Stacking and mobility of cationic amphiphiles in the complex with DNA and DNA transfection activity of the complex

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Cationic amphiphiles, which have a quaternary ammonium head, hydrocarbon tails and a glutamic acid connector with methylene and/or phenylene spacer, have been used to deliver DNA into eukaryotic cells. The relationship between the mobility of various synthetic cationic amphiphiles complexed with DNA and their DNA transfer activities has been examined. When plasmid DNA was added to liposomes formed with cationic amphiphiles and treated with DNase I, DNA was found to be completely resistant to digestion suggesting that DNA is enclosed in the liposomal vesicle formed by the amphiphiles. CD spectra due to a chiral phenylene chromophore of the amphiphiles showed that the amphiphile–DNA complexes with higher transfection activity exhibited disordering of stacking of the amphiphiles by formation of the complex at the culture temperature. Study of the NMR signal of protons in the methylene and terminal methyl groups of the hydrocarbon chain of the amphiphiles indicates that the mobility of the chain was restricted by the binding of the cationic headgroup to DNA at lower than the phase transition temperature of the amphiphiles. Based on these results and those on the gene transfer activity of the complexes, we discuss the relationship between the amphiphile–DNA interaction in the complex and the gene transfection activity of the complex, and conclude that the most important requirement for efficient gene transfection is that amphiphiles in the complex are in a fluid bilayer at the culture temperature of the cells.

Formation of a bilayer structure is not a property specific to amphiphiles of biological origin; rather it is a common feature of many dialkyl amphiphiles.[1] The structure and physicochemical properties of a number of bilayer-forming synthetic amphiphiles, especially dialkylammonium compounds, have been studied.[2] These compounds have basic structural characteristics such as formation of a bilayer of 30–50 Å thickness depending on the length of the hydrocarbon chain, formation of bilayer aggregates of 10^6–10^7 Da, and trapping of aqueous organic substances in the inner hydrophilic core. Because most of these properties are similar to those of bio-membranes, the synthetic bilayer membrane may be of possible use in various biological systems without harmful effects. Use of synthetic amphiphiles in biological systems has the advantage that their physicochemical properties can be controlled by designing specific molecular structures.

The utilization of a dialkyl ammonium compound for DNA transfection into eukaryotic cells was first developed by Felgner *et al.*[3] A variety of different ammonium compounds, including 1,2-dioleol-3-*N*,*N*,*N*-trimethylaminopropane hydrochloride (DOTMA)[3] and 3β[*N*(*N*′,*N*′-dimethylaminoethanyl)-carbamoyl]cholesterol (DC-Chol)[4] have been used successfully to express heterologous genes. We have also synthesized a series of amphiphiles with two hydrocarbon tails, a trifunctional glutamate connector, phenylene and/or methylene spacer, and an ammonium group as the cationic head, and examined the efficiency of the synthetic amphiphiles in gene transfer into mammalian cultured cells and their physicochemical properties.[5,6] We found good correlation between their efficiencies in gene transfer and physicochemical characteristics, such as membrane fluidity and morphology. The effective compounds have a phase transition temperature (T_c) lower than the temperature of the culture system and form small and stable vesicles. How these properties facilitate the introduction of DNA into cells has remained an open question.

The mechanism of interaction of DNA with cationic amphiphiles and the physicochemical characteristics of the resulting amphiphile–DNA complexes are still poorly understood. Electron microscopy suggested that the cationic liposomes are attached like beads on a string, gradually covering the DNA chain until a complete covering of DNA is reached.[7] On the other hand, Sternberg *et al.* showed, using freeze-fracture electron microscopy, that DNA–liposome complexes are observed in at least two distinct features, semi-fused liposome–DNA aggregates and bilayer-covered DNA tubules of *ca.* 7 nm diameter.[8] From an examination of the optimal DNA to amphiphile ratio for transfection and their dimensions, they suggested that the tubular structure is the active DNA–liposome complex.

In the present study, we have investigated the interaction between various synthetic cationic amphiphiles with different fluidity and aggregate morphology and DNA in their complexes, especially in terms of stacking and mobility of the amphiphiles, using CD and NMR spectroscopies, and discuss the relationship between those properties and their transfection efficiency.

Experimental

Materials

A series of double-chain amphiphiles **1** and **2** were synthesized according to the procedure reported by Kunitake *et al.*[9] Amphiphile **3** and cetyltrimethylammonium bromide (CTAB) were purchased from Sogo Pharmaceutical Co. (Tokyo, Japan) and Wako Pure Chemicals (Osaka, Japan), respectively. COS-1, a simian kidney cell line transformed with simian virus 40 (SV40), was maintained in Dulbecco-modified Eagle Medium (DMEM) supplemented with 10% inactivated foetal calf serum under an atmosphere of 10% CO_2 at 37 °C. Plasmid DNA (pCH110) containing the β-galactosidase gene (lac Z) was from PL Biochemicals.

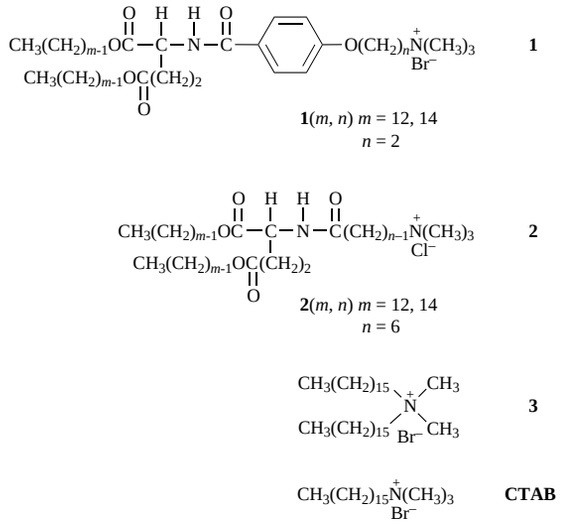

Fig. 1 Structures and nomenclature of the synthetic cationic amphiphiles examined

Amphiphile-mediated DNA transfection and assay of transfection efficiency

Transfection experiments were carried out as described by Felgner *et al.*[3] with some modifications.[5] Growing to just before confluence in 22 mm dishes, COS cells were washed twice with 1 ml of HEPES-buffered saline (HBS; 20 mM HEPES–KOH, 150 mM NaCl, pH 7.4). Plasmid DNA (in 10 mM Tris–HCl buffer, pH 8.0, containing 1 mM ethylenediaminetetraacetic acid, EDTA) and amphiphile suspensions (in water) prepared by sonication were diluted separately with 0.15 ml of HBS. Both solutions were combined and 0.4 ml of the amphiphile–DNA mixtures was poured gently onto the cells. After incubation for 3–5 h at 37 °C, 2 ml of DMEM supplemented with 10% foetal calf serum was added and the cells were further incubated. The medium was replaced with 2 ml of the fresh DMEM medium 12–20 h later, and the cells were incubated for 48 h from the first addition of the medium.

The cells were harvested 48 h after transfection. The efficiency of amphiphiles in the expression of β-galactosidase was determined by assaying the total enzyme activity in the cell extract, using *o*-nitrophenyl-β-D-galactopyranoside[10] as the substrate.[5]

Deoxyribonuclease digestion and agarose gel electrophoresis

After mixing plasmid DNA (pCH110; in 10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and amphiphile suspensions (1.3 mM, prepared by sonication) and allowing them to stand at *ca.* 25 °C for 30 min, the amphiphile–DNA complexes were digested with 0.5 ml of 1 mg ml^{-1} DNase I and 1.0 ml of 73 mM $MgCl_2$ at 37 °C for 1 h, and the reaction was stopped by adding EDTA (25 mM final concentration). DNA was dissolved in sample buffer (pH 7.4) consisting of 20 mM HEPES–KOH, 150 mM NaCl, 25 mM EDTA, and 20% (v/v) glycerol, with bromophenol blue as the marker. Electrophoresis was performed in a horizontal gel consisting of 0.8% agarose in 0.04 M Tris–HCl, 0.013 M sodium acetate and 1 mM EDTA, pH 8.0, with 1 mg ml^{-1} ethidium bromide for detecting DNA.

Physicochemical analyses

Dynamic light scattering. Amphiphiles suspended in water (4.3×10^{-5} M) were sonicated for several minutes to give a clear solution. Aqueous dispersions of amphiphiles (4.3×10^{-5} M) were left *ca.* 25 °C for 0.5 h both in the presence and absence of DNA (pCH110, 2.1×10^{-5} unit M) and 24 h in the absence of DNA. The mixture was used for dynamic light scattering experiments with an Otsuka Electronics Co., Model DLS-700 with a laser light source.

Circular dichroism. Circular dichroism (CD) spectra were measured on a JASCO J-700 spectropolarimeter at various temperatures. To the clear solution of amphiphiles (8.7×10^{-5} M) prepared as described above, DNA (pCH110) was added to a final concentration of 2.90×10^{-9} M, and the mixture used for measurements.

^{1}H NMR. ^{1}H NMR spectra were measured by means of a Bruker AP-250 FT NMR spectrometer (250 MHz) equipped with a temperature control apparatus. The chemical shift was measured relative to an external capillary of 0.3% $SiMe_4$ in $CDCl_3$.

Results and discussion

Amphiphiles and DNA-transfection activities

The first series of amphiphiles used in this study, **1**(12,2) and **1**(14,2), are composed of two hydrocarbon tails (length of tail: $m = 12$ or 14), trifunctional glutamate and phenylene groups as the connector, a methylene spacer (the length of spacer: $n = 2$) and an ammonium group as the cationic head (Fig. 1). The amphiphiles of the second series, **2**(12,6) and **2**(14,6), have the same basic structure as **1**, except for lacking a phenylene group in the connector. Compound **3** consists of two hydrocarbon tails and an ammonium group. CTAB, a cationic surfactant, has one hydrocarbon chain. These compounds, except CTAB, formed vesicular structures in aqueous solution, though they do have various phase-transition temperatures (T_c) (Table 1, ref. 5). No change in T_c values was observed even after addition of DNA, suggesting retention of the bilayer structure.

The efficiency of the compounds in the delivery of DNA into mammalian cells was examined by the transient expression of plasmid pCH110 which has a β-galactosidase gene. The extent of expression was measured by assaying the enzyme activity in the cell extracts. As shown in Table 1, **1**(12,2), **2**(14,6) and **3**, which have T_c values just below 37 °C, showed high efficiency in DNA transfection. The efficiency was much lower in compounds **1**(14,2) and **2**(12,6), which have T_c values slightly higher and considerably lower than 37 °C, respectively. The transfection activity of CTAB was nil. These findings, except that for **2**(12,6), confirm previous results that a fluid vesicular structure is essential for DNA transfection activity.[5]

DNase digestion of the DNA–amphiphile complex

To determine whether DNA is enclosed within liposomes formed by the amphiphiles or attached to the surface of the liposomes, the sensitivity of DNA in the DNA–amphiphile complex to DNase digestion was examined (Fig. 2). After treatment of the plasmid DNA (pCH110)–amphiphile **1**(12,2) complex with DNase I, the reaction mixture was subjected to agarose gel eletrophoresis and undigested DNA was visualized with ethidium bromide [Fig. 2(*a*)]. When 0.2 μg of the plasmid DNA (0.3 nmol phosphate units) was mixed with the corresponding amount of amphiphile (0.3 nmol), the DNA band was observed at the same position as that of the free plasmid, and the intensity slightly decreased [Fig. 2(*a*), lanes 1 and 2]. Upon incubation of the complex with DNase I, the DNA band disappeared (lane 3). When Triton X-100 (final concentration 0.2%) was added to the same sample, however, a faint DNA band appeared (lane 4). As previously reported,[11] the fluorescence of the DNA–ethidium bromide complex is suppressed by the addition of bilayer-forming amphiphiles, probably because of conformational change in DNA induced by the bilayer and recovered from the quenching by disruption of the bilayer structure with Triton X-100. When the amount of amphiphile was increased to 0.6 and 1.3 nmol, the intensity of DNA bands in the absence of Triton X-100 markedly decreased (lanes 5 and 8). DNA bands appearing in the presence of the detergent were increased even after DNase digestion (lanes 7 and 10), although no bands were yet seen in the absence of the detergent (lanes 6 and 9). These observations can be explained as follows: when

Table 1 Physicochemical properties of cationic amphiphiles in the absence and presence of DNA and their gene transfection activities

Amphiphiles	T_c/ °C [a]	β-Galactosidase activity expressed (arbitrary unit) [b]	Vesicle size/10^{-3} Å [c] −DNA 0.5 h	−DNA 24 h	+DNA 0.5 h	NMR linewidth of methylene signal [d] 25 °C −DNA	25 °C +DNA	37 °C −DNA	37 °C +DNA	45 °C −DNA	45 °C +DNA
1(12,2)	24.8	100	1.2	1.5	1.8	41	81	37	41	31	31
1(14,2)	41.1	1.7	1.2	1.3	1.6	47	86	46	54	37	43
2(12,6)	9.0	1.6	1.1	4.0	3.6	22	23	16	18	16	16
2(14,6)	25.4	26.2	0.8	9.4	11.2	42	41	27	32	27	32
3	28.0	22.0	1.5	10.9	9.2	38	40	32	37	32	34
CTAB	N.d. [e]	0	— [f]	—	—	4	18	4	17	4	16

[a] Phase-transition temperature T_c (peak top temperature in the DSC scan). Sample was dissolved in water to 20 mM. Temperature was elevated from 5 °C at a rate of 1 °C min^{-1}. [b] Transfection efficiency of various amphiphiles. The relative activity of β-galactosidase was expressed by setting the enzyme activity in the extract of the cell transformed with **1**(12,2) at 100. [c] The measurements were carried out using a dynamic light scattering spectrophotometer at 25 °C. [d] The half-height linewidths (in Hz) of methylene signal of ^{1}H NMR at 25, 37 and 45 °C. 5.0×10^{-4} unit M pCH110 was added to 1×10^{-3} M amphiphiles of deuterium oxide solution. [e] Not detected. [f] Not determined.

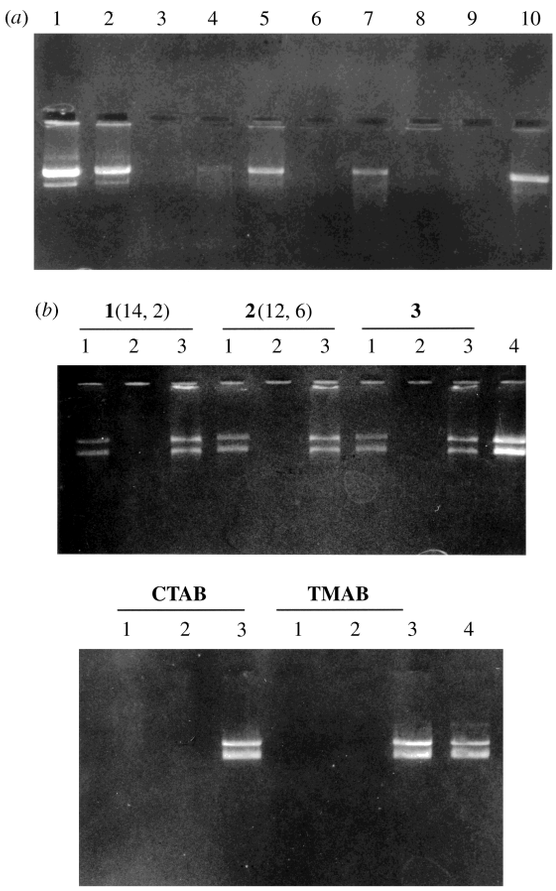

Fig. 2 Digestion of amphiphile **1**(12,2)–DNA complex with DNase I. (*a*) The complex containing pCH110 (0.2 μg, 3.14×10^{-10} unit mol) and various amounts of an amphiphile **1**(12,2) were treated with DNase I for 1 h at 37 °C, and electrophoresed. Lane 1, input free pCH110 plasmid; lanes 2–4, complex with **1**(12,2) (3.14×10^{-10} mol); lanes 5–7, complex with **1**(12,2) (6.3×10^{-10} mol); lanes 8–10, complex with **1**(12,2) (12.6×10^{-10} mol). Lanes 2, 5 and 8, untreated complex; lanes 3, 6 and 9, complex digested with DNase I; lanes 4, 7 and 10, complex digested with DNase I and then treated with Triton X-100. The material remaining in the well would be aggregates of plasmid DNA and/or contaminating genomic DNA. (*b*) The complex containing pCH110 (0.2 μg, 3.14×10^{-10} unit mol) and various amphiphiles (3.14×10^{-10} mol) were treated with DNase I and then electrophoresed. Lane 1, complex digested with DNase I and then treated with Triton X-100; lane 2, complex digested with DNase I; lane 3, untreated complex; lane 4, input pCH110.

plasmid DNA is mixed with amphiphiles, the DNA probably exists in two states, free and liposome-enclosed. The ratio between these DNAs depends on the amount of the amphiphile added, and more than four equivalents (for phosphate groups of DNA) of the amphiphile are required for complete enclosure of the plasmid DNA. The liposome-enclosed DNA would be resistant to DNase digestion, whereas the free DNA would be susceptible to digestion and even partial degradation would not occur. Thus, the sum of DNA in the lane of the complex in the absence of DNase treatment [free DNA, *i.e.* Fig. 2(*a*), lanes 2, 5, 8] and DNA appearing on detergent treatment after DNase treatment (enclosed DNA, *i.e.* lanes 4, 7, 10, respectively) corresponds to the total input DNA (*i.e.* lane 1).

The efficiency in enclosure of plasmid DNA with various cationic amphiphiles is shown in Fig. 2(*b*). Lane 1 shows the DNA resistant to DNase digestion (enclosed DNA) and lane 3 the DNA not enclosed in liposomes (free DNA). Amphiphiles **1**(14,2), **2**(12,6) and **3** had the same efficiency in affording resistance to DNase digestion, whereas in the presence of CTAB, a micellar amphiphile, the DNA was destroyed by DNase I. Thus, only bilayer-forming amphiphiles can enclose DNA molecules so as to be resistant to DNase digestion, and DNA in the amphiphile vesicles cannot be detected by ethidium bromide unless the bilayer structure is destroyed by Triton X-100.[11] Contrary to our observations, Reimer *et al.*[12] reported that a complex of dioleoyldimethylammonium chloride, which is similar to our **3**, with DNA was susceptible to DNase I digestion, although we have no explanation for this discrepancy.

When DNA, a negatively charged polymer, is added to a cationic liposome, the cationic heads of the amphiphiles interact with the phosphate groups of DNA on the surface of the liposome through coulombic forces and arranged themselves helically along the phosphate groups. The distance between ammonium head groups of adjacent amphiphiles in the bilayer membrane is roughly estimated to be 9 Å,[13] whereas phosphate groups are arranged every *ca.* 5 Å along the DNA helix.[14] Because the latter is slightly shorter than the former, the head groups of amphiphiles might arrange themselves in a zig-zag manner to occupy all of the surface area of the DNA molecule. Other amphiphile molecules would cover the monolayer of amphiphiles bound to DNA to form a bilayer membrane.

Change in size of cationic liposomes by DNA

The sizes of the liposomes formed from amphiphiles were measured using a dynamic light scattering apparatus (Table 1). Liposomes formed with these amphiphiles were estimated to be $0.5–2.0 \times 10^3$ Å in size with an average diameter of *ca.* 1.2×10^3 Å, when measured 30 min after forming liposomes. After standing for 24 h at *ca.* 25 °C, the liposome sizes of **1**(12,2) and **1**(14,2) increased by *ca.* 20%. On the other hand, vesicles of **2**(12,6), **2**(14,6) and **3** increased in size by 4–12 times. Almost the same change in size was observed when DNA was added to the liposomes. DNA facilitated fusing the liposomes together, and the sizes at 30 min after addition of DNA were similar to those after standing for 24 h in the absence of DNA. An increase in the diameter of the liposomes of **1**(12,2) and **1**(14,2)

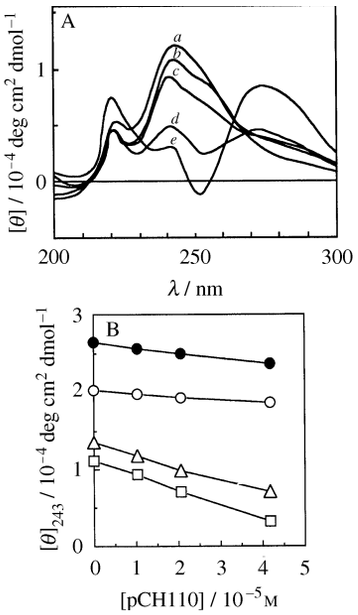

Fig. 3 CD spectra of amphiphile-DNA complex. (A) Effect of plasmid DNA on the CD spectra of amphiphile **1**(12,2). Liposome formed with **1**(12,2) (8.7×10^{-5} M) in water was mixed with various amounts of plasmid DNA (pCH110) and the CD spectrum was measured at 25 °C. The final concentration of pCH110 was: *a*, 0 M; *b*, 1.05×10^{-5} unit M; *c*, 2.09×10^{-5} unit M; *d*, 4.19×10^{-5} unit M; *e*, 10.5×10^{-5} unit M. (B) Effect of temperature on CD spectra of the amphiphile–plasmid DNA complex. CD spectra of the complex formed with **1**(12,2) (8.7×10^{-5} M) or **1**(14,2) (8.7×10^{-5} M) and pCH110 was measured at various temperatures: ○, **1**(12,2) at 15 °C; △, **1**(12,2) at 25 °C; □, **1**(12,2) at 30 °C; ●, **1**(14,2) at 25 °C.

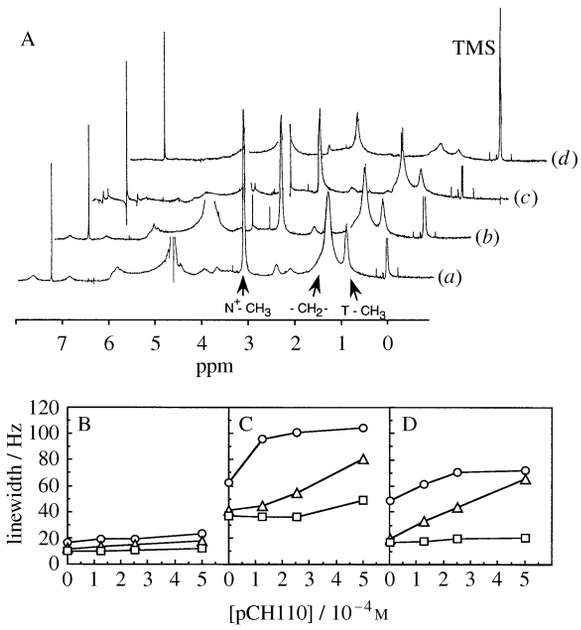

Fig. 4 ^{1}H NMR spectra of amphiphile–DNA complex. (A) Effect of increasing amount of plasmid DNA on the ^{1}H NMR spectra of amphiphile **1**(12,2). Liposome formed with **1**(12,2) (8.7×10^{-5} M) in D_2O was mixed with various amounts of plasmid DNA (pCH110) and ^{1}H NMR spectra were measured at 37 °C. The final concentration of pCH110 was: (*a*), 0; (*b*), 1.26×10^{-4} unit M; (*c*), 2.51×10^{-4} unit M; and (*d*), 5.04×10^{-4} unit M. (B–D) Effect of plasmid DNA on the linewidths of ^{1}H NMR signals derived from amphiphile **1**(12,2). ^{1}H NMR spectra of **1**(12,2) (1×10^{-3} M) was measured in the presence of various amounts of pCH110. (B) *N*-methyl proton; (C) methylene proton; (D) terminal methyl proton. Temperature: ○, 10 °C; △, 25 °C; □, 37 °C.

by *ca*. 40% after the addition of DNA indicates that the surface area of the liposomes was increased about two-fold and that the liposomal structures formed by these amphiphiles were stable and a little fusion occurred between the vesicles, suggesting that plasmid DNA condensed by neutralization of phosphate charges with cationic amphiphiles.[15] On the other hand, the addition of DNA to liposomes formed with **2**(14,6) and **3** led to an increase in diameter by *ca*. 10 times, implying an increase in surface areas of *ca*. 100 times. Therefore, about one hundred liposomes seemed to fuse to form a large liposome even if the vesicle was unilamellar. In such a large liposome, DNA also seemed to be enclosed [Fig. 2(*b*)], though the number of DNA molecules in each liposome was not determined. Thus, the amphiphiles examined here could be classified into two groups with different responses in the fusion of the liposomes to the addition of DNA: amphiphiles forming liposomes which do not fuse, **1**(12,2) and **1**(14,2), and those forming liposomes which fuse, **2**(12,6), **2**(14,6) and **3**.

Stacking of amphiphiles in the complex with DNA

Because the optical activity in CD spectra, which is due to the π–π* transition between chiral chromophores of amphiphiles, is markedly enhanced by formation of the ordered bilayer structure,[9] the change in molecular stacking of the amphiphiles in the complex with DNA can be monitored by CD spectroscopy. The CD spectrum derived from a chiral benzene group of amphiphiles **1**(12,2) is shown in Fig. 3(A). The amphiphile had a CD maximum at 243 nm with a $[\theta]$ value of 1.4×10^4 ° cm^2 dmol^{-1}. When DNA was added to the liposomes at 25 °C, the CD spectrum was greatly suppressed with increasing amounts of DNA and finally exhibited a spectrum similar to that of DNA itself. Fig. 3(B) shows the effect of temperature on change in the $[\theta]_{243}$ value in the CD spectra of the **1**(12,2)–DNA complex. As has been reported,[9] the $[\theta]_{243}$ value in the absence of DNA was decreased with increasing temperature, probably because of increased membrane fluidity. The decrease in this value with increasing amount of DNA was large at 30 and 25 °C and the value reduced from 1.1×10^4 to 0.3×10^4 ° cm^2 dmol^{-1} by addition of *ca*. 0.5 equiv. of DNA at 30 °C, while at 15 °C little spectral change was observed under the same conditions. On the other hand, in the case of an amphiphile **1**(14,2) with a T_c higher than that of **1**(12,2), the $[\theta]_{243}$ value at 25 °C was much higher than that of **1**(12,2) and the spectral change induced by DNA was small, as that of **1**(12,2) at 15 °C. Thus, at a temperature higher than the T_c binding of the amphiphiles to DNA might have altered the chromophore orientation in the bilayer, without lowering the mobility of the hydrocarbon chains (see below), in such a way as to lose the coupling of the electrical transition moment.

These findings suggest that the amphiphiles might be forced to arrange themselves along the phosphate groups of DNA so that stacking of the benzene ring would slightly slide along the helix. Such a change in the arrangement of the amphiphiles inside the liposomes, the inner layer of the liposomes, caused by the addition of DNA could affect their arrangement in the outer layer at a temperature higher than the T_c of the amphiphile, whereas, when the temperature is lower than the T_c, stacking of the amphiphiles seems to be strong enough to maintain their arrangement in the liposomes, even after the addition of DNA. In such a case, the structure of DNA changes instead, as previously reported.[11]

Mobility of amphiphiles in the complex with DNA

NMR signals from amphiphiles forming a bilayer membrane were considerably broadened in comparison with those of micellar solutions and the half-height linewidths of the signals decrease with temperature.[16] Therefore, the change in mobility of amphiphiles in the bilayer membranes by addition of DNA can be detectable at the molecular level by measuring the linewidth of the individual signals using an NMR spectrometer. Fig. 4(A) shows a typical ^{1}H NMR spectrum of a 1 mM

aqueous solution of amphiphile **1**(12,2) at 25 °C. Three absorption lines at 3.09, 1.27 and 0.88 ppm are assigned to protons in the *N*-methyl, methylene and terminal methyl groups, respectively.[16] The lines were broad, an effect attributed to restricted molecular motion in the bilayers. The addition of increasing amounts of plasmid DNA to the vesicles led to further broadening in the NMR signals [Fig. 4(A, *b–d*)]. The effect of DNA on the line broadening of the signals due to the three kinds of protons in **1**(12,2) at various temperatures is shown in Fig. 4(B–D). The half-height linewidth of the absorption signal of the *N*-methyl proton was small and unaltered with increasing temperature [Fig. 4(B)]. Little change in the linewidth of this proton was also observed when DNA was added to the vesicles. On the other hand, the linewidths of the NMR signals from the methylene and terminal methyl protons in the absence of DNA were much larger than that for the *N*-methyl proton, and they decreased with increasing temperatures [Fig. 4(*c*) and (D)]. The addition of DNA led to broadening of these signals, especially at temperatures lower than the T_c of **1**(12,2), 24.8 °C. The linewidths of these signals at 10 °C were considerably increased by the addition of DNA and reached a plateau.

If the line broadening is caused by a difference in chemical shifts induced by the binding of amphiphiles to DNA, the linewidth of the *N*-methyl proton is expected to be larger than those of others. Just the opposite results were obtained. Therefore, the linewidth observed here must be determined mainly by the fluctuation of an anisotropic local magnetic field due to dipole–dipole interaction of the protons, as induced by a reduction in translational and rotational motions of the amphiphile molecules. As DNA binds to the bilayer membrane, the mobility of the amphiphile molecules decreases and fluctuation of the local magnetic field would be induced. Consequently, the linewidth is expected to increase with the addition of DNA.[17] Thus, binding of DNA has little effect on the mobility of the *N*-methyl proton at the temperatures examined. With regard to the mobility of methylene and terminal methyl protons, binding of DNA has no apparent effect at temperatures higher than the T_c, whereas at a lower temperature, the mobility of the protons is markedly restricted.

Because the methylene proton was the most sensitive to DNA addition, the half-height linewidths of the NMR signals of this proton of various cationic compounds at various temperatures were examined to observe motion of the compounds (Table 1). In the absence of DNA, the linewidth of the methylene protons of **1**(12,2), **1**(14,2), **2**(14,6) and **3** was similar (38–47 Hz), though that of **2**(12,6) was much smaller, probably because of its extremely low T_c compared with those of the others. The linewidth of each amphiphile decreased with increasing temperature, indicating an increase in mobility of the hydrocarbon chains of amphiphiles. The linewidths were roughly correlated with their T_c values, particularly at the temperature at which all the amphiphiles are in a fluid state, 45 °C. Addition of DNA caused marked broadening of the signals in the amphiphiles **1**(12,2) and **1**(14,2) at 25 °C. In other amphiphiles the linewidths remained practically unchanged. Because the measurement of the linewidth was rather difficult especially at a low amphiphile concentration, fluctuation in the value was 10–15%. At higher temperatures, little effect of DNA on the linewidth was observed, although a small increase was still found in some amphiphiles such as **1**(12,2), **1**(14,2) and **2**(14,6). CTAB caused a different response from the bilayer-forming amphiphiles. In the absence of DNA, CTAB exhibited a very sharp NMR signal of its methylene proton, while the linewidth of the proton was markedly increased by addition of increasing amounts of DNA at any temperature examined.

These results indicate that, with the addition of DNA, molecular motion of the amphiphiles at 25 °C was more restricted in the bilayer membranes formed by **1**(12,2) and **1**(14,2), but practically no change was observed in the membranes of **2**(12,6), **2**(14,6) and **3**. Based on systematic studies of the relationship between T_c values of various bilayer-forming amphiphiles and their structural elements, such as head, connector, spacer and tails, Kunitake *et al.*[2] noted that an aromatic ring in the spacer region affords a strong intermolecular interaction. After the head groups of **1**(12,2) and **1**(14,2) are bound to DNA through ionic interactions, they seem to be tightly fixed with a benzene ring in the spacer region, whereas with other amphiphiles, binding of the head groups to DNA might have no apparent effect on movement of the hydrocarbon chain tails.

Interaction of amphiphiles with DNA in the complex and their gene transfer activities

The requirements of an amphiphile for efficient DNA transfection are that it has a T_c value lower than the culture temperature, 37 °C, and forms small vesicles with the bilayer membrane.[5] How or why do these properties contribute to the transfection efficiency of an amphiphile? The present findings on the physicochemical properties of amphiphiles in the complex with DNA, though they are still fragmentary, offer a possible answer to the question. At the culture temperature, stacking of **1**(12,2) in the bilayer membrane becomes loose due to DNA, while **1**(14,2) retains a rigid structure even after addition of DNA. Although, because other amphiphiles have no chiral chromophore, it is impossible to obtain information about their stacking in the bilayer, they are probably more disturbed in their stacking by DNA than **1**(12,2) because they have no strong interaction such as that between phenylene rings. NMR data that show a large linewidth value at 37 °C in the presence of DNA also indicate the rigid nature of **1**(14,2) in the complex. Thus, the low gene transfer activity of **1**(14,2) might be due to low fluidity not sufficient for fusion of the liposomes with cellular membranes.

Liposomes formed with **2**(14,6) and **3** tend to fuse to form large liposomes consisting of more than one hundred original liposomes. The number of liposomes per unit amount of the amphiphile is then less than one hundredth of that of **1**(12,2). Thus, the reduction in the number of liposomes available for transfection may explain the poorer efficiency in transfection potential of **2**(14,6) and **3**. Disturbed stacking of the amphiphiles in the membrane by DNA probably induces or accelerates the fusion of the liposomes. There is at present no reliable explanation for the low transfection ability of **2**(12,6), because it is fluid enough at the culture temperature and forms a rather small fused vesicle with DNA. One possible explanation is that the bilayer structure of this compound would be quite unstable at 37 °C and might be partially micellar in nature, because it has an extremely low T_c and the hydrocarbon chains are very mobile even at 25 °C.

The critical micelle concentration of CTAB has been determined to be 0.9 mM and the final concentration of the compound in the culture medium is about 0.1 mM. Most CTAB molecules are, therefore, mono-dispersed in the medium. Each CTAB molecule seems to attach to DNA through both ionic and hydrophobic interactions. The presence of those interactions between CTAB and DNA could explain the temperature-independent increase in the linewidth of the NMR signal of the methylene proton. Our present and previous results[5] indicate that monomeric or micellar forms of amphiphiles have no transfection activity and bilayer structures would be essential for the interaction to cellular membranes, as suggested by Zhou and Huang[18] showing that DC-Chol exhibited a significantly increased transfection efficiency only in combination with a liposome-forming amphiphile, dioleoylphosphatidylethanolamine, although Reimer *et al.*[11] suggested that the binding of monomeric or micellar form of cationic amphiphiles to DNA is the first step of the formation of DNA–amphiphile particles with gene delivery activity.

The present study indicates that the most important requirement for efficient gene transfection is that amphiphiles in the complex with DNA are in the fluid bilayer at the culture

temperature of cells. Fluidity of an amphiphile in the complex primarily depends on its own phase transition temperature T_c, and an amphiphile with slightly lower T_c value than the culture temperature is the best for efficient gene transfer activity. The second requirement is formation of the small stable complex. The ordered stacking of the amphiphile in the complex would prevent the liposomes from fusion to form large aggregates. The π–π interaction by the phenylene group in the spacer region of amphiphiles, in addition to electrostatic and hydrogen bond interactions between the head and connector portions and the phosphate groups of DNA, is effective in retention of the ordered stacking of hydrocarbon chains of the amphiphile, even after complexing with DNA. Based on the information, we can design new compounds with higher DNA transfection ability.[6]

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