

Stabilization of cationic liposome-plasmid DNA complexes by polyamines and poly(ethylene glycol)-phospholipid conjugates for efficient in vivo gene delivery

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Abstract Stable complexes of cationic liposomes with plasmid DNA were prepared by (1) including a small amount of poly(ethylene glycol)-phospholipid conjugate or (2) condensing the DNA with polyamines prior to the formation of liposome-plasmid complexes. These preparations were stable for months at 4°C and gave reproducible high transfection activity for in vivo gene delivery after intravenous injection in mice. Under these conditions, the expression of marker gene (luciferase) was primarily in the lungs (reaching values up to 3 ng expression per mg tissue protein), but also in other tissues to a lesser extent. Non-stabilized formulations lost all their transfection activity in 4 days. In these formulations cholesterol, not dioleoylphosphatidylethanolamine, was the helper lipid effective for sustaining high transfection activity in vivo. These new developments in formulation technology should enhance the potential for liposome-mediated gene therapy.

Key words: Cationic liposome; Luciferase; Gene delivery; Stable liposome-plasmid complex; Dimethyldioctadecylammonium bromide; Cholesterol

1. Introduction

Liposomes which consist of amphiphilic cationic molecules have been considered as a potential non-viral vector for gene delivery (reviewed in [1–5]). In theory, the positively charged liposomes can complex to the negatively charged plasmids via electrostatic interactions. The plasmid-liposome complexes have several advantages as gene transfer vectors. Unlike viral vectors, plasmid-liposome complexes can be used to transfer expression cassettes of essentially unlimited size. Since the complexes lack proteins, they may evoke fewer immunogenic and inflammatory responses. Moreover, they cannot replicate

or recombine to form an infectious agent and have low integration frequency.

There are a number of publications which demonstrate convincingly that cationic liposomes can mediate gene delivery by showing detectable expression of a reporter gene in culture cells in vitro [6–8]. Because liposome-plasmid complexes are not as efficient as viral vectors for achieving successful gene transfer, much effort has been devoted in finding ‘better’ cationic lipids for increasing the transfection efficiency [3–5]. Liposome-plasmid complexes are presently recognized with enthusiasm by researchers at both academic institutions and pharmaceutical industries as a potentially useful tool for gene therapy, although the mechanism of liposome-mediated gene transfection is still not well understood. Few studies have begun to deal with the structure-function relationship of the liposome-plasmid complex [5,9], but to date no clear correlation between complex structure and transfection activity has been shown.

During the last few years, several groups have reported on the use of cationic liposomes for in vivo transfection both in animals, and in humans (reviewed in [5,10–13]). Despite considerable promise, the technical problems for preparation of stable complexes have not been addressed as yet. Unlike viral vector preparations, liposome-plasmid complexes are not stable in terms of particle size [3–5]. It has been difficult to obtain homogeneous liposome-plasmid complexes with size distribution suitable for systemic injection, and metastable preparations of liposome-plasmid complexes were used in most published studies. Frequently, these complexes were used within a short period of time ranging from 30 min to a few hours. In recent clinical trials using cationic lipids as a carrier for DNA delivery, the two components were mixed at the bed-side and used immediately [5]. The structural instability along with the loss of transfection activity of liposome-DNA complex with time have been challenges for the future development of liposome-mediated gene therapy. Development of stable, well-defined formulations is critically needed for facilitating efforts to correlate the structural parameters of liposome-DNA complexes to their transfection activity in vivo.

Here, we report a systematic study describing the formulation of stable plasmid-liposome complexes which maintain high transfection activity after intravenous injection in mice using luciferase as a reporter gene expression. We identified cholesterol, not dioleoylphosphatidylethanolamine (DOPE) as an effective ‘helper’ lipid in dimethyldioctadecylammonium bromide (DDAB) liposomes for in vivo gene delivery. In vivo transfection activity of these stable formulations was maintained in storage at 4°C for up to 2 months.

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Abbreviations: Chol, cholesterol; DC-chol, 3β-[N-(N',N'-dimethylaminoethane)carbamoyl]cholesterol; DDAB, dimethyldioctadecylammonium bromide; DMEPC, dimyristoylglycerol-3-ethylphosphocholine; DODAP, dioleoyl-3-dimethylammonium propane; DOEPC, dioleoylglycerol-3-ethylphosphocholine; DOGS, N,N-dioctadecylamidoglycylspermine; DOPE, dioleoylphosphatidylethanolamine; DOTAP, dioleoyl-3-trimethylammonium propane; DOTMA, N-[2,3-(dioleoyloxy)propyl]-N,N,N-trimethylammonium bromide; DSPE, distearoylphosphatidylethanolamine; DTT, dithiothreitol; ESPM, ethyl sphingomyelin (egg); LYSPE, lysinyl dipalmitoylphosphatidylethanolamine; MMCE, myristylmyristoyl carnitine ester; PEG-PE, N-[ω-methoxypoly(oxyethylene)-α-oxycarbonyl]-DSPE; POEPC, palmitoyl-oleoylglycerol-3-ethylphosphocholine.

2. Materials and methods

2.1. Lipids and other reagents

DOPE was purchased from Avanti (Alabaster, AL). Highly purified cholesterol was obtained from Calbiochem (San Diego, CA). DDAB was purchased from Sigma (St. Louis, MO). DDAB was recrystallized once from acetone-methanol solution. D-Luciferin was obtained from Boehringer Mannheim. PEG-PE was a gift from Sequus Pharmaceuticals (Menlo Park, CA). DC-Chol, MMCE and DOGS were obtained from the UCSF Gene Transfer Vehicle Core of Gene Therapy Center. ESPM, DOTAP, POEPC, DOEPC, DMEPC and DODAP were gifts from Avanti (Alabaster, AL). Chloroform solution of each lipid was stored under argon in sealed ampules at -40°C . Other reagents of the highest possible grade were purchased and used without further purification.

2.2. Preparation of liposomes

Small cationic liposomes were prepared in 5% (w/v) dextrose solution in the following fashion. DDAB or other cationic lipids in chloroform was mixed with DOPE or/and cholesterol in the desired molar ratio, and the solvent was removed slowly under reduced pressure at 50°C on a rotary evaporator. The dry lipid film was hydrated with 5% dextrose solution prewarmed to 50°C and the container was sealed under argon. The hydrated lipid suspension was sonicated in a bath sonicator (Lab Supplies, Hicksville, NY) for 5–10 min at 50°C . The final concentration of liposomes was 5 mM cationic lipid and the size of liposomes was measured by dynamic light scattering to be 195 ± 65 nm. Sonicated liposomes were stored under argon at 4°C until use.

2.3. Luciferase reporter system

Plasmid, pCMV/IVS-luc⁺, was constructed as follows. A fragment containing the CMV promoter and synthetic IgE intron was excised from pBGt2.CAT using *SpeI* and *HindIII* and cloned into pBSIISK⁺. The cDNA encoding the modified firefly luciferase (luc⁺) including SV40 late poly(A) signal was cut from the pGL3-Basic Vector (Promega) with *HindIII* and *SalI* and was put into the pBS-CMV-IVS clone downstream of the splice. Plasmids were purified using alkaline lysis procedures adopted and devised by Qiagen Corp. (Chatsworth, CA). Plasmid purity was measured by the ratio of absorbance at 260 nm vs. 280 nm, and purified plasmids were stored in buffer containing 10 mM Tris-Cl and 1 mM EDTA at pH 8.0 at concentrations of 1–2 mg/ml.

2.4. Preparation of transfection complexes

Prior to the transfection experiments, the optimal DNA/liposome ratio for forming complexes which were not large aggregates was determined by mixing a fixed amount of plasmid with various amounts of liposomes. In general, the transfection complexes were formed by pipetting plasmid into a liposome suspension of equal volume and mixing rapidly. The final DNA concentration of the complexes was 200 $\mu\text{g}/\text{ml}$ for in vivo studies and 10 $\mu\text{g}/\text{ml}$ for in vitro studies. Routinely, liposomes containing 8–12 nmol of DDAB could complex with 1 μg plasmid without forming visible large aggregates. Such complexes have excess positive charge, but still tend to aggregate with time during storage at 4°C and lose transfection activity in 4 days. For in vitro experiments, which called for much dilute complexes, DNA-liposome complexes at 5–12 nmol DDAB per μg DNA could be formed. To keep the plasmid-liposome complexes from forming large aggregates and losing transfecting activity with time, two approaches were taken: (1) incorporating a small amount of PEG-PE (from 0.5 to 2% of cationic lipid) by addition to the DNA-liposome complexes within a few minutes after their preparation; (2) condensing plasmid with polyamines (e.g. 0.5 nmol of spermidine per μg DNA) prior to mixing with liposomes. The optimal amount of polyamines was determined by titrating polyamines to DNA before forming large aggregates. The size of these complexes was estimated by dynamic light scattering to be in the range of 410 ± 150 nm.

2.5. Assay of reporter gene expression

Purified luciferase was purchased from Boehringer Mannheim as a standard for calibrating the luminometer and constructing a control standard for the relative specific activity of luciferase. Reporter gene expression in a tissue extract was presented in nanogram quantities by

converting relative light units measured from a luminometer into weight units according to a standard curve. Luciferase expressed in cells or tissues was extracted with cell lysis solution. Effective lysis buffer consisted of 0.1 M potassium phosphate buffer at pH 7.8, 1% Triton X-100, 1 mM DTT and 2 mM EDTA.

Female CD1 mice (4–6 weeks old, weighing approx. 25 g) were obtained from Charles River Laboratory. Mice received liposome-plasmid complexes by tail vein injection and were killed 24 h later. The anesthetized animals were perfused with cold phosphate-buffered saline (PBS) via heart puncture. Each tissue was dissected and washed in PBS, and then homogenized in 6 ml round-bottomed culture tubes containing 500 μl of lysis buffer. The samples were kept at room temperature for 20 min with occasional mixing. The homogenized samples were centrifuged for 10 min at 3000 rpm in an Eppendorf centrifuge. Luciferase activity of each tissue was measured by mixing 100 μl of the reconstituted luciferase substrate (Promega, Madison, WI) with 20 μl of the supernatant of tissue homogenate in the injection system of a luminometer. Peak light emission was measured for 10 s at 20°C . Relative light units of each sample were converted to the amount of luciferase in the tissue extract by comparing with a standard curve which was established for each set of experiments. The protein content of the extract was determined using protein assay kits (BioRad, Richmond, CA). Background was the count of lysis buffer in tissues of mice without treatment. We have obtained consistent negative controls of tissue samples from mice that were treated with liposomes only or with complexes with β -gal DNA. The luciferase activity of these tissues measured was at the same level as lysis buffer analysis, i.e. 200–300 RLU. After background subtraction, the detection limit of our luminometer was linear on a log scale from 2×10^2 to 9×10^6 RLU, which corresponded to 3×10^{-5} ng up to 3 ng luciferase.

SK-BR-3 cells [14] were cultured in McCoy's 5 A medium supplemented with 10% heat-inactivated bovine calf serum and in 5% CO_2 . SK-BR-3 cells in monolayer culture were plated at 50 000 cells per well in 12-well plates and incubated overnight. Each well received 0.5–1 μg of pCMV/IVS-luc⁺ within 20 min of complex formation. Cells were harvested after 24 h of incubation with complexes at 37°C . Luciferase activity in cells was determined as described above.

3. Results and discussion

The use of cationic liposomes for in vitro gene transfer has become widespread since Felgner et al. [6] reported their early study. It was established later [8] that DOPE is by far the most efficient helper lipid for in vitro gene transfection and this has been confirmed by several laboratories [15,16]. It has been suggested, on the basis of in vitro studies, that DOPE may facilitate cytoplasmic delivery via membrane fusion once positively charged DNA-liposome complexes are bound to the cell membrane [16]. Even though Friend et al. [17] did not obtain any morphological evidence that the DOTMA/DOPE liposome-plasmid DNA complexes fuse directly with the plasma membrane, they do not exclude the possibility of fusion events. They suggested that the complexes are endocytosed and the cationic lipids disrupt the endosomal/lysosomal membranes and then facilitate escape of the DNA complexes into the cytoplasm and eventually into the nucleus.

Contrary to most expectations, the helper role of DOPE established from in vitro studies is not evident for in vivo gene delivery following i.v. injection of the complexes. We found that when DOPE was included in DDAB cationic liposomes, in vivo gene transfection was inhibited. This DOPE-dependent inhibition is shown in Fig. 1. Cholesterol, not DOPE, was found to be effective as helper lipid for in vivo gene delivery. There was a 10-fold reduction in luciferase expression in mouse lungs when half of the cholesterol was replaced with DOPE. Our in vivo results of DDAB and other cationic liposomes are not consistent with the general assumption

tion that DOPE is a suitable helper lipid. On the contrary, DOPE in cationic liposome-plasmid complexes attenuates the *in vivo* transfection to such a great degree that we consider DOPE as an inhibitory agent in formulations for *in vivo* gene delivery. Cholesterol has been chosen for *in vivo* studies in recent published reports [11,12] in which the authors do not elaborate on how and why they selected different helper lipids for their experimental designs, i.e. DOPE for *in vitro* and cholesterol for *in vivo* studies. Stabilization of anionic and neutral liposomes in blood by cholesterol has been known for a long time [18]. It is therefore obvious that for systemic gene delivery, one has to consider the stability of liposome-DNA complexes in blood, various components of which are known to react with macromolecular complexes. In fact, our preliminary study of various formulations of plasmid-liposome complexes using freeze-fracture electron microscopy has shown that the cholesterol-containing complexes were structurally more stable than the DOPE-containing complexes in the presence of serum. In 50% serum, DOPE-containing complexes form very large multilamellar and hexagonal lipid structures, while cholesterol-containing complexes maintain

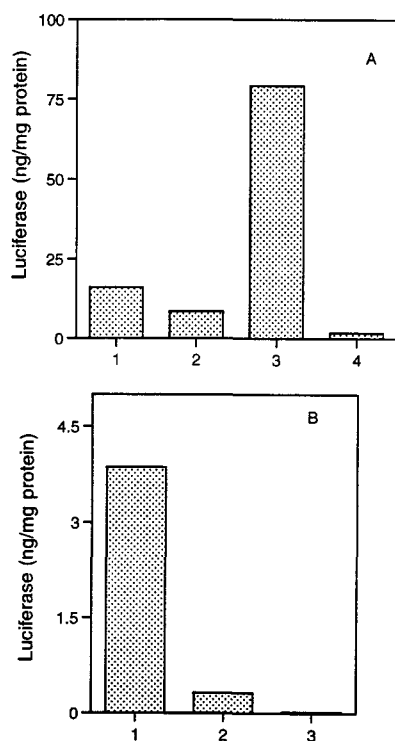


Fig. 1. Role of neutral lipid in gene delivery: cholesterol vs. DOPE. Three liposome formulations were tested for gene delivery to both culture cells (SKBR-3, human breast cancer cell) and mice (CD1, female, 20–25 g). 1, DDAB/Chol (1:1); 2, DDAB/Chol/DOPE (1:0.5:0.5); 3, DDAB/DOPE (1:1); 4, DDAB alone. (A) Cell transfection: SKBR-3 cells were plated at 50 000 cells per well in 12-well plates and incubated overnight. Each well received 1 μ g of P-CMV/IVS-Luc⁺ plasmid which was complexed with liposomes at 8 nmol of DDAB. Cells were harvested after 24 h incubation with complexes at 37°C. Values presented are mean from 2 wells. Values ranged within 10–30% of mean. (B) *In vivo* transfection in mice (lungs): mice received via tail vein injection 60 μ g of P-CMV/IVS-Luc⁺ plasmid which was complexed with liposomes at 8 nmol DDAB per μ g DNA ratio. Values presented are mean from 2 mice. Values ranged within 20–25% of mean. Since it required more concentrated complexes for *i.v.* injection, DDAB alone did not form suitable complexes for this study.

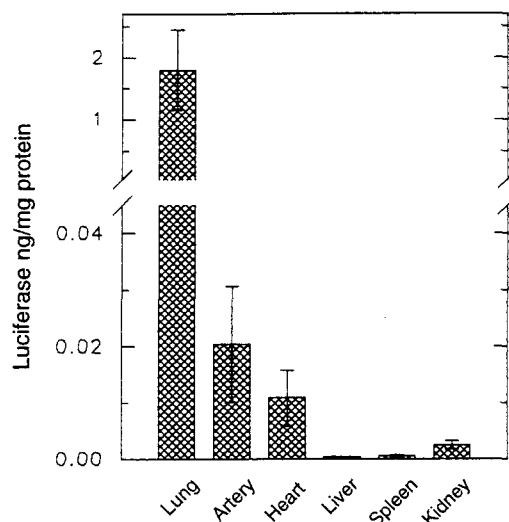


Fig. 2. Reporter gene expression in mouse tissue extracts. Mice received (via tail vein injection) 60 μ g of P-CMV/IVS-Luc⁺ plasmid, which was complexed with DDAB/Chol (1:1) liposomes at 8 nmol DDAB per μ g DNA ratio (without spermidine). Artery was for large blood vessels we could harvest. Values presented were mean from 3 mice.

small liposomal structures showing simple aggregation of liposomes by plasmid. Morphological observations of the structural characteristics of different formulations of liposome-plasmid complexes will be published separately.

Using DDAB/Chol liposome-DNA complexes (8 nmol DDAB/ μ g DNA) for *in vivo* transfection experiments, we have found that detectable luciferase expression in the lung of 25 g mouse required a DNA dose ranging from 30 to 60 μ g. Routinely, 40–60 μ g plasmid DNA per mouse gave consistent gene expression. The amount of DDAB usually associated with 80 μ g DNA (or more) per mouse was found to be too toxic to the animal. The expression of luciferase in various tissues is shown in Fig. 2. As observed before [10–12], maximal expression was found in lung tissue. For 60 μ g plasmid injected, 1–2 ng luciferase per mg tissue protein was routinely obtained. Fig. 3 shows the duration of reporter gene expression in lung tissue. Expression of luciferase decreased rapidly during the first 3 days and reached undetectable levels in 1 week. Zhu et al. [10] reported that following *i.v.* injection of DOTMA/DOPE (1:1)-plasmid complexes into adult mice, the expression of the reporter gene (CAT) is widespread among various tissues and the maximum expression is from complexes with a ratio of 1 μ g plasmid to 8 nmol total lipids. However, at this ratio (corresponding to 1 μ g plasmid to 4 nmol cationic lipid), DDAB/Chol liposome-plasmid complexes tended to aggregate and did not produce measurable gene expression in our investigation.

Since different reporter genes have been used among different laboratories, it has been difficult to attribute the variations in the efficiency of *in vivo* gene delivery to changes in the formulation of liposomes. For a direct comparison of the results in the literature, we have converted the relative light units of luciferase activity measured from a luminometer to a standard of purified luciferase. By doing so, we have found that the peak transfection activity of DDAB/Chol formulations was 3 orders of magnitude higher than values reported recently in comparable experiments [13]. Given that same re-

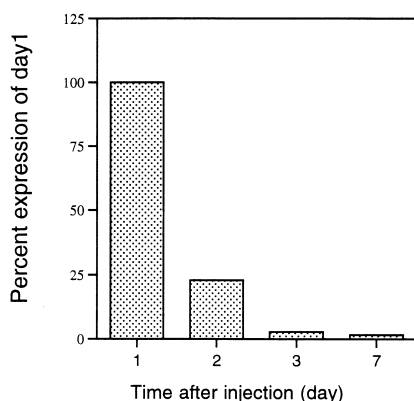


Fig. 3. Duration of reporter gene expression in mouse lungs. Each animal received 40 μ g of P-CMV/IVS-Luc⁺ plasmid which was complexed with DDAB/Chol (1:1) liposomes at 8 nmol DDAB per μ g DNA ratio.

porter gene along with the same promoter in the experimental design, the difference in expression may reflect the selection of liposome formulation. In fact, DDAB/Chol was one of the most efficient gene delivery vehicles among many formulations from 18 different cationic lipids which we screened recently. Preliminary results of expression in mouse lung following i.v. injection indicated that DOTMA/Chol, DOTAP/Chol, MMCE/Chol and ESPM/Chol gave 10–100% transfection activity of DDAB/Chol, DOGS/Chol, POEPC/Chol, LYSPE/DOPE and DC-Chol/DOPE gave 1–10% of DDAB/Chol. DOEPC/Chol, DMEPC/Chol, DODAP/Chol and DDAB/DOPE did not give any measurable activity.

The relationship between structural stability and transfection activity of liposome-DNA complexes has not been detailed in the published reports so far. We have established screening procedures to avoid large aggregates of plasmid-liposome complexes by changing the ratio of DNA to lipid from net negatively charged to positively charged. DNA-liposome complexes of each particular cationic lipid at various ratios of DNA/lipid were prepared and the resulting stable and metastable formulations were used for in vivo transfection. Complexes which contained 8–12 nmol of cationic lipid per μ g DNA were found to have the highest in vivo transfection activity. However, the transfection activity of these complexes decreased with time. Without modifying the procedures for forming the DNA-liposomes complexes, there was a visible aggregation within a few days, and the transfection activity decreased by more than 1000-fold to almost background levels after 1 month storage at 4°C (Fig. 4). Therefore, we undertook to formulate stabilized DNA-liposome complexes which could maintain high in vivo transfection activity during storage.

It has been established recently that PEG-PE incorporation in liposomes produces steric stabilization resulting in longer circulation times in blood [19,20]. We have found that adding PEG-PE (1% of cationic lipid) into the freshly formed plasmid-liposome complexes could prevent the complexes from aggregating during storage. Incorporation of PEG-PE into the complexes is evident in view of the dose-related inhibition of the transfection activity with increasing percentage of PEG-PE: addition of 0.5% PEG-PE reduces activity down to 60%, 1% down to 40% and 2% down to 10% of original luciferase activity in lungs. However, storage of the complexes contain-

ing PEG-PE at 4°C slowly restored the original activity as shown in Fig. 4. Addition of free PEG 2000 in solution of up to 3 mM to comparable complexes did not affect the transfection activity. The mechanistic aspects of the inhibition effect on transfection by PEG-PE, as well as the recovery of the activity following storage at low temperature, are not known at the present time. Further structural studies on these complexes are warranted.

Small polycationic molecules are known to condense DNA via electrostatic charge-charge interactions [21]. The pretreatment of plasmid DNA with polyamines can therefore reduce the number of charge sites for complexing with cationic liposomes. The plasmid-liposome complexes formed with such pretreatment were stable at a lower ratio of lipid to DNA without aggregation. Fig. 4 shows the level of in vivo transfection activity of such preparation, and its fate during storage. Again, we found an increase in transfection activity in aged polyamine-treated liposome-DNA complexes, when compared to that of the samples which were not pretreated with polyamines and used immediately after complexes were formed.

We have determined the transfection activity of some stabilized formulations up to 2 months in storage. The activity of such formulations at that time point did not decrease and was practically the same as that of a 1-month-old sample. A different approach to obtain stable cationic lipid/DNA complexes by complexing plasmid with lipid in lipid-detergent micelles was published recently [22]. However, only 30% of the transfection efficiency was maintained by such complexes in 15% serum for in vitro, and no in vivo results were reported.

We conclude that several of the cationic liposome-plasmid complexes described in this report are stable and can give consistent in vivo transfection activity (ranging from 0.5 to 3 ng luciferase per mg tissue protein) even after long storage at 4°C. Formulations containing cholesterol as the helper lipid generate much higher in vivo transfection efficiency. Stabilizing the complex structure by PEG-PE maintains the complex activity in storage and may prolong the circulation time in blood for targeting to specific tissues. Condensing the DNA

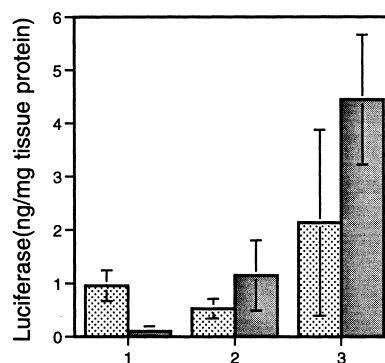


Fig. 4. Gene delivery in mouse lung by various stabilized complexes. Each mouse received 60 μ g of PCMV/IVS-Luc⁺ which was complexed with DDAB/Chol (1:1) liposomes at 8 nmol DDAB/ μ g DNA ratio. Values presented are mean from 3 mice. (Stippled bars) Freshly made complexes; (filled bars) 1-month-old samples. 1, no stabilizing agent was added; 2, PEG-PE was added at 1% of cationic lipid to the formed complexes; 3, spermidine (0.5 nmol per μ g DNA) was added to the plasmid prior to the formation of complexes.

with polyamines before lipid complexation stabilizes the complexes in storage and maintains their activity in vivo. Our methodical approach for producing stable formulations of DNA-liposome complexes exhibiting high transfection activity in vivo should confer advantages for establishing pharmaceutically acceptable preparations, and could therefore facilitate liposome-based gene therapy.

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References

- [1] Crystal, R.G. (1995) *Science* 270, 404–410.
- [2] Blaesé, M., Blankenstein, T., Brenner, M., Cohen-Haguenauer, O., Gansbacher, B., Russell, S., Sorrentino, B. and Velu, T. (1995) *Cancer Gene Ther.* 2, 291–297.
- [3] Behr, J.-P. (1994) *Bioconj. Chem.* 5, 382–389.
- [4] Remy, J.-S., Sirlin, C., Vierling, P. and Behr, J.-P. (1994) *Bioconj. Chem.* 5, 647–654.
- [5] Gao, X. and Huang, L. (1995) *Gene Ther.* 2, 710–722.
- [6] Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.S., Wenz, M., Northrop, J.P., Ringold, M. and Danielsen, H. (1987) *Proc. Natl. Acad. Sci. USA* 84, 7413–7417.
- [7] Loeffler, J.-P. and Behr, J.-P. (1993) *Methods Enzymol.* 217, 599–618.
- [8] Felgner, J.H., Kumar, R., Sridhar, C.N., Wheeler, C.J., Tsai, Y.J., Border, R., Ramsay, P., Martin, M. and Felgner, P.L. (1994) *J. Biol. Chem.* 269, 2550–2561.
- [9] Xu, Y. and Szoka, F.C. (1996) *Biochemistry* 35, 5616–5623.
- [10] Zhu, N., Liggitt, D., Liu, Y. and Debs, R. (1993) *Science* 261, 209–211.
- [11] Liu, Y., Liggitt, D., Zhong, W., Tu, G., Gaensler, K. and Debs, R. (1995) *J. Biol. Chem.* 270, 24864–24870.
- [12] Solodin, I., Brown, C.S., Bruno, M.S., Chow, C.-Y., Jang, E.-H., Debs, R.J. and Heath, T.D. (1995) *Biochemistry* 34, 13537–13544.
- [13] Thierry, A.R., Lunardi-Iskandar, Y., Bryant, J.L., Robinovich, P., Gallo, R.C. and Mahan, L.C. (1995) *Proc. Natl. Acad. Sci. USA* 92, 9742–9746.
- [14] Park, J.W., Hong, K., Carter, P., Asgari, H., Guo, L.Y., Keller, G.A., Wirth, C., Halaby, R., Kotts, C., Wood, W.I., Papahadjopoulos, D. and Benz, C.C. (1995) *Proc. Natl. Acad. Sci. USA* 92, 1327–1331.
- [15] Farhood, H., Xiang, G., Son, K.-H., Yang, Y.-Y., Lazo, J.S., Barsoum, J., Bottega, R., Epand, R.M. and Huang, L. (1994) in: *Gene Therapy for Neoplastic Diseases* (Huber B.E. and Lazo, J.S. eds.) pp. 23–55, N.Y. Acad. Sci.
- [16] Zhou, X. and Huang, L. (1994) *Biochim. Biophys. Acta* 1189, 195–203.
- [17] Friend, D.S., Papahadjopoulos, D. and Debs, R.J. (1996) *Biochim. Biophys. Acta* 1278, 41–50.
- [18] Mayhew, E., Rustum, Y., Szoka, F. and Papahadjopoulos, D. (1979) *Cancer Treat. Rep.* 63, 1923–1928.
- [19] Allen, T.M., Hansen, C., Martin, F., Redemann, C. and Yau-Yong, A. (1991) *Biochim. Biophys. Acta*, 1066, 29–36.
- [20] Papahadjopoulos, D., Allen, T., Gabizon, A., Mayhew, E., Matthey, K., Huang, K., Lee, K.-D., Woodle, M.C., Lasic, D.D., Redemann, C. and Martin, F.J. (1991) *Proc. Natl. Acad. Sci. USA* 88, 11460–11464.
- [21] Plum, G.E., Arscott, P.G. and Bloomfield, V.A. (1990) *Biopolymers* 30, 631–643.
- [22] Hofland, H.E.J., Shephard, L. and Sullivan, S.M. (1996) *Proc. Natl. Acad. Sci. USA* 93, 7305–7309.