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# Polyethylene glycol enhances lipoplex-cell association and lipofection

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**Abstract**

The association between liposome-DNA complexes (lipoplexes) and targeted cell membranes is a limiting step of cationic liposome-mediated transfection. A novel technique was developed where lipoplex-cell membrane association is enhanced by the addition of 2–6% polyethylene glycol (PEG) to the transfection media. Lipoplex-cell association was found to increase up to 100 times in the presence of PEG. Transfection increased correspondingly in the presence of PEG. This increase was found in several cell lines. These results show that lipoplex adsorption to cell membranes is a critical step in liposome-mediated transfection. This step can be facilitated by PEG-induced particle aggregation. 

*Keywords:* Polyethylene glycol; Liposome; Transfection; Fluorescence; In vitro gene expression

## 1. Introduction

An effective vector for gene delivery is essential for gene therapy. Many different methods of gene transfer have been devised, including viral and non-viral-mediated delivery. The search for efficient non-viral techniques for delivery is warranted due to the immune reactions and safety issues that arise when viruses are used as vectors [1,2]. Cationic liposome-mediated delivery is a promising non-viral delivery method [3,4]. However, this technique has several drawbacks, mainly from its low transfer efficiency [5].

Since the first report [6] of the use cationic liposome-DNA complexes (lipoplexes [7]) as gene delivery agents, an intense effort has existed to sort out the mechanisms responsible for efficient delivery. The lipoplex structure [8] has been studied by electron microscopy [9–11], atomic force microscopy [12,13] and X-ray diffraction [14,15]. The relation of the lipoplex structure to the lipofection efficiency has been studied by many different techniques, including associating the lipid composition [16–18], lipoplex size [19,20], lipid hydration [21], lipid mixing [22] and fusion ability [23] to the lipofection efficiency. The mechanism of delivery [11,24,25] has been studied by DNA protection assays [9,26], confocal microscopy [11], transmission electron microscopy [27] and by many differing lipofection protocols. The mechanism has been determined to consist of a lipo-

Abbreviations: BME, basal medium Eagle; CHO, Chinese hamster ovary; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOTAP, 1,2-dioleoyl-3-trimethylammonium propane; FBS, fetal bovine serum; F-PE, L-α-phosphatidylethanolamine-*N*-fluorescein (egg); MEM, minimum essential medium; MLV, multilamellar vesicle; NCS, newborn calf serum; ONPG, *O*-nitrophenyl β-D-galactopyranoside; PEG, polyethylene glycol; QLS, quasi-elastic light scattering; SUV, small unilamellar vesicle

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plex-cell adhesion step [11], followed by endocytosis of the lipoplex [24,25], with fusion occurring between the lipoplex and endocytic vesicle to allow for DNA entry into the cytoplasm [26,28,29]. From here, the DNA makes its way into the nucleus where expression occurs. A limiting step for cytoplasmic delivery is lipoplex-cell adhesion [19,20,30,31]. This is especially true when serum is present in the transfection media [19,32]. Once adhesion occurs, many cells readily endocytose the bound object.

Polyethylene glycol (PEG) is a widely available non-toxic compound and is used for a broad range of biochemical and cell biological processes, including cell aggregation, fusion and protein condensation. The effect of free PEG upon liposomes includes inducing aggregation and fusion through the effects of depletion, destabilization and bilayer dehydration [33–35]. At low PEG (molecular weight (MW) 8000) concentrations (2–5%), surface depletion of the polymer leads to particle/liposome aggregation [33]. At higher PEG concentrations, bilayer disruption occurs upon extreme dehydration and may lead to fusion between membranes [33]. The aggregation potency of PEG is MW dependent. The most effective PEG has a MW of 8000–10 000. Below this range, the polymer depletion effect is absent. Above the range, polymers adsorb to the membrane surface and inhibit particle/cell aggregation [34].

Here, we report the use of PEG to enhance in vitro cationic liposome-mediated transfection. Liposomes, composed of the cationic lipid 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), along with the helper lipid 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and in combination with the plasmid pSV-β-galactosidase, show significantly enhanced lipofection in the presence of 2–6% PEG 8000 (w/v). The efficiency of delivery, as measured by the amount of plasmid needed to produce optimal transfection, is much greater with PEG. This leads to lower concentrations of lipid and plasmid needed for an optimal effect. We attribute the increases in lipofection to the ability of PEG to stimulate lipoplex-cell association by a depletion effect [34]. This finding adds to previous findings that lipoplex-cell association is a limiting step in cationic liposome-mediated transfection [19,30,31]. It also suggests that the lipoplex-cell adhesion step may be enhanced, leading to greatly increased transfection.

## 2. Materials and methods

### 2.1. Materials

All lipids, including DOTAP, DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) and L-α-phosphatidylethanolamine-*N*-fluorescein (egg) (F-PE), were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Cell culture media, including F-10, Dulbecco's modified Eagle medium, basal medium Eagle (BME), minimum essential medium (MEM), RPMI 1640, fetal bovine serum (FBS), newborn calf serum (NCS) and penicillin-streptomycin-neomycin, were purchased from Gibco BRL (Grand Island, NY, USA). Tissue culture dishes (12 and 96 wells) were purchased from Corning (Corning, NY, USA) through VWR Scientific (Rochester, NY, USA). All cell types including Chinese hamster ovary (CHO), Cos, L1210, 10T1/2, K562 and MC2 were from the American Type Culture Collection (Rockville, MD, USA). Reporter lysis buffer and luciferase assay buffer were from Promega (Madison, WI, USA). All other chemicals including *O*-nitrophenyl β-D-galactopyranoside (ONPG), PEG 3300, 8000 and 15 000–20 000 were from Sigma (St. Louis, MO, USA).

### 2.2. Liposome, plasmid and lipoplex preparation

Liposomes were formed by mixing lipids in chloroform, followed by drying under a stream of nitrogen and additional drying by vacuum for 3 h. The dry lipid film was re-suspended with deionized water by vortexing. Multilamellar vesicles (MLVs) resulted from this. Small unilamellar vesicles (SUVs) were formed by sonicating this solution in a bath type sonicator (Laboratory Supply, Hicksville, NY, USA). Sonication was under a nitrogen atmosphere, for 10 min, at which point the solution turned clear. The concentration of lipids at this point was 1.4 mg/ml.

The plasmid pSV-β-galactosidase was grown in *Escherichia coli* and isolated with the mega prep kit from Qiagen (Chatsworth, CA, USA). Isolated plasmids were stored in STE buffer (100 mM NaCl, 10 mM Tris-Cl, pH 8.0, 1 mM EDTA) at a concentration of 1 mg/ml. Plasmid purity was verified by the ratio of absorbance at 260 nm/280 nm. Presence of

the gene was verified by *Hin*dIII (Gibco BRL) and *Pst*I (Boehringer Mannheim, Indianapolis, IN, USA) excision. The plasmid pGL3-Luciferase (Promega) was grown in *E. coli* and isolated with the mega prep kit from Qiagen. It was stored in STE buffer at a concentration of 1 mg/ml. Purity was verified by the ratio of absorbance at 260 nm/280 nm, with presence of the gene verified by *Xho*I and *Sal*I (Boehringer Mannheim) excision.

Lipoplexes were formed by the addition of plasmid DNA (1 mg/ml) to liposomes (1.4 mg/ml total lipid, 0.7 mg/ml cationic lipid), to charge ratios (cationic lipid:DNA) of 2:1 or 1:2. This was followed by addition of serum-free media, water and PEG in water (if present). The total lipid concentration after addition of components equaled 0.093 mg/ml. For solutions at a 2:1 charge ratio, DNA was at 0.014 mg/ml and for solutions at 1:2, DNA was at 0.056 mg/ml. Solutions were pipetted several times to assure mixing. Liposome and lipoplex solutions were made and used on the day of experimentation. The solutions were made and kept at room temperature. Aggregations for all solutions were similar regardless of PEG. Aggregation was limited and no precipitation was found.

### 2.3. Cell culture and lipofection procedures

CHO cells were grown in F-10 plus 13% NCS at 37°C and 5% $CO_2$. K562, L1210 and MC2 cells were grown in RPMI 1640 plus 10% FBS. Cos cells were grown in MEM plus 10% FBS. 10T1/2 cells were grown in BME plus 10% FBS. All cells were passaged twice weekly. For lipofection, suspension cells were seeded at an initial concentration of 20 000 cells per well for 96 well plates and 250 000 cells per well for 12 well plates. Adherent cells were seeded at 5000 cells per well for 96 well plates and 60 000 cells per well for 12 well plates. These cells were allowed to grow for 18–20 h, when adherent cells reached approximately 80% confluence, before experimentation.

Lipoplexes were formed on the day of experimentation, following the lipoplex preparation procedure, and added to cells immediately, unless specified. Transfection media consisted of 10–13% serum before addition of lipoplexes. Lipoplexes were then added at a 7:3 cell media to lipoplex solution ratio. At this point, the total lipid concentration was 0.028 mg/ml. For a 96 well plate, 0.48 or 1.92 μg of plasmid was added per well, for a 12 well plate, 5.7 or 22.8 μg of plasmid was added per well. When lipofection was being assessed, lipoplexes were added to the cells for 4 h, before being removed and the cells being covered with fresh media plus serum.

The lipofection efficiency for CHO and Cos cells was measured by following the Promega protocol TB 097 for β-galactosidase enzyme activity assessment (ONPG hydrolysis). Briefly, cells were incubated for 48 h after lipofection, washed twice with phosphate-buffered saline (PBS) and lysed with Promega reporter lysis buffer. Extract was recovered, vortexed and centrifuged. Extract was then incubated with an equal volume of assay 2× buffer (200 mM sodium phosphate buffer, pH 7.3, 2 mM $MgCl_2$, 100 mM β-mercaptoethanol, 1.33 mg/ml ONPG) for 1 h. 1 M $NaCO_3$ was added and the optical density was measured at 420 nm. Readings were compared with a β-galactosidase standard. Lipofection for K562, L1210, 10T1/2 and MC2 cells was assessed as in Promega protocol TB 161 for luciferase. Extract was recovered as stated. Samples (20 μl) were added to 100 μl luciferase assay buffer and light units were assessed in a luminometer (Berthold (Oak Ridge, TN, USA) Lumat 9501). Samples were compared with a luciferase standard.

Total cellular protein was measured by lysis of cells with Promega lysis buffer. The protein content was analyzed by the bicinchoninic acid protein assay reagent (Sigma) and compared with a bovine serum albumin (Sigma) standard.

### 2.4. Lipoplex-cell association and uptake

Association of lipoplexes with cells and their uptake may be measured by determining the lipid environment of the fluorescent probe F-PE [11]. Association is defined to be the total lipid adherent and endocytosed by the cell. At pH 7.4 (cell surface pH), F-PE is more fluorescent than at lower pH values (such as that found in lower pH endosomes) [36]. In a system where cells have endocytosed many F-PE containing lipoplexes, the total fluorescence will be lower than if all F-PE lipoplexes were on the cell surface. This difference is measurable and shows F-PE lipoplexes endocytosed by the cells. After this measurement is taken, Triton X-100 is added to the

cells. Triton solubilizes all membranes, returning all F-PE to pH 7.4 (the external pH). Re-measurement of fluorescence at this point allows us to detect total fluorescence.

F-PE was added to lipoplexes at 1 mol% and was used to measure the membrane surface pH by the fluorescence sensitivity of fluorescein to pH. Lipoplexes were added to cells in 12 well plates following the lipofection procedure. The cells were incubated for 0 or 2 h and washed twice with PBS. The fluorescence intensity of the intact cells was measured with an excitation of 497 nm and an emission being monitored over the range of 510–550 nm. Triton X-100 (Kodak, Rochester, NY, USA) was then added to cells at 0.1% (v/v) to dissolve all membranes. Fluorescence measurements were then repeated.

At time 0, F-PE lipoplexes were added to cells followed by immediate washing with PBS. These cells were then measured for fluorescence ($F_0$). Triton was added and the samples were re-measured ($T_0$). The total fluorescence due to non-specific sticking is $T_0$, with the relative amount of non-specific initial lipoplex uptake $T_0-F_0$. For the 2 h points, lipoplexes were added to cells and incubated at 37°C and 5% $CO_2$. Total fluorescence was then measured ($F_2$). Triton was added and the samples were re-measured ($T_2$). The fluorescence of all probes minus the 0 h control is $T_2-T_0$. This represents total cellular association of lipoplexes both external and internal. Change in fluorescence resulting from a pH change is $T-F$. Subtracting out the zero time point thus gives $T=T_2-T_0$ and $F=F_2-F_0$, which results in $(T_2-T_0)-(F_2-F_0)$. This represents cellular uptake of the fluorescent lipoplexes.

### 2.5. Lipoplex size measurements

Lipoplex sizes were measured by quasi-elastic light scattering (QLS), using a particle sizer model 370 (Nicomp Particle Sizing Systems, Santa Barbara, CA, USA). The particle sizer was calibrated with latex beads (Interfacial Dynamics Corporation, Portland, OR, USA) between the sizes of 30 nm and 2980 nm and found accurate for all diameters. Lipoplexes were formed as in the lipoplex preparation procedure, with PEG 8000 present at 6%. The sample size was analyzed by multi-modal NICOMP vesicle analysis to detect the sizes of lipoplexes. The sample measurement viscosity was adjusted to allow for the change in viscosity with PEG 8000 present. The viscosity was adjusted to 2.35 for a 6% PEG 8000 solution, with the liquid index of refraction being set to 1.332.

## 3. Results

From our previous findings [19,30], along with other findings [31,32], lipoplex-cell adhesion has been found a limiting factor in transfection. There-

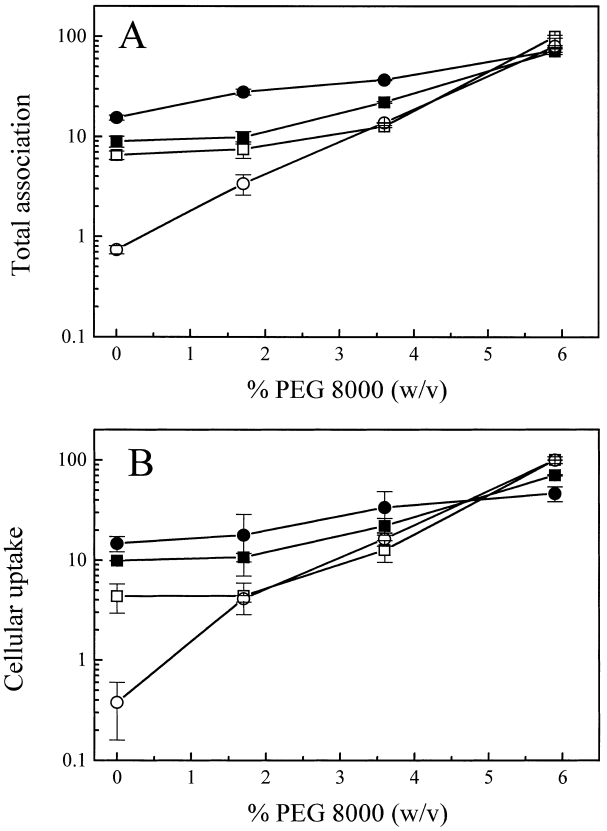


Fig. 1. Lipoplex-cell association and uptake as measured by fluorescence spectroscopy. (A) The total cellular association of lipoplexes incubated with CHO cells in variable amounts of PEG 8000 was found to increase with an increased PEG concentration. (B) Measurement of lipoplex uptake by pH sensitive fluorescence spectroscopy. Lipoplexes incubated with CHO cells, in the presence of variable amounts of PEG 8000, showed increasing levels of cellular uptake with an increasing PEG concentration. Symbols for lipoplex types are MLV:DNA at charge ratios of 2:1 (■), 1:2 (□) and SUV:DNA at charge ratios of 2:1 (●), 1:2 (○). Error bars represent the S.D. Units are arbitrary with the highest point normalized to 100.

fore, we aim at increasing this adhesion to determine its relative importance upon cellular uptake and transfection.

### 3.1. Lipoplex-cell association and uptake in the presence of PEG 8000

The addition of PEG 8000 to lipoplexes composed of the cationic lipid DOTAP and the fusogenic helper lipid DOPE (1:1), in combination with the plasmid pSV-β-galactosidase, markedly enhanced lipoplex-cell association. Lipoplexes were labelled with 1% F-PE and added to CHO cells, in the presence of variable amounts of PEG 8000. The association of lipoplexes with CHO cells after 2 h was much greater when PEG was present in the lipofection media (Fig. 1A). This relationship held for all charge ratios and liposome types tested. The total association of lipoplexes increased with increasing amounts of PEG in the lipofection media. With PEG, the association of lipoplexes and CHO cells was 5–100 times higher than without PEG. At 6% PEG, all lipoplexes associated with cells at approximately the same rate, whereas without PEG, the rates of association differed greatly.

The uptake of lipoplexes labelled with 1% F-PE was also greater in the presence of PEG 8000 (Fig. 1B). The levels of lipoplexes taken up by CHO cells showed that with PEG, uptake is increased 3–100 times over samples without PEG. Uptake, as measured by the change in fluorescence at different pH values, showed the ability of PEG to stimulate internalization of lipoplexes. This result corresponded with the greater association of lipoplexes in the presence of PEG (Fig. 1A). One point to be noted is that lipoplexes composed of SUV:DNA at a 2:1 charge ratio showed only a three times increase in cellular uptake at 6.0% PEG. We believe that uptake would have been higher for this point, but a degree of toxicity was found in this sample.

### 3.2. Enhancement of cationic liposome-mediated transfection by PEG

Lipoplexes were used to transfect CHO cells in the presence of varying levels of PEG. Up to 6.0% PEG (w/v) was used in the lipofection media (Fig. 2). Lipofection with PEG represented a 2–7-fold increase over lipofection without PEG, at the same lipoplex concentrations. The enhancement varies between lipoplexes at differing cationic lipid:plasmid DNA charge ratios, as lipoplexes at charge ratios of 1:2 showed greater increases of lipofection than lipoplexes at charge ratios of 2:1. The best transfecting lipoplexes studied were MLV lipoplexes at 1:2 charge ratios. These lipoplexes showed up to 1.4 mU of β-galactosidase activity per μg of total cellular protein. This level is near saturation for transfection of these cells. Apparently, lipoplexes at 1:2 charge ratios are more efficient. This is because transfections were measured and compared at a constant lipid content. Lipoplexes at 1:2 ratios have four times more plasmid DNA. Lipofection was found to decrease at the highest levels of PEG studied. These decreases were attributed to increased toxicity that resulted from increased lipoplex uptake, as measured by trypan blue exclusion (not shown).

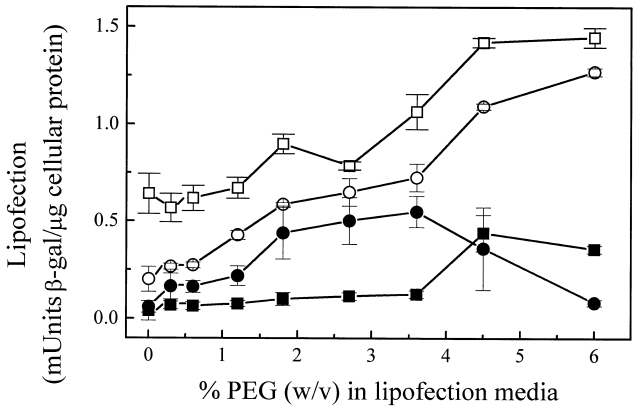


Fig. 2. Lipofection of CHO cells by cationic liposome:DNA complexes. Lipofection, as measured by mU of β-galactosidase produced per μg of cellular protein, varied based upon the concentration of PEG in the lipofection media. Lipoplexes showed an increased lipofection with up to 6.0% PEG 8000 (w/v) in the lipofection media. Symbols for lipoplex types are as in Fig. 1. Error bars represent the S.D. from repeating experiments.

The lipofection efficiency at optimal transfection was increased in the presence of PEG for all lipoplex types studied. When the lipofection data were adjusted to represent the efficiency of delivery, as measured by mU β-galactosidase per μg of pSV-β-galactosidase plasmid, the efficiency of delivery was much greater in the presence of PEG 8000 (Fig. 3). The enhancement of lipofection efficiency (and thus in conservation of plasmid DNA) was 2–10-fold, depending upon the lipoplex type.

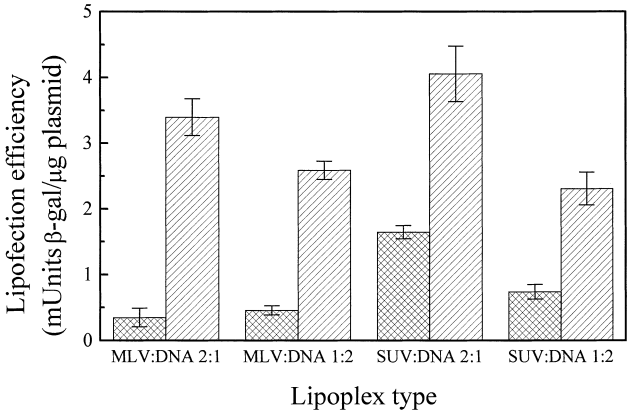


Fig. 3. Lipofection efficiency in CHO cells. The efficiency, as measured by β-galactosidase activity per μg of plasmid DNA, showed that the efficiency of delivery is much higher with PEG (hatched) than without PEG (cross-hatched). Error bars represent the S.D. from repeating experiments.

PEG can increase cellular association of lipoplexes and subsequently lipofection in a variety of cell lines. As shown in Table 1, the lipoplex-cell membrane association with PEG ranges from 1.5 to 14.3 times the association without PEG. The cellular uptake and transfection varied in course with the association.

### 3.3. *Effect of PEG MW on cell association, uptake and transfection*

The cellular association of fluorescent-labelled lipoplexes with CHO cells varied based upon the MW of the PEG present in the transfection media (Fig. 4A). PEG 8000 lead to greater increases of association than PEG 3300 or PEG 15 000–20 000. This was true for all lipoplex types studied. The cellular uptake was also greatest in the presence of PEG 8000 (Fig. 4B).

The enhancement of lipofection depended on the MW of PEG. As Fig. 4C shows, PEG 8000 stimulated higher levels of lipofection than PEG 3300 and PEG 15 000–20 000 for most lipoplex types. However, the differences between PEG 3300 and 8000 were more pronounced than between 8000 and

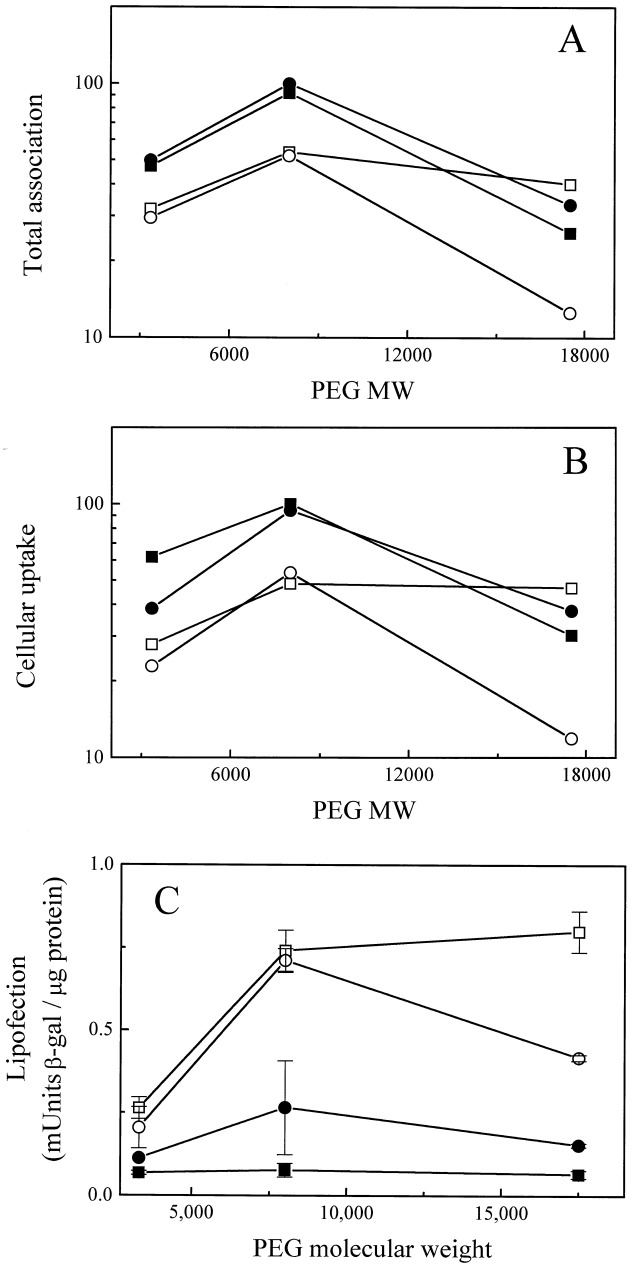


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Fig. 4. Effect of the PEG MW upon lipoplex-cellular association, uptake and transfection of CHO cells. (A) Cellular association of 1% F-PE-labelled lipoplexes. Lipoplexes in a transfection media containing PEG 8000 (3% w/v) showed more cellular association than lipoplexes in transfection media with PEGs (3% w/v) of other MWs. (B) Cellular uptake of 1% F-PE-labelled lipoplexes as measured by pH sensitive fluorescence. Cellular uptake is the highest with PEG 8000 in the transfection media. (C) Lipofection with differing MW PEGs (3% w/v). Lipoplexes showed optimal lipofection at a PEG MW of 8000, except MLV:DNA at 1:2, which had a similar lipofection for PEG of MWs 8000 and 15 000–20 000. Symbols for lipoplex types are as in Fig. 1. Error bars represent the S.D. from repeating experiments. Units for (A) and (B) are arbitrary units with the highest point in each normalized to 100.

15 000–20 000, which sometimes showed a similar lipofection enhancement. This method is wide ranging as both adherent and suspension cell lines showed an increasing association and transfection.

### 3.4. Effect of PEG on the lipoplex size

Incubation of lipoplexes in a cell-free environment in the presence of culture media with PEG 8000, but without serum, for 2 h, lead to increases in the lipoplex size, as measured by QLS (Table 2). Samples did exhibit a degree of heterogeneity. The major peaks in size distribution showed that without PEG, lipoplexes all remained less than 1 μm in size. With the addition of PEG, followed by incubation for up to 2 h, the sizes of all lipoplexes showed growth, with three of the four lipoplex types growing above 1 μm in size.

Pre-incubation of lipoplexes in PEG 8000 before addition to culture cells showed an initial increase in lipofection efficiency (Fig. 5). This large increase was followed by decreases from the optimum with pre-incubation up to 2 h.

## 4. Discussion

The use of cationic liposomes as a vector for gene delivery has been under development for several years. The use of these vectors is limited mainly by their low levels of DNA transfer efficiency. It is believed that through study and refinement of the lipoplex structure, the efficiency of lipoplex-mediated delivery will be enhanced. It must be remembered, however, that although the structure of the lipoplex is important, so is the overall mechanism of DNA delivery. Here, we report that lipoplex-cell membrane association is an important step of the in vitro DNA delivery mechanism. Through the addition of PEG to lipoplexes, we can induce lipoplex-cell membrane association, stimulate the levels of lipoplexes endocytosed by culture cells and ultimately increase the efficiency of DNA delivery. This work is significant for in vitro and ex vivo delivery and research concerned with non-viral gene delivery.

In vitro lipofection has been plagued by low gene delivery. This delivery has been blamed on several factors including inefficient DNA packing by liposomes and a low DNA entry into cellular cytoplasm [32,37,38]. Work in this laboratory [19,30], along with other findings [20,31,39], suggests that the association of lipoplexes with cell membranes is the limiting step in the delivery of DNA into the cellular cytoplasm. The work shown here adds weight to lip-

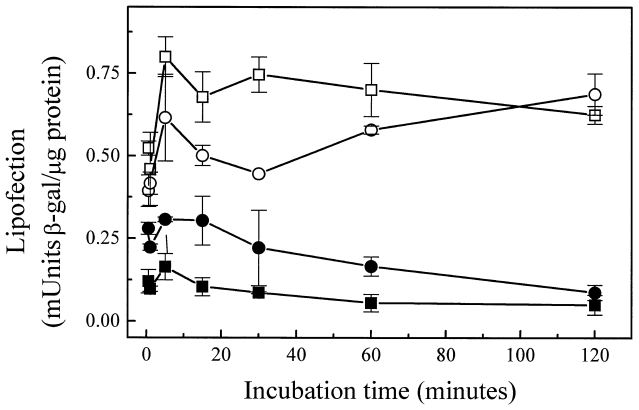


Fig. 5. Measurement of the lipofection ability of lipoplexes pre-incubated with PEG 8000. Lipoplexes were pre-incubated with PEG 8000 for various periods before addition to CHO cells. An increase of transfection with short pre-incubation periods followed by slight decreases of transfection with large pre-incubation periods was found. PEG is present in the lipofection media at 3% (w/v). Symbols for lipoplexes types are as in Fig. 1. Error bars represent the S.D. from repeating experiments.

Table 1
PEG-enhanced transfection, lipoplex-cell association and lipoplex uptake in six different cell lines

| Cell Line[a] | Transfection[b] | Association[c] | Uptake[d] |
|---|---|---|---|
| CHO | 2.2 | 2.0 | 2.9 |
| Cos | 4.2 | 1.9 | 1.2 |
| MC2 | 135.8 | 3.5 | 3.8 |
| K562 | 14.3 | 2.2 | 2.8 |
| L1210 | 216.5 | 14.3 | 19.0 |
| 10T1/2 | 5.9 | 1.5 | 1.3 |

[a]Transfected by MLV:DNA at 1:2 charge ratios in 7–13% serum containing media.
[b]Transfection enhancement in the presence of 3.6% PEG 8000 versus transfection without PEG. β-Galactosidase was the marker enzyme for CHO and Cos cells and luciferase was the marker for MC2, K562, L1210 and 10T1/2 cells.
[c]Qualitative measurement of association of F-PE-labelled lipoplexes with cell membranes. Given as the increase in association with 3.6% PEG 8000 over association without PEG.
[d]Qualitative measurement of F-PE-labelled lipoplex uptake. Given as the increase of cellular uptake in the presence of 3.6% PEG 8000 over uptake without PEG.

Table 2
Particle sizes of lipoplexes before or after addition of PEG 8000

| Lipoplex type[a] | % PEG[b] | Incubation time[c] (min) | Particle size (nm) | % Volume |
|---|---|---|---|---|
| MLV:DNA 2:1 | 0 | – | 200 | 8 |
| | | | 800 | 92 |
| | 6 | 30 | 450 | 10 |
| | | | 750 | 20 |
| | | | 1500 | 70 |
| | 6 | 120 | 250 | 5 |
| | | | 1 375 | 95 |
| MLV:DNA 1:2 | 0 | – | 150 | 10 |
| | | | 500 | 74 |
| | | | 960 | 16 |
| | 6 | 30 | 275 | 8 |
| | | | 950 | 92 |
| | 6 | 120 | 250 | 13 |
| | | | 1 100 | 87 |
| SUV:DNA 2:1 | 0 | – | 100 | 5 |
| | | | 675 | 95 |
| | 6 | 30 | 100 | 2 |
| | | | 1 200 | 98 |
| | 6 | 120 | 115 | 3 |
| | | | 1 250 | 97 |
| SUV:DNA 1:2 | 0 | – | 215 | 100 |
| | 6 | 30 | 250 | 5 |
| | | | 675 | 95 |
| | 6 | 120 | 650 | 100 |

[a]The lipoplex type is represented as precursor liposome:plasmid DNA, followed by the charge ratio.
[b]% PEG represents the percent PEG present upon QLS measurement. For points with 0% PEG, no PEG was present upon measurement, for 6% points, 6% PEG (w/v) was present.
[c]Time between PEG addition and sample measurement.

oplex-cell association as the limiting step in cytoplasmic delivery. Simultaneously, it suggests a method to increase association, by the use of compounds that induce association. Here, we have shown that increasing the association of lipoplexes with cell membranes is possible (Fig. 1A and Table 1). This increase is due to the presence of PEG, as increases in the PEG concentration in the transfection media show increases in cellular association of lipoplexes (Fig. 1A). The result of Fig. 1A is expected, given the role of PEG in mediating cell-liposome aggregation [33,35]. Yang et al. found that low concentrations of PEG (2–5%) induce liposome aggregation. This aggregation is a result of the polymer depletion effect upon membrane surfaces [34]. Using PEG in low concentrations (2–6%), we can increase lipoplex-cell membrane association and study the effect of association upon transfection. We attribute the increase in lipoplex-cell membrane association in the presence of PEG to be due to the polymer depletion effect.

The increases in association are also evident in the uptake (endocytosis) of lipoplexes by cells in the presence of PEG (Fig. 1B). This is expected, as many cell types readily endocytose surface bound components. An increased uptake would allow for increased DNA escape from endosomes into the cytoplasm. Eventually, some of this DNA would make its way to the nucleus where expression will occur. Therefore, it is expected that with an increased uptake, transfection would correspondingly increase. This is what is found (Figs. 2 and 3). These results hold true not only for CHO cells that have active endocytosis, but also for many different cell lines (Table 1). From these results, we can theorize that association must be a limiting step for in vitro lipofection. Through the increase of association, we can increase transfection. We must note that our meas-

urements of lipoplex association and uptake do not directly correspond with lipofection. However, one must remember that lipoplex association and uptake are only two of many factors determining transgene expression. Consequently, the trend of increasing association and uptake along with increasing lipofection with an increasing PEG concentration fits together well.

Transfection efficiency (Fig. 2), cell association (Fig. 1A) and uptake (Fig. 1B) of lipoplexes at 1:2 charge ratios, especially those made from SUVs, are sensitive to PEG. Lipoplexes bearing a net negative charge (1:2 for example) are less likely to associate with cell surfaces due to weak electrostatic repulsion. This is especially noticeable for those smaller SUV lipoplexes that need to be accumulated and aggregated on the cell surface to be recognized and internalized by the cells. Therefore, PEG-induced cell association makes a significant contribution to the transfection efficiency in such cases.

Other factors that may have contributed to increased lipofection in the presence of PEG include the lipoplex size and DNA protection. We have previously found that the CHO cell line, used here for most experiments, is sensitive to the lipoplex size [19]. Larger lipoplexes, up to 2–2.5 μm, are more efficient lipofection agents. Lipoplexes in the presence of PEG are found to increase in size with incubation, as shown in Table 2. Although a correlation exists between the increasing lipoplex size (Table 2) and lipofection at short PEG pre-incubation times (Fig. 5), we do not believe that these increases in size are a determining factor in the effect of PEG. An increases in lipoplex size has been found to increase lipoplex-cell association, leading to increased lipofection. By use of PEG, we can induce lipoplex-cell association, no matter what the lipoplex size or liposome type is. We postulate that lipoplex-cell association leading to the recognition and internalization of lipoplexes by cells is the critical step in lipofection. The sizes of lipoplexes may not be the sole determinant for this step, as granules of lipoplexes may grow at the cell surface once the association is established [11].

DNA protection is similar in the presence or absence of PEG (not shown). From this finding, we assumed that no gross structural rearrangements concerning DNA cationic lipid binding, or overall structure, other than possible aggregation are found. Increases in lipofection can be seen as due to factors other than lipoplex structural rearrangement.

The relation of the PEG MW upon lipofection warrants discussion. The reason that PEG 8000 is the most efficient MW for lipofection is related to the ability of differently sized PEGs to promote adhesion between bilayers and membranes through polymer depletion. As determined by Kuhl et al. [34], PEGs of different MWs have differing membrane aggregating properties. At an MW of 8000–10 000, the depletion-induced adhesion is strongest, as is the cellular association found with PEGs with an MW of 8000 (Fig. 4A). Higher MW PEG adsorbs to the membrane surface and inhibits aggregation [34]. This is shown also in this lipoplex-cell association measurement (Fig. 4A). The depletion-induced adhesion is not as great at a low MW because the polymers are not completely excluded from the membrane surface. Therefore, PEGs with a MW below 8000 and at 20 000 have a lower lipofection due to a decreased ability to induce adherence of lipoplexes to cell membranes (Fig. 4A). It should also be mentioned that the PEG weight percentage is held constant for these experiments. The molar concentration has not been held constant. It is understood that the osmotic pressure of polymer solutions, especially for PEG, is controlled more by the weight percentage than by the molar concentration [34].

The presence of PEG also increases the efficiency of delivery, as measured by the amount of plasmid (Fig. 3). This finding lends credence to our hypothesis of the PEG effect upon lipoplex-cell association in culture. By increasing the levels of lipoplex-cell association, decreasing the levels of plasmid DNA needed to provide a specific effect is possible. This finding validates lipoplex-cell membrane association as the limiting step for DNA delivery. It also provides insights into possible techniques to enhance this limiting step. This technique may be valuable for in vitro and ex vivo applications where DNA is scarce. We suggest the addition of 2–6% PEG to lipoplexes for improvements in overall in vitro DNA delivery.

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