Effect of Non-Ionic Surfactants on the Formation of DNA/Emulsion Complexes and Emulsion-Mediated Gene Transfer

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Purpose. To study the structure-function relationship of non-ionic surfactants in emulsion-mediated gene delivery.

Methods. Four different types of non-ionic surfactants including Tween, Span, Brij and pluronic copolymers were used as co-emulsifiers for preparation of emulsions composed of Castor oil, dioleoylphosphatidylethanolamine (DOPE) and 3β[N-(N′, N′-dimethylaminoethane) carbamoyl] cholesterol (DC-Chol). The effect of different surfactants on the formation of DNA/emulsion complexes and transfection activity were analyzed using plasmid DNA containing luciferase cDNA as a reporter gene.

Results. Non-ionic surfactants containing branched polyoxyethylene chains as the hydrophilic head group were more effective in preventing the formation of large DNA/emulsion complexes than those containing one or no polyoxyethylene chain. All emulsion formulations except those containing Brij 700 exhibited high activity in transfecting mouse BL-6 cells in the absence of serum. In the presence of serum, however, transfection activity of each formulation varied significantly. Emulsions containing Tween, Brij 72, pluronic F68 and F127 demonstrated increased activity in transfecting cells in the presence of 20% serum. In contrast to emulsions containing Span, long chain polyoxyethylene of Brij showed decreased transfection activity. The particle size of the DNA/emulsion complexes and their ability to transfect cells are dependent on the concentration of non-ionic surfactant in the formulation.

Conclusions. The structure of the hydrophilic head group of the non-ionic surfactants in the emulsion is important in determining how DNA molecules interact with emulsions and the extent to which DNA is transferred inside the cell.

KEY WORDS: emulsions; gene transfer; transfection; gene therapy; non-ionic surfactant; cationic lipid.

INTRODUCTION

Delivery of DNA into cells using cationic lipids has drawn increasing attention in the field of gene therapy due to their simplicity, non-immunogenicity, low toxicity and commercial availability. The cationic lipid-mediated gene delivery usually requires additional lipid components for optimal delivery activity. Therefore, the efficiency of gene transfer depends on the chemical structure and properties of the cationic lipid as well as those of the additional components (1–4).

The most commonly used formulations for gene delivery are liposomes composed of cationic lipid and a helper lipid such as dioleoylphosphatidylethanolamine (DOPE). In addition to the function of stabilizing the bilayer of cationic lipids, DOPE is believed to have the function of facilitating the transfer of DNA from endosome to cytoplasm by destabilizing the endosome membranes (4–7). Although gene expression has been shown in cultured cells and in some cases in animals (8, 9), one of the major drawbacks of liposomal formulations is that the DNA/liposome complexes are not stable with frequent and rapid formation of aggregates and flocculates. It is necessary, therefore, to prepare the complex freshly and use it within a short period of time. In addition, it is also known that the transfection activity of cationic liposomes is interfered by serum components which presumably neutralize the unpaired positive charges in the complexes (1, 10, 11) which are essential for binding of DNA/lipid complexes to cell surface. Because of this problem, the use of cationic liposomes for gene transfer is limited to the situation where a minimal amount serum is present.

Recent work in our laboratory has shown that the problems associated with cationic liposomes can be overcome by using oil-in-water emulsions (12). In addition to the cationic lipids and neutral phospholipids, these new formulations contain significant amounts of oil and non-ionic surfactant such as Tween 80. It appeared that Tween 80 plays an important role in preventing the formation of large DNA/emulsion complexes and reducing the serum-mediated inhibition of gene transfer. To elucidate the molecular bases for the activities of Tween 80, we have conducted experiments to examine the structure-function relationship between Tween 80 and the activity of the non-ionic surfactants in blocking the formation of large DNA/emulsion complexes, in affecting the transfection activity of the DNA/emulsion complexes and in providing the serum resistance. The results presented here clearly show that the hydrophilic polyoxyethylene chains in the molecules dominate the overall properties of emulsions.

MATERIALS AND METHODS

Materials

Non-ionic surfactants including Tween series (Tween 20, 40, 60, and 80); Span series (Span 20, 40, 60, and 80); Brij series (Brij 72, 76, 78, and 700) and pluronic surfactants (F68 and F127) and castor oil were obtained from Sigma. DOPE was from Avanti Polar Lipids. 3β-[N-(N′,N′-dimethylaminoethane)carbamoyl]cholesterol (DC-Chol) was synthesized as described by Gao and Huang (11). The luciferase assay kit was purchased from Promega. Cell culture medium was from Life Technologies. Fetal bovine serum (FBS) was from Hyclone Laboratories. Plasmid pCMV-Luc containing cDNA of firefly luciferase under the control of cytomegalovirus (CMV) immediate early promoter was purified by cesium gradient centrifugation according to a standard procedure (13).

Preparation of Cationic Emulsions

Different lipid components and surfactants were dissolved in chloroform and mixed at desired ratios. Chloroform was then evaporated under a stream of nitrogen gas. The lipid film was vacuum desiccated overnight at 4°C to remove the residual

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solvent. The dried lipid film was hydrated in 1 ml of PBS (pH 7.4) for 1 h at room temperature followed by vortex mixing and subsequent homogenization using a tissue tearer (20,000 rpm, 3–4 min). The size of the emulsion particles was measured by 90° laser light scattering using a particle size analyzer (Coulter N4SD).

Measurement of the Particle Size of DNA/emulsion Complexes

Unless specified, emulsions (18 μl containing 13.5 μg DC-Chol) in 125 μl PBS (pH7.4) were mixed with equal volume of PBS containing 2 μg of plasmid DNA. The mixture was incubated at room temperature for 1 h. The average particle size of the mixture was measured by light scattering.

Transfection and Luciferase Assay

Murine melanoma BL-6 cells (5×10^4 cells/well) were seeded in a 48-well plate and cultured in RPMI medium with 10% FBS for 24 hours before transfection. Emulsions (18 μl) diluted to 125 μl with Hank's buffered saline solution were mixed with 2 μg of plasmid DNA in 125 μl of serum free medium and incubated for 10 min at room temperature. For transfection in the presence of 20% serum, 62.5 μl of FBS was added to the mixture and incubated for an additional 5 min before being added to cells right after removing the culture medium. The cells with transfection reagents were incubated for 5 h. The transfection medium was replaced with growth medium containing 10% FBS and the cells were then cultured for additional 2 days for gene expression. For luciferase assay, cells were washed twice with PBS and lysed with 100 μl lysis buffer (0.1 M Tris-HCl/0.05% Triton X-100/2 mM EDTA, pH 7.8). The lysate was centrifuged at 12,000 × g for 10 min. Ten μl of supernatant was taken for luciferase assay using a luciferase assay kit (Promega) with a luminometer (Promega). Protein concentration in the supernatant was determined by a standard Commassi blue assay (Pierce) according to the manufacturer's instruction.

RESULTS

Effect of Non-Ionic Surfactants on the Particle Size of Emulsions and DNA/lipids Complexes

Structures of the four different types of surfactants used in this study are shown in Figure 1. Data in Table I show that all these surfactants serve as good co-emulsifier for preparation of oil-in-water emulsions consisting of castor oil, DC-Chol and DOPE. The average diameter of lipid particles for emulsions containing various surfactants was very similar, ranging from 170 to 250 nm. Upon mixing with DNA, the particle size increased to a different level depending on the type of non-ionic surfactant used for emulsion preparation. A 5 to 14 fold increase in diameter of the lipid particles (DNA/emulsion complexes) was observed when DNA was mixed with emulsions containing Span, Brij 72, 74, 76, pluronic F68 or F127 which display a average diameter of 200–300 nm without DNA added. The largest size of DNA/emulsion complexes was obtained with emulsions containing Brij 72 (>3 μm). Much less increase (~2 fold) in particle size upon mixing with DNA was observed in emulsions containing Tween.

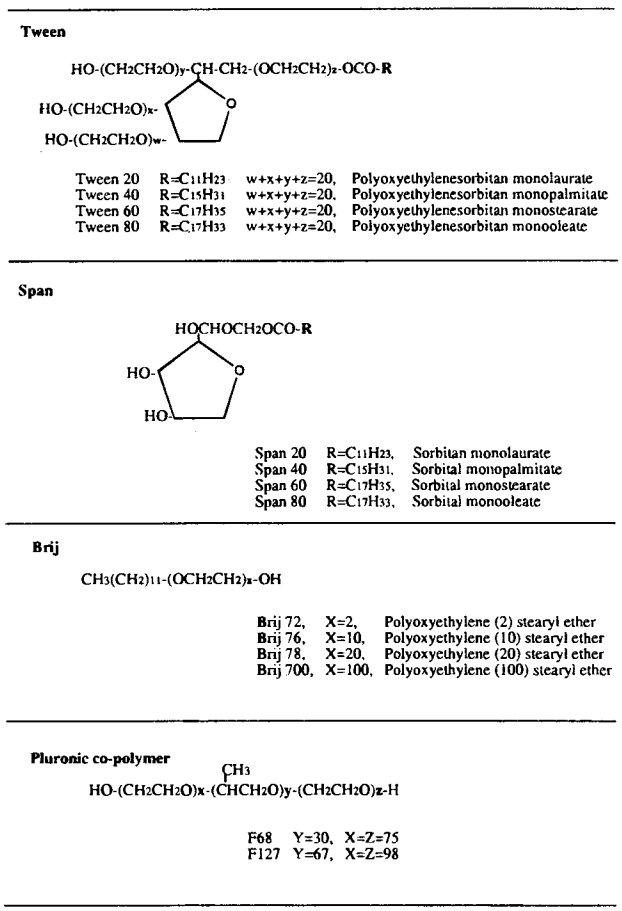

Fig. 1. Structure of non-ionic surfactants used in the study.

Table I. Effect of Non-ionic Surfactants in the Formulation on the Particle Size of Emulsion and DNA/Emulsion Complexes[a]

Non-ionic Detergent	Amount (mg)	Size of Emulsion Particle (nm)[b]	Size of DNA/emulsion Complexes (nm)[c]
Tween 20	0.117	187 ± 9	387 ± 6
40	0.122	184 ± 21	410 ± 38
60	0.125	172 ± 25	393 ± 13
80	0.125	194 ± 14	384 ± 13
Span 20	0.033	210 ± 38	1385 (Broad)
40	0.038	233 ± 30	1238 (Broad)
60	0.041	242 ± 17	1304 (Broad)
80	0.041	242 ± 14	1365 (Broad)
Brij 72	0.034	239 ± 21	3351 (Broad)
76	0.680	195 ± 16	2225 (Broad)
78	0.110	208 ± 12	1937 (Broad)
700	0.446	187 ± 5	177 ± 10
Pluronic F68	0.802	235 ± 31	1490 (Broad)
F127	1.202	218 ± 16	1198 (Broad)

[a] The matrix lipids for emulsions contain Castor oil:DOPE:DC-Chol with a weight ratio of 0.25:0.25:0.75 (mg/mg). The lipids were suspended in 1 ml of PBS (pH7.4) for emulsion preparation.

[b] The particle size represents the mean ± S.D. of three independent experiments.

[c] DNA to DC-Chol ratio was 2:13.5 (μg/μg).

Effect of Non-Ionic Surfactants on Emulsion-Mediated Transfection

The transfection activity of those emulsions is shown in Figure 2. In the absence of serum, all formulations exhibited fairly high activities in transfecting the BL-6 cells, with highest activity observed in emulsions containing Brij 74 and the lowest activity obtained with Brij 700-containing emulsions. Transfection activity for Tween-containing emulsions was approximately same at the level of 2–3 × 10^7 light unit/well. Similar level of gene expression was also obtained in cells transfected with Span-containing emulsions. The transfection activity of each formulation was also tested in the presence of serum (20%). For Tween-containing emulsions, the total amount of luciferase activity obtained was slightly higher than those obtained in the absence of serum. Much lower luciferase activity was obtained from cells transfected with emulsions containing Span. For emulsions containing Brij, it appeared that the serum sensitivity is dependent on the chain length of the polyoxyethylene. For emulsions that contain Brij 72 (2 oxyethylene units), slight increase in luciferase activity in the presence of serum was observed. In contrast, serum decreased the transfection activity of emulsions containing either Brij 74 or 76. No obvious difference in the level of gene expression was caused by the presence of serum in transfection using emulsions containing pluronic polymers (F68 and F127).

The relative toxicity of each emulsion formulation to the cells as determined by the total amount of proteins extracted from the transfected cells, is shown in Figure 2B. It is clear that all formulations tested under the experimental conditions did not have any appreciable toxic effect on cells as indicated by the total amount of proteins extracted. This would suggest that the difference in luciferase activities detected among these formulations is due to the transfection activity of the formulation, not the toxic effect on these cells.

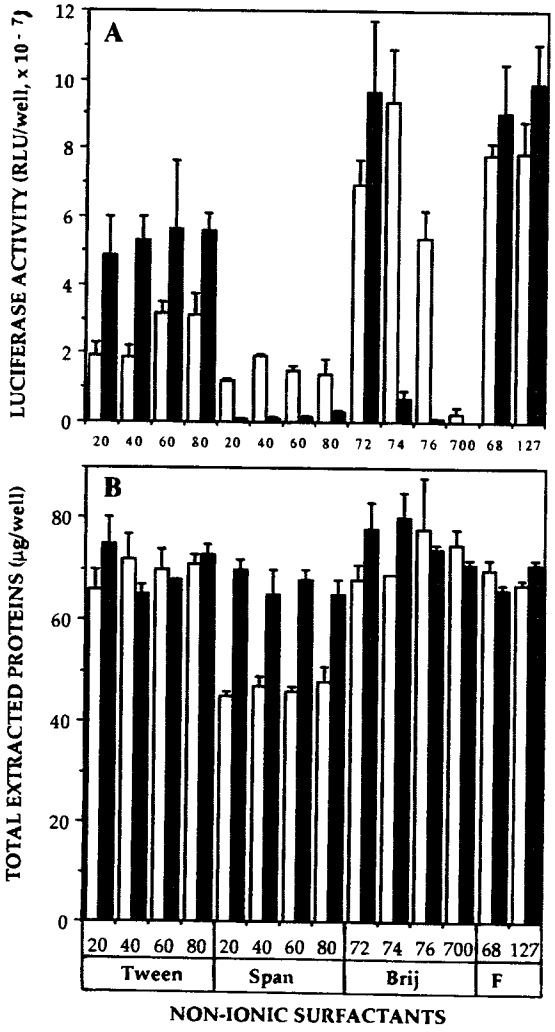

Fig. 2. Transfection activity of emulsions containing different non-ionic surfactants. Transfection was performed with (■) and without (□) serum. Results are mean ± S.D. (n = 3).

Effect of Surfactant Concentration

It is evident from the results in Table I and Figure 2 that inclusion of non-ionic surfactants in the emulsions significantly affects the size of DNA/lipid complexes, transfection activity and serum sensitivity. To test whether such activities are dependent on the concentration of the non-ionic surfactants, we have chosen Tween-80 as an example for this study. The effect of Tween 80 concentration on the size of DNA/lipid complexes was examined at two DNA and emulsion concentrations and the results of such experiment were summarized in Figure 3. It is clear from these results that the size of DNA/lipid complexes varies with the concentrations. When the DNA/lipid complexes were prepared at relatively low concentration (DNA, 8 μg/ml, DC-Chol, 54 μg/ml), the average diameter of the complexes was around 250 nm regardless of the Tween 80 concentration in the emulsions. However, at higher concentration (DNA, 108 μg/ml and DC-Chol, 729 μg/ml), the average size of complexes varied with the concentration of Tween 80 in the formulation. The size of DNA/lipid complexes was around 10 μ for emulsions containing about 9.1% Tween 80 but decreased to about 250 nm when the concentration of Tween 80 in the formulation was 26% or higher.

Transfection activity of the emulsions containing different amount of Tween 80 is shown in Figure 4. These experiments were done in the presence of 20% serum under the identical conditions as described in the method section, except that the DNA/emulsion complexes were prepared using different concentrations of DNA and emulsions. As shown in Figure 4, the total luciferase activity for DNA/emulsion complexes prepared at low concentration was about same for emulsions containing Tween 80 ranging from 9.1% (Oil:DOPE:DC-Chol:Tween 80 = 0.25:0.25:0.75:0.125, mg/mg) to 19.9% (Oil:DOPE:DC-Chol:Tween 80 = 0.25:0.25:0.75:0.31, mg/mg) of the total lipids, but it decreased by about 3 fold as its concentration increased to 23.0% (Oil:DOPE:DC-Chol:Tween 80 = 0.25:0.25:0.75:0.38, mg/mg) or greater. Compared to the transfection activity of DNA/emulsion complexes prepared at low concentration, the activity for those DNA/emulsion complexes prepared at high concentration was quite different. First of all, significantly lower enzyme activity was detected at low concentration of Tween 80. Secondly, the transfection activity increases with increasing Tween 80 concentration. Finally, the gene product detected from the cells transfected with emulsions containing 29% Tween 80 was about 4 fold higher than that of emulsions prepared at low concentration.

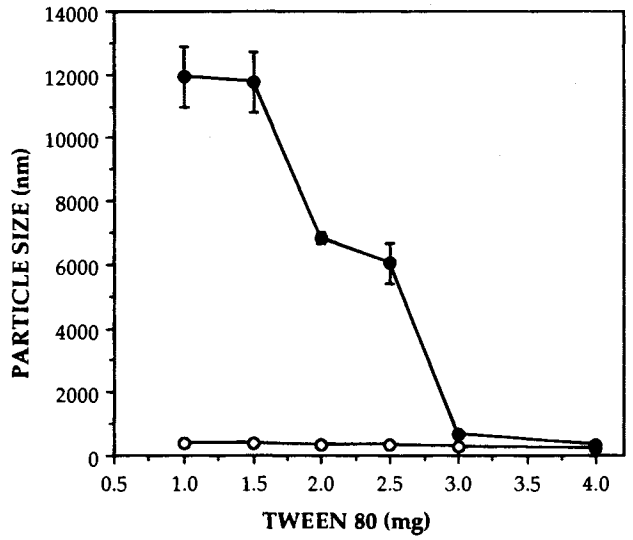

Fig. 3. Effect of Tween 80 concentration in emulsions on the particle size of DNA/emulsion complexes. Emulsions composed of Castor oil:DOPE:DC-Chol (0.25:0.25:0.75, weight ratio) and the amount of Tween 80 indicated in *x* axes was mixed with DNA at weight ratio (DC-Chol:DNA) of 6.75. Complexes were prepared at DNA concentration of 8 μg/ml (○) or 108 μg/ml (●). Results are mean ± S.D. (n = 3).

DISCUSSION

A few conclusions can be drawn from this study. Firstly, the function of non-ionic surfactants on emulsion-mediated DNA stabilization and transfection is mainly determined by the hydrophilic head groups (Table I and Figure 2A). The hydrophobic segment did not seem to play important role although essential for emulsion stabilization. Among the types of surfactants tested, branched polyoxyethylene in Tween is most effective

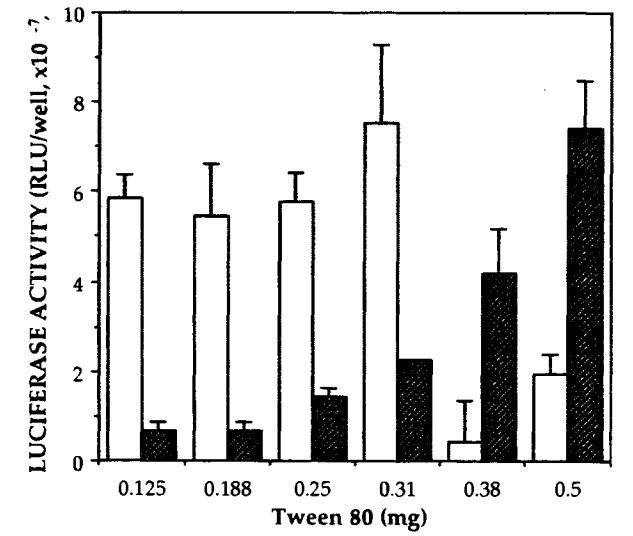

Fig. 4. Effect of Tween 80 concentration in the emulsions on the transfection activity in the presence of serum. Same conditions as described in Figure 3 legend were used except that DNA/emulsion complexes were incubated for 5 min before serum was added. Two μg of plasmid DNA/well was used for transfection. DNA/emulsion complexes were prepared at DNA concentration of 8 μg/ml (□) or 108 μg/ml (■). Results represent mean ± S.D. (n = 3).

in inhibiting DNA-induced aggregation (Table I). Surfactants without polyoxyethylene moiety (Span series), with single polyoxyethylene chain (Brij series) at one end, or those located at both ends of the hydrophobic segment of the molecules (pluronic series) did not show much activity in blocking the formation of large DNA/emulsion complexes. Secondly, it appears that there is no clear relationship between the transfection activity and the property of the hydrophilic head groups of different surfactants (Figure 2). All emulsions except for those containing Brij 700 showed relatively high activity in transfecting cells in the absence of serum. However, the transfection activity of these emulsions in the presence of serum (20%) varied significantly, depending on the structure of hydrophilic portion of the non-ionic surfactants. Span series which do not contain polyoxyethylene head groups appear not able to provide the serum resistant activity to the emulsions; neither the Brij series with long polyoxyethylene chains (Bij 74, 76, 700). Both pluronic F68 and F127-containing emulsions were serum resistant. Thirdly, all formulations tested did not show any significant cell toxicity even at fairly high surfactant concentration. Fourthly, the transfection activity of a given formulation depends on not only the ratio of DNA to positively charged lipids but also the concentrations at which the DNA complexes were prepared. This is important, concerning in vivo transfection where DNA complexes are normally prepared at high concentrations in order to maximize the total amount of DNA for administration with a reasonable volume.

It is clear from our results that different types of non-ionic surfactants have a range of activities in blocking the DNA-induced size increase of emulsion particles, in affecting the transfection activity and in resisting the inhibition effect of serum components. However, the detailed mechanism for such activities is not fully understood. Based on the current understanding for cationic lipid-mediated transfection (1–3), it is essential for DNA molecules to form a DNA/emulsion complex in order to enter the cells and express the gene. To form such a complex, DNA molecules need to bind to the surface of the emulsion particles via electrostatic interactions between the negatively charged phosphate groups of DNA and positively charged cationic lipids. Working like an anionic polymer, one DNA molecule may bind more than one emulsion particles such that large aggregates are formed. The formation of these large aggregates is likely to be facilitated if the positive charges on the surface of the particles are readily accessible (e.g. Oil/DOPE/DC-Chol/Span emulsions). However, the formation of such large aggregates may not occur if there are structures on the surface that sterically prevent each DNA molecule from binding to more than one particles. We believe that the polyoxyethylene moieties in Tween series provided steric stabilization activity. Ideally, steric hindrance provided by the surface structure should be adequate to block the DNA induced particle aggregation without interfering with the reach of DNA to the oil/water interface. The most likely reason that Brij, pluronic F68 and F127 failed to provide such steric stabilization is that there are not enough polyoxyethylene molecules to cover the entire surface of the emulsion particles. The decrease of DNA/emulsion particle size with increasing the polyoxyethylene chains in Brij-containing formulations suggests that longer polyoxyethylene chains have better steric stabilization activity. However, the hydrophilic moiety could not be too long

because the steric barrier that is designed to prevent emulsion aggregation may block the access of DNA to the oil-water interface. Thus the DNA/emulsion complexes could not be formed easily. This is likely the reason that emulsions containing Brij 700 show very low transfection activity (Figure 2A).

This model also predicts that the density of the hydrophilic moiety that provides the steric hindrance activity is one of the most important factors. Shorter chains will work better than the longer ones concerning the probability of preventing the formation of DNA/emulsion complexes. At a given concentration, the molecules with branched hydrophilic head groups will work better than the ones with a single chain. The branched hydrophilic segments would cover a larger area on the surface than the single chain due to their umbrella type structure. By the same principle, this hypothesis would also predict that prevention of large aggregate formation depends on the concentration of DNA and emulsions. For emulsions (such as those containing Tween 80) that do not have very long polyethylene oxide chains, increasing the concentration of DNA and the emulsions will physically increase the close proximity among the emulsion particles and DNA. This will result in the formation of large DNA/emulsion aggregates. High concentration of non-ionic surfactants in the emulsions should provide a stronger steric stabilization activity in each particle such that large DNA/emulsion aggregates are no longer formed even at higher concentration of DNA and emulsions. Results presented in Figure 3 support such hypothesis.

The lack of activity in forming small DNA/emulsion complexes for pluronic F68 and F127, even though they may have different configuration on the surface of the emulsion from those of other types of non-ionic surfactants due to their unique structure (relatively hydrophobic moiety in the middle and a hydrophilic segment at each end), may be because the number of polyoxyethylene per molecule of pluronic polymer is less than that of Tween.

Unlike the mechanism by which polyoxyethylene segments of non-ionic surfactants block the formation of large DNA/emulsion complexes, the difference in transfection activity of these surfactant-containing emulsions may be more complicated. It is possible that the transfection activity is not dependent on the size of the DNA/emulsion complexes. However it should be noted that the particle size used in the transfection is not as large as those presented in Table I. This is because emulsions were only incubated with DNA for about 10 min, compared to 1 h for size measurement, before they were added to cells. Cells may take the DNA complexes inside before the size of the complexes become fairly large. The relationship between the size of DNA/emulsion complexes and the transfection activity is currently under the investigation.

Concerning the serum sensitivity of cationic lipid mediated transfection it is generally believed that the loss of transfection activity of cationic liposomes is due to the neutralization of the positive charges on the DNA complex. These unpaired positive charges are thought to be important for the binding of the DNA/lipids complexes to the cell surface. This is likely the case for emulsions containing Span (Figure 2). However, such mechanism can not explain why long chain polyoxyethylene in Brij 76 and 78 have low transfection activity in the presence of serum, compared to that of emulsions containing Brij 72 which has only two units of polyoxyethylene. It is not clear for the time being why emulsions containing either pluronic F68 or F127 have strong resistance to the serum. Furthermore, difference (approximately 10 fold) in transfection activity observed among different emulsions is also not well understood. It is possible that the optimal ratio between DNA and cationic lipid in different emulsion formulations is different. Alternatively, the mechanism by which DNA molecules are taken into cells by these emulsions may differ from that for liposome mediated transfection.

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