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Physico-chemical characterisation and transfection efficiency of lipid-based gene delivery complexes

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Abstract

Cationic liposomes spontaneously interact with negatively charged plasmid DNA to form a transfection competent complex capable of promoting the expression of a therapeutic gene. This work aims to improve the understanding of the poorly defined mechanisms and structural rearrangements associated with the lipid–DNA interaction. Specifically, dimethyl dioctadecylammonium bromide (DDAB):dioleoyl phosphatidylethanolamine (DOPE) and 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) liposomes were mixed with a reporter plasmid (pADβ or pCMVβ) to form lipid–DNA complexes. The size and charge characteristics of the complexes as determined by photon correlation spectroscopy and microelectrophoresis were found to be dependent on the lipid:DNA ratio, with both DDAB:DOPE–DNA and DOTAP–DNA complexes aggregating at around neutral zeta potential. Negative stain transmission electron microscopy demonstrated at least three distinct complex structures being formed at the same DOTAP:DNA ratio. We postulate that two of these aggregates are structural moieties involved in the formation of the efficient transfection particle. Gel electrophoresis was used to determine the efficiency and extent of lipid–DNA complex formation. Results showed that only DOTAP liposomes were capable of preventing ethidium bromide intercalation with DNA and protecting the enclosed plasmid from nuclease digestion. When a range of lipid–DNA complexes were transfected into in vitro cell lines, the efficiency of reporter gene (β-galactosidase) expression was found to depend on the type of liposome used in the complex, the ratio of lipid:DNA and the transfected cell line. Our results challenge the requirement for DOPE to be included in the formulation of cationic lipid vectors, especially in the case of DOTAP containing liposomes.

Keywords: Cationic liposome; Gene therapy; Physico-chemical characterisation; Transfection; DNA

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1. Introduction

If gene therapy is to succeed the corrective plasmid DNA (pDNA), constructs must be delivered to the cell targets in a form that will preserve their function, penetrate the numerous barriers to cell invasion and promote the expression of the therapeutic protein (Brigham and Schreier, 1993; Tomlinson and Rolland, 1996; Lee and Huang, 1997). Cationic liposomes have intrinsic properties which make them attractive as vehicles for gene delivery; they are synthetic and, as such, manufacturable to drug standard, biodegradable, non-immunogenic and able to interact with DNA to promote its transfection into both replicating and non-replicating cells (Felgner et al., 1987; Gao and Huang, 1995). For these reasons, cationic lipid-based systems represent the most investigated approach to non-viral gene therapy (Rolland, 1998). Liposome–pDNA complexes are formed by self-assembly through liposomes undergoing electrostatic interaction with the negatively charged phosphate backbone of plasmid DNA. With the majority of cationic lipids, except 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), the liposomes are formulated with neutral lipids such as dioleoyl phosphatidylethanolamine (DOPE) or cholesterol. Under physiological conditions, the molecular configuration of DOPE is considered to encourage the formation of inverted hexagonal (H_{II}) phases (Litzinger and Huang, 1992; Koltover et al., 1998). It is believed that these non-bilayer phases promote fusion between the lipid–pDNA complex and cell membranes such as the endosomal membrane. This interaction is considered to improve the transfer of internalised cationic liposome–DNA complexes from endocytic cellular uptake compartments into the cell cytoplasm (Farhood et al., 1995).

When forming a complex with DNA, a profound structural rearrangement of the liposome structure occurs. Gershon et al. (1993), using the Kleinschmidt method of rotary shadowing electron microscopy, visualised the structures formed when DNA was complexed with cationic liposomes comprising the cationic lipid DOTMA and the neutral species DOPE. These authors suggested an interaction in which the cationic liposomes bind initially to DNA molecules to form clusters of aggregated vesicles along the nucleic acid strand. At a critical lipid:DNA charge ratio (around 1:1 +/−), any further increase in lipid causes a synergistic DNA-induced liposome fusion and lipid-induced DNA collapse. The result is the formation of a condensed DNA structure completely entrapped within a fused lipid bilayer; the DNA being no longer accessible to ethidium bromide intercalation or susceptible to nuclease attack. Freeze-fracture electron microscopy has been used to study complexes formed between DC-Chol:DOPE liposomes and pDNA (Sternberg et al., 1994, 1995). The resulting structures were found to vary as a function of the time adopted for the complexation process and the DNA concentration. Based on such experimental observations, Felgner et al. (1995) proposed a general model for the interaction of cationic liposomes with DNA. According to the model, the cationic liposomes approach opposite sides of the anionic DNA strand and fuse with each other. The final rearrangement of the liposome–DNA complex involves coating of the DNA strand with a single lipid bilayer.

Despite the existence of these studies, the precise mechanisms and structural rearrangements associated with the interaction between cationic liposomes and DNA are still poorly defined. Physico-chemical characterisation investigations including electron microscopy and cellular based studies are often viewed in isolation. Therefore, with this study, we have aimed to combine a range of physico-chemical characterisation techniques and in vitro transfection studies to provide a considered, rational evaluation of lipid–DNA complexes as pharmaceutical entities. We selected DOTAP and dimethyl dioctadecylammonium bromide (DDAB):DOPE (0.4:1 w/w) liposomes as our cationic lipid gene delivery vectors, as both of these liposomes have been shown to be effective at delivering pDNA in vitro (Rose et al., 1991; McLachlan et al., 1995; Crook et al., 1996; McLachlan et al., 1996) and in vivo (Philip et al., 1993; McLachlan et al., 1995).

2. Materials and methods

2.1. Materials

DDAB was purchased from Sigma-Aldrich Company (Poole, UK); DOPE and DOTAP were purchased from Avanti Polar Lipids (Alabama, USA).

The following compounds were used as received: agarose LE (Promega, Southampton, UK), deoxyribonuclease I from bovine pancreas (DNaseI) (Fluka, Gillingham, UK), ethidium bromide solution (Pharmacia Biotech, St. Albans, UK), Luria Bertani (LB) agar (Sigma-Aldrich), LB broth (Sigma-Aldrich), β-mercaptoethanol (Pharmacia Biotech), *o*-nitrophenol-β-D-galactoside (ONPG) (Sigma-Aldrich), phosphoric acid (Sigma-Aldrich), Reporter Lysis Buffer 5X (RLB) (Promega) sodium dodecyl sulphate (SDS) (Pharmacia Biotech), Tris base (Sigma-Aldrich). All other chemicals were of analytical grade and purchased from Fisher Scientific UK (Loughborough, UK).

Cell culture flasks and 24-well clusters were obtained from Costar UK (High Wycombe, UK). Dulbecco's Modified Eagle's Medium (DMEM 25 mM HEPES), foetal bovine serum, penicillin–streptomycin solution and trypsin–EDTA solution 1X were purchased from Gibco.

2.2. Preparation of plasmid DNA

The 7.1 kb pADβ plasmid construct containing the adenovirus 2 major late promotor and β-galactosidase reporter gene was provided by Megabios Corporation (Burlingame, USA).

The 7.2 kb plasmid pCMVβ containing the human cytomegalovirus immediate early gene promoter/enhancer and β-galactosidase reporter gene was amplified and purified from a commercial construct (Clontech, Palo Alto, USA). The plasmid was propagated using a transformed DH5α strain of *Eschericia coli*, colonised onto an ampicillin selective LB agar plate and cultured overnight at 37°C. The plasmid DNA was harvested and purified using a Qiagen Plasmid Mega Kit (Qiagen, Crawley, UK).

2.3. Preparation of cationic liposomes

The method of liposome preparation was adapted from New (1990). Solutions of lipid(s) (DOTAP or DDAB:DOPE 0.4:1w/w) in chloroform were evaporated to dryness under high vacuum. After all traces of the organic solvent had been removed, the lipid film was purged with nitrogen for 15 min. Multilamellar liposomes were formed when the dried lipid film was resuspended in deionised water heated above the T_c of the lipid(s). Vesicle size was reduced by nine cycles of extrusion through a 100 nm pore size polycarbonate Isopore® membrane (Millipore UK, Watford, UK). Liposomes were either prepared immediately prior to use or stored under nitrogen at 4°C for no longer than 5 days. The particle size of the liposomes was assessed immediately before use using the technique described in Section 2.5.

2.4. Formation of liposome–DNA complexes

The cationic liposome–DNA complexes were prepared by adding the appropriate amount of aqueous liposome suspension to dilutions of the DNA stock solution (1 mg/ml in distilled water). Immediately after mixing, the complex suspension was gently agitated by a brief period of pipetting and left for 20 min at room temperature to allow complex formation to proceed. Complexes were analysed immediately following the incubation period.

2.5. Analysis of particle size and zeta potential of liposome–pDNA complexes

The diameter of the DNA–liposome complexes was measured by photon correlation spectroscopy (PCS) (Coulter N4MD submicron particle analyser; Coulter Electronics, Luton, UK). Samples were analysed at 25°C using a 4mW laser and a scattering angle of 90°. The diameter of the complexes was determined in triplicate and expressed in unimodal analytical mode.

The zeta potential of the cationic liposome–DNA complexes was measured by microelectrophoresis (Malvern Zetasizer 3; Malvern Instruments, Malvern, UK). The instrument was

calibrated by washing with degassed deionised water and injecting the Malvern AZ55 Electrophoretic Standard prior to each series of measurements. Freshly prepared complexes were injected into the AZ10 standard sample cell and the measurement performed at 25°C and 150 mV using 1mM sodium chloride as the electrolyte solution. Ten measurements were taken for each sample.

2.6. Electron microscopy

The method used to visualise the lipid–DNA complexes was adapted from Zabner et al. (1995). The freshly prepared samples were placed onto 100 mesh nickel grids. After 3 min, the excess solution was wicked off with filter paper and replaced with freshly filtered and centrifuged 2% aqueous uranyl acetate (UA). The UA solution was removed after 30 s, and the grids were washed twice with distilled water and allowed to dry. Rotary shadowing was performed using platinum wire and the grids were imaged on a Philips 208 transmission electron microscope.

2.7. Ethidium bromide fluorescence

The degree of DNA condensation and restrictive access to ethidium bromide (EtBr) intercalation offered by complexation was assessed using gel electrophoresis. A 0.8% agarose gel was prepared in 0.5% Tris–borate EDTA (TBE) buffer. The gel was submerged in 400 ml of TBE buffer containing 0.5 μg/ml EtBr. Liposome–DNA complexes were mixed with gel loading buffer (6 × strength, blue–orange dye containing 0.25% bromophenol blue, 40% glycerol in TBE buffer) and carefully added to the wells of the gel at a volume representing 1 μg of DNA per well. The gel was run at 100 V for 1 h, removed from the tank and visualised under UV light with quantitation by Molecular Analyst software (Bio-Rad Gel Doc 1000; Bio-Rad Laboratories, Hercules, CA).

2.8. Nuclease digestion of plasmid DNA

The method used for assessing the resistance of the complexed plasmid to nuclease degradation was adapted from Ruysschaert et al. (1994). Complexes formed between pADβ and either DOTAP or DDAB:DOPE liposomes were incubated with DNase solution (0.32 U/μg DNA) at 37°C for 15 min. The enzyme reaction was stopped by addition of a solution containing 3% SDS, 45% glycerol and 0.25% bromophenol blue. The samples were carefully added to the wells of a 0.8% agarose gel at a volume representing 1 μg of DNA per well. The gel was run in TBE buffer containing 0.5 μg/ml EtBr at 100 V for 1 h. Subsequently, the gel was removed from the tank and visualised under UV light with quantitation by Molecular Analyst software.

2.9. Cell culture

A549 (human lung epithelial carcinoma) and COS-7 (African green monkey kidney fibroblast) cells were obtained from the European Collection of Animal Cell Cultures, Salisbury, UK. The cells were cultured in 24-well plates in DMEM with 10% foetal bovine serum, penicillin (100 IU/ml) and streptomycin (100 μg/ml). For transfection experiments, 100 000 cells were grown to 85% confluency at 37°C in a humid atmosphere containing 5% CO_2.

2.10. Cell transfection

Defined volumes of sterile filtered 1 mg/ml lipid samples were made up to 100 μl with DMEM in the wells of a 96-well plate. In another 96-well plate, 5 μg of pCMVβ was made up to 100 μl with DMEM. The liposome samples were added to the plasmid samples and the complexes were allowed to form at room temperature for 20 min. Meanwhile, the 85% confluent cells were washed twice with PBS and submerged in 1 ml of DMEM. The DNA–liposome complexes were added to the cells and the plates were incubated at 37°C for 4 h to allow uptake of the DNA. After incubation, the lipid–DNA complexes were removed, the cells were surface rinsed thoroughly and fed with 1 ml of culture medium. The cells were returned to the incubator for a further 44 h to allow intracellular gene expression to proceed.

2.11. Quantification of gene expression

The quantitative β-galactosidase enzyme assay was developed from Promega Technical Bulletin No. TB097. The transfected cells were washed twice with PBS and lysed with RLB. The cells were scraped from the wells using a pipette tip and transferred in suspension into 1.5 ml microcentrifuge tubes. The tubes were vortexed briefly and centrifuged to liberate the cell extract supernatant from the sedimented cell debris. The diluted cell extracts were vortexed with 2 × Assay Buffer (200mM sodium phosphate buffer, pH 7.3, 2mM $MgCl_2$, 100mM β-mercaptoethanol, 1.33 mg/ml ONPG) and incubated at 37°C for 1 h. The reaction was stopped with 1 M Na_2CO_3 and the UV–visible spectroscopic absorbance of the samples read at 420 nm. β-Galactosidase activity was calculated using the following equation (MacGregor et al., 1991):

$$\beta - \text{Gal activity (U)} = \frac{380 \times A_{420}}{\text{Reaction time (min)}}$$

The results were standardised for protein content using a Bradford protein assay (Kruger, 1991).

3. Results

The size and charge of lipid–DNA complexes will influence their physical stability, in vivo distribution, cellular interaction and extent of cell uptake (Mahato et al., 1995a,b; Lasic and Templeton, 1996; Tomlinson and Rolland, 1996). Fig. 1 illustrates the extent to which both the size and zeta potential of both DOTAP–DNA and DDAB:DOPE–DNA complexes depend on the cationic lipid to DNA charge ratio. A significant increase in the size and variance in size of the complexes (mean ± SEM: 214.7 ± 9.9 nm for DDAB:DOPE–DNA 1.5:1 +/− and 1187.6 ± 136.3 nm at DDAB:DOPE–DNA 0.75 +/−) was observed when their zeta potential approached zero. This is the point at which the net charge on the surface of the complexes would be neutral or 'isoelectric' and therefore there would be no electrical barrier to aggregation between complexes.

Negative stain electron microscopy was used to visualise the lipid–DNA assemblies. Fig. 2A shows complexes that have been freshly prepared from DOTAP liposomes and pADβ at a lipid:DNA ratio of 0.7:1 +/−. Fig. 2A clearly shows DOTAP vesicles (extruded to approximately 100 nm) free in suspension. A number of liposomes appear to have approached the plasmid and attached to its surface to form a new aggregated structure. The micrograph shows visible DNA and surface adsorbed cationic liposomes forming a structure not inconsistent with a zeta potential close to zero (Fig. 1). The adsorbed liposomes lie in close proximity with each other with the DNA acting as a fusogenic agent, neutralising the repulsive electrostatic forces on the cationic lipid and drawing them together to form semi-fused liposomes (Li et al., 1996). Fig. 2B shows the same formulation at a 0.7:1 +/− charge ratio but in which the DNA is no longer visible. The unilamellar liposomes have attached to the surface of the DNA to form an aggregated liposome complex. The size of the structures seen in Fig. 2A,B are representative of the large aggregates measured by PCS (Fig. 1). To further demonstrate the heterogeneity within a single lipid–DNA formulation, Fig. 2C shows yet another structure observed at the 0.7:1 +/− charge ratio. DNA is not visible on the surface of this complex, where structural rearrangement of the lipids has formed a new multilamellar configuration. This 'fingerprint-like' internal structure has been considered to represent lipid bilayers that have interacted with and coated the DNA into a colloidal particulate system (Tomlinson and Rolland, 1996). The structures seen in Fig. 2A–C are not seen in samples of DOTAP in the absence of DNA. Fig. 2A–C demonstrate that the heterogeneity of cationic liposome–DNA complexes within the same formulation can be quite profound, and it clearly raises the question of which of the various forms of complex from within a single formulation is the most efficient for transfection. Methods designed to produce homogeneous complexes and to identify the complex that is most effective at delivering the gene could provide an important advance in improving gene transfer (Zabner et al., 1995). Caution is required,

however, as Zabner (1997) has found that preparations of cationic lipid/DNA that are enriched in the small homogenous particles are less transfection efficient than the heterogeneous cationic lipid/DNA complexes represented in Fig. 2A–C.

The appearance of DOTAP–DNA complexes containing an excess of lipid (4.7:1 +/−) are shown in Fig. 2D. At this ratio, free DNA or

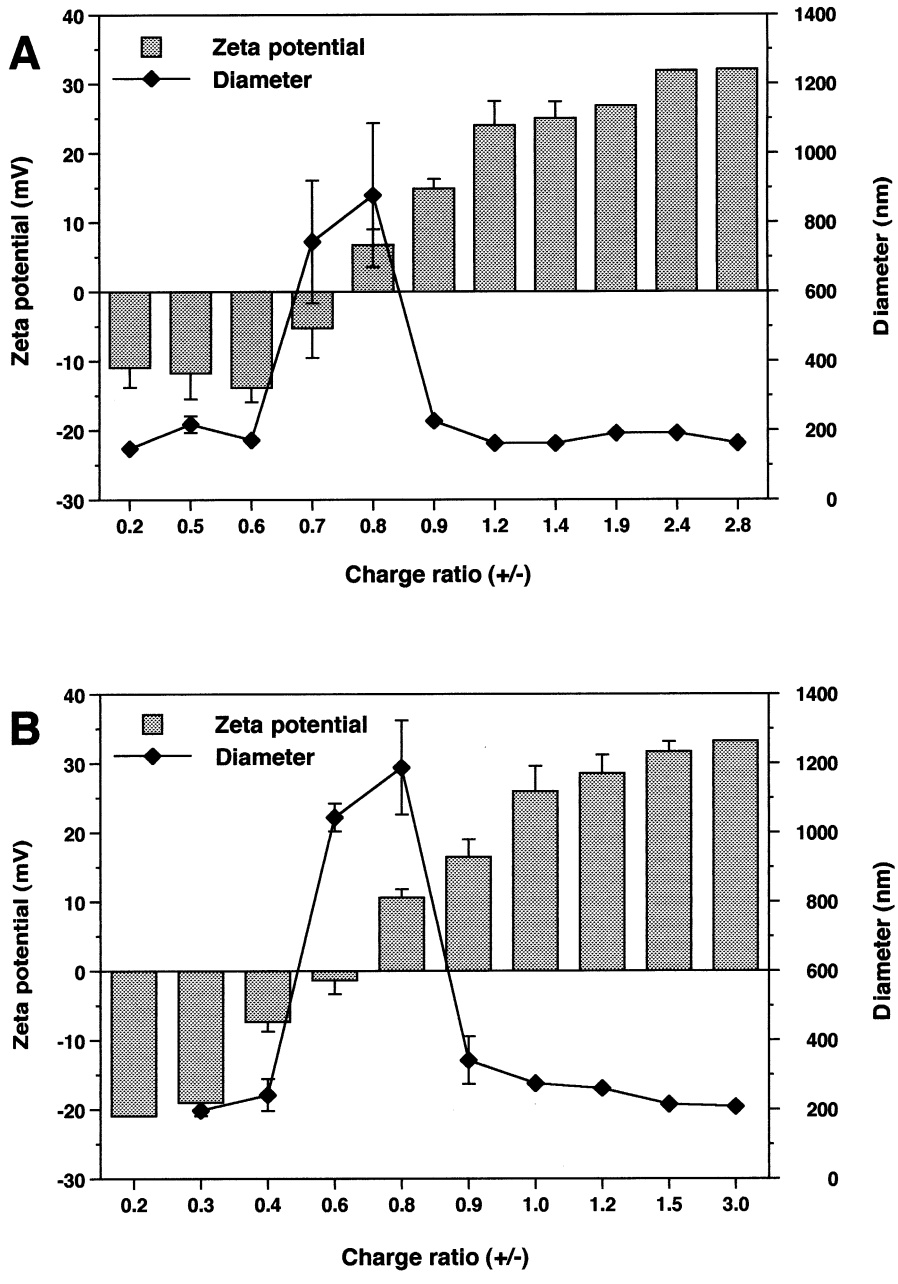


Fig. 1. Effect of charge ratio on diameter and zeta potential of DOTAP–DNA (A) and DDAB:DOPE (0.4:1 w/w)–DNA (B) complexes. Different ratios of lipid–pADβ plasmid (100 μg/ml DNA) in distilled water were analysed by photon correlation spectroscopy (line graph) and microelectrophoresis (column graph). Mean ± SE ($n = 3$).

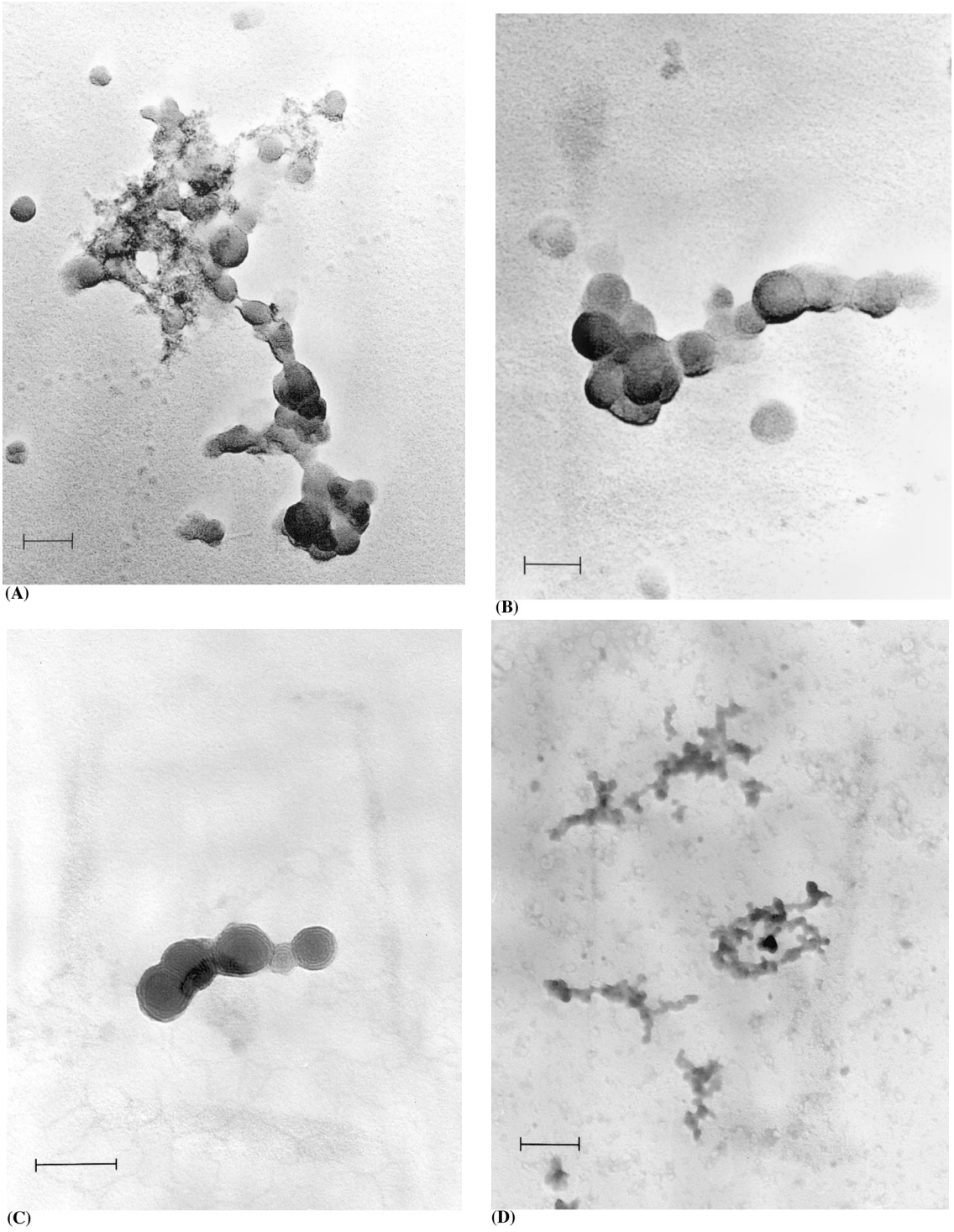
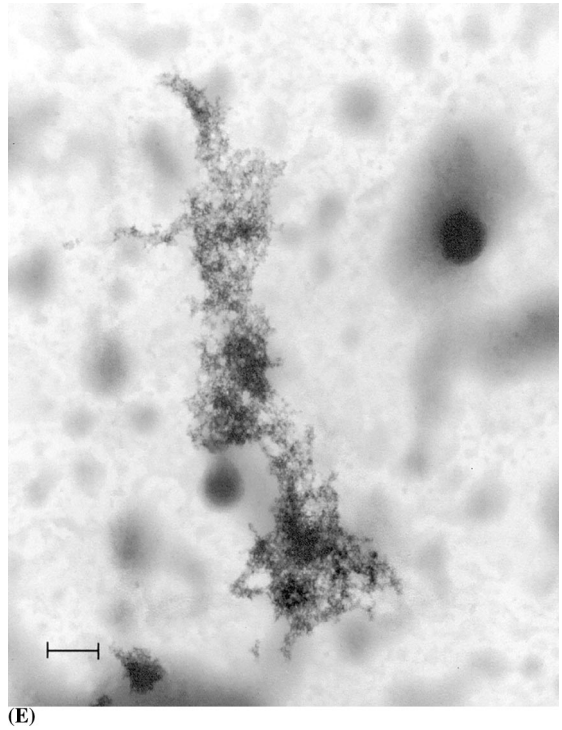


Fig. 2. Negative stain transmission electron microscopy of DOTAP–DNA and DDAB:DOPE(0.4:1 w/w)–DNA complexes. Freshly prepared samples were stained with aqueous 2% uranyl acetate and imaged using a Phillips 208 TEM: DOTAP–pADβ 0.7:1 + / − (A–C), bar = 100 nm; DOTAP–pADβ 4.7:1 + / − (D), bar = 1 μm; DDAB:DOPE–pADβ 3:1 + / − (E), bar = 300 nm.



(E)

Fig. 2. (*Continued*)

unilamellar cationic liposomes are no longer visible. The cationic lipid appears to have completely enclosed the plasmid inside a dense lipid matrix. The lipid density of these aggregates is demonstrated by their sensitivity to the electron beam (Gustafsson et al., 1995). These large aggregates have been observed by other investigators using a similar electron microscopy method (Zabner et al., 1995; Fasbensder et al., 1997).

Fig. 2E shows a typical electron micrograph of DDAB:DOPE–DNA complexes. The presence of the fusogenic lipid DOPE appears to discourage the formation of well defined lamellar lipid structures. These lipid assemblies are not seen to structurally enclose the pDNA, which remains uncomplexed and free in suspension over a range of lipid:DNA ratios (0.3:1 to 3:1 + / −).

Agarose gel electrophoresis was used to examine the efficiency of complexation of pDNA by cationic liposomes; specifically, how complexation with cationic lipid affects EtBr intercalation with DNA (Fig. 3). Lane 1 shows the fluorescence emitted by EtBr when it is able to intercalate between DNA base pairs in the absence of lipid. Access of EtBr to DNA binding sites was still

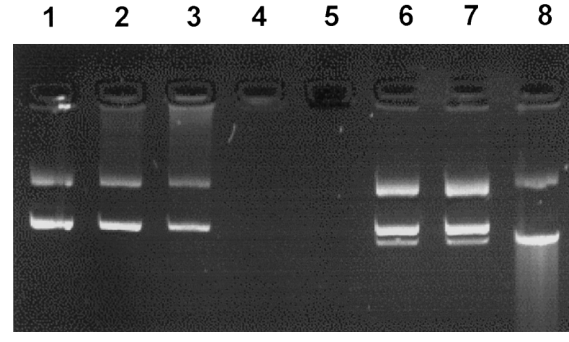


Fig. 3. Agarose (0.8%) gel of DOTAP–DNA and DDAB:DOPE(0.4:1 w/w)–DNA complexes stained with ethidium bromide. Naked pADβ plasmid DNA (lane 1), DOTAP–DNA 0.2:1 + / − (lane 2), DOTAP–DNA 0.7:1 + / − (lane 3), DOTAP–DNA 2.4:1 + / − (lane 4), DOTAP–DNA 4.7:1 + / − (lane 5), DDAB:DOPE–DNA 0.6:1 + / − (lane 6), DDAB:DOPE–DNA 1.5:1 + / − (lane 7), DDAB:DOPE–DNA 3:1 + / − (lane 8).

evident by the appearance of a fluorescent signal when the plasmid was complexed with sub-optimal ratios of DOTAP. At a DOTAP:DNA ratio of 0.2:1 +/−, approximately 95% (by imaging densitometry) of the DNA was available for intercalation, whereas only approximately 65% (by imaging densitometry) of the DNA was accessible at DOTAP:DNA 0.7:1 +/− (lanes 2 and 3). Lane 3 shows signs of DOTAP:DNA 0.7:1 +/− complexes failing to migrate far from the well and this phenomenon has been observed in other experiments (data not shown). The reduced mobility of these low ratio lipid:DNA complexes are a consequence of charge neutralisation and/or increases in the molecular size of the complex (Reimer et al., 1995). When the DOTAP:DNA ratio reached 2.4:1 +/−, there was no detectable fluorescent signal (lane 4). This infers that at this 2.4:1 +/− DOTAP:DNA ratio, the lipid has enclosed the plasmid to such an extent that EtBr can not access the DNA structure.

The results of the electron microscopy, microelectrophoresis and EtBr exclusion studies are consistent. At low DOTAP:DNA ratios ($\leq$ 0.7:1 +/−), the complex possesses a negative zeta potential indicative of free non-condensed DNA. Electron microscopy investigations demonstrate structures where some DNA remains visible among liposomes that have aggregated in close association. At these low lipid:DNA ratios, up to 65% of the DNA is still accessible to EtBr intercalation (Fig. 3). At higher DOTAP:DNA ratios ($\geq$ 2.4:1 +/−), the complex carries a net positive charge. Transmission electron microscopy shows structures where the liposomes have interacted to totally enclose the negatively charged DNA inside a dense lipid matrix. Fig. 3 confirms that none of this enclosed DNA is available for EtBr intercalation.

DDAB:DOPE liposomes failed to enclose the plasmid with 100% of the fluorescent signal apparent at all lipid–DNA ratios (lanes 6–8). These observations correlate with the electron microscopy experiments, which failed to demonstrate any physical interaction between the DDAB:DOPE liposomes and pDNA.

A more biological endpoint with regard to the protection afforded to pDNA by complexation with lipid is the protection against nuclease digestion. DOTAP–DNA and DDAB:DOPE–DNA complexes containing variable amounts of lipid and a constant amount of plasmid were exposed to DNaseI. After cessation of the nuclease activity, the appearance of the remaining DNA was analysed on an agarose gel (Fig. 4). When the plasmid was complexed with a low ratio of DOTAP liposomes (0.2:1 +/−), up to 70% (by imaging densitometry) of the fluorescent signal was retained. The same DOTAP:DNA complexes were inefficient at excluding ethidium bromide (Fig. 3). This is because the DNA, although not totally enclosed, is associated in such a way with the lipid that the DNase molecule cannot interact totally with the bases, whereas a smaller molecule like EtBr can access the DNA structure (Crook et al., 1996). At DOTAP:DNA ratios of 2.4:1 and 4.7:1 +/−, the DNA was totally protected from nuclease digestion as judged by retention of the fluorescent signal. Depending upon the lipid condensing agent, this may have important implications for cystic fibrosis (CF) gene therapy clinical trials. Pre-administration of DNase could be used to aid clearance of the cellular debris that obstructs the CF airway prior to the delivery of a DOTAP–CFTR construct. This could improve the delivery efficiency of the plasmid and enhance

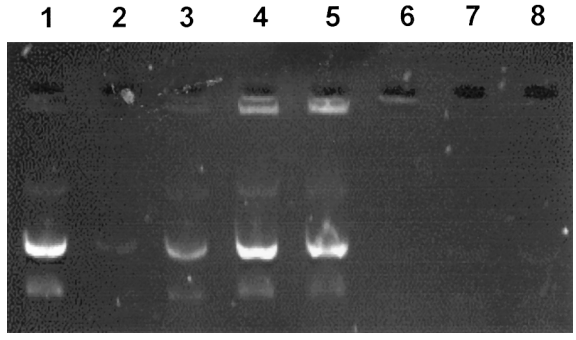


Fig. 4. Agarose (0.8%) gel of DOTAP–DNA and DDAB:DOPE(0.4:1 w/w)–DNA complexes incubated with DNase1 at 37°C for 15 min. Naked pADβ plasmid DNA (lane 1), DNA plus DNase1 (lane 2), DOTAP–DNA 0.2:1 +/− plus DNase1 (lane 3), DOTAP–DNA 2.4:1 +/− plus DNase1 (lane 4), DOTAP–DNA 4.7:1 +/− plus DNase1 (lane 5), DDAB:DOPE–DNA 0.6:1 +/− plus DNase1 (lane 6), DDAB:DOPE–DNA 1.5:1 +/− plus DNase1 (lane 7), DDAB:DOPE–DNA 3:1 +/− plus DNase1 (lane 8).

clinical outcome (Crook et al., 1996). DDAB:DOPE liposomes failed to prevent nuclease digestion of the plasmid even at high lipid:DNA ratios.

The results of the transfection of A549 and COS-7 cells with the DNA–lipid complexes are shown in Fig. 5. DOTAP liposomes are able to

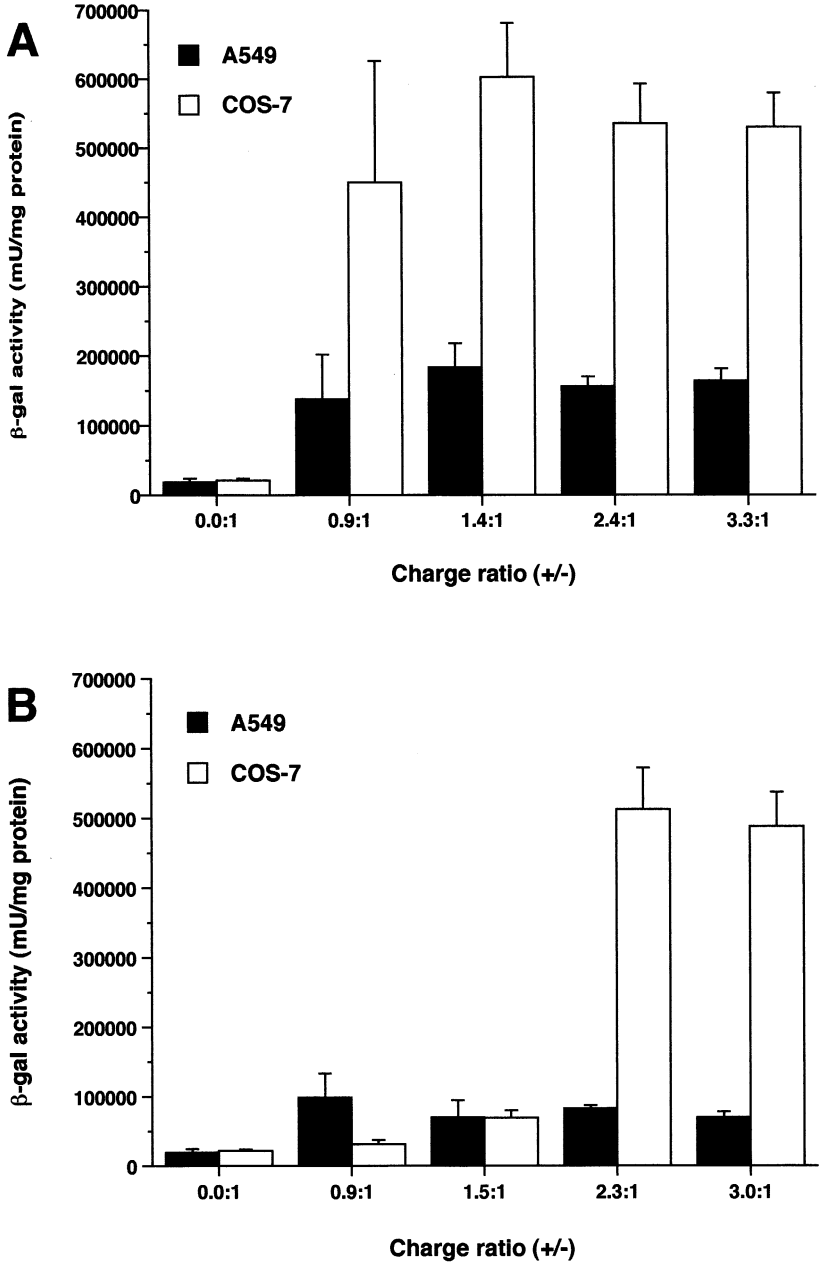


Fig. 5. Transfection of A549 (black columns) and COS-7 cells (white columns) with DOTAP–pCMVβ (A) and DDAB:DOPE–pCMVβ (B) complexes. Freshly prepared complexes were incubated at 37°C with 85% confluent cells in the absence of serum for 4 h. β-Galactosidase activity was determined 48 h after transfection and standardised against cellular protein content. Mean ± SE ($n = 3$).

mediate a significant increase (one-way analysis of variance and Duncans multiple range test, $P = 0.05$) in pCMVβ expression relative to naked DNA control in both A549 and COS-7 cells. The increase in transfection did not significantly depend on the ratio of lipid–DNA in the complex. DDAB:DOPE liposomes were also capable of significantly increasing the transfection efficiency of plasmid DNA in A549 and COS-7 cells. This increase was only apparent at charge ratios above $2.3{:}1+/-$ in COS-7 cells.

The lower transfection efficiencies observed in A549 cells may be caused by their endogenous ability to synthesise and secrete phosphatidylcholine (PC), a major component of pulmonary surfactant (Smith, 1977). Pulmonary surfactant has been shown to inhibit cationic lipid-mediated gene transfer to epithelial cells in vitro (Duncan et al., 1997; Tsan et al., 1997) by mixing with the cationic lipid and disrupting the lipid–DNA complex (Duncan et al., 1997). It is probable that DDAB:DOPE–DNA complexes would be more sensitive to PC-mediated destabilisation than DOTAP–DNA complexes, as our physico-chemical characterisation experiments demonstrated that a less structured complex is formed between this mixed lipid system and pDNA.

4. Discussion

The work in this study set out to define the structure and physico-chemical properties of cationic lipid–DNA gene delivery complexes. We investigated whether there were any definable differences in the way two commercially available liposome systems interacted with and promoted the expression of plasmid DNA.

The diameter and surface charge of the complexes formed between our liposome samples and pADβ plasmid DNA was found to depend on the ratio of lipid:DNA used in the formulation but not the type of liposome used in the preparation. Preparation of neutral zeta potential lipid–DNA complexes caused the samples to form heterogeneous aggregates. Electron microscopy allowed us to visualise these heterogeneous complexes. We postulate that the 200 nm diameter colloidal complex shown in Fig. 2C will predominate when the complexes are prepared at positive charge ratios. The two larger structures observed in Fig. 2A,B therefore act as essential structural alternatives in the formation of the smaller multilamellar lipid–DNA complexes. Transfection studies have shown that this ratio of DOTAP:DNA ($0.7{:}1+/-$) is not as efficient at giving rise to reporter gene expression in vitro as DOTAP:DNA ratios above $0.9{:}1+/-$ (data not shown). This could be due to the fact that these complexes contain an excess of lipid which promotes formation of the colloidal particle. These smaller colloidal complexes would be of a size capable of cell internalisation by endocytosis (Mahato et al., 1997) and would afford more protection to the internalised plasmid DNA as demonstrated by ethidium bromide fluorescence and nuclease digestion.

DDAB:DOPE liposomes did not appear to interact with the plasmid DNA in the same way as DOTAP liposomes. Electron microscopy, ethidium bromide fluorescence and nuclease digestion studies all suggest that the liposomes do not structurally enclose the plasmid DNA. Loosely associated lipid is more likely to be lost from the complex prior to interaction with the cell membrane. This may explain why more DDAB:DOPE liposomes are required to achieve comparable levels of transfection in COS-7 cells. It is likely that even more DDAB:DOPE liposomes would be required to achieve efficient DNA delivery in vivo, as the presence of serum proteins destabilise lipid–DNA complexes (Gao and Huang, 1995; Zelphati and Szoka, 1996; Mahato et al., 1997; Yang and Huang, 1997). We have confirmed that DOTAP liposomes in the absence of DOPE can effectively deliver plasmid DNA to certain cell lines in vitro. The inclusion of DOPE in non-viral gene delivery formulations may therefore not always be required and may ultimately restrict the development of more stable and efficient non-viral vectors containing a single lipid component.

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