*-DDAB/DNA returns a model with the parameters summarized in Table 2. Because of the rapid rate of interaction and to allow the time-dependent change in reflectivity to be observed, data were collected in only one H/D solvent contrast. This means that the solvent volume present within each layer of the model cannot be determined. However, given the

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Table 1. Model Parameters Required to Fit the Neutron Reflectivity Curves of a Model Membrane after 25 h of Exposure to *h*-DDAB/DNA Lipoplexes[a]

layer (description)	thickness (Å)	scattering-length density ($\times 10^{-6}$ Å^{-2})	roughness (Å)	% solvent
layer 1 (SiO_2)	14 ± 1	3.4 ± 0.2	2 ± 1	0
layer 2 (Hg)	11 ± 1 (8)	4.5 ± 0.2 (5.4)	3 ± 2	45 ± 9 (35)
layer 3 (Hc)	25 ± 1 (27)	6.5 ± 0.1 (6.8)	3 ± 2	37 ± 3 (25)
layer 4 (Hg)	11 ± 1 (8)	4.5 ± 0.2 (5.4)	3 ± 2	45 ± 9 (35)

[a] SiO_2 = silicon oxide layer, Hg = headgroup region of the model membrane, Hc = hydrocarbon core region of the model membrane. Values in parentheses respresent the values for a model membrane before exposure to lipid/DNA complexes.[11]

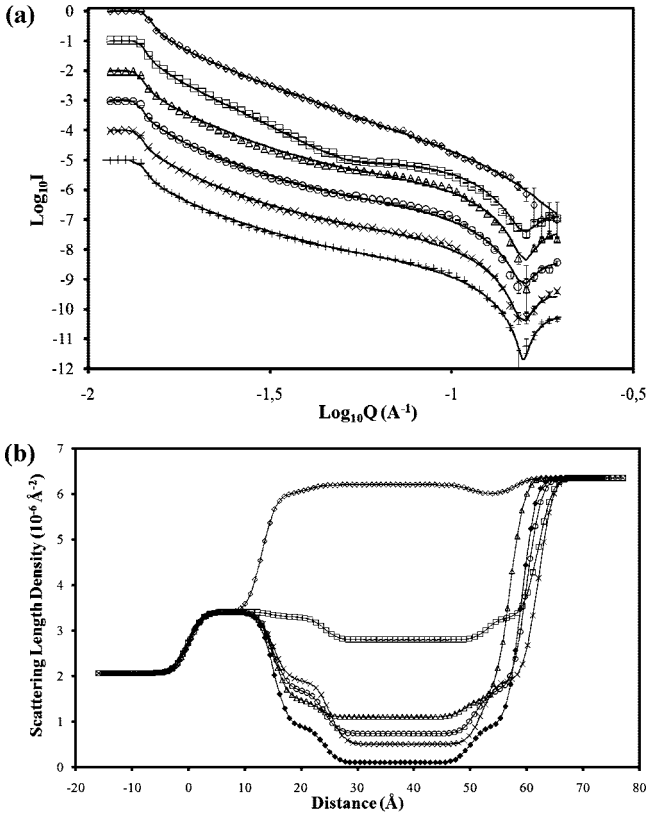

Figure 2. (a) Neutron reflection curves of a model membrane exposed to *h*-DDAB/Chol/DNA lipoplexes in D_2O as a function of time: (◇) model membrane in D_2O, (□) after 1 h of exposure, (Δ) after 2 h, (○) after 3 h, (×) after 4 h, and (+) after 13 h. Data are offset for reasons of clarity. Solid lines represent fits to experimental data. (b) Scattering-length density profiles for the model fits for a model membrane exposed to *h*-DDAB/Chol/DNA lipoplexes in D_2O as a function of time: (◇) model membrane in D_2O, (□) after 1 h of exposure, (Δ) after 2 h, (○) after 3 h, (×) after 4 h, and (♦) after 13 h.

time-dependent decrease in ρ for the layers corresponding to the model membrane (layers 2−4), the exchange of lipid must again take place between the deuterated components of the membrane and the *h*-DDAB/Chol/DNA complexes.

Model Membrane Exposed to h-DDAB/DOPE/DNA Complexes. Similar observations to those described above were made when a model membrane was exposed to a D_2O solution of *h*-DDAB/DOPE/DNA complexes. Even within the first hour of exposure there were significant changes seen in the neutron reflectivity profile (Figure 3).

Results of model fitting of the reflectivity data (Table 3) show a rapid decrease in ρ for layers 3−5, consistent with an exchange of lipid between the deuterated components of the membrane and the hydrogenous lipids in the *h*-DDAB/DOPE/DNA complexes, as observed for *h*-DDAB/Chol/DNA complexes and *h*-DDAB/Chol and *h*-DDAB/DOPE vesicles in the absence of DNA.[11]

The rate of exchange between the *h*-DDAB/DOPE/DNA complexes and the membrane lipid is extremely fast. Indeed, during the first hour of exposure of the membrane to the *h*-DDAB/DOPE/DNA complexes, the ρ of layer four (representing the hydrocarbon core of the lipid bilayer) decreases from 6.8×10^{-6} to 1.4×10^{-6} Å^{-2} (Table 3). This corresponds to the replacement of ∼70% of the deuterated hydrocarbon chains of the membrane with the hydrogenous hydrocarbon chains of *h*-DDAB and *h*-DOPE molecules. The speed of the lipid exchange is thus significantly faster than the ∼55% exchange observed after 9 h for *h*-DDAB/DOPE vesicles in the absence of DNA.

Interestingly, after 5 h of exposure of the model membrane to *h*-DDAB/DOPE/DNA complexes it is necessary to include a solvent layer between the silicon oxide surface and the headgroups of the lipid bilayer to successfully model the minimum that appears in the reflectivity curve (Figure 3). Because this behavior is observed only for DOPE-containing complexes where high levels of lipid exchange have occurred between the model membrane and the lipid/DNA complexes, we conclude that the incorporation of DOPE into the model membrane results in well-defined headgroup regions of the model membrane, which allows us to resolve the trapped solvent layer.

Effects of Long-Term Exposure of Model Membranes to Lipid/DNA Complexes. To provide for a more detailed characterization of the model membranes after their exposure to the various lipid/DNA complexes, D_2O solutions of each were injected into the subphase above a membrane (characterized as earlier[11]), and the system was then left for a period of several days. The solution containing the lipid/DNA complexes was subsequently removed, and neutron reflectivity data were collected with the solvent replaced (in sequence) by pure D_2O, $CMSiO_2$, CMSi, and (for the DDAB/DNA and DDAB/DOPE/DNA studies only) H_2O. These reflectivity data were then fitted simultaneously, in all cases using an initial model based on that which gave a good fit to the reflectivity data for the initial model membranes.

Prolonged Exposure of a Model Membrane to h-DDAB/DNA Complexes. The model used to simulate the reflectivity curves after 5 days of exposure of the membrane to *h*-DDAB/DNA complexes (Figure 4 and Table 4) is interpreted as a 13-Å-thick silicon oxide layer at the surface of the silicon block, with an adsorbed lipid layer above this and an additional layer of ∼20-Å-thickness at the membrane surface. Because different silicon surfaces were used here and for the time course study described above (Table 1), the variation in solvent volume present within the modeled layers after 25 h and here after 5 days of exposure to *h*-DDAB/DNA complexes is considered to be a result of variations in the characteristics of the substrate.

The lipid layer is well modeled as a single layer of 44 Å thickness with a scattering-length density of 3.0×10^{-6} Å^{-2}. Given that the scattering-length density of the hydrocarbon core of the initial model membrane is 6.8×10^{-6} Å^{-2}, it is inferred (as earlier) that there is a significant exchange of lipid between the *h*-DDAB/DNA complexes and the model membrane, with the membrane ultimately containing a mixture of approximately 50% deuterated and 50% hydrogenous lipid (with *h*-DDAB

Table 2. Model Parameters Required to Fit the Neutron Reflectivity Curves of a Model Membrane during 13 h of Exposure to *h*-DDAB/Chol/DNA Lipoplexes in D_2O (Figure 2)[a]

layer	no vesicles	0−1 h	1−2 h	2−3 h	3−4 h	12−13 h
layer 1	13 ± 1.0	15 ± 1	17 ± 1	16 ± 1	16 ± 1.0	16 ± 1
(SiO_2)	3.4 ± 0.1	3.4 ± 0.1	3.4 ± 0.1	3.4 ± 0.1	3.4 ± 0.1	3.4 ± 0.1
layer 2	8 ± 2	9 ± 2	8.0 ± 2	9 ± 2	10 ± 2	9 ± 3
(Hg)	6.0 ± 0.3	3.3 ± 0.2	1.5 ± 0.4	1.7 ± 0.4	1.9 ± 0.3	0.9 ± 0.2
layer 3	29 ± 1	29 ± 1	26 ± 1	27 ± 1	27 ± 1	26 ± 1
(Hc)	6.2 ± 0.2	2.8 ± 0.1	1.1 ± 0.1	0.74 ± 0.1	0.5 ± 0.1	0.1 ± 0.1
layer 4	8 ± 2	9 ± 2	8 ± 2	9 ± 2	10 ± 2	9 ± 3
(Hg)	6.0 ± 0.3	3.3 ± 0.2	1.5 ± 0.4	1.7 ± 0.4	1.9 ± 0.3	0.9 ± 0.2

[a] For each layer, the thickness (Å) is quoted above ρ = scattering-length density ($\times 10^{-6}$ Å^{-2}). SiO_2 = silicon oxide layer, Hg = headgroup region of the lipid bilayer, and Hc = hydrocarbon core region of the lipid bilayer.

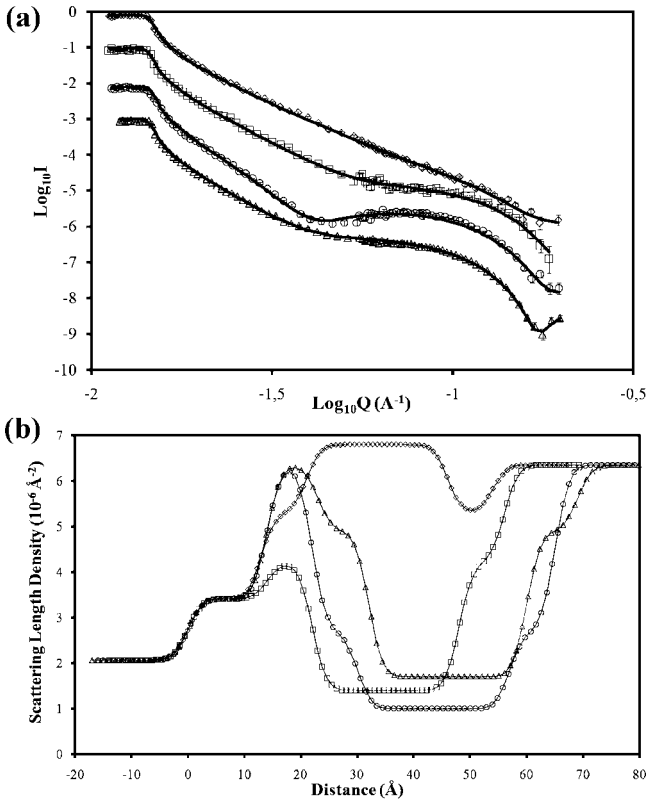

Figure 3. (a) Neutron reflection curves of a model membrane exposed to *h*-DDAB/DOPE/DNA lipoplexes in D_2O as a function of time: (◇) model membrane in D_2O, (□) after 1 h of exposure, (○) after 6 h, and (Δ) after 6 days. Data are offset for reasons of clarity. Solid lines represent fits to the experimental data. (b) Scattering-length density profiles for the model fits for a model membrane exposed to *h*-DDAB/DOPE/DNA lipoplexes in D_2O as a function of time: (◇) model membrane in D_2O, (□) after 1 h of exposure, (○) after 6 h, and (Δ) after 6 days.

exchanged with *d*-DMPC). If the lipid exchange between the membrane and the *h*-DDAB/DNA complexes is similar to that described for the interaction between the membrane and *h*-DDAB vesicles in the absence of DNA,[11] then the increase in the lipid bilayer thickness can be explained by the intrusion of the longer C18 hydrocarbon chains of the *h*-DDAB molecules into the mixed C14/C16 hydrocarbon chain region of the membrane. It should be noted that when *h*-DDAB molecules intercalate into a model membrane in the absence of DNA, the resulting membrane has a thickness of 37 Å, which is similar to that observed for the bilayers of *h*-DDAB vesicles.[18] In the presence of DNA, however, the thickness of the membrane after exposure to *h*-DDAB/DNA complexes is 44 Å. It appears, therefore, that the presence of the DNA molecules leads to a different lipid packing arrangement

Table 3. Model Parameters Required to Fit the Neutron Reflectivity Curves of a Model Membrane as a Function of Exposure Time to *h*-DDAB/DOPE/DNA Lipoplexes (Figure 3)[a]

layer	no vesicles	0−1 h	5−6 h	144 h
layer 1	13 ± 1	14 ± 1	14 ± 1	13.2 ± 0.4
(SiO_2)	3.4 ± 0.1	3.4 ± 0.1	3.4 ± 0.1	3.4 ± 0.1
layer 2	N/A	N/A	9 ± 1	8 ± 1
(Sol)			N/A	N/A
layer 3	8 ± 2	8 ± 1	9 ± 2	8 ± 1
(Hg)	5.3 ± 0.2	4.2 ± 0.4	4.9 ± 0.2	2.7 ± 0.2
layer 4	25 ± 1	26 ± 1	28 ± 1	27 ± 1
(Hc)	6.8 ± 0.1	1.4 ± 0.1	1.7 ± 0.1	1.0 ± 0.1
layer 5	8 ± 2	8 ± 1	9 ± 2	8 ± 1
(Hg)	5.3 ± 0.2	4.2 ± 0.4	4.9 ± 0.2	2.7 ± 0.2

[a] For each layer, thickness (Å) is quoted above ρ = scattering-length density ($\times 10^{-6}$ Å^{-2}). SiO_2 = silicon oxide layer, Sol = trapped solvent layer, Hg = headgroup region of the lipid bilayer, and Hc = hydrocarbon core region of the lipid bilayer.

at the surface of the silicon block, resulting in an increased membrane thickness.

The scattering-length density of the additional layer formed at the membrane surface after its exposure to the *h*-DDAB/DNA complexes (3.9×10^{-6} Å^{-2}) is consistent with a layer comprising DNA. We note too that the thickness of this layer (∼20 Å) corresponds with the diameter of a DNA double helix.[22] Work performed by Harvie et al.[23] has demonstrated that the interaction of cationic lipid/DNA lipoplexes with anionic lipid vesicles results in rapid destabilization of the lipid/DNA complexes. This destabilization is characterized by lipid exchange and DNA release from the cationic lipid/DNA complex. The interaction of the *h*-DDAB/DNA lipoplexes with the model membrane (which contains 10 mol % of the anionic lipid *h*-DPPS) will have a similar effect, with the released DNA becoming bound to the surface of the increasingly positively charged model membrane.

Such a model, with a lipid bilayer adsorbed to the surface of a silicon block, with DNA bound to its outer surface (Figure 5), is similar to that described by Kago et al.[24] for a monolayer binding DNA at the air/liquid interface. The exchange of lipid between the membrane and the *h*-DDAB/DNA complexes in the subphase has the effect of changing the charge at the surface from net negative to net positive. Once the membrane becomes sufficiently positively charged there is the potential for the

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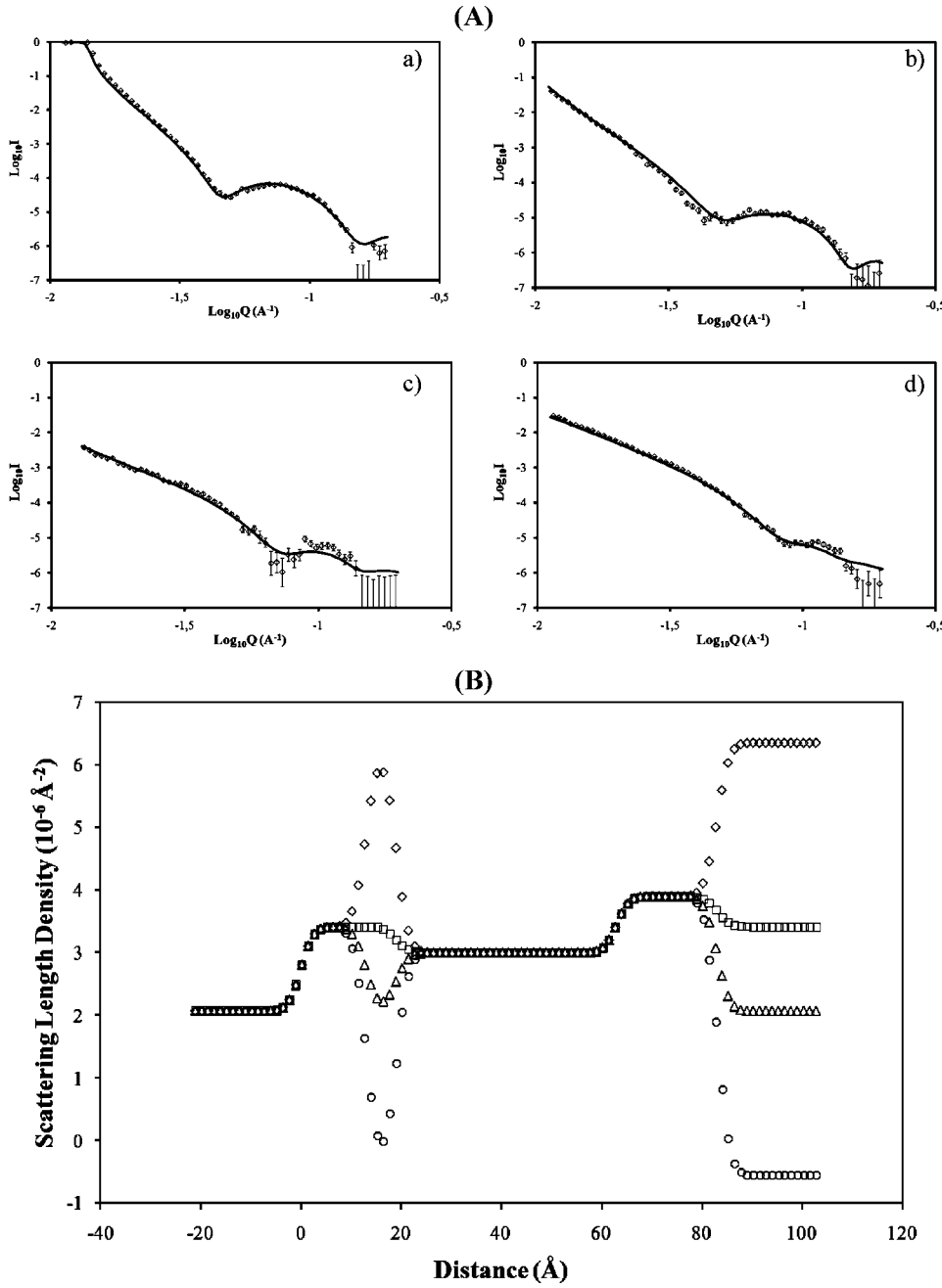

Figure 4. (a) Model membrane exposed to *h*-DDAB/DNA lipoplexes for 5 days. Four H/D contrasts of solvent were used in the subphase for the collection of these data: (a) D_2O, (b) $CMSiO_2$, (c) CMSi, and (d) H_2O. Solid lines represent fits to the experimental data. (b) Scattering-length density profiles for the model fits of a model membrane exposed to *h*-DDAB/DNA lipoplexes for 5 days (Table 2) and characterized in four solvent contrasts; D_2O (◇), $CMSiO_2$ (□), CMSi (Δ), and H_2O (○).

negatively charged DNA to become bound to the surface, in a similar manner to DNA interacting with the *h*-DDAB vesicles in solution (and forming *h*-DDAB/DNA complexes).

If one considers the model membrane to represent a cell membrane, then these results demonstrate that the process of gene transfection, as mediated by the cationic lipid *h*-DDAB, involves the intercalation of the *h*-DDAB molecules from the *h*-DDAB/DNA complex into the cell membrane of the gene transfection target. The presence of these cationic lipids results in the binding of DNA to the surface of the cell membrane from where it enters the cell by endocytosis, as suggested by a number of researchers such as Almofti et al.[25]

Whereas this process is a reasonable interpretation of the structures derived from the model fitting of the reflectivity curves collected for the model membrane exposed to *h*-DDAB/DNA complexes, the time scales required to visualize the exchange of lipid are extremely long. For significant changes in the (ρ of the) model membrane to be observed, it has to be exposed to the *h*-DDAB/DNA complexes for periods >25 h. Certainly for in vivo transfection, this period of time would allow the removal of the *h*-DDAB/DNA complexes from the blood stream by the immune system of the subject. However, low levels of DDAB intercalation into the cell membrane may cause increased endocytosis by changing the lipid packing within the membrane.

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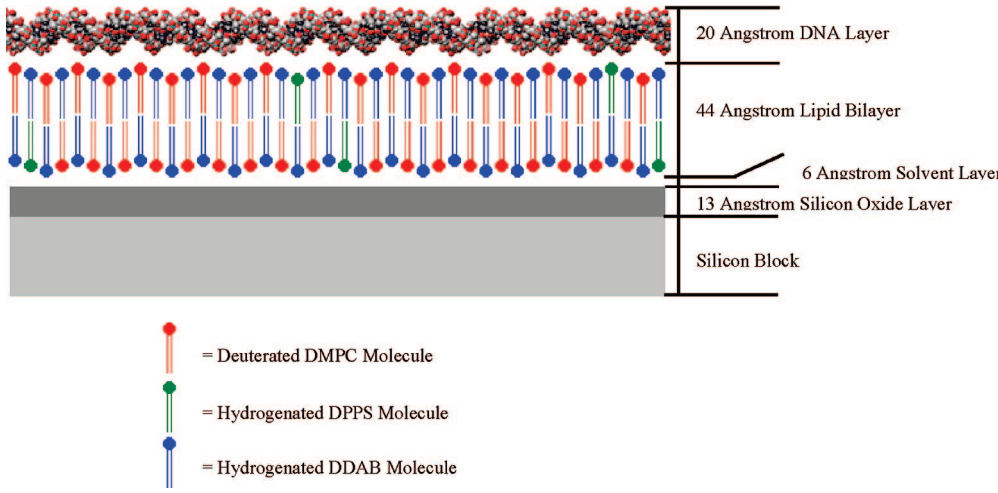

Figure 5. Interpretation of the model parameters, described in Table 4, used to fit the neutron reflectivity curves (Figure 4) collected for a model membrane exposed for 5 days to *h*-DDAB/DNA lipoplexes.

Table 4. Model Parameters Required to Fit the Neutron Reflectivity Curves of a Model Membrane after 5 Days of Exposure to *h*-DDAB/DNA Lipoplexes (Figure 4)[a]

layer (description)	thickness (Å)	scattering-length density ($\times 10^{-6}$ Å^{-2})	roughness (Å)	% solvent
layer 1 (SiO_2)	13 ± 2	3.4 ± 0.2	2 ± 1	0
layer 2 (Sol)	6 ± 1	N/A	2 ± 2	100
layer 3 (DDAB)	44 ± 1	3.0 ± 0.1	2 ± 2	13 ± 2
layer 4 (DNA)	20 ± 3	3.9 ± 0.6	2 ± 2	75 ± 6

[a] SiO_2 = silicon oxide layer, Sol = trapped solvent layer, DDAB = mixed lipid bilayer of DDAB molecules with *d*-DMPC and *p*-DPPS, and DNA = layer of DNA molecules.

Table 5. Model Parameters Required to Fit the Neutron Reflectivity Curves of a Model Membrane after 13 h of Exposure to *h*-DDAB/Chol/DNA Lipoplexes[a]

layer (description)	thickness (Å)	scattering-length density ($\times 10^{-6}$ Å^{-2})	roughness (Å)	% solvent
layer 1 (SiO_2)	18 ± 1	3.41 ± 0.2	2 ± 1	0
layer 2 (Hg)	13 ± 1	0.75 ± 0.1	2 ± 1	16 ± 2
layer 3 (Hc)	21 ± 1	0.27 ± 0.1	2 ± 1	5 ± 1
layer 4 (Hg)	13 ± 1	0.75 ± 0.1	2 ± 1	16 ± 2

[a] SiO_2 = silicon oxide layer, Hg = headgroup region of the lipid bilayer, and Hc = hydrocarbon core region of the lipid bilayer.

This, in conjunction with increasingly positive charge of the membranes, may result in the improved transfection rates observed for *h*-DDAB/DNA complexes compared to those for free DNA.

Prolonged Exposure of a Model Membrane to h-DDAB/Chol/DNA Complexes. After an 8 day exposure of a model membrane to *h*-DDAB/Chol/DNA complexes, the lipid/DNA complexes were removed and neutron reflectivity data were collected using the three different H/D solvent contrasts: D_2O, $CMSiO_2$, and CMSi. The model parameters required to fit these data simultaneously show some differences regarding the values of thickness, ρ, or roughness of the layers from those used in modeling the system after a 13 h exposure to the same type of complex (Tables 5 and 6); however, the general characteristics of the models remain unchanged.

The model after 8 days of exposure to the lipid/DNA complexes has more solvent present, suggesting that the coverage of the silicon surface is less complete. Because different silicon surfaces were used in these two experiments, this is considered to be a result of variations in the characteristics of the substrate. Even

Table 6. Model Parameters Required to Fit the Neutron Reflectivity Curves of a Model Membrane after 8 Days of Exposure to *h*-DDAB/Chol/DNA Lipoplexes[a]

layer (description)	thickness (Å)	scattering-length density ($\times 10^{-6}$ Å^{-2})	roughness (Å)	% solvent
layer 1 (SiO_2)	15 ± 1	3.41 ± 0.2	2 ± 1	0
layer 3 (Hg)	8 ± 1	0.32 ± 0.3	3 ± 1	25 ± 3
layer 4 (Hc)	29 ± 1	−0.1 ± 0.1	3 ± 1	16 ± 1
layer 5 (Hg)	8 ± 1	0.32 ± 0.3	3 ± 1	25 ± 3

[a] SiO_2 = silicon oxide layer, Hg = headgroup region of the lipid bilayer, and Hc = hydrocarbon core region of the lipid bilayer.

after this prolonged exposure of the membrane to the *h*-DDAB/Chol/DNA complexes, there are no indications of either the formation of additional layers or the adsorption of DNA at the membrane surface.

It would appear, therefore, that the most significant effect of incorporating cholesterol in the DDAB vesicles is to increase the rate of interaction of the complexes with the membrane, with 90% of the membrane being replaced by a mixture of DDAB and cholesterol after 13 h (compared to 50% exchange after 5 days in the absence of cholesterol). Whereas the binding of DNA to the surface is not apparent from the model fitting of the neutron reflectivity curves, it is reasonable to propose that some negatively charged DNA (possibly at very low concentrations) will become bound to this new bilayer in much the same way as seen following the exposure of the supported bilayer to DDAB/DNA lipoplexes. We propose, therefore, that the role of the cholesterol as a helper lipid, which increases the in vivo transfection efficiency of DDAB/DNA complexes,[26] is to increase the rate of lipid exchange between the DDAB/DNA complexes and the cellular membrane of the transfection target, resulting in the binding of DNA to the cell surface.

Exposure to h-DDAB/DOPE/DNA Complexes. Following a 5 h and then a 6 day exposure of model membranes to *h*-DDAB/DOPE/DNA complexes, the complexes were removed (by flushing with D_2O), and the adsorbed layer structures were characterized using the four H/D solvent contrasts provided by subphases of D_2O, $CMSiO_2$, CMSi, and H_2O. As in the case of the membrane exposed over 8 days to *h*-DDAB/Chol/DNA complexes, the model fitting of the reflectivity curves for the membranes exposed to the *h*-DDAB/DOPE/DNA complexes (both the one exposed for 5 h and that exposed for 6 days) gave clear evidence of lipid exchange between the

Table 7. Model Parameters Required to Fit the Neutron Reflectivity Curves of a Model Membrane after 5 h of Exposure to *h*-DDAB/DOPE/DNA Lipoplexes[a]

layer (description)	thickness (Å)	ρ ($\times 10^{-6}$ Å^{-2})	roughness (Å)	% solvent
layer 1 (SiO_2)	18 ± 1	3.4 ± 0.1	2 ± 1	0
layer 2 (Hg)	8 ± 1	1.8 ± 0.3	2 ± 1	8 ± 3
layer 3 (Hc)	26 ± 1	0.7 ± 0.2	2 ± 1	5 ± 2
layer 4 (Hg)	8 ± 1	1.8 ± 0.3	2 ± 1	8 ± 3

[a] SiO_2 = silicon oxide layer, Hg = headgroup region of the lipid bilayer, and Hc = hydrocarbon core region of the lipid bilayer.

Table 8. Model Parameters Required to Fit the Neutron Reflectivity Curves of a Model Membrane after 6 Days of Exposure to *h*-DDAB/DOPE/DNA Lipoplexes[a]

layer (description)	thickness (Å)	ρ ($\times 10^{-6}$ Å^{-2})	roughness (Å)	% solvent
layer 1 (SiO_2)	13 ± 1	3.4 ± 0.1	2 ± 1	0
layer 2 (Sol)	10 ± 1	N/A	2 ± 1	100
layer 3 (Hg)	7 ± 1	2.2 ± 0.7	2 ± 1	45 ± 9
layer 4 (Hc)	31 ± 1	-0.3 ± 0.2	2 ± 1	30 ± 2
layer 5 (Hg)	7 ± 1	2.2 ± 0.7	2 ± 1	45 ± 9

[a] SiO_2 = silicon oxide layer, Sol = trapped solvent layer, Hg = headgroup region of the lipid bilayer, and Hc = hydrocarbon core region of the lipid bilayer.

membranes and complexes but no evidence of the deposition of DNA at the membrane surface (Tables 7 and 8).

The absence of DNA at the surface of the model membranes after their exposure to *h*-DDAB/DOPE/DNA complexes is surprising, but as was noted for the system exposed to *h*-DDAB/Chol/DNA complexes, it is reasonable to propose that *some* negatively charged DNA may become bound to the membrane. However, if this is the case, then it occurs only at a concentration that is below the threshold for detection by neutron reflectivity.

Comparing the rate of lipid exchange between the model membrane and *h*-DDAB/Chol/DNA and *h*-DDAB/DOPE/DNA complexes shows that the cholesterol-containing complexes replace ~50% of the membrane with hydrogenous lipid within the first hour (Table 2) whereas those containing DOPE replace ~70% within the same period (Table 3). Therefore, the rate of exchange is faster for *h*-DDAB/DOPE/DNA than for the *h*-DDAB/Chol/DNA complexes.

Conclusions

The results of these neutron reflectivity experiments define a clear role for the neutral helper lipid when lipid/DNA complexes interact with model membranes. We have previously shown that in the absence of DNA the rate of interaction between the membrane and lipid vesicles appears to be determined by the helper lipid present.[11] Both *h*-DDAB/Chol and *h*-DDAB/DOPE vesicles interact at a faster rate than pure *h*-DDAB vesicles whereas the presence of DOPE results in a faster rate than the presence of cholesterol. Interestingly, there are no novel structures formed at the surface of the silicon block or at the surface of the membrane. Instead, there is exchange of lipid(s) between the vesicles and the membrane.

In the presence of DNA, a similar process is observed with lipid exchange occurring between the lipid/DNA complexes and the membrane. The rate of lipid exchange is faster for the lipid/DNA complexes than for lipid vesicles, whereas the order of increasing rate remains the same, *h*-DDAB/DNA < *h*-DDAB/Chol/DNA < *h*-DDAB/DOPE/DNA.

The lipid/DNA complexes appear to form ordered structures in solution,[18] with lipid bilayers arranged in stacks with the DNA molecules sandwiched between the lipid sheets. However, these solution structures do not translate to similar structures at the surface of the silicon block. This is because, whereas lipid exchange between the model membrane and the lipid/DNA complexes results in the surface of the membrane becoming positively charged, DNA does not become bound in significant quantities to the lipid structure at the silicon surface. Therefore, the formation of lipid/DNA stacks at the surface is not possible.

If one considers that the model membrane represents a cell membrane, it would appear that during gene transfection the key process at the surface of the cell membrane is the exchange of lipid between the lipid/DNA complexes and the cell membrane. Although this does not result in the binding of significant quantities of DNA to the membrane surface (in the presence of *h*-Chol or *h*-DOPE), it may encourage endocytosis, which has been shown to be important in DNA entering the cell.[25]

As to why there is DNA deposited above the model membrane after its exposure to DDAB/DNA complexes but no (detectable) deposition of the nucleic acid when the membrane is exposed to complexes involving either of the two helper lipids may perhaps be explained in terms of a dilution effect. That is, in the case of the helper lipid systems, the DDAB is mixed either with DOPE or cholesterol, both of which are *neutral* molecules. Thus, when the lipid from these complexes exchanges with the lipid in the model membranes, the proportion of *cationic* molecules exchanged will be less than would be exchanged from a complex involving just DDAB, so the positive charge imparted to the model membrane will be correspondingly lower. This in turn would mean that there is a reduced level of attraction between the membrane and the anionic DNA, with the result being that there is no significant deposition of the nucleic acid on the membrane surface.

From the fact that there is such pronounced lipid exchange observed between the model membranes and all forms of lipid vesicles and all forms of lipid/DNA complexes, it would seem that the gain in entropy afforded to the system by the lipid mixing outweighs the strong electrostatic interaction between the DDAB and DNA. Given that the exchange process is completed over so long a period, however, would indicate that there must be a significant activation barrier associated with it.

Relating the results of these neutron reflectivity experiments to the observations of Sternberg et al.[26] shows that the rate of exchange of lipid between the cell membrane and the lipid/DNA complex is vital in determining transfection efficiency. The *h*-DDAB/DOPE/DNA complexes have the fastest rate of interaction with the model membrane, whereas Sternberg et al.[26] have shown that these complexes have a high transfection efficiency *i*n vitro but a low transfection efficiency in vivo. The *h*-DDAB/Chol/DNA complexes interact at a slower rate and have been shown by Sternberg et al.[26] to have a higher transfection efficiency in vivo than in vitro.

An explanation for this behavior is that whereas the *h*-DDAB/DOPE/DNA complexes interact with the cell membrane rapidly, increasing the positive charge at the surface and potentially binding DNA, which is an efficient process in vitro, the complexes are not stable enough for high transfection efficiency in vivo. The opposite is true for the *h*-DDAB/Chol/DNA lipoplexes, which may be stable enough to circulate successfully in vivo with their slower rate of interaction with cell membranes but with a rate of exchange

fast enough to have an effect before their removal from the bloodstream. These characteristics may be less efficient in vitro, where the highest rate of interaction may be the optimum condition for high transfection efficiency.

Acknowledgment. We acknowledge the assistance of Simon Wood in the fabrication of the sample holders that were used; the ESRF optics laboratory for polishing the silicon surfaces; the ILL for providing access to their D17 neutron reflectometer; and ISIS for access to their SURF neutron reflectometer. P.C. gratefully acknowledges the financial support provided by EPSRC and ILL.

Supporting Information Available: Model membranes exposed to lipoplexes. This material is available free of charge via the Internet at http://pubs.acs.org.

LA802847H