

Structure of Complexes of Cationic Lipids and Poly(Glutamic Acid) Polypeptides: A Pinched Lamellar Phase

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Abstract: Complexes of cationic lipids with negatively charged biological polyelectrolytes such as DNA and proteins have elicited much interest recently because of their potential applications in gene delivery and in developing novel biomolecular materials. We report on the structure of complexes made from the anionic polypeptide poly-L-glutamic acid (PGA) and a positively charged lipid mixture consisting of the cationic lipid didodecyl dimethylammonium bromide (DDAB) and the neutral lipid dilauroyl-sn-glycero phosphocholine (DLPC). Small-angle X-ray scattering (SAXS), small angle neutron scattering (SANS), and optical microscopy of the complexes are consistent with a condensed multilamellar structure with PGA macromolecules sandwiched between the bilayers of the lipids. At the isoelectric point of the complex, lipid dilution experiments at increasing ratios of the neutral lipid to the cationic lipid resulted in an unexpectedly large increase in the interlamellar “d” spacing from 39 Å for the pure DDAB membrane to 60 Å at very high dilutions. Significantly, SAXS data shows that the lamellar complexes remained single phase which indicates that PGA interchain interactions are repulsive with their average spacing increasing with increasing lipid dilution. The data are consistent with a model of a “pinched lamellar” phase of the lipid–PGA complex where the PGA macromolecule and DDAB associate to form localized pinched regions. Between PGA–membrane “pinches” large pockets of water stabilized by hydration repulsion are contained and the system behaves as a nearly pure DLPC membrane with a large equilibrium spacing of 60 Å. These results suggest that biologically active molecules could be incorporated in the large hydration domains between “pinched” regions for delivery applications.

Introduction

It has been realized in recent years that ordered microstructures formed through a noncovalent self-assembly process lead to materials with unusual optical, electrical, mechanical, and biological properties. Polyelectrolyte–surfactant complexes represent a class of ordered microstructured materials exhibiting a rich variety of phase morphology which makes them promising materials for template-directed synthesis of inorganic zeolites, for layer-by-layer creation of organic thin films and novel biomolecular materials, and in particular, for developing nonviral gene delivery systems.^{1–3} These complexes are formed by spontaneous self-assembly of the surfactants with oppositely charged polyelectrolytes in aqueous solutions. The dominant driving force for the formation of such complexes is the release into solution of counterions which were one-dimensionally bound to the polyelectrolyte and two-dimensionally bound to the charged surfactant self-assembled structure.⁴ Since the self-assembly process is noncovalent, the macroscopic properties of the complexes can be suitably tailored by varying the

molecular interactions of the self-assembly process by changing temperature, solvent conditions, and surfactant charge, for example. The understanding that the study of these systems can bring to the problem of self-assembly and the wide array of surfactants and polyelectrolytes available have elicited considerable interest in this area.

To date work in this area has mainly focused on the complexation between synthetic polyelectrolytes and single-chain surfactants. Extending this approach by using charged lipids and biopolymers such as proteins, DNA, and polysaccharides as the polyelectrolyte offers several advantages in the areas of biotechnology, for example, in nonviral drug and gene delivery applications.

Gene therapy refers to the successful transfer and expression of extracellular DNA to mammalian cells with the aim of replacing a defective or adding a missing gene. Both viral and nonviral vectors such as cationic liposomes, DEAE–dextran etc. have been employed for gene therapy. Recently, there has been increasing interest in the use of nonviral methods for gene delivery owing to their lower immunogenicity compared to viral vectors and the ability to package large amounts of DNA in these systems. Felgner et al.⁵ were the first to demonstrate that complexes of DNA and cationic liposomes with a slight overall positive charge can effectively transfer DNA to cells (which usually possess a slight negative charge). Other groups soon

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demonstrated that these complexes could be used for in vivo gene expression in targeted organs^{6,7} and in human clinical trials.^{8,9} Compared to other nonviral vectors, cationic liposomes seem to hold the most promise for gene therapy as indicated by their higher transfection efficiencies in vivo.¹⁰ There have also been recent reports on the use of protein-cationic lipid complexes for protein delivery.¹¹ Moreover, proteins are also used as target-selective agents in cationic liposome-mediated delivery of DNA to cells.¹²

Apart from their obvious medical applications in gene therapy, complexes of cationic liposomes with biopolymers such as DNA and proteins hold potential as novel biomaterials. Since the biopolymers adopt well-defined ordered structures (such as α -helices, β -sheets, double helices etc.), the complexes in principle could form higher-ordered self-assembled structures where the biopolymers organize into liquid crystalline arrays in a self-assembled lipid matrix. Such a higher-ordered structure was recently reported by Raedler et al.^{4,13,14} who studied complexes of a linear DNA with mixtures of a cationic (DOTAP) and a neutral lipid (DOPC) by means of high-resolution X-ray scattering. It was found that the complex adopts a lamellar liquid crystalline morphology with alternating layers of lipid bilayers and DNA. In addition, the DNA between the bilayers was found to be ordered in a two-dimensional (2-D) smectic array. The structure of these complexes was also verified by detailed line-shape analysis of high-resolution small-angle X-ray scattering data of the complexes.^{15,16} Other groups have also reported similar structures in DNA-cationic liposome complexes.^{17,18}

The experimental investigations of these systems have been complemented by several recent theoretical studies which investigate the structure and thermodynamic stability of these complexes. Analytical and numerical studies of DNA-DNA interactions bound between membranes show the presence of a novel long-range repulsive electrostatic interaction.^{19–21} Moreover, a novel “sliding columnar phase” has been predicted where the positional coherence between polyelectrolyte molecules in adjacent layers is lost without destroying orientational coherence of the chains from layer to layer.^{22,23}

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The aim of the present investigation is to systematically characterize the structure of protein-cationic lipid complexes. There have been very few studies on polypeptide-cationic lipid complexes and such studies were mostly restricted to the solid-state properties of such systems.^{24–26} In this study we mainly deal with the structure of these complexes in the solution state because the presence and the nature of the solvent can significantly affect the phase morphology of these systems.

Since natural proteins interact and fold specifically depending on the exact amino acid sequence and the local environment, we chose to study a relatively well-defined model anionic polypeptide, sodium salt of poly-L-glutamic acid (PGA). Most of the experiments were carried out with a commercially available sodium salt of PGA of molecular weight 81 500. Commercially available PGA seldom has a molecular weight higher than 100 000 and is not quite monodisperse ($M_w/M_n = 1.15$). Attempts to synthesize PGA with higher molecular weights and narrower molecular weight distributions using existing synthetic methodologies have been unsuccessful. We have devised a new route to synthesize a high-molecular weight PGA with $M_n \approx 320\,000$ and a polydispersity of 1.06. The high-molecular weight PGA was used to study the effect of molecular weight and the polydispersity of the PGA on the microstructure of the complexes. The cationic lipid used was a double-chained amphiphile didodecyldimethylammonium bromide (DDAB) whose phase behavior is well-known.²⁷ Complexes were usually made with PGA and mixtures of the cationic lipid (DDAB) and the neutral lipid dilauroyl-sn-glycero phosphocholine (DLPC). The neutral lipid was used to systematically vary the charge density of the mixed lipid system. Both DDAB and DLPC contain 12 carbon atoms in their hydrophobic tails and are thus closely matched in their hydrophobic regions. However, the headgroup of DLPC is larger by 3 Å. Both DDAB and DLPC predominantly form lamellar phases in aqueous solutions and hence serve as a good model system to study the influence of anionic polyelectrolytes such as PGA on their morphology.

The structure of these complexes was characterized by X-ray scattering, small angle neutron scattering (SANS), ζ potential measurements, and optical microscopy. X-ray scattering, and SANS of these complexes revealed a multilamellar structure of the lipids with the PGA intercalated between the layers, similar to the structure observed in DNA-cationic liposome complexes. However, there was no evidence of any in-plane ordering of the PGA macromolecules in contrast to the 2-D smectic ordering of DNA molecules found in complexes of DNA and cationic liposomes.^{4,13,14} Rather, the PGA chains confined between and attached to neighboring charged membranes appear to be orientationally and positionally disordered. At low membrane charge densities (i.e., high DLPC/DDAB ratios) the data indicate the formation of a “pinched lamellar” phase of the lipid-PGA complex where large pockets of water stabilized by hydration repulsion are contained between PGA chains which locally pinch opposing membranes (Figure 9).

Materials and Methods

Materials. PGA was obtained from Sigma Chemical Co and used as received. The weight-average molecular weight of PGA was 81 500 Da with a polydispersity (M_w/M_n) of 1.15. DDAB (MW = 464 Da) was

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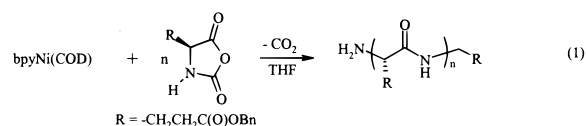
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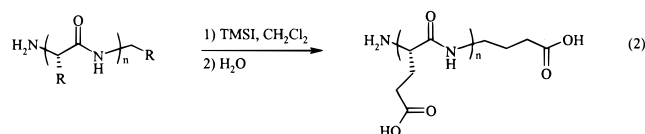
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obtained from Fluka and DLPC (MW = 622 Da) was obtained from Avanti Polar lipids. The lipids were used as received.

Synthesis of High-Molecular Weight of Poly-L-glutamic Acid Sodium Salt. High-molecular weight PGA was synthesized in two steps. First poly- γ -benzyl glutamate (PBLG) with a number-average molecular weight of (M_n) of 400 000 and a polydispersity (M_w/M_n) of 1.03 was synthesized as described previously²⁸ (reaction 1). The PBLG



was then converted to the acid form by removing the benzyl-protecting group. Deprotection was accomplished by dissolving the PBLG (100 mg) in dry CH₂Cl₂ (5 mL) followed by addition of excess Me₃SiI (40 μ L) (reaction 2). The resulting pale yellow solution was stirred under



nitrogen at 40 °C for 12h. The PGA was then isolated by addition of the solution to hexanes saturated with water (50 mL). The gelatinous polymer was isolated by centrifugation, washed with water (2 \times 40 mL) and then with acetone (2 \times 25 mL), and then dried. To convert the acid form of the polymer to its sodium salt, the polymer was suspended in doubly distilled water and reacted with 1 equiv of NaOH per acid side chain. The resulting solution was filtered and then freeze-dried to isolate the PGA as the sodium salt. No residual benzyl resonances were observed for this polymer by ¹H NMR spectroscopy (D₂O). GPC analysis of the polymer after rebenzylation showed that no chain cleavage had occurred. The number average molecular weight (M_n) of the deprotected polymer was found to be \sim 320 000 with a polydispersity of 1.06.

Complex Formation. The PGA–lipid complexes were either made from liposomal stock solutions of the lipids (25 mg/mL concentration of total lipids) or from lamellar phases at 20 wt % total lipids in water. The structure of the complexes was similar for both types of stock solutions used. The liposome stock solutions were used at high ratios of DLPC to DDAB because they demixed at 20 wt % concentration of the lipids. The complexes were made by mixing the appropriate lipid stock solutions with 30–40 mg/mL PGA stock solutions. Samples for X-ray experiments were directly made in 1.5 mm quartz capillaries and flame-sealed. Gentle centrifugation was employed to ensure good mixing of the samples in the capillaries.

Charge of the Complexes. The surface potential (and hence the sign of the charge) of the complexes was determined from measurement of the electrophoretic mobility of the complexes using a Zetaplus potentiometer. Samples for ζ potential measurements were typically made from 0.1 mg/mL lipid stock and 0.1 mg/mL PGA solutions.

Optical Microscopy. Optical micrographs of the complexes under bright field as well as under crossed polarizers were obtained with a Nikon optiphot microscope with a 40 \times objective. The concentrations used were 25 and 5 mg/mL for the lipid and PGA, respectively.

X-ray Scattering. X-ray scattering is the definitive probe for elucidating the bulk structure of the complexes from a few angstroms and up to more than 1000 Å using high-resolution small-angle X-ray scattering (HRSAXS) techniques. Low-resolution experiments were conducted in-house at the Materials Research Laboratory X-ray facility using a 18 kW Rigaku rotating anode generator, while high-resolution experiments were done at the Stanford Synchrotron Radiation Laboratory. The resolution of the X-ray beam was set by slits both before and after the sample. Typical in-plane resolution (fwhm) for in-house rotating anode experiments was \sim 0.01 Å⁻¹. At Synchrotron the resolution was \sim 0.001 Å⁻¹. The energy of X-rays used in-house was 8 keV (corresponding to a wavelength of 1.54 Å), whereas in

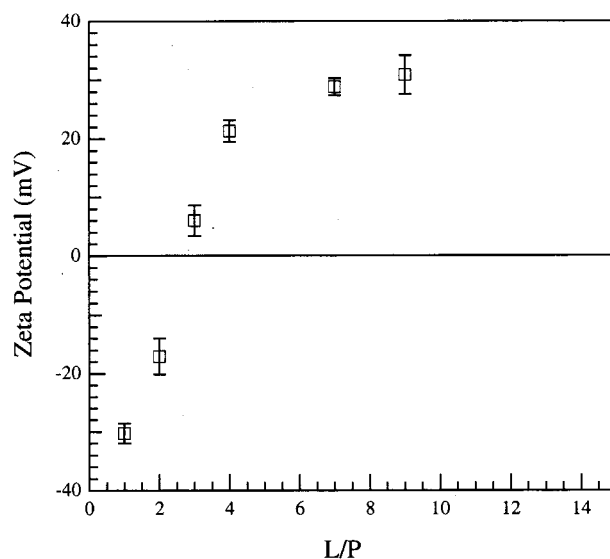


Figure 1. Zeta Potential of the complexes as a function of L/P . The complexes were made from stock solutions of 1 mg/mL DDAB and 0.2 mg/mL PGA. The horizontal line indicates charge neutrality condition (ζ potential \sim 0) and occurs close to the theoretical value of $L/P = 3$.

Synchrotron the energy used was 10 keV. For typical powder samples, $\theta - 2\theta$ scans were performed which give the scattered intensity as a function of the scattering vector of magnitude $q = 4\pi/\lambda \sin(2\theta/2)$.

Small-Angle Neutron Scattering (SANS). Neutron scattering experiments were conducted at the Low Q diffractometer (LQD) at the Manuel Lujan Jr. Neutron Scattering Center, Los Alamos National Laboratory. LQD is a broad-band time-of-flight diffractometer using neutrons from a liquid hydrogen moderator (20 K) with a spectral peak of neutron wavelength at about 2.4 Å and a usable wavelength range of 1.5–15 Å. The magnitude of the incident neutron momentum was measured by time-of-flight techniques.²⁹ The great advantage of using SANS is that the contrast variation between the scattering objects and the solvent is easily accomplished by changing the ratio of water to deuterium oxide (D₂O) in the mixture. This is possible because of the huge difference in the scattering cross sections of hydrogen and deuterium. Samples were prepared in quartz cells of either 1 mm or 2 mm path length. The complexes were made in solvents containing various ratios of water to D₂O.

Results and Discussion

We measured the surface potential (hence the sign of the charge) by ζ potential measurements. The surface potential was determined by measuring the electrophoretic mobility of the complexes in an external field. Measurement of the ζ potential is a convenient way to determine the stoichiometric point of the complexes. Figure 1 shows the results of ζ potential measurements of complexes made with pure DDAB and PGA. At large lipid to protein mass ratio (L/P), the sign of the ζ potential is positive because of the presence of excess cationic lipids while at low L/P , the ζ potential is negative, indicating the presence of excess PGA. At the stoichiometric point of the complexes, the ζ potential should vanish because of charge neutralization. The observed L/P at charge neutrality is indeed very close to the theoretical estimate of 3, corresponding to one positively charged DDAB molecule for every negatively charged PGA monomer.

High-resolution X-ray diffraction was used to elucidate the microstructure of the complexes. A typical high-resolution X-ray diffraction pattern of a stoichiometric complex made from 20

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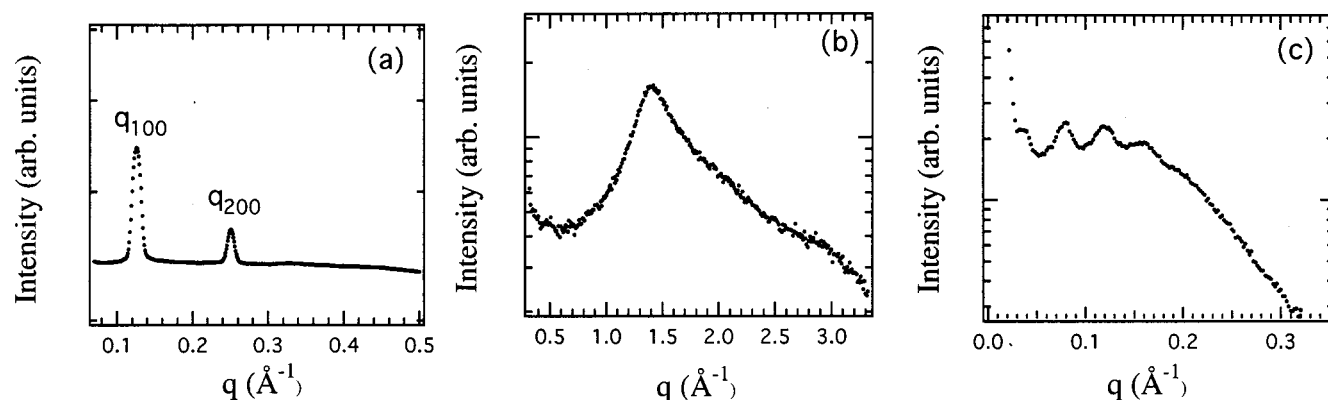


Figure 2. (a) Synchrotron small-angle X-ray scattering (SAXS) of a typical DDAB/DLPC–PGA complex made from 20 wt % DDAB/DLPC (1:1 w/w) and 4% PGA stock solutions. The two sharp Bragg reflections at $q = 0.126 \text{ \AA}^{-1}$ and $q = 0.252 \text{ \AA}^{-1}$ indicate a lamellar structure with a spacing of $d = 49.9 \text{ \AA}$. There are no other evident peaks in the data. The scans are offset for clarity. MW of PGA is 81 500 Da. (b) In-house high-angle scan of the complex in (a) showing a broad peak at $q = 1.5 \text{ \AA}^{-1}$. The broad peak indicates the liquidlike nature of the lipid chains. The membrane is thus in its L_α phase. (c) In-house SAXS of 20 wt % DDAB/DLPC (1:1 w/w) shows the first few lamellar peaks corresponding to a spacing of $d = 157 \text{ \AA}$.

wt % DDAB/DLPC (1:1 w/w) and 40 mg/mL (MW 81 500 Da) PGA solutions is shown in Figure 2a. The two sharp Bragg reflections at $q_{001} = 0.126 \text{ \AA}^{-1}$ and $q_{002} = 0.252 \text{ \AA}^{-1}$ are consistent with a multilamellar structure of the complex with a repeat distance d ($d = 2\pi/q_{001}$) of 49.9 \AA . High-angle X-ray scans of the same complex (Figure 2b) reveal a broad peak around $q = 1.5 \text{ \AA}^{-1}$, indicating that the lipid chains are in the disordered fluid state.^{30,31} To test the effect of PGA on the structure of complexes, we performed an X-ray scan of the pure lipid sample (1:1 w/w DDAB/DLPC) at a concentration of 20 wt % total lipids (Figure 2c). The data show four Bragg peaks corresponding to a lamellar structure with a spacing of $d = 157 \text{ \AA}$. From the “d” spacing and the volume fraction, Φ , of the lipids, the membrane thickness, δ_m , can be determined from the expression, $\delta_m = d/\Phi$ assuming a linear swelling behavior of the lamellar structure. The calculated membrane thickness (1:1 w/w DDAB/DLPC membrane) from the above expression is 31.4 \AA . The “d” spacing of the pure lipid system is to be contrasted with that of the complex. The pure lipid has a spacing of 157 \AA , and the addition of PGA to the pure lipid immediately reduces the spacing to 50 \AA while still preserving the multilamellar structure. Thus, complexing of PGA and cationic lipids results in a condensed multilamellar liquid crystalline structure.

It has been shown from circular dichroism measurements that the PGA macromolecules adopt an α -helical conformation on complexing with cationic surfactants.²⁵ The diameter of PGA macromolecules is known to be 13 \AA in the α -helical state.²⁵ The membrane thickness of 1:1 DDAB/DLPC was found to be 31.4 \AA from a water dilution study of pure DDAB/DLPC samples. The remaining water gap of 18.5 \AA is sufficient to accommodate a layer of PGA macromolecules taking into account a hydration shell around the PGA macromolecules. The observed lamellar spacing is thus consistent with a *multilamellar assembly of the lipid membranes with the PGA macromolecules intercalated between the membranes*. This structure is quite similar to the one observed by Raedler et al.⁴ for DNA–cationic lipid complexes. The significant difference between the structure of DNA–cationic lipid complexes and PGA–cationic lipid complexes is the presence of an additional peak between the lamellar peaks in the complexes containing DNA. This ad-

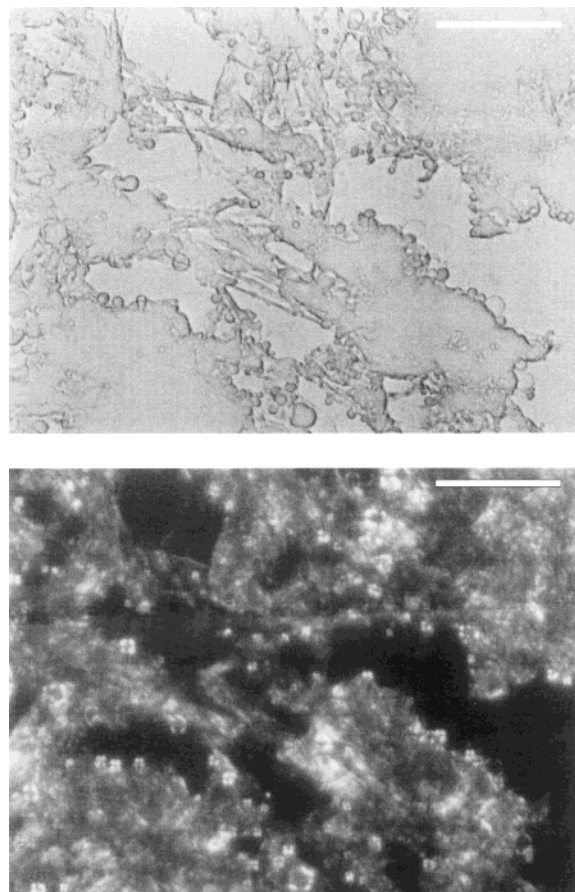


Figure 3. Optical micrographs of the complexes under crossed polarizers (top) and bright field (bottom) of complexes made from 25 mg/mL DDAB/DLPC (1:1 w/w) and 5 mg/mL PGA solutions. The spherulites in the top micrograph are characteristic of multilamellar structure. Scale bar is 10 micrometers.

ditional peak was attributed by Raedler et al.^{4,13,14} as arising from the 2-D ordering of the DNA macromolecules in planes parallel to the membrane planes. Salditt et al. later carried out a detailed line-shape analysis of the DNA–DNA correlation peak with both DOTAP/DOPC and DDAB/DLPC lipid mixtures, unambiguously confirming that the extra peak originated from a 1-D lattice of DNA chains confined in two dimensions.^{15,16}

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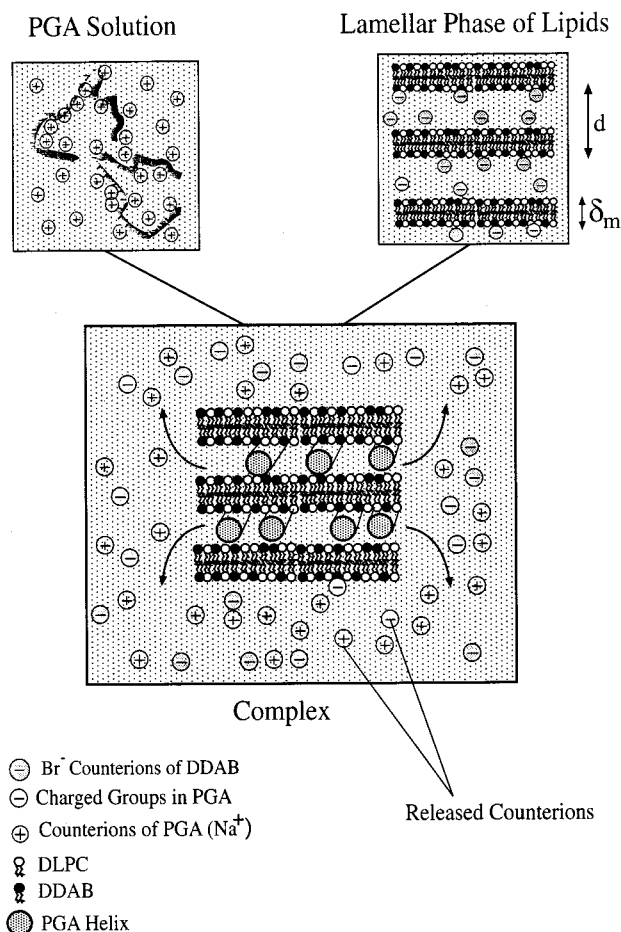


Figure 4. Schematic of the complex formation between PGA and DDAB/DLPC mixtures. The complexation of PGA and DDAB/DLPC membranes results in the formation of a condensed multilamellar structure with PGA intercalated between alternating lipid bilayers. The driving force for the complex formation arises from entropy increase associated with counterion release.

The liquid crystalline nature of the complexes was also confirmed by optical microscopy. Optical micrographs of the complex under crossed polarizers revealed numerous spherulitic defect structures characteristic of multilamellar structures (Figure 3 top). Bright field microscopy of the same regions shows patches of the complexes (Figure 3 bottom).

The self-assembly of PGA and the cationic lipids is driven predominantly by electrostatics. According to Manning's theory of counterion condensation,³² 78% of the anionic groups of PGA will be neutralized by condensation of counterions. Counterion condensation occurs whenever the mean distance between the charged groups is smaller than the Bjerrum length l_B defined by $l_B = e^2/ek_B T$ where ϵ is the dielectric constant. l_B is equal to 7.1 Å for water at room temperature. For the case of PGA, the mean spacing between unit anionic charges, l_0 , on the backbone is 1.54 Å. Counterions will condense on the PGA backbone till the renormalized line charge density is equal to l_B^{-1} . More specifically, the renormalized charge Q^* of a single PGA macromolecule including the bound counterions is given by $Q^* = Q/\xi_M$ where Q is the bare charge of the PGA macromolecule (without bound counterions) and ξ_M is the Manning parameter defined as the ratio of the Bjerrum length, l_B , to l_0 . The reciprocal of the Manning parameter determines the fraction of the charges of the PGA macromolecule that is *not* neutralized by the bound

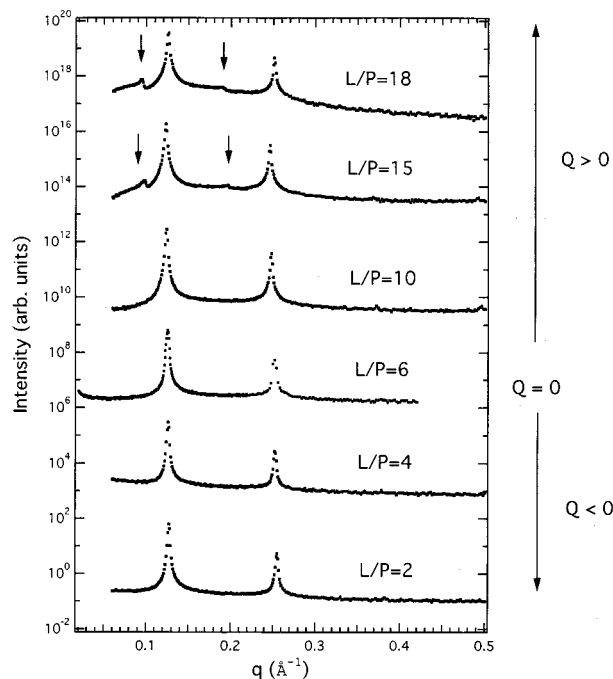


Figure 5. Synchrotron SAXS of complexes at a constant DDAB/DLPC ratio of 1:1 but at various ratios of L/P . L/P refers to the mass ratio of total lipid (DDAB + DLPC) to PGA. $L/P = 6$ corresponds to charge neutrality. At high L/P ratios phase separation occurs whereby the excess lipids excluded from the complexes form multilamellar assemblies. The scans are offset for clarity. MW of PGA is 81 500 Da.

counterions. For PGA, the fraction of the counterions that is bound to the PGA backbone can be calculated to be 78%. The self-assembly between the PGA and the cationic lipids occurs from the replacement of these condensed counterions by the cationic lipids. This releases the bound counterions into the bulk, thereby increasing their entropy. The process is shown schematically in Figure 4.

Significantly, electrostatic repulsive forces stabilize the pure lipid membrane, even though counterions condense on the pure lipid membrane. This electrostatic repulsion between adjacent membranes is strong enough to stabilize the lamellar system even at high water dilutions.²⁷ Moreover, our data indicate that over the concentration domain examined here, the phase behavior of the pure lipids is the predominant factor in determining the phase morphology of the complexes. The phase behavior of the pure lipids is dictated by their headgroup packing parameters, spontaneous curvature, electrostatic charges, hydration repulsion, and van der Waals attraction. Both DDAB and DLPC tend to form bilayers either in the form of vesicles or lamellar phase. Thus, we expect the complexes to form a multilamellar structure composed of bilayers of the lipid. This is indeed what is observed because the addition of PGA to the pure lipids does not induce a phase transition from a lamellar phase to other phases.

Figure 5 shows the X-ray data for a fixed DDAB/DLPC ratio of 1:1 with the DDAB/DLPC–PGA complexes at various L/P ratios. Here, the L/P ratio refers to the mass ratio of total lipid (DDAB + DLPC) to PGA. It can be seen from Figure 5 that for $L/P < 10$, the samples are in a single lamellar phase with a constant “ d ” spacing of 50 Å. $L/P = 6$ corresponds to stoichiometric ratio that is, the isoelectric point of the complexes. For $L/P > 10$, (positive side of the complexes) the complexes phase separate as evidenced by the appearance of extra low-angle peaks in the X-ray scans (indicated by arrows in the figure). These extra peaks are possibly due to excess multila-

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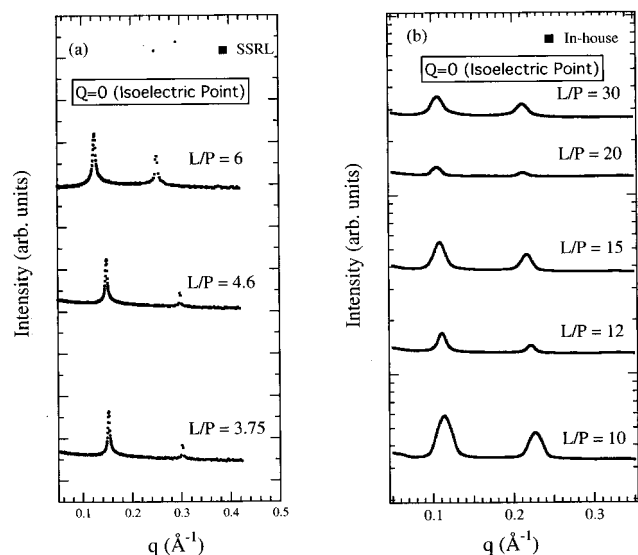


Figure 6. SAXS of complexes at the stoichiometric point ($L_{\text{DDAB}}/P = 3$) but at various ratios of DDAB to DLPC. The complexes remain single-phase even up to DLPC/DDAB ratios of 9:1. The structure of the complexes is multilamellar at all ratios of DLPC/DDAB. MW of PGA is 81 500 Da.

mellar assemblies of the lipids that are not complexed with the PGA. For L/P much greater than the isoelectric point, there is excess lipid in the system. The complexes accommodate a certain amount of lipids excluding the rest. The excess lipids could form a separate phase composed of multilamellar assemblies. Note that the peak positions of the complexes (strong peaks in Figure 5) do not vary with the L/P ratio. None of the scans showed any evidence of PGA ordering on the plane of the membrane.

To confirm the role of the membrane charge in the self-assembly, we varied the charge density on the membrane by changing the ratio of DDAB to DLPC. This lipid dilution experiment enabled us to systematically alter the surface charge density of the membranes while keeping constant the stoichiometric balance between the cationic lipid and the PGA. As the charge density is lowered by adding more DLPC, the average spacing between the PGA macromolecules was expected to increase because of the reduced electrostatic interaction between the PGA and the cationic lipids. The data showed (Figure 6) that over the entire range of lipid dilutions, the complexes adopted a multilamellar structure and that the complexes remained in a single phase even at very high dilutions ($L/P = 30$). Again, there was no evidence of a peak arising from PGA–PGA correlations on the plane of the membrane.

A significant result of the lipid dilution experiments is the steep monotonic increase in “d” spacing of the complexes from 39 Å at $L/P = 3$ to 58 Å at $L/P = 15$. There is little or no change of the “d” spacing (~ 60 Å) at very high dilutions (Figure 7). One reason for the increase in “d” spacing could be the stretching of the DDAB chains on lipid dilution. The fully stretched thickness of pure DDAB bilayer is 36 Å, assuming that the hydrocarbon chains are in their *trans* configuration. Also it is known²⁷ that at room temperature, the area per headgroup of DDAB is 65 Å² and the molecular volume is 784 Å³. This gives a bilayer thickness of 24 Å. These values imply that in pure DDAB bilayers the positively charged headgroups are forced apart by the electrostatic repulsions, leading to an expanded area of the headgroups. Assuming the molecules to have a constant molecular volume, this implies a reduction of the bilayer thickness. This effect could be significantly reduced

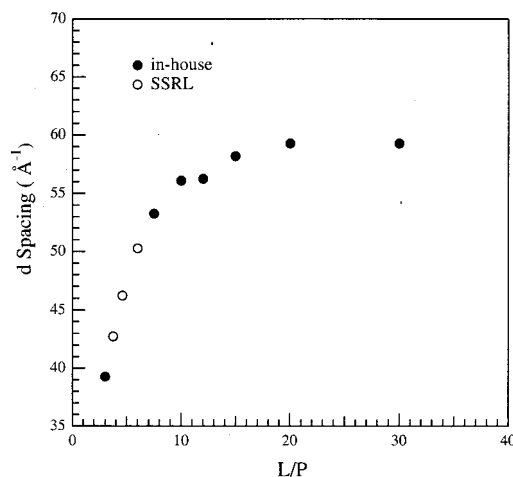


Figure 7. Plot of the d spacing obtained from Figure 6 as a function of L/P . In the absence of DLPC, the d spacing corresponds to the sum of DDAB membrane (24 Å) and a PGA macromolecule (diameter of 13 Å). MW of PGA is 81 500 Da.

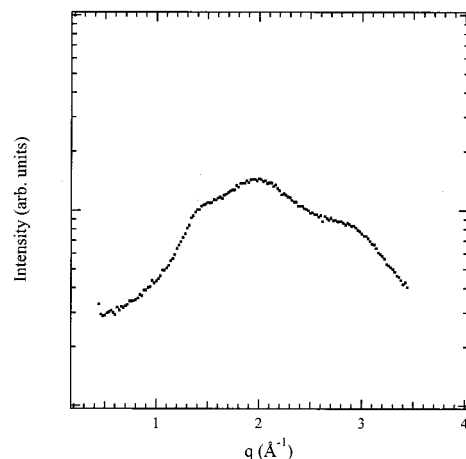


Figure 8. In-house high-angle scan of the $L/P = 15$ complex showing a broad peak at $q = 1.5 \text{ \AA}^{-1}$. The broad peak indicates the liquid like nature of the lipid chains. The membrane is thus in its L_α phase. MW of PGA is 81 500 Da.

when the neutral lipid DLPC is added, resulting in dilution of the charge density on the membrane (assuming ideal mixing of the lipids). Thus, lipid dilution could result in less electrostatic repulsion, reducing the headgroup area and changing the hydrophobic tail packing. The membrane might then thicken. However, this mechanism could account for an increase in the “d” spacing of only 12 Å. Further, such a fully extended state of the DDAB chains is unlikely. Moreover, the high-angle X-ray patterns of these membranes at $L/P = 15$ (Figure 8) did not show the sharp Bragg peaks expected from a hexagonal or distorted hexagonal gel phase with all of the lipid chains in the *trans* configuration. Rather, the high angle X-ray scan of complexes showed a broad liquidlike peak indicating that the lipid molecules are in the chain-melted L_α phase. Thus, the “d” spacing increase of as much as 20 Å (Figure 7) cannot be completely explained by stretching of DDAB chains.

Clearly other factors must also come into play at higher dilutions. For pure DDAB complexed with PGA, the “d” spacing is 39 Å (Figure 7). Since the thickness of DDAB bilayer is 24 Å, the remaining space is just sufficient to pack a monolayer of PGA macromolecules (diameter 13 Å), including a hydration layer. Thus, for pure DDAB bilayers, the PGA macromolecules are tightly packed between the bilayers (see below for more on

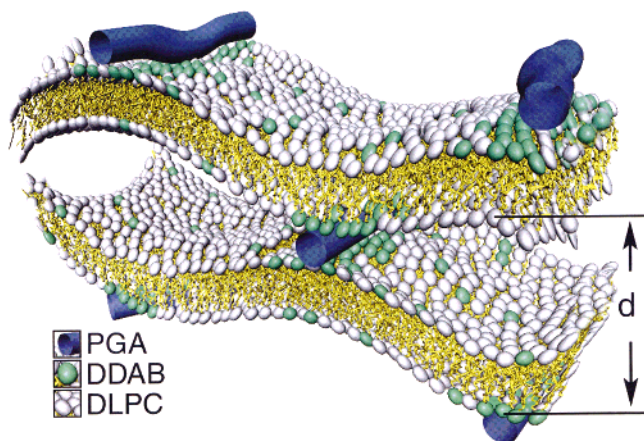


Figure 9. Schematic of the local lipid–PGA complex structure showing “pinching” mechanism. At larger length scales, the PGA macromolecules are positionally and orientationally disordered.

this point). The reduced charge density of the membranes at moderate dilutions ($L/P < 12$) reduces the electrostatic interaction between the PGA and the membrane. Assuming that the PGA–membrane association is predominantly driven by charge interactions, the reduced electrostatic interaction could result in a less tightly packed PGA layer and could manifest itself as an increase in the “d” spacing. At very high dilutions (i.e., at high ratios of DLPC to DDAB), the attractive van der Waals interaction and the repulsive hydration forces between the membranes play the dominant role in setting the equilibrium spacing of the system. It is known that pure DLPC membranes can be diluted up to a maximum of 60 Å determined by the balance of van der Waals and hydration forces.^{30,31,33} This corresponds to a volume fraction of water of 0.4. When more water than this is added to pure DLPC membranes, the system phase separates into an excess water phase and a lamellar phase with a repeat spacing of 60 Å. These observations combined with the observation that the “d” spacing of the complexes saturates at 60 Å at very high dilutions indicate that the balance between the repulsive hydration forces and attractive van der Waals of the membranes play the dominant role in determining the spacing.

To explain the data we propose the onset of a pinched lamellar phase of the lipid–PGA complex at high lipid dilutions consistent both with the observation of (i) a dramatic increase in “d” and (ii) the single phase nature of the complex as DLPC/DDAB ratio increases at the isoelectric point of the complex. In the pinched lamellar phase, the PGA and the DDAB molecules are localized in regions and interact electrostatically to form a localized packed layer (“pinch”). Away from this region the properties of the system are predominantly controlled by DLPC. The mechanism is schematically depicted in Figure 9. There are localized regions where the electrostatic interactions between the PGA and DDAB govern the properties of the system, but the average properties of the system such as the “d” spacing are controlled by DLPC. Recently, Schiessel³⁴ has done theoretical studies on the interactions between charged membranes in the presence of oppositely charged polyelectrolytes using the linearized Poisson–Boltzmann equation. He predicted that, depending on the parameters such as charge density of membrane, bending rigidity, and the Debye screening length, pinching is indeed possible in these systems.

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At charge neutral conditions (stoichiometric point of the complex) we can estimate the average spacing d_{PGA} between the PGA macromolecules as a function of the DDAB/DLPC ratio by using a simple packing argument.⁴ Assuming that all PGA macromolecules are complexed with the lipids at the stoichiometric point, we get:

$$d_{\text{PGA}} = A_{\text{PGA}} \left(\frac{\rho_{\text{PGA}}}{\rho_{\text{L}}} \right) \left(\frac{1}{\delta_{\text{m}}} \right) \left(\frac{L}{P} \right) \quad (1)$$

where A_{PGA} is the area of the PGA macromolecules, ρ_{PGA} and ρ_{L} are the densities of the PGA and the lipids respectively, and δ_{m} the membrane thickness. L/P again corresponds to the weight ratio of total lipids (DDAB + DLPC) to the PGA. Note that at the stoichiometric point, $L_{\text{DDAB}}/P = 3$. The area of the PGA macromolecule can be estimated from its molecular weight, density, and the contour length. For PGA with a molecular weight of 81 500, the contour length (in the α -helical state) is 832 Å. The membrane thickness δ_{m} varies between 25 and 34 Å in the two limits of 0 and 100% DLPC in the membrane. For a 1:1 DDAB:DLPC membrane $L/P = 6$ for charge neutrality of the complex; d_{PGA} is then equal to 31.4 Å, assuming a δ_{m} of 31.4 Å. For a 10:90 DDAB/DLPC membrane with $L/P = 30$ and $\delta_{\text{m}} = 34$ Å, the corresponding d_{PGA} is 145 Å. If the PGA macromolecules on the plane of the membrane are correlated and exhibit a smectic or nematic ordering, then we would observe an additional peak corresponding to d_{PGA} in addition to the lamellar peaks. As noted previously, the X-ray data do not indicate any peaks arising from PGA–PGA correlations.

The lipid dilution experiments of PGA–cationic lipid complexes at the isoelectric point reveal a very different behavior compared to DNA–cationic lipid complexes. While the PGA–cationic lipid complexes remain single phase even up to high dilutions and exhibit a large increase in bilayer spacing (~ 20 Å), the DNA–cationic lipid complexes phase separate at high fractions of the neutral lipid and exhibit only a moderate increase in bilayer spacing.⁴ Moreover, the DNA–cationic lipid complexes exhibit a 2-D smectic ordering of DNA-helices with a continuously adjustable inter-helical spacing, whereas such ordering of PGA helices are not evident from the X-ray scattering data of the PGA–cationic lipid complexes. Recent experiments by Salditt *et al.*¹⁵ on complexes of DNA with DDAB/DLPC lipid mixtures, however, reveal that the bilayer spacing increases by ~ 18 Å on lipid dilution at the isoelectric point. It is quite possible that pinching may also be operative in that system although phase separation still occurs at high fractions of DLPC. Intuitively it is clear that pinching is favored for membranes with lower bending constants. Owing to their smaller hydrophobic region, DDAB/DLPC membranes have a lower bending constant compared to those of DOTAP/DOPC membranes. Consequently, “pinching” is more likely to be observed in complexes with DDAB/DLPC membranes.

The absence of any peaks arising from PGA–PGA correlations may be due to any of the following reasons: (a) the area fraction of the PGA macromolecules is lower than the critical concentration needed for ordering; (b) the low molecular weight and relatively high polydispersity of PGA preclude the formation of an ordered state; (c) the contrast between the PGA macromolecules and the surrounding water space is not high enough to produce any appreciable X-ray signal; or (d) the PGA macromolecules are in a disordered state.

The area fraction α of the PGA macromolecules can be estimated using a simple geometrical argument, assuming that all of the PGA macromolecules are intercalated between the

membranes at the stoichiometric point. The expression is given by

$$\alpha = 2l_p d_p \left(\frac{MW_{lip}}{MW_{PGA}} \right) \left(\frac{1}{A_{lip}} \right) \left(\frac{L}{P} \right) \quad (2)$$

where l_p and d_p denote the contour length and diameter of the PGA macromolecules respectively; MW_{lip} and MW_{PGA} the molecular weights of the lipid and PGA respectively; A_{lip} is the area per headgroup of the lipids ($\sim 65 \text{ \AA}^2$). Using values of 831 \AA , 13 \AA , and $81\,500$ Daltons for l_p , d_p , and MW_{PGA} , respectively, we can calculate α at various L/P ratios. Note that for the lipid dilution experiments at charge neutral conditions, the smallest possible L/P is 3; this situation corresponds to the complexation of PGA to a pure DDAB membrane. At low L/P ratios it is seen that the area fraction can be quite large (0.63 at $L/P = 3$) and would be expected to induce a nematic or smectic ordering of the PGA macromolecules on the plane of the membranes.

Both the molecular weight and the polydispersity of the polyelectrolyte can affect the tendency of the molecules to self-assemble into liquid crystalline phases. Molecular weight directly influences the aspect ratio of the molecules, which is a crucial parameter in nematic or smectic phase formation. Recently Yu et al.³⁵ described the effect of polydispersity in inducing smectic phase in solutions of poly- γ -benzyl glutamate (PBLG) in an organic solvent. The presence of the smectic phase was attributed to the very low polydispersity of the PBLG polymer. We studied the effect of molecular weight and polydispersity of PGA on the morphology of the complexes. Since commercially available PGA seldom has a molecular weight above $100\,000$ Da and moreover is not highly monodisperse, we synthesized a high-molecular weight PGA ($M_n \approx 320\,000$) with a very narrow distribution ($M_w/M_n = 1.06$). X-ray diffraction patterns of the complexes on lipid dilution are presented in Figure 10. The complexes exhibit two strong peaks that are consistent with a multilamellar structure. There are no additional peaks that can be attributed to the ordering of PGA macromolecules on the plane of the membrane. Moreover, the complexes remain single phase even at very high dilutions ($L/P = 30$). These data are very similar to those of the lower-molecular weight analogue (Figures 6 and 7). Hence it is evident that higher molecular weights or improved polydispersity of the PGA macromolecules do not induce any ordering of the PGA macromolecules in the complexes.

To test the effect of contrast on the elucidation of PGA–PGA correlations, we performed small angle neutron scattering experiments. Neutron scattering is a very powerful technique employed to study the structure of biomolecules, particularly by contrast variation. Contrast variation technique takes advantage of the very different scattering cross sections of hydrogen and deuterium. The simplest way to change the contrast between the sample and the surrounding aqueous medium is by changing the ratio of D_2O to water in the medium. Another way to enhance contrast is by isotopic substitution of hydrogen atoms in the sample by deuterium.

Figure 11 shows the neutron scattering data of complexes made from 1:1 DDAB/DLPC and PGA ($MW\ 81\,500$ Da) at various ratios of D_2O to water in the solvent medium. The solvent compositions were chosen to selectively mask the signal either from the lipids or from the PGA macromolecules. Table

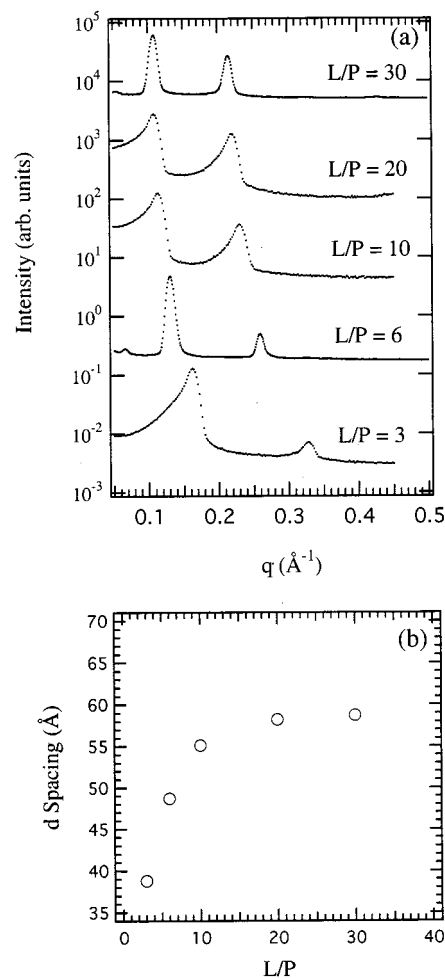


Figure 10. (a) SAXS of complexes at the stoichiometric point ($L_{DDAB}/P = 3$) but at various ratios of DDAB to DLPC. The complexes remain single-phase even up to DLPC/DDAB ratios of 9:1. The structure of the complexes is multilamellar at all ratios of DLPC/DDAB. (b) Plot of the d spacing obtained from (a) as a function of L/P . MW of PGA is $320\,000$ Da.

1 lists the scattering length densities of various solvent compositions. The average scattering length density of PGA over the various H_2O/D_2O ratios was taken to be $3.3 \times 10^{10} \text{ cm}^{-2}$, assuming fully protonated groups.³⁶ The hydrocarbon chains in the lipids have a scattering length density of $-0.36 \times 10^{10} \text{ cm}^{-2}$, while the headgroups typically have a value of $1.2 \times 10^{10} \text{ cm}^{-2}$.³⁶ At high water compositions, the scattering cross section of the lipids and the solvent are closely matched. Thus, at these compositions, the predominant signal is expected to be from the arrangement of PGA macromolecules. At low D_2O fractions, the data clearly show the absence of any peaks arising from the ordering of PGA macromolecules. As the amount of D_2O is increased in the solvent medium, the contrast between the lipids and the medium increases. This is evident from the appearance of the sharp Bragg peak at $q = 0.125 \text{ \AA}^{-1}$. The peak also becomes more prominent as the D_2O fraction is increased, consistent with the increasing contrast between the lipids and the solvent medium. This peak is consistent with the multilamellar assembly of the lipid molecules in the complex. Note that the peak position is identical to that found from X-ray scattering, further confirming that the peak arises due to the lipid assembly.

The experimental data presented reveal the following information about the structure of DDAB/DLPC PGA complexes: (a) the complexes form a multilamellar structure with the PGA

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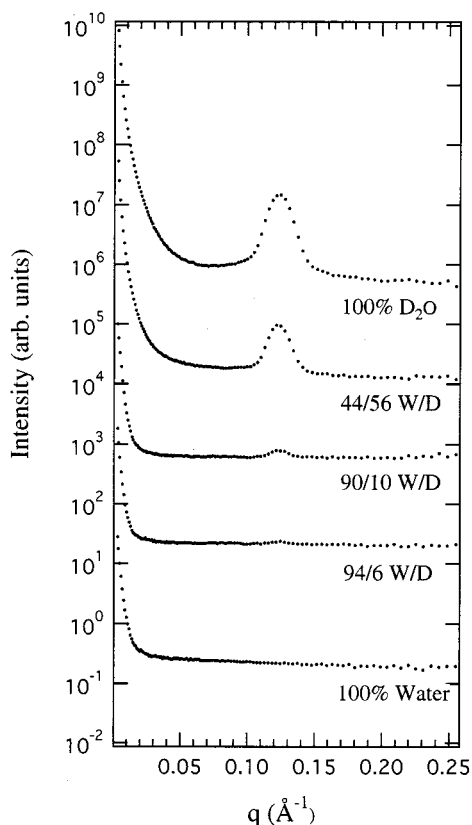


Figure 11. Small angle neutron scattering (SANS) of complexes made from 20 wt % DDAB/DLPC (1:1 w/w) and 4% PGA solutions at various solvent scattering densities. Solvent scattering density was varied by changing the ratio of water to deuterium oxide (D_2O). The Bragg peak corresponding to the bilayer becomes stronger as the amount of D_2O in the solvent is increased. Maximum contrast of the lipids and the solvent occurs in pure D_2O . There is no evidence of peaks arising from PGA–PGA correlation. MW of PGA is 81 500Da.

Table 1

water/ D_2O vol/vol	solvent scattering length density ($1 \times 10^{10}/\text{cm}^2$)
100/0	-0.56
94/6	-0.142
90/10	0.136
44/56	3.34
0/100	6.4

macromolecules intercalated between the membranes; (b) the PGA macromolecules do not exhibit any order in the plane of the membrane irrespective of their molecular weight or polydispersity; (c) the lipid–PGA complex forms a pinched lamellar phase at low membrane charge densities.

The apparent absence of any ordering of the PGA macromolecules may be attributed to its low persistence length (~ 20 Å). Low persistence length of the molecules induces kinks and bends in the molecules, thereby reducing the tendency of the molecules to order. Complexes of other synthetic polyelectrolytes such as poly(acrylic acid)³⁷ with cationic surfactants also display a multilamellar morphology but without any additional ordering of the polyelectrolyte chains on the plane of the membrane. The structure of PGA–lipid complexes is to be

(37) Antonietti, M.; Conrad, J.; Thunemann, A. *Macromolecules* **1994**, *27*, 6007–6011.

contrasted with those of DNA–lipid complexes. In addition to the multilamellar structure, the DNA–lipid complexes also exhibit a 2-D smectic organization of the DNA molecules. Since both DNA and PGA have comparable charge densities, the only attribute of DNA that contributes to its ordering is the large persistence length (~ 500 Å). These experiments provide strong evidence that the persistence length of the polymer is the crucial parameter in inducing order in these complexes. The PGA–lipid complexes, moreover, remain single phase even at high lipid dilutions at the isoelectric point ($L/P = 30$) and exhibit a large increase in the bilayer “d” spacing (~ 20 Å).

The high lipid dilutions achievable in these systems result in the formation of the pinched lamellar phase of lipid–PGA complexes where a large fraction of water is incorporated between the membranes in regions between the PGA macromolecular chains. The large fraction of water makes feasible the incorporation of host molecules in the complexes. One possible application is the use of these complexes as templates in the synthesis of porous inorganic materials. Another particularly exciting application of these complexes could be as novel targeted drug delivery systems whereby the drug could be dissolved in the water domains with the polypeptides providing the requisite targeting.

Conclusions

We have carried out a systematic investigation of polypeptide–cationic lipid complexes using high-resolution X-ray and neutron scattering. The structure of complexes made from poly-L-glutamic acid, DDAB, and DLPC was found to be a multilamellar liquid crystalline structure with alternating layers of lipid membranes and PGA macromolecules. Lipid dilution experiments on these complexes revealed a monotonic increase in the “d” spacing up to moderate dilutions with subsequent saturation at 60 Å at very high dilutions. Two significant differences are found between these lipid–PGA complexes and lipid–DNA complexes studied previously by Raedler *et al.*⁴ First, the PGA chains confined between membranes do not exhibit orientational or positional order, while the DNA molecules are ordered in a finite sized 2-D smectic array. Second, the lipid–PGA complexes remain single phase at very low membrane charge densities giving rise to the onset of a novel pinched lamellar phase.

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