Lipoplexes are composed of lipids and nucleic acids and have an ordered multilamellar structure with a periodic 1D array of parallel nucleic acid strands in the lipid bilayers. We report a low-angle X-ray diffraction study on solid-supported lipoplex films composed of synthetic single-stranded oligodeoxynucleotides (ssODN) and lipids. The ssODN molecules distribute sparsely in the headgroup regions when the weight concentration of the ssODN in the lipoplex is low. The lipoplex separates into two phases, an ODN-poor phase and an ODN-rich phase, when the weight concentration of the ssODN is increased beyond a level at which the ssODN molecules contact each other so that some regions of the lipid bilayers must accommodate two layers of the ssODN. The phase separation is a result of the fusion of such regions to minimize the total interfacial energy of the system. The ssODN molecules distort the lipid bilayers in the ODN-poor phase. The local area per lipid molecule is increased by the distortions so that the interbilayer distance of the lipoplex film is smaller than that of the lipid film without the ssODN. The ODN-rich phase has a much larger interbilayer distance because two layers of ssODN are intercalated into each lipid bilayer. The ssODN molecules are tightly compacted, and the lipid bilayers are not distorted in the ODN-rich phase.

**Introduction**

Short-chain nucleic acids play important roles in some biological processes. For example, they can be introduced into cells to interfere with the functions of endogenous genes. Because the short-chain nucleic acids can be easily degraded, their protection and storage are important. Liposomes have been extensively used for gene delivery and gene carrier storage. When an aqueous solution of DNA is mixed with a suspension of cationic liposomes, a composite called a lipoplex is formed in which the nucleic acids are confined in the lipid bilayers. In some special cases, the lipoplexes exhibit a hexagonal liquid-crystalline structure.

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Lipoplexes are composed of lipids and nucleic acids and have an ordered multilamellar structure with a periodic 1D array of parallel nucleic acid strands in the lipid bilayers. We report a low-angle X-ray diffraction study on solid-supported lipoplex films composed of synthetic single-stranded oligodeoxynucleotides (ssODN) and lipids. The ssODN molecules distribute sparsely in the headgroup regions when the weight concentration of the ssODN in the lipoplex is low. The lipoplex separates into two phases, an ODN-poor phase and an ODN-rich phase, when the weight concentration of the ssODN is increased beyond a level at which the ssODN molecules contact each other so that some regions of the lipid bilayers must accommodate two layers of the ssODN. The phase separation is a result of the fusion of such regions to minimize the total interfacial energy of the system. The ssODN molecules distort the lipid bilayers in the ODN-poor phase. The local area per lipid molecule is increased by the distortions so that the interbilayer distance of the lipoplex film is smaller than that of the lipid film without the ssODN. The ODN-rich phase has a much larger interbilayer distance because two layers of ssODN are intercalated into each lipid bilayer. The ssODN molecules are tightly compacted, and the lipid bilayers are not distorted in the ODN-rich phase.

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solid-supported ssODN—lipid lipoplexes by using a drop-casting method24,25 and apply the X-ray diffraction technique to characterize the structure of the air-dried lipoplexes. The low-angle X-ray diffraction data show that the lipid membranes in the air-dried lipoplexes are all parallel to the solid surface. This enables us to gain a deeper insight into the structure of the lipoplexes. Two different lipoplexes are observed. One is poor in ssODN and another is rich in ssODN. Individual ssODN molecules are sparsely distributed in the ODNPoor phase, but two tightly compacted ssODN layers are sandwiched into each lipid bilayer in the ODNRich phase.

Experimental Section

**Materials and Sample Preparation.** Neutral lipid 1,2-dioleoyl-snglycero-3-phosphocholine (DOPC) and positively charged lipid 1,2-dioleoyl-3-trimethylammonium-propane (choline salt) (DOTAP) powders were purchased from Avanti Polar Lipids Inc. Lipid mixtures were prepared with various weight fractions of DOTAP, Φ(TAP) (from 0.1 to 1.0), defined as the ratio of the weight of DOTAP to the total weight of DOTAP and DOPC. The lipids were dissolved in a mixed solvent of chloroform and methanol, dried under nitrogen flow, and subsequently stored in vacuum for more than 12 h. The dried liposome films were then dissolved again in Millipore water (18.2 MΩ·cm) to the final concentration (10 mg/mL) and sonicated to clarify in a 300 K water bath. Freshly prepared liposomes were used immediately.

The ssODN molecules were synthesized by the Beijing SBS Genetech Company. The sequences are 5′-AAAGG GGG GGG GGG G-′ (25 bases) and 5′-AAAAA AAA AAA AGG GGG GGG GGG G-′ (39 bases). Such special sequences were used to ensure that the ssODN molecules remain single-stranded and do not form secondary structures in the experiments so that the explanation would not be complicated by these possible structures. We had also checked other ssODN molecules with random sequences and found that the results are independent of the sequences. The ssODN molecules were dissolved in 10 mM HEPES from Alfa Aesar (pH 7.4, 100 mM NaCl) as the stock solution to a final concentration of 100 μM. The liposome and the ssODN stock solutions were mixed together to form the lipoplex solutions. They were stored at 4 °C for several days to equilibrate before they were cast onto the surfaces of silicon wafers. The wafers were cleaned in 5:1:1 v/v/v H₂O/H₂O₂/H₂SO₄ at 90 °C for 20 min and rinsed with distilled water several times. A solid-supported film was prepared as follows. A drop of the solution was pipetted onto the silicon wafer (4 × 10 mm²). The solution spread spontaneously on the hydrophilic surface and the solvent evaporated slowly in a chamber over a period of 12 h. A multilamellar film was formed during the spreading process.24–26 The film was then kept in a desiccator at room temperature before X-ray measurements were made. It can be estimated that the film contains about 120 bilayers according to the area per molecule, the total spread area, and the volume of the solution pipetted.

**X-ray Diffraction Measurements.** The low angle X-ray diffraction measurements were performed on a Bruker D8-Advance diffractometer equipped with a Goebel mirror to get parallel X-ray beams and to suppress the Cu Kβ radiation. Cu Kα radiation was used. The incident beam was confined by a 0.1 mm slit 300 mm in front of the sample, and the scattering beam was confined by a 0.2 mm slit in front of the detector. The diffraction measurement was performed by keeping the incidence angle θ₁ equal to the exiting angle θ₂.

Results and Discussion

The drop-casting procedure results in multilayered lipoplexes in which the lipid bilayers are parallel to the surface of the solid support. The intensities of the Bragg peaks of the lipoplexes are greatly enhanced because all of the bilayers coherently scatter X-rays in the direction of the Bragg diffraction. Figure 1a shows the X-ray diffraction patterns of the air-dried ssODN/DOTAP/DOPC lipoplexes containing various weight concentration of the 25-base ssODN molecules. The weight concentration of ssODN, C(OHDN), which is defined as the ratio of the weight of ssODN to that of the whole lipoplex, is such that the total negative charges on ssODN are equal to the total positive charges of the cationic DOTAP. The structure of the lipoplexes is the best when the total negative charges of the ssODN are equal to the total positive charges of the cationic DOTAP. Several sharp Bragg peaks can be seen in the diffraction curves. The interbilayer distances of the lipoplexes are shown in Figure 1b.

When C(OHDN) is lower than 0.16, only a homogeneous phase (named the ODNPoor phase) is observed (Figure 1a). When C(OHDN) is increased to >0.16, an ODNRich phase emerges (curves 4–9 in Figure 1a). The ODNPoor phase fades out eventually as C(OHDN) is increased. Its existence is not detectable when C(OHDN) is higher than 0.30. The interbilayer distance can be determined from the position of the Bragg peaks (Figure 1b). The weight fraction of DOTAP, Φ(TAP) (see above for its definition), in the lipid mixture is so designed as to balance the negative charges of the ssODN in the lipoplex. The interbilayer distance decreases with increasing C(OHDN) or equivalently with increasing Φ(TAP) because the size of the headgroup in DOTAP is smaller than that of DOPC. Meanwhile, the interbilayer distance of the lipoplex is smaller than that of the lipid mixture with the same Φ(TAP) but without ssODN. We propose that the ssODN molecules are embedded sparsely in the headgroup regions of the bilayers. The insertion of ssDNA molecules, whose diameter is ~0.1 nm, into the soft lipid bilayers causes severe distortions to the bilayers.
The local area per lipid molecule is increased by the distortions so that the interbilayer distance of the lipoplex becomes smaller than that of the lipid mixture at the same $\Phi_{\text{TAP}}$ but without ssODN. The lipoplexes with higher ssODN become more disordered, as can be seen from the gradually reduced number of Bragg peaks from curve 1–3 in Figure 1a.

In contrast to the ODN-poor phase, the structure of the ODN-rich phase is independent of $C_{\text{ODN}}$, meaning that the weight concentrations of the ssODN and the lipids are the same in all of the ODN-rich phases. (See the discussion below.) In other words, most of the DOPC molecules are expelled from the ODN-rich phase so that the structure of the ODN-rich phase is independent of the initial value of DOPC. As a consequence, the weight concentration of DOPC in the ODN-poor phase is higher than the initial value during sample preparation. The decrement of the interbilayer distance, $\Delta d$, increases almost linearly with the weight concentration of ssODN when $C_{\text{ODN}} < 0.16$ (Figure 2). The decrement rate becomes smaller in the two-phase coexistence region ($0.16 < C_{\text{ODN}} < 0.30$). It is consistent with the conclusion that the DOPC molecules, which are longer than the DOTAP molecules, are left in the DNA-poor phase. But this is not the only reason. We also measured the structures of the ssODN/DOTAP lipoplexes, namely, without DOPC. The decrement rate of the interbilayer distance also becomes smaller in the two-phase coexistence region (Figure 2). This can be explained by the assumption that some ssODN molecules are attracted into the ODN-rich phase so that the apparent concentration of ssODN in the ODN-poor phase becomes smaller than the value in the initial solution. To summarize, not only are the DOPC molecules left in the ODN-poor phase, but also the ssODN molecules are drawn out of the ODN-poor phase. Therefore, the apparent concentration of the ssODN molecules in the ODN-poor phase is lower than it seems to be according to the initial value of the ssODN in the solution before being cast onto the solid support.

To demonstrate that the phase transformation is not caused by the geometric effect arising from the difference in length of the DOPC and the DOTAP, we studied the lipoplexes composed of ssODN and DOTAP, that is, without DOPC. The X-ray diffraction results are shown in Figure 3a. The phase behavior of the ssODN/DOTAP lipoplexes is very similar to that of the ssODN/DOTAP/DOPC lipoplexes (Figure 3b). The only difference between the two types of lipoplexes is that the quality of the ssODN/DOTAP/DOPC lipoplexes is higher than that of the ssODN/DOTAP lipoplexes. The neutral lipids hence act as assistant lipids in our experiments. The charge balance between ssODN and DOTAP is not necessary in the air-dried lipoplexes. The extra positive charges of the DOTAP molecules can be balanced by the cationic counterions when the total negative charges of the ssODN molecules are smaller than those of the DOTAP molecules. The interbilayer distance of the ODN-rich phase is, again, independent of $C_{\text{ODN}}$. Its value, 51.0 ± 0.5 Å, is the same as that of the ODN-rich lipoplexes nominally containing DOTAP/DOPC mixtures (Figure 1b), which means that the ODN-rich phase contains an undetectable amount of DOPC whether or not the DOPC has existed in the initial solution.

We conducted similar experiments with longer ssODN molecules. Lipoplexes with 39-base ssODN have been studied. The X-ray diffraction patterns of the lipoplexes containing different lengths of ODN are compared in Figure 4. The X-ray structures of
samples containing the 39-base-ssODN are almost the same as those containing the 25-base-ssODN, which suggests that the ssODN molecules do not form three-dimensional globules in the lipid bilayers. Otherwise, the size of the globules would increase with the contour length of the ssODN, enlarging the interbilayer distance of the lipoplexes. This helps us to find the driving force of the phase transformation. When the concentration of ssODN in the lipoplexes is low, the ssODN molecules disperse sparsely inside the 2D headgroup regions of the lipid bilayers. The ssODN molecules begin to contact each other when the concentration is increased to \( C_{\text{ssODN}} = 0.16 \), beyond which some regions in the lipid bilayers must accommodate two layers of ssODN (Figure 5).

This procedure creates interfaces between the single-layer regions and the double-layer regions. Fusion of the double-layer regions reduces the total interfacial energies, leading to the emergence of the ODN-rich phase. Note that the ssODN molecules distort the lipid bilayers in the ODN-poor phase. However, as we will show, the lipid membranes are less distorted in the double-layer regions. Some of the ssODN molecules in the single-layer region are hence attracted into the double-layer region to reduce the bending energy of the lipid membranes; consequently, more cationic DOTAP molecules are absorbed into these regions to neutralize the charges. This explains why the interbilayer distance of the lipoplexes does not change much in the two-phase coexistence region (Figure 2) even when the concentration of ssODN seems to increase according to the amount of DOPC added to the solution initially.

The phase transformation of the air-dried lipoplexes on solid supports is different from that in solutions. The interfacial energies between the single-ssODN layer regions and the double-ssODN layer regions are the key controlling parameter for the transformation from the ODN-poor phase to the ODN-rich phase when the weight concentration of ssODN in the lipoplex increases. Both of the ODN-poor phase and the ODN-rich phase are lamellar phases. In contrast, the phase transformation of the lipoplexes in solutions is between the lamellar phase and the columnar inverted hexagonal phase. \(^{13,18}\)

The electron density profiles (EDP) of the lipid bilayers in different phases can be reconstructed using a method proposed by Salditt et al. \(^{27,28}\) Briefly, the EDP of a bilayer can be refined from the X-ray diffraction data via

\[
\rho(z) = \sum_{n=1}^{M} f(q_m) \cos(2\pi mz/d)
\]

where \( f(q_m) \) is the form factor of the bilayer, \( d \) is the interbilayer distance of the multilamellar membrane system, and \( m \) running from 1 to \( M \) is the index of the Bragg peaks. The magnitudes of \( f(q_m) \) are determined from the intensities of the Bragg peaks,

\[
I(q) \propto \sum_{n=0}^{N} f_n \delta_{\text{solid}}^2 \left| f_n \right|^2
\]

where \( f_0 = f_s \) is the reflection of the substrate and \( f_n = f(q) \exp(-i\theta/2) \). Because of the mirror plane symmetry of the bilayers, the phases of \( f(q_m) \) are reduced to their positive/negative signs only, facilitating the solution of the phase problem enormously. The phases are determined by locating the zero crossings of the form factor. Comparing the EDP of the lipoplex film (curve 1 in Figure 5a, \( C_{\text{ssODN}} = 0.16 \)) to that of the lipid film (curve 2 in Figure 5a), one sees that the headgroup regions in the lipoplex film are wider than that of the lipid film. Also, it is hard to see the interface between the phosphorous groups and the tails, whereas such an interface is clear in the lipid film. Such a disordering arises from the rearrangements of the lipid molecules caused by the ssODN. More importantly, there is not a layer that can be clearly attributed to the ssODN, meaning that the ssODN molecules are embedded in the headgroups of the lipids. In contrast to this, the tails of the lipids can be clearly seen in the EDP of the ODN-rich lipoplex (curve 1 in Figure 5b). The ssODN can also be readily recognized. The increased intensity and the large number of Bragg peaks should be attributed to the enhanced electron density contrast between the ssODN layer and the alkyl layer (Figure 5b).

The interbilayer distance of the ODN-rich lipoplex is 51.0 ± 0.5 Å (Figure 1a), which is 14.5 ± 0.5 Å thicker than that of the lipid film. The extra thickness is due to the double-layered ssODN. To quantify the electron density of the ssODN layer in the ODN-rich lipoplex, we assume that the electron density of the alkyl layer is the same as that in the lipid film without ssODN. The resulting electron density of the ssODN layer is about 0.50 e/Å\(^3\), which is only slightly lower than the electron density of dry DNA, 0.52 e/Å\(^3\), as calculated according to the value of the DNA dry specific volume, 0.57 mL/g, given by Luzzati et al. \(^{29,30}\) Therefore, the ssODN molecules must be tightly compacted in the 2D space in the lipid membranes. The driving force for such a tight arrangement may arise from the release of distortions of the lipid membranes as the ssODN molecules come out of the headgroup regions (Figure 6b). The concentration of ssODN plays a key role in the phase transformation of the lipoplex. A structural model is depicted in Figure 6. The ssODN molecules are dispersed sparsely in the ODN-poor lipoplexes, and they condense into a double

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layer in each lipid bilayer in the ODN-rich lipoplexes. The condensation might be induced by the electrostatic interactions between the charges on the lipids and the nucleic acids.

Conclusions

We produced via a drop-casting method multilamellar lipoplexes containing single-stranded oligodeoxynucleotides (ssODN) and cationic lipids on solid supports and applied low-angle X-ray diffraction to characterize their structures. The highly aligned multilamellar samples enabled us to gain deeper insight into the structure and phase transformation of the lipoplexes. The lipoplex is homogeneous when the weight concentration of ssODN is lower than 0.16, beyond which the lipoplex separates into an ODN-poor phase and an ODN-rich phase. The electron density profiles deduced from the X-ray diffraction patterns reveal that the ssODN molecules are embedded in the headgroup regions so that the lipid bilayers are severely distorted in the ODN-poor phase. The ssODN molecules condense into a double-layer in each lipid bilayer in the ODN-rich phase. The lipid membranes are less distorted in the ODN rich-phase. This new form of the air-dried lipoplex contains more than 30 wt % of the nucleic acids and is a good medium for their storage.

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Figure 6. Structural models of (a) the ODN-poor lipoplexes and (b) the ODN-rich lipoplexes.