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Correlation between Physicochemical Characteristics of Synthetic Cationic Amphiphiles and Their DNA Transfection Ability

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Liposomes formed with synthetic double-chain ammonium amphiphiles were used for DNA transfection into eukaryotic cells. We studied the correlation between the efficiency of various cationic amphiphiles in DNA transfection and their physicochemical properties. The efficiency of amphiphiles in the transfection was examined by the transient expression of β-galactosidase from its cDNA in COS cells. Amphiphiles with a phase-transition temperature (T_c) lower than 37 °C, as measured by differential scanning calorimetry, could introduce DNA into the cells. Electron microscopic observation indicated that amphiphiles possessing DNA transfection ability form vesicular structures in aqueous solution. Thus, fluid and vesicular bilayer structures were much higher than rigid and helical bilayer structures regarding the effectiveness of amphiphiles in DNA transfection. The efficiency of didodecyl *N*-[*p*-(2-trimethylammonioethoxy)benzoyl]-L-glutamate bromide was the highest of all the synthetic amphiphiles examined.

In 1977 it was found that stable bilayer membranes were formed from dialkyldimethylammonium bromides[1] and a large number of cationic amphiphiles with long dialkyl chains were shown to form bilayer membranes.[2] As these synthetic bilayer membranes have physicochemical characteristics, such as an aggregate morphology,[3] phase transition,[4] fusio,[5] and phase separation,[5] similar to those of the biolipid bilayer membranes, possibly they can be used for various biological systems, without harmful effects. Use of synthetic amphiphiles in biological systems has the advantage in that the physicochemical characteristics of amphiphiles can be controlled by designing specific molecular structures.

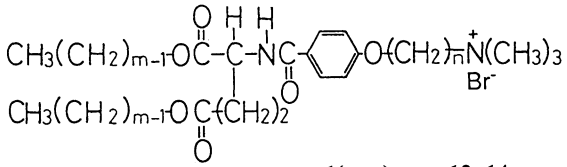
Cationic liposome-mediated transfection is one of effective methods of introducing DNA into eukaryotic cells. The liposomes spontaneously interact with DNA, fuse with cultured cells and facilitate the delivery of functional DNA into the cells. A cationic liposome formed from [2,3-bis(oleoyloxy)propyl]-trimethylammonium chloride (DOTMA) was first reported to be effective in DNA transformation.[6,7] In the foregoing paper we showed that one of dialkyl ammonium amphiphiles, didodecyl *N*-[*p*-(2-trimethylammonioethoxy)-benzoyl]-L-glutamate bromide, which can form a stable bilayer membrane, had a higher DNA transfection potential than did DOTMA.[8,9]

We have examined the correlation between the efficiency of synthetic amphiphiles in DNA transfection and their physicochemical characteristics, such as membrane fluidity and aggregate morphologies. We show that amphiphiles forming fluid and vesicular bilayer are highly effective in DNA transfection.

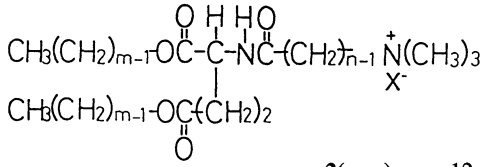
Results and Discussion

Two series of chiral dialkylammonium amphiphiles, **1** and **2** (Fig. 1), were synthesized and the correlation between physicochemical properties and DNA transfection ability was examined. A first series of amphiphiles, **1**(*m,n*), consists of two tails (length of "tail", *m*), trifunctional glutamate and phenylene groups as a connector, methylene spacer (the length of "spacer", *n*) and ammonio group as a cationic head. A second series of amphiphiles, **2**(*m,n*), has the same basic structure as **1**, but there is no phenylene group in the connector.

Transfection Efficiency of Amphiphiles. To examine the relative efficiency of amphiphiles in the delivery of DNA into cells, we used the transient expression of plasmid pCH110 with a β-galactosidase gene in COS cells. The extent of the expression was determined



1(*m,n*) $m=12, 14$ $n=2, 4, 6, 8$



2(*m,n*) $m=12, 14$ $n=2, 4, 6$

X=Cl (n=2, 4)
=Br (n=6)

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

by measuring the percentage of cells expressing β-galactosidase and by assaying enzyme activity in the cell extracts. Figure 2 shows the efficiency of various amphiphiles in the expression of β-galactosidase. COS cells were transformed with 1 to μg of pCH110 DNA using 10 μg (13 nmol) of liposomes prepared from the amphiphiles, as described under Experimental. Since less than 5% of the total cells were detached from the dish during the culture periods, in all the experiments, the amphiphiles were not cytotoxic, under the condition used. As can be seen in the figure, two measurements of the expression of β-galactosidase, the number of cells expressing the enzyme and the total enzyme activity in the cell extract, correlated fairly well, hence all the cells incorporating plasmid DNA seem to express the gene and produce the enzyme protein, to almost the same extent. In a series of **1**, the transfection efficiency varied greatly according to differences in lengths of a tail and spacer of the amphiphiles. An increase in spacer length of a series of **1**(12,*n*) caused a remarkable decrease in the transfection efficiency. A more dramatic difference was observed between series of **1**(12,*n*) and **1**(14,*n*). Change of dialkyl chains from dodecyl to tetradecyl groups resulted in an almost complete loss of transfection ability. In the second series, **2**(12,*n*) and **2**(14,*n*), all compounds listed in Fig. 1 had the ability to transfer a gene, though their efficiencies were less than a half of **1**(12,2). There seems to be no correlation between the efficiency of the amphiphiles of series **2** in gene transfection and their structural characteristics, lengths of a tail and spacer (Fig. 2). In the series of **1**(12,*n*) and **2**(12,*n*), 1-2 μg of DNA was optimum for the transfection, and 5 μg of DNA led to a marked decrease in the efficiency. Since a preliminary experiment showed that about 2 μg of DNA could bind to 10 μg of the amphiphiles, free DNA might interfere with the liposome-mediated transfection of DNA into the cells when 5 μg of DNA was used. Among the amphiphiles examined, **1**(12,2) showed the highest efficiency in DNA transfection.

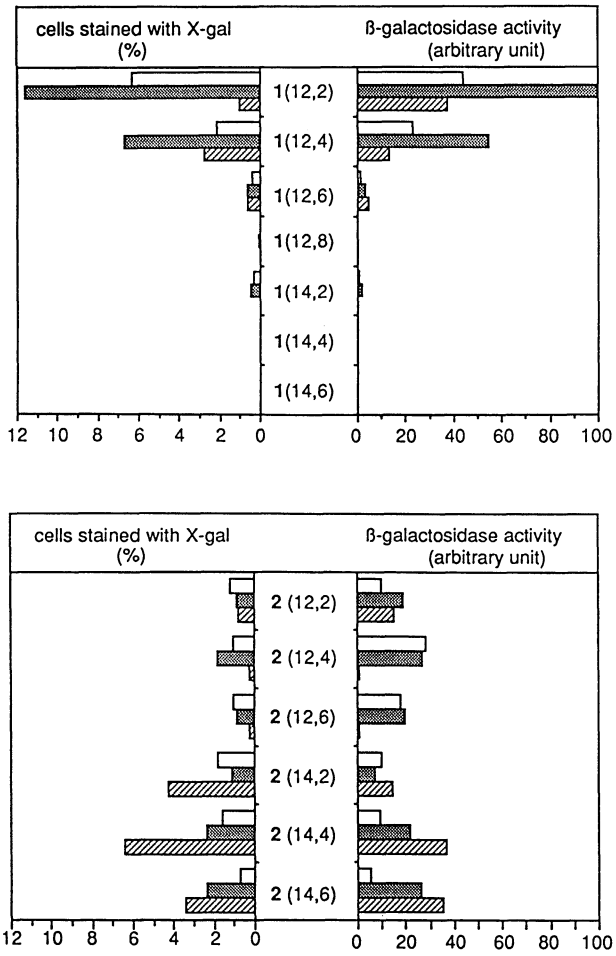


Fig. 2. Transfection efficiency of various amphiphiles. Cells were transfected with amphiphiles/DNA complexes containing 13 nmol of amphiphiles and μg (open bar), 2 μg (dotted bar), 5 μg (oblique line bar) of plasmid DNA, pCH110, per 22 mm dish. After incubation of the cells for 48 h, the cells were harvested and subjected to assay of transfection, as described under Experimental. 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.

Table 1. Phase Transition Temperature T_c and Aggregation Morphology of Amphiphiles **1** and **2**

Amphiphile (*m*, *n*)	T_c[a] (DSC peak)/°C	Aggregate morphology[b]
1 (12, 2)	24.8	Vesicle
1 (12, 4)	29.4	Vesicle
1 (12, 6)	39.4	Lamella
1 (12, 8)	39.9	Filament
1 (14, 2)	41.1	Vesicle
1 (14, 4)	45.0	Vesicle & lamella
1 (14, 6)	51.2	Filament
2 (12, 2)	N. D.[c]	Vesicle & lamella
2 (12, 4)	N. D.	Vesicle & lamella
2 (12, 6)	9.0	Vesicle & lamella
2 (14, 2)	22.0	Vesicle
2 (14, 4)	21.8	Vesicle
2 (14, 6)	25.4	Vesicle

a) Sample, 20 mM in water. Temperature was elevated from 5 °C at a rate of 1 °C min^{-1}.
b) Sample, 10 mM in water. c) N. D.: not detected.

Physicochemical Characteristics of Amphiphiles. To determine which physicochemical properties of amphiphiles are responsible for transfection efficiency, we measured thermal and morphological behavior, of amphiphiles in aqueous solution and searched for a possible correlation between transfection efficiencies and physicochemical characteristics.

Phase Transition. The transition between the crystal and liquid-crystal phases is one of the most fundamental characteristics of a bilayer membrane. The phase transition can be detected directly by differential scanning calorimetry (DSC).[4] The phase-transition temperature T_c (peak top temperature in the DSC scan) is summarized in Table 1. As pointed out d by Kunitake and Ando,[4] T_c increased with an increase in tail and spacer lengths of the amphiphiles. A series of **2** without a phenylene unit in the connector region had much lower T_c values than did a series of **1**. In a series of **1**, enhanced aromatic stacking may suppress molecular disordering in the liquid crystalline state. T_c values of **2**(12,2) and **2**(12,4) were too low to be determined by DSC measurements. These results indicate that all the amphiphiles possessing measurable DNA transfection ability, **1**(12,2), **1**(12,4), and a series of **2**, had T_c values lower than 37 °C. Since the transfection experiment was carried out at 37 °C, these amphiphiles were in the fluid liquid crystalline state in the culture medium. Thus, the primary requirement for the transfection of DNA appears to be fluidity of the liposomes.

Aggregate Morphology. Electron microscopy is a useful tool for the direct observation of bilayer formation and aggregate morphology of the amphiphiles. Some representative electron micrographs are shown in Fig. 3. All the amphiphiles had bilayer structures. However, the aggregate morphology varies with the molecular structure of the component amphiphile. Amphiphile **1**(12,2) formed well-developed vesicles with a diameter of 300—600 Å, as shown in Fig. 3a. Similar morphologies were observed for **1**(12,4), **1**(14,2), **1**(14,4), **2**(14,2), **2**(14,4), and **2**(14,6), but with different diameters of 500—3000, 400—1000, 300—600, 800—1500, 1000—2500 and 500—3000 Å, respectively. Figure 3b shows multiwalled vesicles and/or lamellae aggregates formed from **2**(12,2), with a diameter of 400—2000 Å. Amphiphiles **2**(12,4) and **2**(12,6) had similar morphologies. Amphiphile **1**(12,6) gave fragmentary lamellae (Fig. 3c), and **1**(12,8) and **1**(14,6) gave helical filaments with a length of 1—3 μm (Fig. 3d). Aggregate morphology of amphiphiles **1** and **2** are summarized in Table 1. These morphological observations indicate that amphiphiles forming vesicular structure had DNA transfection ability and that the smaller vesicles appeared to be more

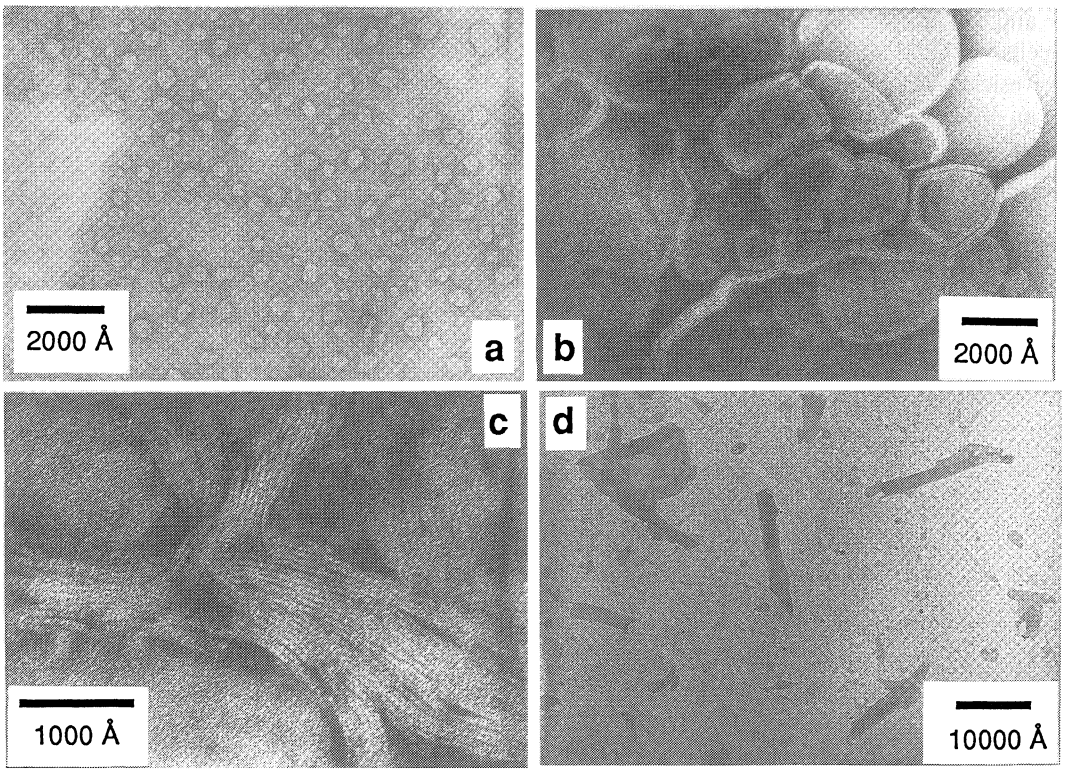


Fig. 3. Electron micrographs of aggregates formed from **1** and **2**. Amphiphiles **1** and **2** were suspended in water (10 mM), sonicated, and stained with uranyl acetate.
a. **1**(12,2), original magnification×50000
b. **2**(12,2), original magnification×50000
c. **1**(12,6), original magnification×150000
d. **1**(14,6), original magnification×10000

effective than larger ones.

Aggregate Size. Stability of the aggregates was studied by measuring change in size while standing. Aqueous dispersions of the amphiphiles were kept at room temperature (about 25 °C) for 0.5 and 24 h and size of the aggregates was measured using a dynamic light scattering apparatus (Table 2). Average size of the aggregates of the amphiphiles **1** and **2** was much the same as those determined by electron microscopy. After leaving for 24 h at room temperature, aggregate sizes of **1**(12,2), **1**(12,4), **1**(14,2), and **1**(14,4) increased by less than 20% of the original size, hence that the vesicular structures formed by these amphiphiles were stable and little fusion occurred between the vesicles. On the other hand, aggregates of **1**(12,6), **1**(12,8), and of a series of **2** increased in size by 4—20 times. In these aggregates, fusion and/or morphological change from vesicular structure to lamella, tubular, or filamentous structure, of the bilayer membranes seemed to readily occur. These results suggest that amphiphiles forming stable aggregates had a higher DNA-transfection ability.

In conclusion, we found a good correlation between the efficiency of the synthetic amphiphiles in gene transfection and their physicochemical characteristics, such as membrane fluidity and aggregate morphologies. The primary requirement of an amphiphile for DNA transfection is that the T_c value be lower than 37 °C so that it is in the fluid liquid crystalline state at the culture temperature and can interact with plasma membranes of the culture cells. The second requirement is to form a small stable vesicle. The vesicular structure might be required for an effective interaction between aggregates and DNA and/or the cells. Thus, amphiphiles that form small stable vesicles in the fluid state are suitable for DNA-transfection, and **1**(12,2) is highest in efficiency of all the amphiphiles we tested.

Table 2. Aggregate Size of Amphiphiles **1** and **2**

Amphiphile (m, n)	Aggregate size[a]/10^3 Å	
	0.5 h	24 h
1 (12, 2)	1.2	1.5
1 (12, 4)	1.6	1.9
1 (12, 6)	1.6	6.9
1 (12, 8)	4.7	25.9
1 (14, 2)	1.2	1.3
1 (14, 4)	1.3	1.4
1 (14, 6)	1.5	—[b]
2 (12, 2)	0.8	16.3
2 (12, 4)	1.3	18.7
2 (12, 6)	1.1	4.0
2 (14, 2)	0.7	11.0
2 (14, 4)	0.9	8.8
2 (14, 6)	0.8	9.4

a) Aqueous dispersion of amphiphiles (0.043 mM) were left at room temperature (about 25 °C) for 0.5 and 24 h. The measurements were carried out using dynamic light scattering spectrophotometer at 25 °C. b) Not determined.

Experimental

Chemicals. Double-chain amphiphiles **1** and **2**, listed in Fig. 1, were synthesized according to the method of Kunitake et al.[10]

Cells and Plasmids. Cos-1, a simian kidney cell line transformed with simian virus 40 (SV40), was maintained in a Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% inactivated fetal calf serum in an atmosphere of 10% CO_2 at 37 °C. Plasmid DNA (pCH110) has a β-galactosidase gene (lac Z) and SV40 early promoter.

Amphiphiles-Mediated Transfection. Transfection experiments were carried out as described by Felgner et al.[6,7] with some modifications.[8,9] COS-1 cells were grown to just before confluency in 22 mm dishes and washed twice with 1 ml of Hepes-buffered saline (HBS; 20 mM Hepes-KOH buffer containing 150 mM NaCl (1 M=1 mol dm^{-3}), pH 7.4). Plasmid DNA (in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and amphiphiles suspension (13 nmol, prepared by sonication) were diluted separately with 0.15 ml of HBS. Both solutions were combined and 0.4 ml of amphiphiles-DNA mixture was poured gently onto the cells. After incubation for 3—5 h at 37 °C, 2 ml of DMEM supplemented with 10% fetal calf serum was added and the detached cells became suspended. The medium was replaced with 2 ml of a fresh medium after 12—20 h, and the cells were incubated for 48 h from the first addition of DMEM.

Transfection Efficiency. Monolayer cells were harvested after a 48 h culture. The efficiency of amphiphiles in the expression of β-galactosidase was measured by counting the number of the cells visualized by histochemical staining of the enzyme activity and by assaying of the total enzyme activity in the extract of the cultured cells using 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal)[11] and *o*-nitrophenyl β-D-galactopyranoside[12] as the substrate, respectively.

Phase-Transition Temperature. An aqueous solution of amphiphile (20 mM) was sealed in a silver sample pan. Measurements were carried out using a Seikosha differential scanning calorimeter Model DSC-210 heating rate, 1 °C min^{-1}.[4]

Electron Microscopy. Amphiphiles suspended in distilled water (10 mM) were sonicated using a Tomy Sonifier for several minutes to give a clear solution and this solution was applied to carbon-coated grids and dried in vacuo. The sample was stained with a 2% aqueous uranyl acetate solution and subjected to electron microscopic examination[13] using a Hitachi electron microscope, Model H-7000.

Dynamic Light Scattering. Amphiphiles suspended in water (0.043 mM) were sonicated for several minutes to give a clear solution. After standing at room temperature (about 25 °C) for 0.5 and 24 h, the suspension was subjected to dynamic light scattering experiment[13] using an Otsuka Electronics Co., Model DLS-700 with a laser light source.

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