Changes in Interfacial Tension of a Lipid Membrane Formed at the Water/Chloroform Interface upon DNA Complex Formation

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Changes in the interfacial tension of a lipid monolayer membrane formed at the water/chloroform interface upon DNA addition were measured using the quasi-elastic laser scattering (QELS) method. A cationic lipid, *N*-[1-(2,3-dioleoyloxy)-propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTAP), as well as zwitterionic lipids, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), were used to form lipid monolayer membranes at different calcium ion concentrations. A rapid decrease of the interfacial tension resulting from electrostatic interactions between DOTAP and DNA was observed within 10 s. However, such rapid decreases were not observed for DOPE or DOPC. A decrease in the interfacial tension was exhibited by DOPE after 1000 s from the addition of DNA, which may be due to an overall structural change in the DOPE membrane. A DOTAP/DOPE complex system showed behaviors attributable to both DOTAP and DOPE, whereas the behavior of the DOTAP/DOPC system resembled that of DOPC alone. The current results provide a model for the so-called lipoplex carriers used in gene therapy.

Keywords Quasi-elastic laser scattering (QELS) method, DNA complexation, lipid membrane, water/chloroform interface

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Introduction

Studies of the interactions between lipid membranes and DNA have garnered interest in fields ranging from fundamental life science research (interactions between DNA and lipid membranes in cells) to medical applications (non-viral gene transfer technology with lipoplexes).[1-3] DNA is a polyanion with a negatively charged main chain due to the presence of ionized phosphate groups; thus, to form lipoplexes, cationic amphiphiles are often used as lipid membranes and liposomes. To increase the transfer efficiency of DNA into cells, zwitterionic lipids are often added as helper lipids in addition to the cationic amphiphiles.[4] Although the transfer efficiency is improved when 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) is used in the lipoplexes instead of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) as the helper lipid,[4,5] the reason for this is unclear. Many studies are being conducted on the interactions of lipid membranes and DNA in terms of physical properties and structures.[6-8] Lipid membrane–DNA interactions are controlled electrostatically over long distances, whereas at short range, the interactions are strengthened by the entropy increase accompanying the release of counter ions and the water of hydration in the vicinity of the lipid membrane, DNA surface, or both.[6] Additionally, Koltover *et al.* analyzed a DNA-lipid complex and showed that the DNA-cationic liposome complex was transformed from a lamellar structure into an inverted hexagonal phase by the addition of DOPE.[7]

In recent years, many reports have featured a method that measures the free energy change that occurs upon the adsorption of DNA to a lipid membrane, which results in changes in membrane tension.[9-11] It is technically difficult to measure tension changes in a micro region at the cellular level; thus, as a model system, a Langmuir–Blodgett (LB) membrane formed at an air/water interface is often used. Many of these studies base their discussions on changes in the surface pressure–area (π-A) isotherms in the presence/absence of DNA, and examples that have measured changes in tension over time are limited.

Hansda *et al.* added DNA solution to the subphase of an octadecylamine LB membrane formed at the air/water interface, and studied the interaction of the lipid and DNA *via* the temporal changes in the surface pressure and Brewster angle microscopy (BAM) images.[9] Cárdenas *et al.* observed the changes in BAM images accompanying surface pressure responses when DNA was added to a cationic lipid (dioctadecyldimethylammonium bromide) membrane formed at the air/water interface, as well as changes in the domains of a complex at the air/water interface.[10] The time scale of these studies investigating the tension changes associated with complex formation was on the order of minutes. A stopped-flow fluorescence study also showed that DNA interacted with a lipid membrane surface; the time scale for the growth of the DNA-lipid complex was milliseconds to seconds.[8] To measure the tension changes accompanying DNA-lipid interactions, the ability to monitor temporal changes on the scale of seconds becomes important.

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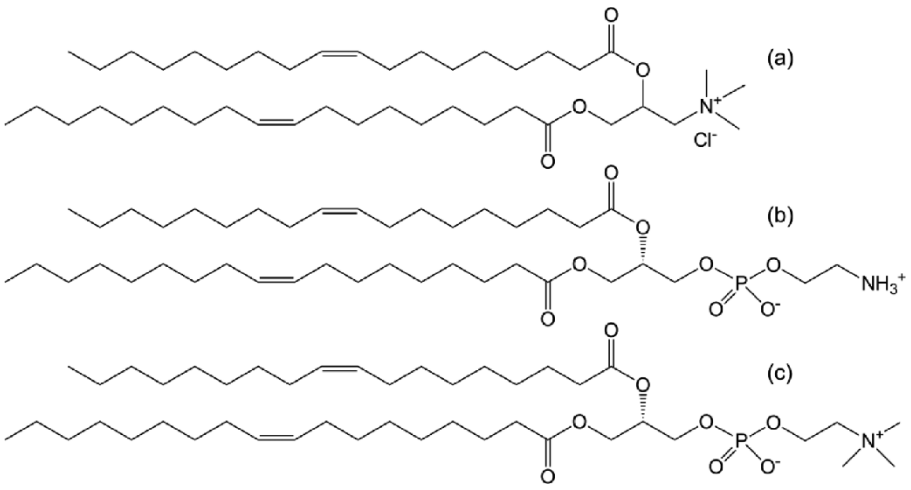

Fig. 1 Structural formulas of (a) DOTAP, (b) DOPE, and (c) DOPC.

Ohki's group discussed the structural relationship between lipid monolayer and bilayer membranes.[12] The hydrocarbon chains of a lipid monolayer at the air/water interface are assumed well oriented. On the other hand, the hydrocarbon chains of a lipid monolayer at the oil/water interface would be less oriented and the hydrocarbon tails are diffused because the hydrocarbon chains interact with the solvent molecules. From the energetic consideration, they stated that the monolayer formed at the oil/water interface stood for the physical state of bilayer membranes in the liquid-crystalline state very well. In biological environments, where the lipid membranes are in the liquid-crystalline state, the evaluation of DNA-lipid membrane interactions at an oil/water interface is preferable to that at an air/water interface. On the contrary, there are no reports of studying tension changes in a lipid membrane on a scale of seconds. We focused on the quasi-elastic laser scattering (QELS) method that allows non-contact measurements of interfacial tension and has a time resolution of seconds.[13,14] We have used the QELS method to measure interfacial tension changes for the process of lysosome adsorption to a lipid membrane formed at a water/chloroform interface.[15] It has been found that the changes of the interfacial tension were observed within a few seconds through the electrostatic interaction between lysozyme and lipid membranes, and that adsorption behaviors of DOPC and DOPS membranes were different, depending on the charges of lipid membranes. In this study, using the QELS method, we measured the interfacial tension changes upon the addition of DNA to a lipid membrane in which adsorption is saturated at the liquid–liquid interface, with the intention of studying the differences in the complexation behavior of DNA depending on the type of lipid. As lipids, cationic *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTAP) and zwitterionic DOPC and DOPE were used to investigate effects of DNA on the tension of a lipid membrane formed at the water/chloroform interface. We also measured the tension changes of a lipid membrane formed at the air/water interface, which have been studied before, for comparison. In addition, we considered the differences in the complexation behaviors of DOPE and DOPC as a function of Ca^{2+} ion concentration. By conducting experiments with mixed membrane systems of the cationic and zwitterionic lipids (DOTAP/DOPC and DOTAP/DOPE), we argued on the complexation dynamics of DNA with mixed membranes from the perspective of interfacial tension changes.

Experimental

Reagents and chemicals

Salmon sperm DNA (500 – 1000 bp) and DOPE were purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). DOPC and DOTAP were obtained from Avanti Polar Lipids (Alabaster, AL). Figure 1 shows the relevant lipid structures. Sodium chloride, calcium chloride, hydrochloric acid, and chloroform were obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). Tris(hydroxymethyl)aminomethane was obtained from Sigma-Aldrich (Germany). Water was purified and deionized using a Milli-Q system (Nihon Millipore, Tokyo, Japan). The purity of the DNA was confirmed by UV/Vis absorbance spectroscopy: The ratio of the absorbance, A_{260}, at the absorption maximum wavelength of DNA, 260 nm, to the absorbance, A_{280}, at the absorption maximum wavelength of protein, 280 nm, *i.e.*, A_{260}/A_{280}, should be 1.80 or higher for pure DNA.[16] The A_{260}/A_{280} value for the DNA used in this study was 1.81; thus, the effects of protein contamination do not have to be considered.

Apparatus

The apparatus used for the QELS measurement has been described in detail elsewhere.[13,15] Samples were irradiated with a beam from a Nd-YVO_4 laser (JUNO 100, Showa Optronics Co., Japan) that was separated into 95 and 5% power beams by a beam splitter, and the 95% segment was introduced to a water/chloroform or water/air interface through a focusing lens. In all experiments, measurements were conducted in a quartz cell of 23 mm × 23 mm × 49 mm. A capillary wave at the interface scatters the irradiation beam quasi-elastically. The frequency of the capillary wave is determined by measuring the frequency of the scattered light, and the interfacial tension of the water/chloroform interface or surface tension of the water/air interface is approximated by the dispersion relationship of the capillary wave, as shown in Eq. (1).[17]

$$f = \frac{1}{2\pi}\left(\frac{\gamma}{\rho_1 + \rho_2}\right)^{1/2} k^{3/2}, \quad (1)$$

where f is the frequency of the capillary wave, γ is the interfacial tension, k is the wave number of the capillary wave, and ρ_1 and ρ_2 are the densities of the two phases.

The scattered light was detected by an avalanche photodiode (C5331-11, Hamamatsu, Japan) with a reference beam originating from the 5% segment of the Nd-YVO_4 laser, the frequency of which was transformed from 0 Hz to 80 MHz by means of an acousto-optic modulator (1205C-2, ISOMET) through a dielectric mirror and lens. The frequency of the scattered light was analyzed by a digital fast Fourier transform (FFT) analyzer (RSA 5103A, Tektronix, Beaverton, OR). The reproducibility of the surface tension values at the air/water interface obtained with this system was estimated to be within 0.2 mN m^{-1}. The measurement of temporal changes in the interfacial tension upon the addition of DNA was started at the time of DNA addition. The measuring position was the center of the quartz cell, although we have never observed any interfacial tension differences with the position. The elapsed time of the QELS measurement was set to 5 s. The plotted time in this study was the midpoint of the elapsed time. All experiments were conducted at 25°C.

Methods

Surface tension measurement of the air/water interface. A lipid membrane was formed at the air/water interface as follows. To a quartz cell was added the aqueous phase (5 mL) consisting of 3 mM $CaCl_2$ and 50 mM Tris–HCl (pH 7.6). An amount of 0.1 mM lipid solution dissolved in chloroform was added to the aqueous phase, and the sample was allowed to rest until the chloroform had evaporated and the interfacial tension values reached equilibrium. For the surface concentration of the lipid, the amount of lipid added was divided by the area of the cell. An aliquot of DNA (10 mg mL^{-1}, 50 mM Tris–HCl pH 7.6, 50 μL) solution was introduced to the lower part of the aqueous phase using a microsyringe (the DNA concentration in the aqueous phase at equilibrium was 0.1 mg mL^{-1}), and temporal changes in the surface tension from that point were observed via the QELS method.

Interfacial tension measurement at the water/chloroform interface. A chloroform solution with dissolved lipid (5 mL) and aqueous phase (5 mL) were added to the quartz cell. The height of the aqueous phase in the cell was approximately 10 mm. The aqueous phase, a mixture of 3 mM $CaCl_2$ and 50 mM Tris–HCl (pH 7.6), was used unless otherwise noted. When measuring the Ca^{2+} ion concentration dependence, Ca^{2+} concentrations were set at 0.3, 3, 33, and 100 mM. The formation of a lipid monolayer membrane at the water/chloroform interface was confirmed when the interfacial tension became constant. To introduce the DNA, an aliquot of DNA solution (10 mg mL^{-1}, 50 mM Tris–HCl pH 7.6, 50 μL) was added to the aqueous phase surface (upper layer) using a pipet (the DNA concentration in the aqueous phase at equilibrium was 0.1 mg mL^{-1}). Temporal changes in the interfacial tension were measured upon DNA introduction using the QELS method.

Results and Discussion

Temporal changes in the surface tension upon the addition of DNA to a saturated lipid membrane at the air/water interface

We first tried to measure the temporal changes in the surface tension upon the addition of DNA when there was no lipid membrane at the air/water interface, however, no changes could be observed. Similar results have been obtained previously by Frommer and Miller.[18] They stated that DNA had the low surface activity because the negative surface excess of a low molecular weight salt balanced the positive excess of the DNA. Chattoraj and Mitra also stated that rod-shaped DNA molecules were not able to orient its hydrophobic part in air and hydrophilic region towards water.[19]

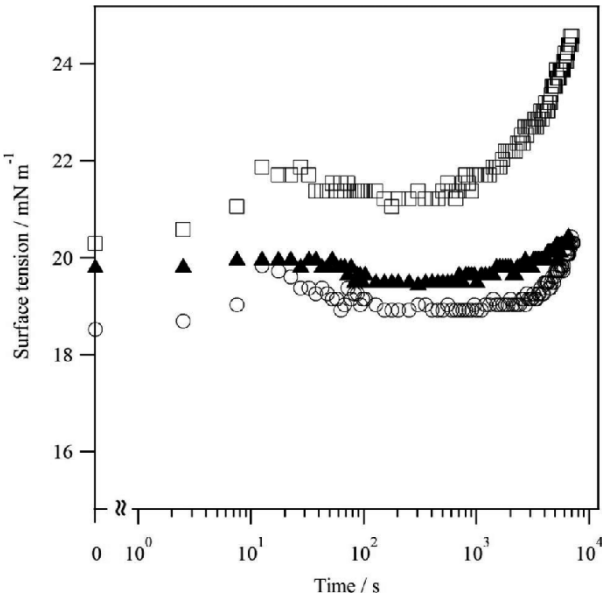

Fig. 2 Temporal changes in surface tension accompanying the complexation of DNA and a saturated lipid membrane at the air/water interface (○ DOPE, □ DOPC, and ▲ DOTAP). Surface tension prior to the addition of DNA is plotted on the vertical axis of the graph.

We conducted an experiment in which DNA was added when the lipid was at saturation absorption at the air/water interface. A lipid membrane was formed at the air/water interface at each lipid surface concentration, and surface tensions were measured. The lipid surface concentration at which the surface tension value no longer changed, even with increased lipid surface concentration, was defined as the saturation adsorption concentration at the air/water interface. The saturation adsorption concentrations of DOPE, DOPC, and DOTAP were 3.3, 2.7, and 3.3 μmol m^{-2}, respectively. The surface tension changes upon the addition of DNA when the lipid is at the saturation adsorption level at the air/water interface are shown in Fig. 2.

For each lipid, there were some increases/decreases in the surface tension up to about 100 s after the addition of DNA; however, it was observed that, even when DNA was added, the surface tension remained about the same, and slowly increased later. The surface tension started to increase for DOPC, DOTAP, and DOPE at about 700, 1000, and 1200 s, respectively. The reason is considered that lipid is released from the air/water interface or that the membrane structure changed due to complexation. The zwitterionic lipid form the complex with DNA through Ca^{2+} salt bridges, and as the membrane surface is positively charged, the DNA is adsorbed through electrostatic interactions.[20,21]

Oberle *et al.* added pCAT plasmid (4.7 kbp) to the monolayer membrane composed of cationic lipid, *N*-methyl-4-(dioleyl) methylpyridinium chloride (SAINT-2), at the air/water interface, and observed changes in the surface pressure.[22] The surface pressure decreased immediately after adding the plasmid (*i.e.*, the surface tension increased), and reached equilibrium at ~1800 s. This behavior was ascribed to the desorption of the

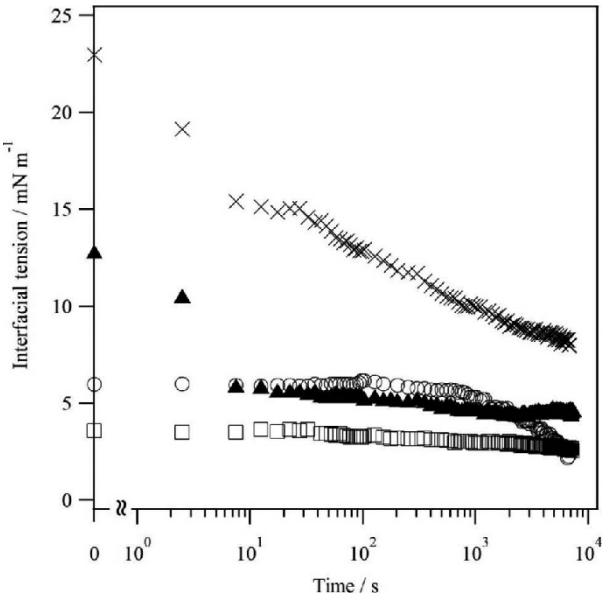

Fig. 3 Temporal changes in interfacial tension accompanying the complexation of DNA and a saturated lipid membrane at the water/chloroform interface (× no lipid, ○ DOPE, □ DOPC, and ▲ DOTAP). Interfacial tension prior to the addition of DNA is plotted on the vertical axis of the graph.

cationic lipid from the air/water interface by its local clustering and complexation with the plasmid. In our experimental system, the lipid membrane and DNA interact at the air/water interface, but the increase in the interfacial tension due to this interaction is small, and it occurs slowly after about 1000 s.

Temporal changes of interfacial tension upon the addition of DNA to a lipid membrane formed at the water/chloroform interface

To conduct an experiment at the saturation adsorption of the lipid at a water/chloroform interface, we examined the concentration at which the saturation adsorption of the lipid occurs. At each lipid concentration, the interfacial tension at the water/chloroform interface was measured. The concentration at which the interfacial tension values no longer changed, even with further increases in lipid concentration, was defined as the saturation adsorption concentration at the water/chloroform interface. The saturation adsorption concentrations of DOPE, DOPC, and DOTAP were determined to be 20, 10, and 10 μM, respectively. Above these concentrations, the water/chloroform interface became saturated with lipid molecules. Thus, we measured temporal changes in the interfacial tension immediately after adding DNA while the lipid concentration was set to 100 μM for DOPE, DOPC, and DOTAP. Figure 3 shows the interfacial tension changes upon the addition of the DNA solution to the saturated lipid membrane formed at the water/chloroform interface.

Since the interfacial tension decreases even without a lipid membrane, we found that DNA was adsorbed onto the water/chloroform interface. Approximately 10 s after adding DNA, the interfacial tension value decreased by about 7 mN m^{-1}. For approximately 20 s, the interfacial tension value barely changed, but then it slowly decreased. This phenomenon is not seen for the air/water interface. Beaman *et al.* examined the adsorption behavior of a polyelectrolyte, polymethacrylic acid (PMA), at the water/carbon tetrachloride interface, using interfacial tension measurement and total internal reflection vibrational sum-frequency spectroscopy.[23] When PMA solution was added to the water/carbon tetrachloride interface, the interfacial tension value immediately decreased by several mN m^{-1}, and slowly decreased thereafter. The proposed mechanism suggested that PMA is adsorbed as a monolayer to the water/carbon tetrachloride interface, and then additional PMA molecules are slowly adsorbed onto the PMA adsorption layer. The DNA used in our experiment, which is a type of polyelectrolyte, diffuses to the interface after addition to the aqueous phase, and since chloroform is a polar organic solvent (dipole moment: 3.47×10^{-30} C m), the adsorption of a DNA monolayer to the interface occurs first. Then, DNA is likely adsorbed onto the adsorption layer.

The equilibrium values of interfacial tension for each lipid before adding DNA to the water/chloroform interface were 12.6, 6.0, and 3.6 mN m^{-1} for DOTAP, DOPE, and DOPC, respectively. The interfacial tension of DOTAP, which is a cationic lipid, was relatively large compared to those of zwitterionic DOPE and DOPC. This is due to electrostatic repulsion between the lipids, which results in lower adsorption to the interface. Depending on the type of lipid, the decreasing behavior in the interfacial tension after the addition of DNA varied. For each lipid, the interfacial tension decreased upon the addition of DNA, and lipid-DNA complexation was likely occurring at the water/chloroform interface. For DOTAP, the interfacial tension value rapidly decreased from 12.6 mN m^{-1} prior to the addition of DNA to 5.7 mN m^{-1} in about 10 s. Thereafter, the interfacial tension slowly decreased and reached an equilibrium value of 4.5 mN m^{-1} about 800 s later.

In contrast, with zwitterionic DOPE and DOPC, rapid decreases in the interfacial tension were not observed immediately after DNA addition. The interfacial tension barely changed for DOPE for as long as 1000 s after adding DNA, but started to decrease thereafter; at ~7000 s, a value of 2.3 mN m^{-1} was observed. The interfacial tension did not change for the first ~40 s after adding DNA to the DOPC system. The decrease in the interfacial tension that followed was gentle, and at ~7000 s, it had decreased to 2.5 mN m^{-1}. As shown, periods in which the interfacial tension values did not change after the addition of DNA were observed for the zwitterionic DOPE and DOPC. In contrast, the interfacial tension decreased rapidly in DOTAP in about 10 s. The strong electrostatic interactions between DOTAP and DNA make it energetically favorable for the added species to interact immediately upon addition, leading to complexation at the interface.

We also found that the time scale for DNA complexation to a lipid membrane at a water/chloroform interface was different from that at an air/water interface. At the latter, after about 1000 s, interfacial tension changes due to complexation were observed. In contrast, for a DOTAP lipid membrane at the water/chloroform interface, interfacial tension changes were observed in as fast as 10 s. Based on this, it is supposed that the complexation of DNA and the lipid membrane does not involve simple DNA adsorption on the membrane surface, but is affected by the structure and fluidity of the lipid membrane. Using the neutron reflectivity method, Wu *et al.* indicated that at the lipid monolayer at the air/water interface, DNA is partially inserted among the hydrophilic groups of the lipid membrane.[24] Also, Benatti *et al.* used spin label ESR techniques to show that the fluidity of a membrane decreases when DNA is adsorbed.[25] In contrast to lipid molecules at an air/water interface which can only move on the two dimensional surface of the membrane, lipid molecules at a water/chloroform interface can move into the chloroform phase in addition to two dimensional surface of

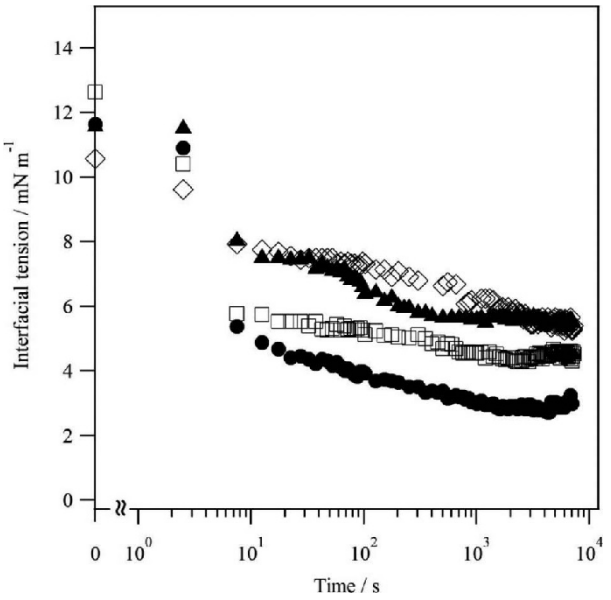

Fig. 4 $CaCl_2$ concentration dependence of temporal changes in interfacial tension accompanying the complexation of DNA and a DOTAP lipid membrane at the water/chloroform interface (● 0.3 mM, □ 3 mM, ▲ 33 mM, and ◇ 100 mM $CaCl_2$). Interfacial tension prior to the addition of DNA is plotted on the vertical axis of the graph.

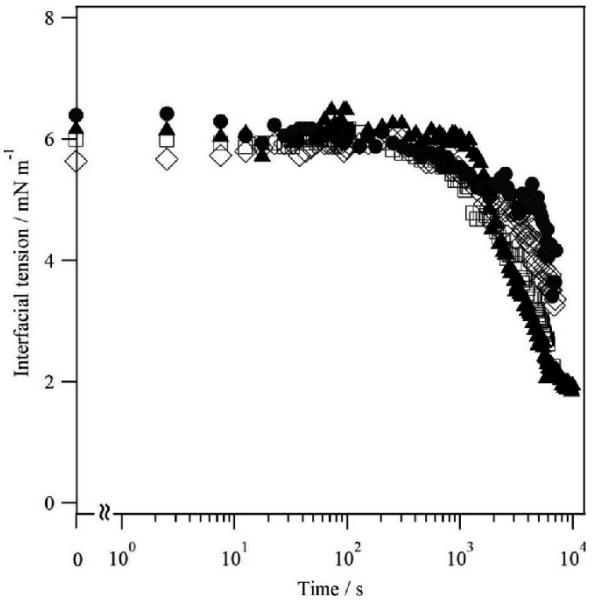

Fig. 5 $CaCl_2$ concentration dependence of temporal changes in interfacial tension accompanying the complexation of DNA and a DOPE lipid membrane at the water/chloroform interface (● 0.3 mM, □ 3 mM, ▲ 33 mM, and ◇ 100 mM $CaCl_2$). Interfacial tension prior to the addition of DNA is plotted on the left edge of the graph.

the membrane. The reason that complexation behavior was observed over a short time span at the water/chloroform interface may have been due to differences in its structure from that of a lipid membrane at the air/water interface, in addition to the fluidity of the membrane at the interface.

When carefully examining the interactions of DNA and lipids at the water/chloroform interface, especially the difference between DOPC and DOPE, electrostatic interactions become an important parameter and are dependent on ionic strength, among other factors. Accordingly, dependence on Ca^{2+} concentration was discussed in the next section.

$CaCl_2$ concentration dependence of temporal change in interfacial tension upon the addition of DNA to a lipid membrane formed at the water/chloroform interface

Figure 4 shows the temporal changes in the interfacial tension with DOTAP at $CaCl_2$ concentrations of 0.3, 3, 33, and 100 mM in the aqueous phase. Before adding DNA, the interfacial tension values were 11.6, 12.6, 11.5, and 10.6 mN m^{-1} for 0.3, 3, 33, and 100 mM, respectively. At all concentrations, the interfacial tension values rapidly decreased after about 10 s from the DNA addition. The extents of the decreases in interfacial tension during that period were 6.7, 6.4, 4.0, and 3.1 mN m^{-1} for 0.3, 3, 33, and 100 mM, respectively. By increasing the $CaCl_2$ concentration, the slope of the interfacial tension decrease became smaller. In addition, the equilibrium values of the interfacial tension after about 7000 s were 3.0, 4.5, 5.5, and 5.5 mN m^{-1}, respectively. Below 33 mM, the lower the $CaCl_2$ concentration, the smaller the equilibrium value of the interfacial tension. Positively charged DOTAP exhibits an electrostatic interaction with negatively charged DNA, but as the electrolyte concentration increased, more of the positive charge of DOTAP was shielded, and the interactions between DOTAP and DNA were reduced. Based on this conjecture, we believe that as the concentration of $CaCl_2$ rises, the amount of the decrease in the interfacial tension during the 10 s after DNA addition declines, and the equilibrium value of the interfacial tension grows.

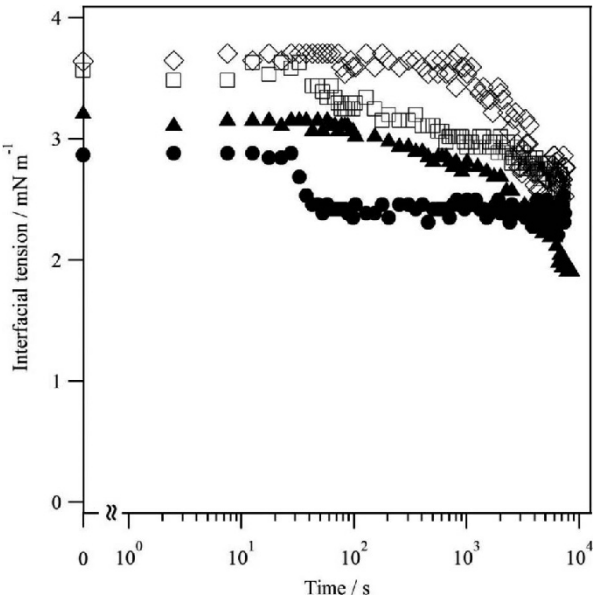

Fig. 6 $CaCl_2$ concentration dependence of temporal changes in interfacial tension accompanying the complexation of DNA and a DOPC lipid membrane and DNA at the water/chloroform interface (● 0.3 mM, □ 3 mM, ▲ 33 mM, and ◇100 mM $CaCl_2$). Interfacial tension prior to the addition of DNA is plotted on the vertical axis of the graph.

For the zwitterionic lipids DOPE and DOPC, the Ca^{2+} concentration dependence of the temporal changes in interfacial tension upon DNA addition is shown in Figs. 5 and 6. The interfacial tension values for DOPE prior to DNA addition were very similar. The adsorption behaviors were also independent

of $CaCl_2$ concentration, and remained nearly the same. The reason of these behaviors is considered that the structure of the membrane is barely influenced by the change of the concentration of $CaCl_2$ because of hydrogen bonds between the ammonium group of DOPE with the oxygen atom of phosphate group of neighboring DOPE molecules, while the trimethylammonium group of DOPC cannot make such hydrogen bond.[26] For the DOPC system prior to the addition of DNA, the interfacial tension values were also nearly the same at different Ca^{2+} levels. However, the behavior after the addition of DNA differed. Compared to the other conditions, with 100 mM $CaCl_2$, the interfacial tension values did not change for a prolonged period which lasted for ~500 s after the addition. At 0.3 mM, the interfacial tension values decreased very slightly, by about 0.4 mN m^{-1}, between ~30 to 50 s, and then became constant.

The main factors behind the different $CaCl_2$ concentration dependence behaviors for DOPE and DOPC are the salt bridge between the phosphate groups and Ca^{2+} ions, salting out effects, and the deprotonation of the DOPE terminal amino group. Zwitterionic DOPE and DOPC both form positively charged membranes with the coordination of Ca^{2+} ions to the phosphate groups; through these, the lipids can interact electrostatically with DNA. Up to a certain level, the higher the $CaCl_2$ concentration, the easier it is for the membrane to gain a positive charge and form a complex with DNA due to the salt bridge effect. However, at the same time, the salting out effect can inhibit complex formation. As the $CaCl_2$ concentration increases, the water of hydration on the surface of the lipids decreases and the electrostatic interactions between lipids become greater, which consequently inhibits complexation with DNA. Also, the higher the concentration of $CaCl_2$, the weaker the electrostatic interactions between charged entities are.[27,28] This hampers the electrostatic interaction between negatively charged DNA and positively charged lipid membrane. Furthermore, the strengthening of the bonds between the Ca^{2+} ions and the lipid phosphate groups may also inhibit complexation with DNA. For DOPC, as the $CaCl_2$ concentration increased, a longer time was observed in which the interfacial tension value did not change after the addition of DNA. This trend is likely due to the large inhibitory salting out effect.

On the other hand, with DOPE, in addition to the salting out and salt bridge effects of the Ca^{2+} ions, deprotonation of the DOPE terminal amino group should be considered. Different from DOPC with its quaternary choline group, DOPE contains a primary amine in its ethanolamine group, and it is possible that the $-NH_3^+$ group is deprotonated for DOPE molecule, the polar head of which becomes negatively charged.[29,30] Tsui *et al.* used vesicles containing mixed DOPC and DOPE, and reported that the intrinsic pK_a of DOPE in the double membrane state was 9.6.[29] This value changes depending on the surface potential of the lipid membrane, but in this experimental system, the DOPE lipid membrane may be less positively charged compared to the DOPC lipid membrane. By this mechanism, electrostatic interactions with DNA would be suppressed more than in DOPC, which would increase the time required until interfacial tension begins to decrease. It is, moreover, that hydrogen bonds between the ammonium group of DOPE and hydrated water molecules delay the interaction between DNA and the membrane surface.[26]

It is known that a DNA and cationic liposome complex adopts a multi-lamellar structure when DOPC is used as the helper lipid in addition to DOTAP, whereas with DOPE, an inverted hexagonal structure is obtained.[7] Further, it has been reported that if Ca^{2+} is present, DOPE, by itself, forms an inverted hexagonal complex structure with DNA.[31] In this inverted hexagonal DNA-DOPE complex, DNA is surrounded by a DOPE monolayer that has a cone-like shape due to its small hydrophilic group.[32] At the water/chloroform interface, DOPE is thought to undergo a large structural change as if to wrap around the DNA molecule. In contrast, DOPC tends to form a multi-lamellar complex structure with DNA; since this structure is difficult to achieve at the water/chloroform interface, the complexation can only occur at an extremely slow rate.

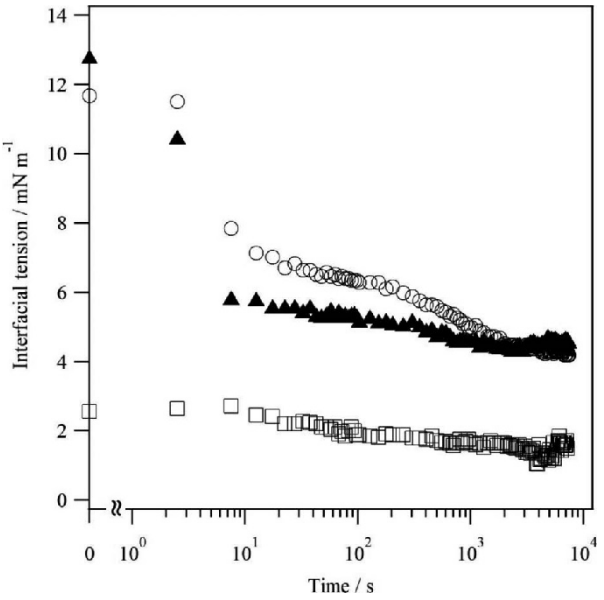

Fig. 7 Temporal changes in interfacial tension accompanying the complexation of DNA and a cationic/zwitterionic ion mixed lipid membrane at the water/chloroform interface (○ DOTAP/DOPE, □ DOTAP/DOPC, and ▲ DOTAP). Interfacial tension prior to the addition of DNA is plotted on the vertical axis of the graph.

Interfacial tension changes due to complexation in mixed lipid systems with DNA

To prepare lipoplexes for use in gene therapy, zwitterionic lipids are used with cationic lipids. Thus, in Fig. 7 we present the results of interfacial tension change measurements made after the addition of DNA to lipid monolayers in mixed DOTAP/DOPC and DOTAP/DOPE systems. It has been reported that when the molar ratio of the cationic lipid and DOPE is 1:1, the transfer of DNA to cells is most efficient;[4] therefore, the ratio of DOTAP and zwitterionic lipid (DOPE or DOPC) was set as 1:1 (each lipid concentration was set at 50 μM).

The equilibrium values of the interfacial tension at the water/chloroform interface for the DOTAP/DOPE and DOTAP/DOPC mixed systems were 11.8 and 2.3 mN m^{-1}, respectively. These values were different from individual interfacial tension values for DOTAP, DOPE, and DOPC; namely, it is believed that each mixed membrane was formed at the water/chloroform interface.

For the DOTAP/DOPE mixed system, upon adding DNA, the changes in interfacial tension were observed to take place in three steps. After adding DNA, the interfacial tension rapidly decreased by about 5 mN m^{-1} in the first ~20 s. However, between 50 and 200 s, it barely changed. Thereafter, the interfacial tension value slowly decreased, and after about 2000 s, it reached equilibrium at 4.2 mN m^{-1}. As shown, the system exhibits behavior similar to the single DOTAP system up to ~50 s, and resembles the single DOPE system thereafter. The amount of decrease in the interfacial tension in the initial stage

was smaller than in the single DOTAP system. This is likely because of the weakening of the electrostatic interactions by DOPE or the decreased amount of DOTAP at the interface.

On the other hand, for the DOTAP/DOPC system, the interfacial tension decrease upon adding DNA was observed to occur in two steps. After addition, the interfacial tension did not change for the first ~10 s, but a gradual decrease was observed thereafter. As such, the mixture exhibited a behavior similar to the single DOPC system. However, the period where the interfacial tension remained constant decreased from ~30 to ~10 s for the mixture. Since the cationic DOTAP was present in the lipid membrane, electrostatic interactions with DNA were more easily accomplished compared to a membrane comprising DOPC alone.

As described, for the mixed system of cationic DOTAP and a zwitterionic lipid, depending on the nature of the zwitterionic species (DOPC or DOPE), the complexation behavior with DNA varies. The transfer efficiency of DNA into cells is better when using DOPE as the helper lipid in a lipoplex, compared to DOPC.[4] We consider the correlation of the difference of the efficiency to the results obtained in this study as follows. When the DNA-lipid complex interacts with the cell, the cell membrane molecules become mixed during the transfer process (endocytosis and membrane fusion) and the charge balance is disturbed (a cell membrane is generally negatively charged). Here, comparing the free energy gain accompanying complexation, mixing effect of DOPE against DOTAP is significantly larger than DOPC; thus, the DNA and DOTAP/DOPE complex becomes easier to dissociate. The details of these effects are still unknown, but this modeling research could advance our understanding of the applied research at the cellular level.

Conclusions

We measured the complexation behavior of DNA with a lipid membrane formed at the water/chloroform interface using the time-resolved QELS method. With cationic DOTAP, a rapid decrease in interfacial tension was observed within 10 s, which was attributed to electrostatic interactions with the negatively charged DNA. Such rapid interfacial tension changes were not observed at the air/water interface. On the other hand, membranes of the zwitterionic lipids DOPE and DOPC did not exhibit interfacial tension changes for several tens to hundreds of seconds, even after DNA addition. In these cases, electrostatic interactions are not likely to be as strong as with DOTAP. The DOPC and DOPE lipids also exhibited different complexation behaviors. Compared to DOPC, DOPE was slower to start a decrease in the interfacial tension. This was assumed to be a result of the deprotonation of the DOPE hydrophilic group. The reason that the interfacial tension decreases after about 1000 s with DOPE was discussed to be due to a significant structural change that occurs in the DOPE membrane.

For the mixed DOTAP and zwitterionic lipid systems, the behavior changed depending on the zwitterionic lipid. With the DOTAP/DOPE mixed membrane, characteristics of both DOTAP and DOPE were observed during a three-stage process for the decrease in interfacial tension. The first step was the decrease based on the electrostatic interactions of DNA with DOTAP, but thereafter, the system's behavior was similar to that of DOPE. On the other hand, the DOTAP/DOPC mixed membrane exhibited a similar behavior to DOPC, and a two-stage decrease in the interfacial tension was observed. In this system, DOTAP-like behavior due to the DOTAP/DOPE mixed membrane was not confirmed. Based on our research with the QELS method, we were able to elucidate the role of the helper lipid in non-viral gene transfer by lipoplexes, as well as better understand the interactions between DNA and lipid membranes. Using this knowledge, we hope to contribute to advances in basic life science research and medical applications.

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