Phase Behavior of DPPC in a DNA−Calcium−Zwitterionic Lipid Complex Studied by Small-Angle X-ray Scattering

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In the presence of DNA and calcium, multilamellar liposomes of DPPC when in excess form complexes in which two distinct lamellar lipid phases coexist. The structure and phase behavior of both of these have been investigated using high-resolution small-angle X-ray scattering. The coexisting lipid phases exist within the same precipitated condensate with the phase behavior of the unbound lipid being affected by the complexed lipid phase. While the thermotropic phase transitions of the uncomplexed lipid from $L_{\beta'}$–$P_{\beta'}$–L_{α} occur as expected, the transitions of the bound lipid are substantially altered. This is manifested as an increase in the main transition temperature, T_m, of the bound lipid, which has been observed before (Tarahovsky, Y. S.; Khusainova, R. S.; Gorelov, A. V.; Nicolaeva, T. I.; Deev, A. A.; Dawson, K. A.; Ivanitsky, G. R. *FEBS Lett.* **1996**, *390*, 133. Kharakoz, D. P.; Khusainova, R. S.; Gorelov, A. V.; Dawson, K. A. *FEBS Lett.* **1999**, *446*, 27). However, we also observe an apparent disappearance of the ripple phase ($P_{\beta'}$) with increasing calcium concentration in the bound lipid phase. In addition we are in a position to comment on the mechanism of formation for complexes prepared in this way.

Introduction

The phase behavior of DPPC is now reasonably well understood, with recent papers detailing its structure in both the gel and fluid phases.[3−5] Even the less well understood ripple phase has been investigated in great detail, throwing light on its structure.[5−9] We have investigated the phase behavior of DPPC in the presence of calcium and DNA using high-resolution small-angle X-ray scattering. The study of these DNA−lipid complexes has been relatively limited since the interaction of calcium with DPPC is generally accepted to be weak.[10,11] We describe a structure in which calcium binding to DPPC is greatly enhanced by the presence of DNA, and we determine how this interaction affects the phase behavior of the lipid. More specifically we concentrate on the phase behavior of DPPC in a complex formed from multilamellar vesicles.

The characterization of many types of DNA−cationic lipid complexes has received much attention in recent years, since their potential for use in gene delivery applications was identified.[12,13] These studies have ranged from structure determination using electron microscopy and small-angle X-ray scattering to examination of complex phase behavior and transfection experiments in an attempt to discover a structure−function relationship.[14−24] It has been proposed that there is a correlation between the two. Complexes that form inverted hexagonal lipid phases, for example, are believed to be more efficient in delivering DNA to cells than the lamellar structures more commonly observed.[25] These experiments are usually carried out on complexes in which mixtures of cationic and zwitterionic lipids condense DNA, since the inclusion of a zwitterionic lipid was found to enhance transfection efficiencies in vitro.[26]

Complexes composed exclusively of neutral lipids offer an alternative to cationic lipids, in that they are completely noncytotoxic. There is no direct electrostatic interaction between zwitterionic lipids and DNA, but the interaction

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can be mediated by divalent cations.[27,28] In this study, the complex formed from the interaction of DPPC, DNA, and calcium has been considered. The phase behavior and stoichiometry of these complexes have been investigated using DSC and temperature-scanning ultrasound techniques.[1,2,29] The complex formed with DNA, calcium, and egg lecithin exhibits a variety of structures.[29] SAXS experiments and electron microscopy have shown that well-ordered lamellar complexes form with DPPC, calf-thymus DNA, and either calcium[30] or magnesium.[31] A lamellar structure for complexes formed from DOPC, DNA, and manganese has also been identified.[32] The formation of a complex from DNA and zwitterionic lipids only has been studied by SAXS recently.[33]

The purpose of this investigation is to study the effect of calcium on the structure and phase behavior of the complex formed when it is prepared in excess lipid from multilamellar vesicles. DSC work has shown that, in equilibrated samples prepared from multilamellar vesicles, a 2-phase region of lipid is present.[1] Uncomplexed DPPC has a T_m, as expected, at ~41.5 °C, and bound lipid has a second T_m at a higher temperature of ~43.1 °C. This second peak was attributed to complexed DPPC, since the intensity of the peak increased with increasing DNA concentration. What has not been established is the function, if any, of the uncomplexed DPPC and the nature of the role of calcium in the complex structure.

Experimental Methods

Preparation of Materials. Calf-thymus DNA was purchased from Sigma and before purification was dissolved in 1 M NaCl, 10 mM Tris, and 2 mM EDTA buffer at 4 °C with gentle stirring over a number of days. Before use, phenol was distilled using an air condenser. The distilled phenol was then equilibrated to pH 7.8 by the following method. The phenol was melted at 68 °C. To the melted phenol an equal volume of Tris buffer (0.5 M, pH 8.0) was added. The two were mixed vigorously in a separating funnel, and the aqueous layer was removed. An equal volume of Tris buffer prepared at a concentration of 100 mM (pH 8.0) was then added to the phenol. Again the solution was mixed vigorously in the separating funnel and the aqueous phase removed. This was repeated until the pH of the phenolic phase reached >7.8, measured using pH indicator paper. To the equilibrated phenol, 0.1 wt % 8-hydroxyquinoline was added. The phenol was mixed in a ratio of 25:24:1 with chloroform and isoamyl alcohol. This solution was stored at −20 °C before use. The phenol/chloroform mixture was mixed with an equal volume of the DNA solution and vortexed until an emulsion was formed. This was then centrifuged at 10 000 rpm for 10 min and the upper aqueous (DNA) layer removed. This procedure was repeated 3−4 times until no protein was observed at the interface of the two phases. Finally chloroform was added to an equal volume of DNA and the centrifuging step repeated. Adding 2 volumes of cold ethanol to the solution precipitated the DNA. The precipitate was redissolved in citrate buffer (pH 7.5). Any remaining ethanol was removed by dialysis against 50 mM citrate, 10 mM EDTA at pH 7.5 over 3 days with a change of buffer each day. Dialysis against 0.5 mM HEPES (pH 7.5) gave a final DNA concentration of about 1 mg/mL. The dialysis membrane used was a Spectro/Por 1 (Spectrum Medical Industries, Houston, TX), which had a molecular weight cutoff of 6000−8000 daltons. The 260/280 nm ratio, which is a measure of DNA purity, was greater than 1.8 measured by UV absorbance ($\epsilon_{260\ nm} = 6600\ M^{-1}\ cm^{-1}$) and is expressed in moles/nucleotide throughout this paper.

For the SAXS experiments, DPPC, dipalmitoyl-*sn*-glycero-3-phosphatidylcholine was dissolved in chloroform and a thin film cast under a stream of nitrogen. The film was then dried in a vacuum oven overnight to remove any remaining traces of solvent. Millipore water was added to the dried film to give a final concentration of 25 mg/mL and the lipid rehydrated at 50 °C for 2 h and then left at 4 °C for 2 days before the preparation of the samples. Multilamellar vesicles were prepared by vortexing the lipid suspension at 50 °C. All buffer salts were analytical grade and were used without further purification.

Small-Angle X-ray Scattering. Since the volumes in which the samples are prepared are small, to prepare the DNA for SAXS experiments, purified DNA samples (which are stored at relatively dilute concentration (1 mg/mL)) were freeze-dried and redissolved in 0.5 mM HEPES to give a final concentration of 5 mg/mL. The samples for X-ray analysis were prepared by adding DNA, lipid, and calcium from concentrated stocks to the wide part of a 1.0 mm X-ray capillary (Hilgenberg, Germany). The total volume in each capillary was kept constant, with all of the samples prepared in excess water. The lipid concentration was kept a constant at 22.7 mM. The concentrations of DNA and calcium were varied to give the appropriate mole ratio of DNA to lipid and the required calcium concentration. In the present experiments a DNA to lipid mole ratio of 1:8 was chosen. The calcium concentrations were varied between 1 and 100 mM. This DNA:lipid ratio was used because it has been calculated that under these conditions the number of tightly bound lipids/DNA phosphate is 4.5−5.[2] Adding 8 lipid molecules/phosphate means that there is adequate uncomplexed lipid to examine this phase simultaneously. The components were mixed in the wide part of the X-ray capillary and left for 1 h at room temperature. The resulting complex was centrifuged into the narrow part of the X-ray tube. The samples were equilibrated by varying the temperature between 25 and 55 °C in a water bath over at least 3 days. The samples were then stored at 4 °C until the experiments were carried out at the ID2 beamline at the ESRF, Grenoble, France. The q-axis for the SAXS was calibrated using a silver behenate standard. The detector distance was 1.5 m, the wavelength being 0.1 nm (12.4 keV), with a wavelength resolution $\Delta\lambda/\lambda = 2 \times 10^{-4}$, a horizontal resolution of $\Delta q = 0.025\ nm^{-1}$, and a vertical resolution of $\Delta q = 0.014\ nm^{-1}$. SAXS scans were carried out from 25 to 55 °C by increasing the temperature in 1.5 °C steps. The temperature was controlled by an external circulating water bath to ±0.5 °C. The exposure time for each sample at each temperature was 100 ms. No evidence of sample degredation was observed in any of the samples at this exposure.

Results

SAXS data presented here are for samples prepared in excess lipid at a DNA:lipid mole ratio of 1:8, where the concentration of DNA is expressed in moles/nucleotide. The samples are prepared by mixing multilamellar vesicle suspensions and DNA and then adding calcium to give the required final concentration. The resulting complex is centrifuged into the narrow part of the X-ray capillary, leaving a condensed precipitate at the end. Annealing of the samples then takes place over a number of days. Two distinct DPPC lamellar phases are present in this equilibrated complex. We identify these as the same two phases observed in the DSC work,[1] one as lipid bound to DNA in a complex and the other as uncomplexed lipid. A typical result can be seen in Figure 1. Each lamellar phase was treated independently for the purpose of calculating the lamellar repeat distances. A sample prepared from pure lipid was used as a control. The measured repeat distance (d spacing) for DPPC in 5 mM HEPES buffer in this sample was 6.34 nm, which is consistent with the value determined by Nagle et al.[3] The lamellar repeat

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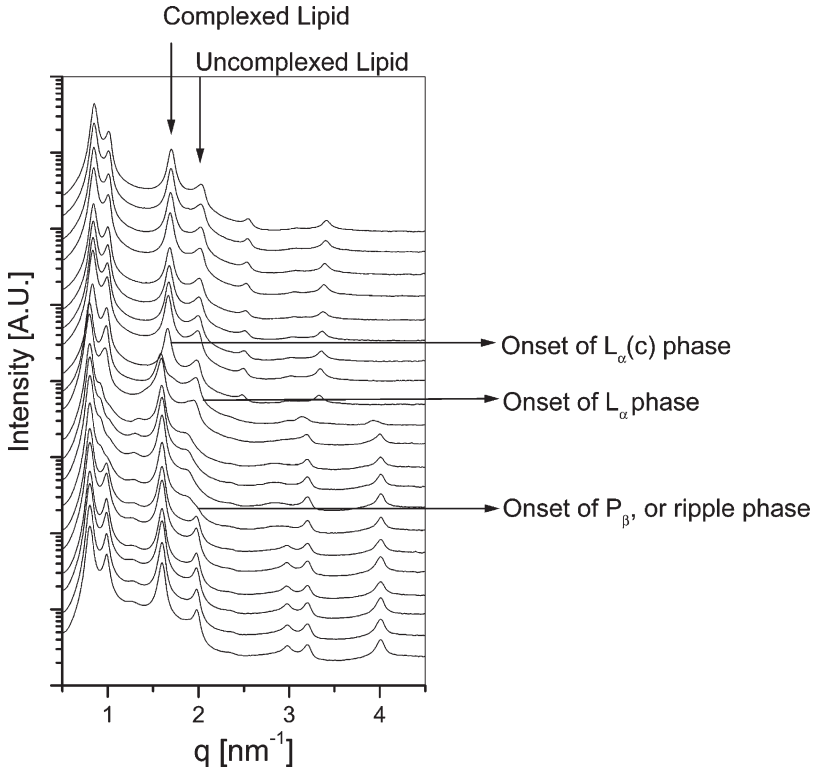

Figure 1. Typical SAXS for a complex prepared at a DNA:lipid mole ratio of 1:8. There is a two-phase coexistence of DPPC lamellar phases, one corresponding to uncomplexed lipid with a lamellar repeat distance of ~6.3 nm and a second corresponding to lipid bound to DNA via calcium with a lamellar repeat of ~7.8 nm. The SAXS shown are at increasing temperature, starting from 25 °C at the bottom and ascending in 1.5 °C steps to 55 °C at the top. The thermotropic phase transitions of each lipid phase are indicated. The broad peak between the (001) and (002) lipid lamellar peaks is the DNA−DNA in-plane correlation. This corresponds to a DNA−DNA in-plane distance of 5.14 nm, calculated as $d_{DNA} = 2\pi/q_{DNA}$.

distance, d, is calculated as $d = 2\pi h/q$, where h is the order of the Bragg peak 1, 2, ..., etc. The value of q was determined by fitting a Lorentz function to each of the peaks. The lamellar repeat distance is the sum of the membrane thickness δ_m and the water space between the layers, δ_w. Above the main transition temperature (41.3 °C) of the pure lipid sample, this spacing increases to 6.72 nm, again consistent with the value determined by Nagle et al.[4] This increase in repeat distance is a consequence of thinning of the bilayer due to an increase in the undulatory fluctuations because of a temperature-dependent decrease in bilayer bending modulus and an increase in the water space between the lamellae. The lamellar repeat distances at each temperature, for both lipid phases, are shown in Figure 2. The lamellar repeat distance of the uncomplexed lipid in the complex formed from DNA, DPPC, and calcium was then monitored as a function of calcium concentration. At 25 °C with increasing calcium concentration, the lamellar repeat distance of the uncomplexed lipid ($L_{\beta'}$) increases slightly to ~5 mM $CaCl_2$ after which it decreases slightly over the calcium concentration range studied (Figure 3b). Above the transition temperature, however, a far greater change is observed. With increasing calcium concentration, the DPPC lamellar spacing decreases from 6.72 nm in the absence of $CaCl_2$ to 5.65 nm in 100 mM $CaCl_2$ at 55 °C (L_α). The onset of the pretransition or ripple phase ($P_{\beta'}$) occurs as is usually observed at ~34 °C in all of the samples prepared between 1 and 20 mM $CaCl_2$. The samples prepared at 50 and 100 mM $CaCl_2$ showed an increase in both the pretransition temperature and main transition temperature.

In the presence of DNA and calcium the DPPC lamellar spacing increases to 7.84 nm at 25 °C, in 20 mM $CaCl_2$ (L_β^C). For convenience this lipid will be referred to as bound lipid. Again in this case, the lamellar repeat distance is the sum of the membrane thickness, δ_m and the water spacing between the bilayers, δ_w, which in the case of bound lipid includes the space occupied by the DNA strand. Figure 4 suggests a possible local arrangement for lipid, DNA, and calcium within the complex structure. Increasing the concentration of calcium from 1 to 4 mM causes a very slight increase in the bound DPPC lamellar repeat distance, which after 5 mM begins to increase again (Figure 3a). At 5 mM there is a decrease in the lamellar repeat distance to 7.86 nm. Above the transition temperature the effect is a little easier to see.

Discussion

Phase Behavior of the Bound Lipid. In the presence of DNA the lamellar repeat distance of the bound lipid phase increases to 7.84 nm. If the bilayer thickness for DPPC is taken as 4.34 nm,[34] this leaves plenty of room to accommodate a hydrated B-DNA strand between the bilayers.[35] The change in lamellar repeat distance between 1 and 4 mM $CaCl_2$ is most probably due to binding of the lipid to the DNA surface. It is difficult to suggest a single reason why this affects the lamellar repeat distance. Binding of calcium will affect the electrostatic interactions on the surfaces of both species, but there is likely to be a reorientation of the lipid headgroup as more calcium is bound. The minimum at 5 mM represents the point at which the lipid becomes fully bound. Specifically for the complex prepared at DNA:lipid mole ratio of 1:8, a Ca^{2+}

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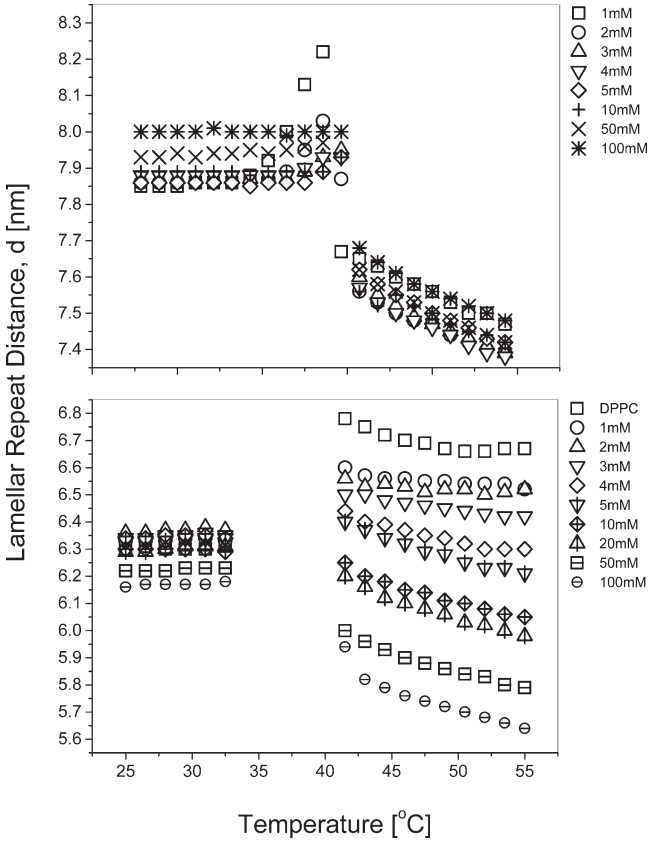

Figure 2. Lamellar repeat distance vs temperature data for bound lipid (top) and uncomplexed lipid (bottom) for the 2 phases in the complex (see Figure 1) at a number of different calcium concentrations. The values between 34 and 41.5 °C, corresponding to the ripple phase for the uncomplexed lipid, can be seen in Figure 7.

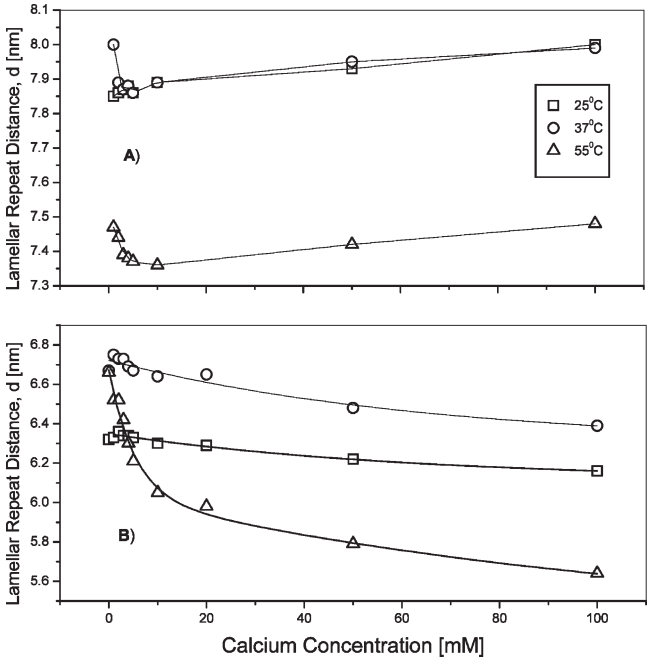

Figure 3. Lamellar repeat distance vs calcium concentratrion for (A) uncomplexed lipid and (B) bound lipid in the complex.

concentration of 5 mM is the point at which there are two DPPC molecules for each calcium atom in the bound lipid portion of the complex, if we assume 4.5−5 strongly bound DPPC molecules/DNA phosphate. The reasons for this conclusion are discussed in detail elsewhere.[38] SAXS data

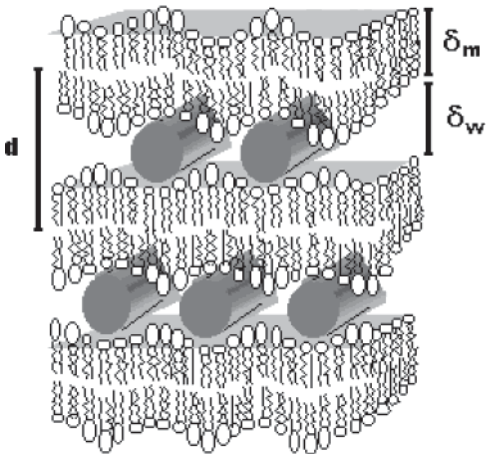

Figure 4. Expected structural arrangement of DPPC and DNA within the complex. The membrane width is indicated by δ_m and the water spacing between the layer, in which the DNA is accommodated, is indicated by δ_w.

have shown that a compact structural arrangement is reached at a concentration *C**; in this case *C** is 5 mM. This stoichiometric ratio suggests that the calcium bridges adjacent lipids and forms an effective cationic moiety, which then binds to DNA. Beyond this point there is a slight increase in the lamellar repeat distance, but this increase is not large. There are many reasons why this may occur. Further calcium binding beyond the minimum at 5 mM will disturb the compact orientation reached at this point. The overall charge on the lipid surface will become positive within the complex, and the bilayer may try to swell due to electrostatic repulsions, as is usually observed in the DNA free system.[37−39]

DNA in-Plane Ordering within the Bound Lipid Phase. In the many samples examined for this study only a small number exhibited DNA−DNA in-plane correlations. These correlations are almost always observed in cationic lipid DNA complexes. We would also expect to find them in our complex since calcium is known to cause DNA condensation when confined to a surface, specifically in a complex with cationic lipids.[40] Since both lipid phases are present in each of the SAXS patterns observed, any DNA peaks which may occur are probably obscured or masked by the peaks arising from the two lipid phases. DNA peaks usually observed in cationic lipoplexes are weak in comparison to the lipid peaks. Complexes prepared from small unilamellar vesicles display only one lipid phase. In these samples, DNA−DNA in-plane correlations are regularly observed.

Uncomplexed Lipid Phase Behavior. Our system comprises two coexisting lamellar phases. The unbound lipid, present because the complexes are prepared in excess lipid, should not be involved in complex formation. It is not unreasonable then to assume that this portion of the lipid should behave in the same way as in the ternary DPPC, calcium, and water system. Therefore, our results are a little unexpected. The work done on DPPC in the presence of calcium has indicated that, in calcium concentrations higher than 1 mM, the bilayers swell into

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excess water.[37−39] This is due to electrostatic repulsion between the bilayers as a result of bound calcium on the surface of the lipids. At much higher calcium concentrations, an ordered lamellar structure returns, since screening of the charge occurs. All of our experiments are carried out at concentrations that in a DNA free system would swell. There are two possible explanations for what we see. The work of Lis et al.[38,39] involved preparing multilamellar structures in the presence of calcium from dried lipid powder. The preparation of our complexes requires that MLVs are prepared in water, with the later addition of calcium and DNA. Ion transport across membranes is entropically unfavorable due to the large hydrophobic region at the center of the bilayer; therefore, the concentration of calcium found on internal layers of lipid within the MLV structure may well be lower than in bulk solution. However, since the complexes are equilibrated for at least a week before measurements, this is unlikely to be significant. The second explanation is that the uncomplexed lipid layers are trapped within the complex structure. There are a couple of reasons for assuming this. Since both lamellar phases are present in the SAXS scans, both are present in the precipitated portion of the complex mixture, suggesting that the mechanism of complex formation traps the uncomplexed lipid within the bound lipid phase, forming a precipitate in which domains of bound lipid with domains of unbound lipid trapped within exist. There is evidence that the bound lipid phase directly affects the unbound lipid. At 5 mM $CaCl_2$ there is a maximum in the lamellar repeat distance for unbound lipid. This corresponds directly with the simultaneous minimum observed for the complexed lipid at the same concentration (Figure 2).

The decrease in the observed lamellar repeat distance with increasing calcium to our knowledge has not been observed before. A partial explanation is that the pressure exerted by the bound lipid phase at calcium concentrations higher than 10 mM as it swells slightly may "squeeze" the lamellae of the uncomplexed lipid forming the thin lamellar phase observed. This alone, however, does not account for the magnitude of the decrease. Another possibility has been outlined in a theory by Rouzina and Bloomfield,[41] based on a theory first suggested by Oosawa.[42] This theory predicts macroion attraction due to electrostatic correlation between screening counterions, at small separations on planar surfaces. They considered the reduced parameter $G = Z_l^2 l_b / a_z$, where $l_b = e^2/4\pi\epsilon_0\epsilon_r kT$ is the Bjerrum length and $a_z = [Z_1/(\sigma/e)]^{1/2}$ is the average distance between the two neighboring counterions at the charged surface characterized by the surface charge density σ. The authors concluded that the attraction appears when $\Gamma = \Gamma^* \approx 2$ and that the attraction is short ranged, important only at $h \leq a_z$, where h is the distance between the charged surfaces. This theory has also been developed for like charged spherical surfaces, and the results are consistent with the findings of Rouzina and Bloomfield.[43,44] Linse and Lobaskin performed simulations on macroions with three different counterion valences and found that attractions were only observed in cases where divalent and trivalent counterions are present.

The question then is if the DPPC surfaces with bound calcium (but not DNA) trapped within this complex satisfy the condition that $h \leq a_z$. It is impossible to know the precise concentration of calcium present in the uncomplexed lipid phase. We believe that calcium is more likely to bind in the complex with DNA, with any remaining calcium being present in the uncomplexed lipid phase, so the calcium concentrations shown in Figures 2 and 3 refer to the concentration of calcium the complexes were prepared in. Furthermore the binding constants reported for calcium and DPPC have varied between 1 and 100 M^{-1} (ref 11 and references therein), depending on how the values were determined. In any case the binding of calcium to DPPC membranes does not exceed a few percent at any calcium concentration.[10] The area/headgroup for DPPC has been reported as 0.47 nm^2.[3] If we take the case in 10 mM $CaCl_2$ at 25 °C, there are between 3% and 6% bound calcium atoms[11] on the lipid surface. We will assume 5% for convenience. We need to estimate a_z, the average distance between the two neighboring counterions at the charged surface characterized by the surface charge density σ. Since there are 5% of lipids with condensed counterions, 1 lipid in 20 is occupied by a calcium ion. The surface area occupied by 20 lipid molecules is 8.4 nm^2. We can define this area as 1 unit, A_u. The distance between condensed counterions can then be estimated as the distance from a point in one unit to the same point in the next unit, $\sqrt{A_u}$, which is 2.9 nm. At 25 °C the lamellar repeat distance in 10 mM $CaCl_2$ of the unbound lipid confined with the complex is 6.3 nm. Taking a bilayer thickness of 4.34 nm, the distance between the bilayers, h, is 1.96 nm. The uncomplexed lipid will not swell as is usually observed in DPPC−calcium systems, since it is trapped in the complex. Even bearing in mind the variation in the determined binding constants and that there is probably some small variation in the area/headgroup for DPPC lipid with bound calcium, the condition of $h \leq a_z$, where h is the distance between the charged surfaces and a_z is the average distance between the two neighboring counterions at the charged surface characterized by the surface charge density σ, is satisfied. The only remaining question then is why the effect is so much more evident above the main transition temperature of the lipid.

We can speculate that this may be explained by the fact that the bilayer thickness decreases from 4.34 nm in the gel phase to 3.43 nm in the fluid phase, which can explain why the lamellar repeat distances are lower but in fact does not mean that the surfaces are actually approaching closer to each other in the fluid phase. The lamellar repeat distance at 10 mM in the fluid phase, for example, is 6.05 nm. The bilayer thickness is this phase is 3.43 nm; therefore, h, the distance between the charged lipid surfaces, is 2.62 nm. The binding of calcium to lipids in this phase is weaker, with the binding constant estimated at one-fourth that of the gel phase. Therefore, the lamellar repeat distance can decrease even further without the lipid surfaces even reaching the same distance as in the gel phase (1.96 nm at 10 mM $CaCl_2$). This may explain the differences in the magnitude of the decrease in lamellar repeat distances between the gel and fluid phases. The effect is in fact not different but a consequence of the change in the calcium binding constant and bilayer thickness. Note carefully the distinction between the lipid domains (not DNA bound) in the presence of calcium here are trapped (or pinned) by the nearby lipid−DNA−lipid domains and the usual case of pure lipid in the presence of Ca^{2+}. In the case of the former, all long-range repulsive effects (undulation repulsions etc) are inoperative and one can develop novel interaction scenarios.

Ripple ($P_{\beta'}$) Phase. DPPC forms a ripple phase between the gel ($L_{\beta'}$) and liquid crystalline (L_α) states. Over the years many studies have concentrated on describing its structure using freeze-fracture electron microscopy and X-ray diffraction data.[5−9] It is believed to occur as an

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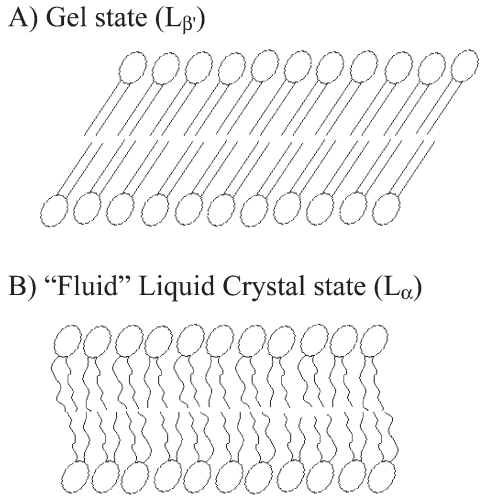

Figure 5. Orientation of lipid tail groups in DPPC. In the gel phase (A) the lipid tails are predominantly gauche or "tilted". On heating the lipid tails become perpendicular after the main transition temperature of ~41.5 °C.

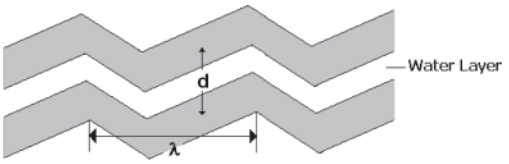

Figure 6. Ripple phase of DPPC indicating the lamellar repeat distance, *d*, and the ripple wavelength, *λ*.

Table 1. Lamellar Repeat Distance and Ripple Wavelength Values at Various Calcium Concentrations for the Ripple Phase of Uncomplexed Lipid within the Two-Lipid Phase Precipitate at 38.5 °C

calcium concn (mM)	lamellar repeat dist (nm)	ripple wavelength (nm)
0	6.77	13.8
1	6.82	14.1
2	6.78	13.9
3	6.77	14.1
4	6.76	14
5	6.81	
10	6.67	14.3
20	6.65	14
50	6.50	14.6
100	6.39	14.6

intermediate in the transition of the tail portion of the lipids from a tilted conformation in the gel phase to perpendicular conformation in the liquid crystalline state (Figure 5). The $P_{\beta'}$ phase conforms to the symmetry of a 2D monoclinic lattice. The low-angle diffraction peaks for this phase require two indices, (*h*,*k*), where *h* is the lamellar repeat distance and *k* is the ripple wavelength *λ* (Figure 6). Two different ripple phases for DPPC have been identified. The occurrence of the ripple phase is very much influenced by the history of the sample. On heating from the gel phase, a ripple phase with a short ripple and a wavelength of ~14.0 nm is observed. On cooling from the liquid crystalline phase, the ripple phase exhibits a long ripple in addition to the short ripple. Some disagreement has existed about the structure of this ripple phase, but the most recently published paper on the subject[9] confirms the two ripple coexistence. In our study data were only collected on a heating cycle, so the ripple phase data we report apply only to samples heated from the $L_{\beta'}$ phase.

Ripple Phase in Uncomplexed Lipid. Many researchers have described the structure of the ripple phase

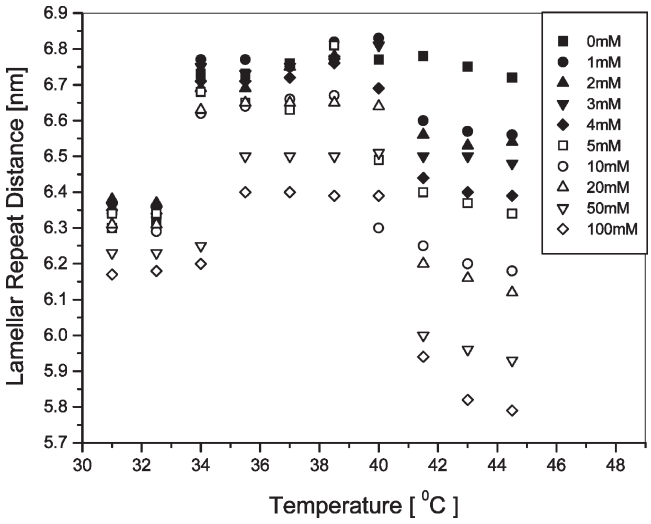

Figure 7. Lamellar repeat distance vs temperature for uncomplexed lipid within the multilamellar structure of the complex, in the vicinity of the ripple phase at increasing calcium concentrations.

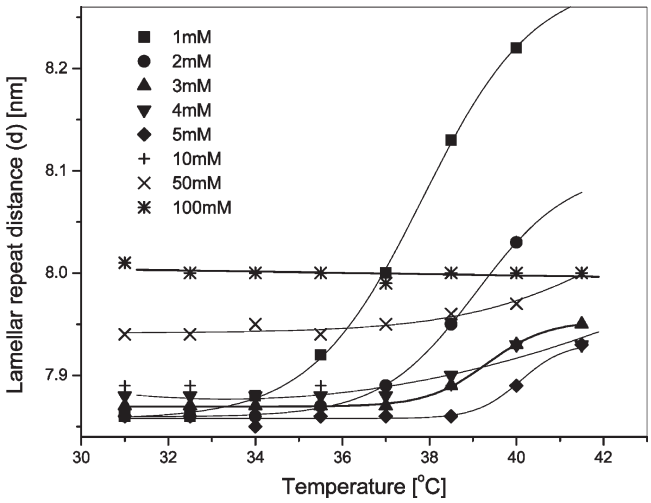

Figure 8. Lamellar repeat distance vs temperature for bound lipid in the expected ripple phase region with increasing calcium concentration. Lines are for guiding the eye and do not represent fits to the data.

for DPPC in water.[5−9] Usually information is obtained from wide-angle X-ray diffraction data or by small-angle X-ray data collected from aligned samples.[9] Information about the ripple phase can be obtained from powder diffraction data obtained on high-resolution instruments,[6] such as the instrument used for our study. We have determined the lamellar repeat distance for the ripple phase in the uncomplexed lipid within the complex and determined the ripple wavelength over a range of calcium concentrations (Table 1). The values we determine for the lamellar repeat distance and the ripple wavelength, *λ*, for DPPC in HEPES are reasonably consistent with the accepted values[9] for DPPC in water. With increasing calcium concentration the lamellar repeat distance of the lipid decreases. This is consistent with the decrease in lamellar repeat distance observed in both the gel and liquid crystalline states over the same calcium range and is probably a direct consequence of that effect.

Ripple Phase in Bound Lipid. Contrary to the observations in the uncomplexed lipid, the occurrence of the ripple phase in the bound lipid seems to be greatly influenced by the presence of DNA and calcium. At low calcium concentrations (1−2 mM) the transition temper-

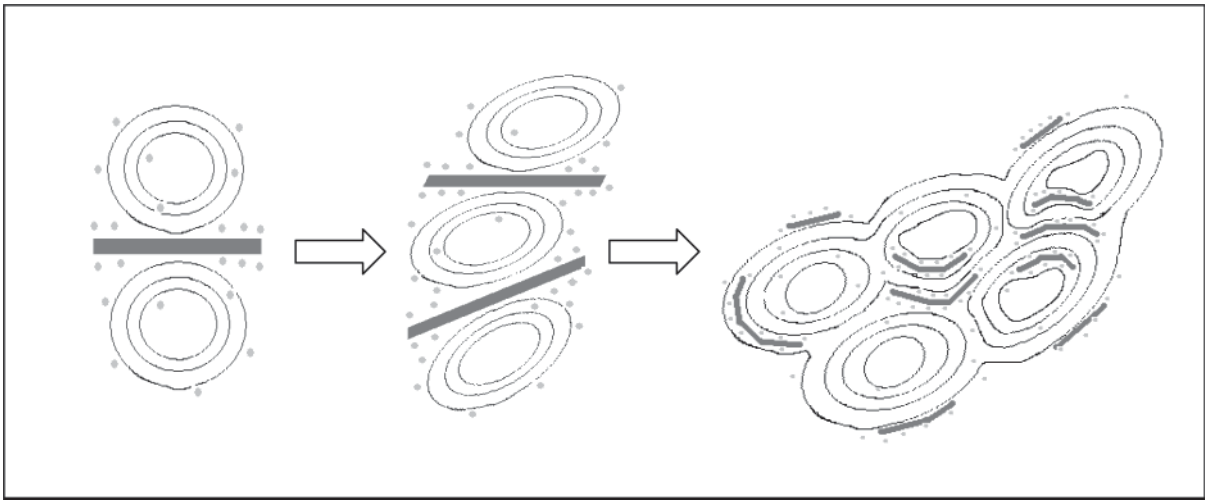

Figure 9. Schematic of proposed mechanism of formation for complexes prepared from multilamellar vesicles. Complexes are formed at room temperature, i.e., while DPPC is in the gel state. (A) DNA binds to the lipid surface. (B) This leads to aggregation of the liposomes, which are bound together by DNA and calcium. (C) Annealing of the aggregated complex allows lipid reorganization and consequent intercalation of DNA to outer layers of lipid, trapping the uncomplexed layer within the complex structure.

ature of the bound lipid is the same as that for the uncomplexed lipid. It seems that although the lamellar repeat distance has increased to accommodate a DNA strand, the lipid is only loosely bound since its thermotropic phase behavior is similar to that of the uncomplexed lipid. Between the pretransition at ~34 °C and the main transition at ~41.5 °C there is an increase in the lamellar repeat distance reaching a maximum just before the main transition temperature. The increase in lamellar repeat distance is usually sharp after the pretransition temperature (Figure 7). In the case of bound lipid, however, the increase is gradual (Figure 8). After 2 mM $CaCl_2$ the main transition temperature of the bound lipid increases to the value determined previously by DSC and temperature-scanning ultrasound techniques.[1,2] The pretransition behavior of the lipid also changes. The increase in lamellar repeat distance in the pretransition range becomes less pronounced. At 5 mM $CaCl_2$ there is no increase in lamellar repeat distance until 40 °C and even then the increase is by less than 0.1 nm. By 100 mM $CaCl_2$ there is no pretransition increase at all. This suggests that there is an elimination of the DPPC ripple phase in the presence of DNA and this effect increases with increasing calcium concentration. Tarahovsky et. al.[29] observed ripples in freeze fracture TEM for these complexes with a ripple wavelength of 25 nm over an extended temperature range. Their DSC results however noted a significant decrease or disappearance of the pretransition maximum for the complexed lipid. The *q*-range of our SAXS does not allow us to determine if a ripple wavelength could be observed at 25 nm. We believe from our results that a ripple phase for the complexed lipid may only exist at low calcium concentrations. It seems on binding the movements of the lipid bilayer become restricted. This restriction does not allow the "kinking" of the bilayer usually observed in the ripple phase. Another possible explanation is that the binding alters the tilted conformation of the lipid tails in the gel phase so that the ripple is not necessary before chain melting occurs. Wide-angle X-ray scattering data will be required to confirm this.

Mechanism of Complex Formation. Very little work has been carried out to identify all of the steps involved in lipoplex formation, although some progress in this regard has been made.[45,46] We can however postulate the mechanism involved in the formation of the complex described in this study on the basis of the structures formed. Initially, DNA must bind to the surface of the liposomes. In complexes formed from DNA, calcium, and small unilamellar vesicles of DPPC, lipid fusion is induced on the DNA surface.[47] Fusion of DNA on the MLV surface will be relatively slow since MLVs are large, making the liposomes kinetically stable. Intercalation of the DNA between the layers is likely to take place during the annealing of the samples. Lipid reorganization takes place in the fluid liquid crystalline state (L_α), facilitating this. This intercalation will however take place from the outside inward, therefore leaving the internal layers of the MLVs uncomplexed to DNA but trapped within the precipitate (Figure 9). This accounts for the structures we have observed in this study. This theory is supported by the fact that complexes prepared from unilamellar vesicles exhibit only one lamellar lipid phase in SAXS patterns. This lamellar lipid phase is the same DNA bound lipid as observed in the complexes described here. A different mechanism of formation occurs in which fusion of DNA on the lipid surface, which is more rapid than in the MLV case, takes place, precipitating the complexed lipid with DNA and leaving the uncomplexed lipid in the supernatant.

Conclusions

DPPC multilamellar liposomes in the presence of calcium and DNA form complexes in which two coexisting lipid lamellar phases are present. The thermotropic phase transitions of the uncomplexed lipid within this complex are the same as for pure DPPC. The effect of calcium on this lipid phase, however, suggests that it is trapped within a precipitate of DNA bound lipid. The effect of calcium on this phase then is different from that observed in the DNA-free system. We observe a novel attractive interaction between the like charged surfaces of the DPPC with bound calcium. Since the DPPC bilayers do not swell under the

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conditions of these experiments, the ripple phase for DPPC in calcium has also been observed. The lamellar repeat distance and ripple wavelength have been calculated at a number of different calcium concentrations for this phase. In the presence of DNA and calcium the ripple phase for DPPC appears to disappear. This effect increases with increasing calcium concentration.

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