Effect of Size and Serum Proteins on Transfection Efficiency of Poly ((2-dimethylamino)ethyl Methacrylate)-Plasmid Nanoparticles

Jong-Yuh Cherng,[1] Petra van de Wetering,[1] Herre Talsma,[1] Daan J. A. Crommelin,[1] and Wim E. Hennink[1,2]

Received February 9, 1996; accepted April 10, 1996

Purpose. The aim of this study was to gain insight into the relation between the physical characteristics of particles formed by a plasmid and a synthetic cationic polymer (poly(2-dimethylamino)ethyl methacrylate, PDMAEMA) and their transfection efficiency.
Methods. The PDMAEMA-plasmid particles were characterized by dynamic light scattering (size) and electrophoretic mobility measurements (charge). The transfection efficiency was evaluated in cell culture (COS-7 cells) using a pCMV-lacZ plasmid coding for β-galactosidase as a reporter gene.
Results. It was shown that the optimal transfection efficiency was found at a PDMAEMA-plasmid ratio of 3 (w/w), yielding stable and rather homogeneous particles (diameter 0.15 μm) with a narrow size distribution and a slightly positive charge. Particles prepared at lower weight ratios, showed a reduced transfection efficiency and were unstable in time as demonstrated by DLS measurements. Like other cationic polymers, PDMAEMA is slightly cytotoxic. This activity was partially masked by complexing the polymer with DNA. Interestingly, the transfection efficiency of the particles was not affected by the presence of serum proteins.
Conclusions. PDMAEMA is an interesting vector for the design of in vivo and ex vivo gene transfection systems.

KEY WORDS: DNA; plasmid; gene therapy; transfection; β-galactosidase; poly(2-dimethylamino)ethyl methacrylate.

INTRODUCTION

Gene therapy is gaining growing attention for curing genetic deficiencies and for the treatment of life threatening diseases (1). More than 100 clinical trials have been approved by the Recombinant DNA Advisory Committee in the USA and many are presently performed (2). For the efficient introduction of foreign DNA into cells, a carrier system is required. Both viral and non-viral vectors (cationic lipids (lipofectin (3), DC-chol (4)) and cationic polymers (e.g., polylysine (5), polyethylenimine (6)) are presently under investigation. Although viral vectors have superior transfection properties over the present generation of non-viral systems, viral vectors have inherent disadvantages (immune response against the transfection systems, limits for the size of the plasmid to be incorporated, the possibility of recombination, difficulties in scaling up). Therefore, there is an urgent need for safe and efficient non-viral gene carriers.

It can be expected that apart from the polymer characteristics (charge density, molecular weight) the physical properties of the formed DNA(plasmid)-polymer particles play an important role in the transfection efficiency. Only a limited number of papers which deal with the physical characteristics of non-viral transfection systems have been published so far (7–9).

The aim of this study was to investigate the correlation between the in vitro (cell culture) transfection efficiency and physical characteristics of particles of plasmid and a synthetic cationic polymer (Poly(2-dimethylamino)ethyl methacrylate). This correlation can shed light on the preferred in vitro properties of gene-carrier particles to achieve optimum transfection efficacy.

MATERIALS AND METHODS

Materials

2-(Dimethylamino)ethyl methacrylate (DMAEMA) was obtained from Fluka. RPMI 1640 medium and plain DMEM (Dulbecco's modified Eagle's medium) were obtained from Gibco, Breda, The Netherlands. Fetal Calf Serum (FCS) was from Bocknet. RSC medium was prepared by supplementing RPMI 1640 with FCS (final concentration 2%) and chloroquine phosphate (an endosome-disruptive agent; final concentration 100 μM). Cells were cultured in complete DMEM medium which was prepared by supplementing plain DMEM medium with FCS (final concentration 5%), penicillin (final concentration 100 IU/ml), streptomycin (final concentration 100 μg/ml) and amphotericin B (final concentration 0.25 μg/ml). X-Gal (5-bromo-4-chloro-3-indoyl-β-galactopyranoside) was from Gibco, Breda, The Netherlands. XTT reagent (proliferation Kit II) was a product of Boehringer, Mannheim, Germany.

Synthesis and Characterization of PDMAEMA

PDMAEMA was synthesized by a radical polymerization of an aqueous solution of 2-(dimethylamino)ethyl methacrylate. The freshly distilled monomer (5 ml) was dissolved in water (20 ml) and the pH was adjusted with 1.0 M HCl to 5. This solution was flushed with N_2 for 30 minutes. Then, the initiator (ammonium peroxydisulphate; 68 mg) was added. The polymerization was carried out for 24 hours at 60°C under a nitrogen atmosphere. The polymer was purified by extensive dialysis against water and collected by lyophilization (yield 70%). The molecular weights and molecular weight distribution were determined by gel permeation chromatography using two thermostated (35°C) columns (Shodex Ollpak KB-802 and KB80 M; Showa Denko, Japan) in series. Degassed 0.7 M $NaNO_3$, 0.1 M Tris-HCl pH 7.0 in Milli Q water was used as the mobile phase. The columns were calibrated using dextran standards of known molecular weights (Fluka). The number average molecular weight (M_n) and weight average molecular weight (M_w) were 45 $*10^3$ and 36 $*10^4$ g/mol respectively. The observed M_w/M_n ratio is not uncommon for polymers synthesized by a radical polymerization.

[1] Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Participant in the Research School Groningen-Utrecht Institute for Drug Exploration (GUIDE), Utrecht University, PO Box 80082, 3508 TB Utrecht, The Netherlands.
[2] To whom correspondence should be addressed.

0724-8741/96/0700-1038$09.50/0 © 1996 Plenum Publishing Corporation

Preparation of Plasmid

pCMV-lacZ plasmid contains a bacterial lacZ gene preceded by a Nuclear Location Signal under control of the CMV promoter (10). The plasmid was amplified in E. Coli (strain DH5a) and purified by column chromatography (QIAGEN-tip 2500, QIAGEN, Germany) (11). The purity of the plasmid was established by UV-spectroscopy (E260 nm/E280 nm ratio 1.87–1.89). Agarose (0.7%) gel electrophoresis analysis using restriction enzymes (11) showed that the plasmid was in supercoiled the form. Further, the same analysis showed that using a restriction enzyme, one band corresponding with a size of 7.8 kB was visible.

Preparation of PDMAEMA-plasmid Particles

PDMAEMA was dissolved in RSC or RC (medium without serum) and aliquots of these solutions were added to plasmid dissolved in the same medium and mixed well (5 seconds, Vortex Genie 2). The final volume was 200 μl. In the first series of transfection experiments the plasmid concentration was fixed at 5 μg/ml, while the PDMAEMA concentration varied from 0–60 μg/ml. In a second series of transfection experiments both the plasmid concentration and the PDMAEMA concentration were varied from 1.6–25 μg/ml and 4.8–75 μg/ml, respectively, fixing the PDMAEMA-plasmid ratio at 3 (w/w). For the electrophoretic mobility measurements, the PDMAEMA-plasmid particles were prepared at low ionic strength (5 mM HEPES buffer pH 7.4).

Particle Size and Electrophoretic Mobility Measurements

The Z-average particle size and polydispersity index (pd) of the PDMAEMA-plasmid particles were determined by dynamic light scattering (DLS) at 25°C with a Malvern 4700 system using a 25 mW He-Ne laser (NEC Corp., Tokyo, Japan) and the automeasure version 3.2 software (Malvern Ltd, Malvern, UK). For the data analysis, the viscosity (0.8905 mPa.s) and refractive index (1.333) of pure water at 25°C were used.

The electrophoretic mobility of the particles was determined in a PC-4 cell with a Malvern zeta-sizer 2C unit (Malvern Ltd, Malvern, UK) at a temperature of 25°C. The instrument was calibrated using a latex with known zeta potential.

Cell Culture and Transfection

For the gene transfer studies COS-7 (cells of SV-40-transformed African green monkey kidney) cells were used. The COS cells were cultured in complete DMEM medium at 37°C and 5% CO_2. 24 hours before transfection, the cells were seeded in a flat bottom 96-well plate (1×10^4 cells per well (0.38 cm^2)). The monolayer of cells (70–80% confluency) was washed with PBS (phosphate buffered saline) shortly before the transfection. Thereafter, the PDMAEMA-plasmid particles (volume 200 μl) were added to the cells and incubated for 1 hour at 37°C and 5% CO_2. Next, the incubation medium was replaced by complete DMEM medium and the cells were cultured for an additional 38 hours (37°C; 5% CO_2).

Expression of the pCMV-lacZ gene was established by incubation of fixed cells (0.25% glutaraldehyde; 5 minutes; 4°C) with X-gal (0.8 mg/ml in phosphate buffer pH 7.4) for 30 minutes at 37°C (12). Using a light microscope, transfected cells were made visible as blue spots and were quantified by counting the number of blue spots in each well (0.38 cm^2). Since the plasmid used contains a nuclear localization signal, the β-galactosidase was expressed in the nucleus allowing a good discrimination between transfected and non-transfected cells. Besides evaluation of the number of transfected cells, also the relative number of living cells was determined using the XTT assay (13). The XTT value for untreated cells (cells not exposed to transfection systems) was taken as 100%.

RESULTS AND DISCUSSION

Effect the PDMAEMA-Plasmid Ratio and Serum on Gene Expression and Cell Viability

Fig. 1a shows the results of transfection experiments which were carried out at a fixed plasmid concentration (5 μg/ml) and a varying PDMAEMA concentration. Since plasmid has a net negative charge, it binds electrostatically to the cationic PDMAEMA polymer yielding polymer-plasmid particles. The

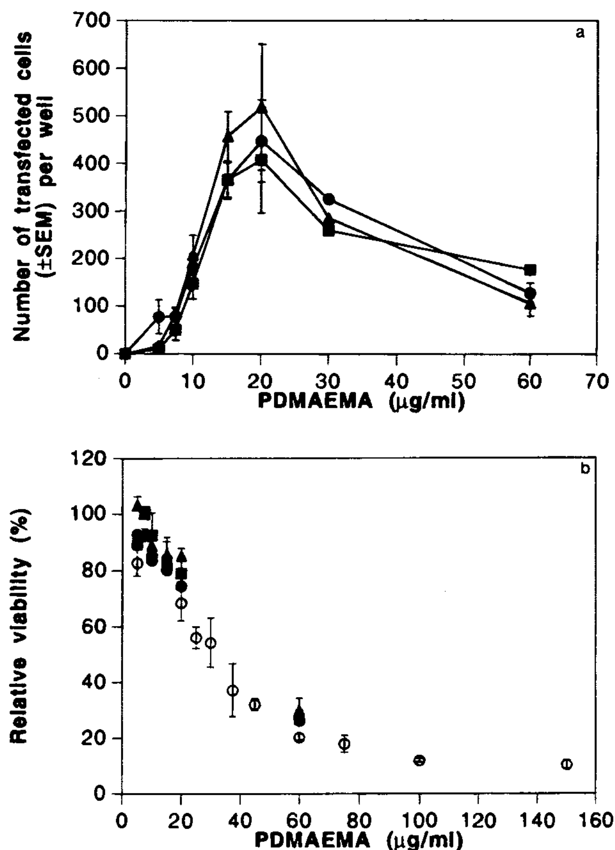

Fig. 1. Effect of PDMAEMA concentration at a fixed plasmid concentration (5 μg/ml) on the number of transfected cells (1a) and on the relative cell viability (1b). The particle formation time was 75 minutes. The results are expressed as mean values (± SEM) of 3–5 experiments. (●) both particle preparation and transfection were carried out in the absence of serum; (■) particles were prepared in the absence of serum, transfection was done in the presence of serum; (▲) both the preparation of the particle and the transfection was carried out in the presence of serum. In Fig. 1b, (○) the effect of PDMAEMA on the relative cell viability in the absence of serum and plasmid.

preparation of the particles and transfection were done both in RSC (serum containing medium) and RC (medium without serum). It appeared that in the absence of PDMAEMA only a low number of transfected cells were observed (usually between 0 and 2 cells per well). From this figure it can be clearly seen that PDMAEMA promoted the cellular uptake and subsequent expression of the trans gene. The number of transfected cells showed a bell shape dependence on the polymer concentration. Such a dependence is observed frequently for both lipid based transfection systems (14) and cationic polymers (5). At an optimal PDMAEMA concentration of 15–20 μg/ml, corresponding with a PDMAEMA/plasmid ratio of 3–4 (w/w), around 500 cells per well were transfected indicating that 1–2% of the cells were actually transfected.

Interestingly, the number of transfected cells was not dependent on the presence of serum neither during the preparation of the PDMAEMA-plasmid particles nor during transfection (two side ANOVA, $p > 0.05$). This is in contrast to the findings with cationic lipid-plasmid systems, where transfection efficiency is sensitive to the presence of serum proteins, and in agreement with the observation that lipophilic polylysines can mediate transfection in the presence of serum (15). Further, within the investigated time range (30–135 minutes) the PDMAEMA-plasmid incubation time did not affect the number of transfected cells at the optimal ratio of PDMAEMA/plasmid.

Due to adverse interactions with the negatively charged cellular membranes cationic polymers are cytotoxic (16). The cytoxicity of PDMAEMA in the presence and absence of plasmid was determined using the XTT assay (Fig. 1b). As can be seen, PDMAEMA was indeed cytotoxic (IC_{50} value around 30 μg/ml). However, in the presence of plasmid, the cytoxicitiy of PDMAEMA was slightly reduced. This was confirmed in a separate experiment where cells were exposed to a toxic dose of PDMAEMA (100 μg/ml) in the presence of a varying concentration of plasmid. An increase of viable cells was observed from 15–25 % with an increasing concentration of plasmid from 0–100 μg/ml. This result shows that the cytotoxicity of the polymer was partially, however not completely, masked by binding to the plasmid. The masking effect of the cytotoxicity of cationic compounds by DNA has been found previously by others (14,6). Moreover, in the concentration range of PDMAEMA with maximum transfection efficiency (15–20 μg/ml), the cytotoxicity of PDMAEMA was marginal (Fig. 1a and 1b). In addition, the cytotoxicity of PDMAEMA was not affected by serum proteins (Fig. 1b).

Effect of the PDMAEMA-plasmid Particle Concentration on Gene Expression and Cell Viability

As demonstrated in Fig. 1, the number of transfected cells showed a maximum at a PDMAEMA-plasmid ratio around 3 (w/w). Therefore, the transfection efficiency of PDMAEMA-plasmid particles, which were prepared at an increasing PDMAEMA/plasmid concentration and at a fixed PDMAEMA-plasmid ratio, 3 (w/w), was studied (Fig. 2). As expected, initially the number of transfected cells increased with an increasing concentration of PDMAEMA-plasmid particles. Obviously, an increasing concentration of PDMAEMA-plasmid particles yielded more transfected cells. However, the number of transfected cells sharply decreased at a plasmid concentration higher than 10 μg/ml (and a PDMAEMA concentration of 30 μg/ml). It was demonstrated that this reduced transfection

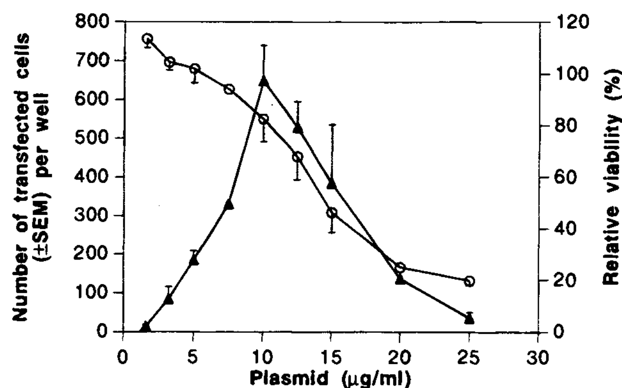

Fig. 2. Effect of the plasmid concentration at a fixed PDMAEMA/plasmid ratio of 3 (w/w) on the number of transfected cells (▲) and on the relative cell viability (○). The results are expressed as mean values (± SEM) of 3 experiments. The particle formation time was 75 minutes. Particle preparation and transfection were carried out in the presence of serum.

efficiency was not caused by an increase in particle size (data not shown). From Fig. 2, it appears that the decrease in transfection efficiency was associated with cytotoxicity. This can be ascribed to the high concentration of PDMAEMA present either in free form or complexed with plasmid (Fig. 1b).

Zeta-potential Measurements

Figure 3 shows the results of the zeta potential of PDMAEMA-plasmid particles. As expected, naked plasmid possesses a negative zeta potential (−22 mV). In the presence of PDMAEMA a positive zeta potential was observed, which levelled off (+30 mV) at high PDMAEMA concentrations. Obviously, the plasmid is becoming fully occupied with PDMAEMA molecules at high PDMAEMA concentration. Already at the lowest polymer concentration used (5 μg/ml; corresponding with a PDMAEMA-plasmid ratio of 1(w/w)), a positive zeta potential was observed. This can be very well explained when the mass of a repeating unit of the plasmid bearing one negative charge (±300 D) is compared with the mass of the repeating unit of the PDMAEMA bearing one

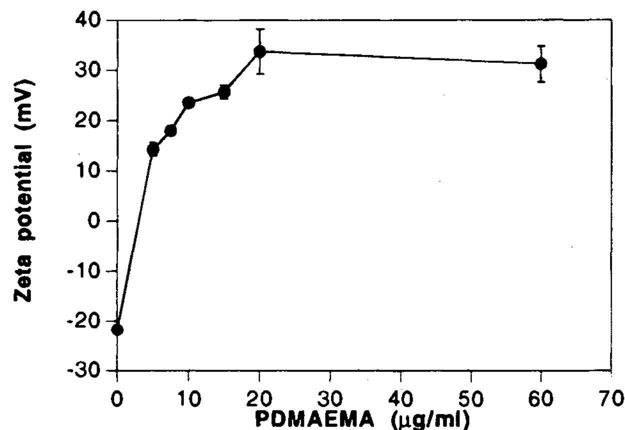

Fig. 3. Zeta potential of PDMAEMA-plasmid particles at a fixed plasmid (5 μg/ml) concentration and a varying PDMAEMA concentration. The particle formation time was 75 minutes. The results are expressed as mean values (± SD) of 3 experiments.

positive charge (191 D), taking into account the pK_a of PDMAEMA at physiological ionic strength (about 8) and the molecular weight of the monomer (157 D).

Since the electrophoretic mobility measurements were carried out at low ionic strength, the zeta potential of the particles under the conditions where transfection was observed will be lower than the zeta potential values shown in Fig. 3. Thus, the zeta potential data should not be evaluated in absolute terms; they should be considered as indications for the net charge of the particles.

Particle Size Measurements of PDMAEMA-plasmid Particles

The extent of cellular uptake depends strongly on the size of the particulate system in question (17,18). Therefore, the effect of the PDMAEMA-plasmid ratio on the size of the formed particles was studied by dynamic light scattering measurements (Fig. 4). Naked plasmid has a rather large hydrodynamic size (0.3–0.4 μm) under the same conditions (pH, ionic strength) as used for the preparation of the polymer-plasmid particles. In the presence of PDMAEMA (>8 μg/ml) smaller particles were formed, demonstrating that the cationic polymer is able to condense the extended structure of plasmids, as suggested by Wagner (19) for polylysine and by Huang (7) for cationic lipids. Moreover, in the absence of serum small particles (diameter around 0.15 μm) were formed at a PDMAEMA/plasmid ratio >2 (w/w). In addition, at a PDMAEMA/plasmid ratio around 3 (w/w)) where the particles had the highest transfection efficiency (fig. 1a), the size distribution was rather homogeneous as demonstrated by the polydispersity index (0.16). At higher PDMAEMA/plasmid ratios the polydispersity index showed a tendency to increase (0.2–0.4) indicating that the particle size distribution widened. It was also observed that particles which showed the highest transfection efficiency, were rather stable for periods up to 28 hours at 25°C; only a slight increase in particle size in time was observed (from 0.17–0.19 μm after 5 minutes and 28 hours respectively). However, at PDMAEMA/plasmid ratios < 1.5 (w/w), rather large particles with a high polydispersity index (0.5) were formed. Probably at these ratios, PDMAEMA molecules act as crosslinker for plasmid molecules yielding large aggregates. These particles are too voluminous to be taken up by cells via endocytosis, resulting in a low number of transfected cells (Fig. 1a). In addition, these particles were not stable in time; a substantial increase in size (from 0.5–1.8 μm) with an increasing incubation time (30–135 minutes) was found. This can very well explain the decreasing transfection efficiency in time which was observed for these particles (results not shown).

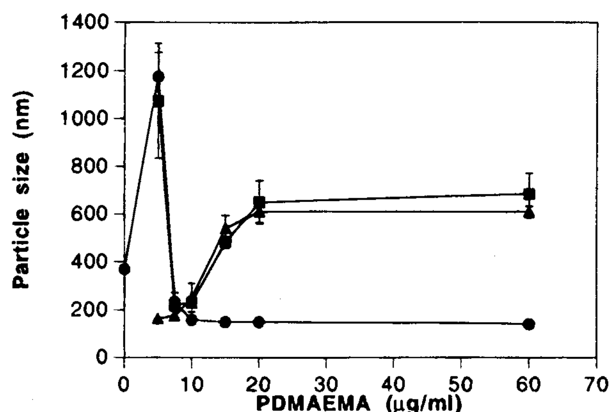

Fig. 4. Size of PDMAEMA-plasmid particles as determined by DLS at a fixed plasmid concentration (5 μg/ml) and a varying PDMAEMA concentration. The particle formation time was 75 minutes. (●) particles prepared in the absence of serum; (▲) particles prepared in the presence of serum; (■) particles prepared in the absence of serum and subsequently exposed to serum.

Surprisingly, large particles were detected when PDMAEMA-plasmid particles were prepared in the presence of serum or when particles preformed in the absence of serum were exposed to serum. This effect occurred especially at high PDMAEMA concentrations (Fig.4). However, as shown in Fig. 1a, serum did not affect the transfection efficiency of the PDMAEMA-plasmid particles. Therefore, a control experiment was carried out in which PDMAEMA was brought into contact with serum. It was shown that when polymer (final concentration 60 μg/ml) was added to a serum solution (2%), particles with a diameter around 0.6 μm were formed. These particles are probably formed due to electrostatic interaction between the positively charged PDMAEMA and negatively charged serum proteins. Thus, the large aggregates detected when PDMAEMA-plasmid particles were prepared in the presence of serum were probably caused by PDMAEMA-protein particles and not by PDMAEMA-plasmid particles. Most likely the affinity of the PDMAEMA for the plasmid is greater than for serum proteins, since at a PDMAEMA-plasmid ratio of 1.5–2 (w/w) no significant effect of serum on particle size was observed. Probably, at these PDMAEMA concentrations all PDMAEMA molecules are complexed with plasmid and no free polymer is present. On the other hand, at higher polymer/plasmid ratios besides PDMAEMA-plasmid particles (size around 0.15 μm) also free polymer is present which gives large aggregates with serum proteins. The small PDMAEMA-plasmid particles were not detected by DLS measurements when large PDMAEMA-protein aggregates were present (20).

Further it was shown that at the PDMAEMA-plasmid ratio where the particles had the highest transfection efficiency, the particle size (0.14–0.17 μm) was hardly dependent on the PDMAEMA/plasmid concentration used for the preparation of the particles. This demonstrating that the decreasing transfection efficiency at high PDMAEMA concentration (Fig. 2) is not due to an increasing particle size, but can be ascribed to the cytotoxic effect of the polymer (free or complexed with DNA) at this concentration.

In conclusion, PDMAEMA is able to interact electrostatically with DNA and to condense its size to such an extent that these positively charged particles (size <0.2 μm) can be taken up by cells. At high PDMAEMA-plasmid ratios, besides transfecting PDMAEMA-plasmid particles also free PDMAEMA is present, which is partially responsible for the observed cytotoxicity. Therefore, our current efforts are directed to separate the PDMAEMA-plasmid particles from free PDMAEMA and to study the (pharmaceutical) stability and transfection efficiency/cytotoxicity of these particles.

ACKNOWLEDGMENTS

We like to thank Dr. A. Bout (Introgene, The Netherlands) for his gift of the plasmid and valuable discussion; we acknowledge Mrs. M. M. G. van Herwaarden, Dr. I. G. W. Bijlsma, and Mr. M. J. Steenbergen for their technical assistance.

REFERENCES

1. F. Herrmann. Cancer gene therapy: principles, problems, and perspectives. *J. Mol. Med.* **73**:157–163 (1995).
2. C. Coutelle. Gene therapy approaches for cystic fibrosis. *Biologicals* **23**:21–25 (1995).
3. P. L. Felgner, T. R. Gadek, M. Holm, R. Roman, H. W. Chan, M. Wenz, J. P. Northrop, G. M. Ringold, and M. Danielsen. Lipofectin: A highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Nat. Acad. Sci. USA* **84**:7413–7417 (1987).
4. N. J. Caplen, E. Kinrade, F. Sorgi, X. Gao, D. Gruenert, D. Geddes, C. Coutelle, L. Huang, E W F W Alton, and R. Williamson. In vitro liposome-mediated DNA transfection of epithelial cell lines using the cationic liposome DC-Chol/DOPE. *Gene Therapy* **2**:603–613 (1995).
5. D. T. Curiel, E. Wagner, M. Cotten, M. L. Birnstiel, S. Agarwal, C-M. Li, S. Loechel, and P.-C. Hu. High-efficiency gene transfer mediated by adenovirus coupled to DNA-polylysine complexes. *Human Gene Therapy* **3**:147–154 (1992).
6. O. Boussif, F. Lezoualc'h, M. A. Zanta, M. D. Mergny, D. Scherman, B. Demeneix, and J.-P Behr. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: Polyethylenimine. *Proc. Nat. Acad. Sci. USA* **92**:7297–7301 (1995).
7. B. Sternberg, F. L. Sorgi, and L. Huang. New structures in complex formation between DNA and cationic liposomes visualized by freeze-fracture electron microscopy. *FEBS Lett.* **356**:361–366 (1994).
8. H. Gershon, R. Ghirlando, S. B. Guttman, and A. Minsky. Mode of formation and structural features of DNA-cationic liposome complexes used for transfection. *Biochemistry* **32**:7143–7151 (1993).
9. A. Rolland, J. Duguid, M. Barron, L. Gong, J. Levin, and E. Eastman. Characterization of non-viral gene medicines. *Proceed. Intern. Symp. Control. Rel. Bioact. Mater.* **21**:240–241 (1994).
10. A. Bout, D. Valerio, and B. J. Scholte. In vivo transfer and expression of the lacZ gene in the mouse lung. *Exp. Lung Res.* **19**:193–202 (1993).
11. J. Sambrook, E. F. Fritsch, and T. Maniatis. Molecular cloning; a laboratory manual, 2nd ed., Cold Spring Harbor, Laboratory Press, Cold Spring Harbor, (1989).
12. K. Lim and C.-B Chae. A simple assay for DNA transfection by incubation of the cells in culture dishes with substrates for β-galactosidase. *Biotechniques* **7**:576–579 (1989).
13. D. A. Scudiero, R. H. Shoemaker, K. D. Paull, A. Monks, S. Tierney, T. H. Nofziger, M. J. Currens, D. Seniff, and M. R. Boyd. Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. *Cancer Res.* **48**:4827–4833 (1988).
14. X. Gao and L. Huang. A novel cationic liposome reagent for efficient transfection of mammalian cells. *Biochem. Biophys. Res. Commum.* **179**:280–285 (1991).
15. X. Zhou, A. L. Klibanov, and L. Huang. Lipophilic polylysines mediate efficient DNA transfection in mammalian cells. *Biochim. Biophys. Acta* **1065**:8–14 (1991).
16. S. Choksakulnimitr, S. Masuda, H. Tokuda, Y. Takakura, and M. Hashida. In vitro cytotoxicity of macromolecules in different cell culture systems. *J. Controlled Release* **34**:233–241 (1995).
17. J. Zabner, A. J. Fasbender, T. Moninger, K. A. Poellinger, and M. J. Welsh. Cellular and molecular barriers to gene transfer by a cationic lipid. *J. Biol. Chem.* **270**:18997–19007 (1995).
18. M. J. Poznanski and R. L. Juliano. Biological approaches to the controlled delivery of drugs: a critical review. *Pharmacol. Rev.* **36**:277–336 (1984).
19. E. Wagner, M. Cotten, R. Foisner, and M. Birnstiel. Transferrin-polycation-DNA complexes: the effect of polycations on the structure of the complex and DNA delivery to cells. *Proc. Nat. Acad. Sci. USA* **88**:4255–4259 (1991).
20. H. Talsma, G. Gooris, M. J. V. Steenbergen, M. A. Salomons, J. Bouwstra, and D. J. A. Crommelin. The influence of the molar ratio of cholesteryl hemisuccinate/dipalmitoylphosphatidylcholine on "liposome" formation after lipid film hydration. *Chem. Phys. Lipids.* **62**:105–112 (1992).