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Gene transfection efficiency of tracheal epithelial cells by DC-Chol–DOPE/DNA complexes

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Abstract

We evaluated the transfection efficiency of five different cationic liposome/plasmid DNA complexes, during the in vitro gene transfer into human epithelial tracheal cell lines. A dramatic correlation between the transfection efficiency and the charge ratio (positive charge of liposome to negative charge of DNA) has been found. DC-Chol–DOPE was found to be the most effective liposome formulation. Therefore, a morphological and structural analysis of DC-Chol–DOPE liposomes and DC-Chol–DOPE/DNA complexes, has been performed by transmission electron microscopy (TEM) and by confocal laser scanning microscopy (CLSM), respectively. The process of interaction between DC-Chol–DOPE/DNA complexes and human epithelial tracheal cells has been studied by CLSM. These results raise some issues for in vivo gene therapy.

Keywords: Cationic liposome; Gene therapy; Tracheal epithelial cell

1. Introduction

Non-viral gene transfection provides a safer alternative to viral systems in gene therapy. In this context, cationic liposomes represent the most current tools used in vitro and vivo gene therapy experiments, particularly for inherited and inflammatory pulmonary diseases [1,2]. Different cationic liposome/DNA complexes have been tested in vitro to evaluate transfer efficiency of the recombinant gene in airway epithelial cells [3–6]. However, the majority of these studies were focused to evaluate the transfection efficiency of single liposome formulations based on different parameter variations, including type of transfected cells, amount of DNA used, liposome to DNA concentration ratio, and liposome concentration [7–9].

Abbreviations: BSA, bovine serum albumin; CLSM, confocal laser scanning microscopy; CMV, cytomegalovirus; CPRG, chlorophenol red galactopyranoside; DC-Chol, 3β[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl]cholesterol; DPPC, dipalmitoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DOSPA, 2,3-dioleyloxy-*N*-[2(sperminecarboxamido)ethyl]-*N*,*N*-dimethyl-1-propanaminium trifluoroacetate; DOTAP, 1,2-bis-(oleoyloxy)-3-(trimethylammonium)propane; DOTMA, *N*-[1-(2,3-dioleyloxy)propyl]-*n*,*n*,*n*-trimethylammonium chloride; TEM, transmission electron microscopy

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We have tested the in vitro gene transfer efficiency of five different cationic liposome formulations, including DC-Chol/DOPE, DOTAP/DOPE, lipofectin (DOTMA/DOPE), lipofectamine (DOSPA/DOPE) and LipoTAXI, in a human epithelial tracheal cell line (56FHTE8o$^-$), by varying in parallel experiments the liposome/DNA concentration ratios. It was found that the best level of induced gene expression at the optimal liposome/DNA concentration ratio in 56FHTE8o$^-$ epithelial cells was obtained by DC-Chol–DOPE/DNA complexes. We have therefore examined the morphology and size of DC-Chol–DOPE liposomes by transmission electron microscopy (TEM), morphology of DC-Chol–DOPE/DNA complexes by confocal laser scanning microscopy (CLSM). Moreover, we have studied DC-Chol–DOPE/DNA complexes uptake by epithelial tracheal cells by CLSM and kinetics of transfection, for different positive charge of liposome to negative charge of DNA concentration ratios.

2. Materials and methods

2.1. Cell lines

Simian virus 40 (SV40)-transformed tracheal (56FHTE8o$^-$) epithelial cell line was used [10]. Cells were cultured in Dulbecco's modified Eagle's/Ham's F12 medium (DMEM/F12; 1:1) (Gibco-BRL) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin (Hyclone), 100 μg/ml streptomycin (Hyclone) and 2 mM L-glutamine (Hyclone) at 37°C in 5% CO_2–95% air.

2.2. Plasmid preparation

The eukaryotic expression plasmid pCMV-gal (Clontech, Palo Alto, CA, USA) [11] containing the *E. coli*-galactosidase (*lacZ*) reporter gene was used to assess transgene expression after cell transfection. Plasmid DNA was prepared removing the bacterial endotoxins, which copurify with the DNA, using the EndoFree Qiagen kit [12].

2.3. Cationic liposomes preparation

Lipofectin (DOTMA/DOPE 1:1) and lipofectamine (DOSPA/DOPE 3:1) liposomes were purchased from Gibco-BRL (Life Technologies, Gaithsburg, USA). LipoTAXI liposomes were obtained from Stratagene (La Jolla, USA), DOTAP and DOPE were purchased from Avanti Polar Lipid, (Alabaster, USA). Vesicles were prepared as previously described [13]. DC-Chol/DOPE liposomes (3:2 molar ratio) were prepared according to Gao and Huang [14] at two different concentrations (1.2 and 3.6 mg/ml).

2.4. Transmission electron microscopy

The morphology of liposome preparations has been studied by negative staining electron microscopy, as described elsewhere [15]. Briefly, small aliquots of liposomes were collected on formvar carbon-coated grids and negatively stained, using a 2% phosphotungstic acid (Agar Scientific (pH 7.00) solution. Samples were observed in a Philips CM12 TEM at 80 kV. The diameter of vesicles was measured on randomly taken micrographs, using the Image Analysis System Cambridge Q570 (Cambridge, UK), equipped with Monocrome Solid State TV camera.

2.5. Transfection procedure for assessing the transgene expression

Cells were plated in 96-well microtiter plates (Costar, Cambridge, USA) at a density of 10^4 cells/well and grown overnight at 37°C in 5% CO_2 (to approximately 80% confluency) in their appropriate growth media. Liposome and DNA amounts (expressed as M/M) were complexed for 30 min at room temperature in 0.1 ml of RPMI medium without serum and then added to the cells. Transfection was allowed to proceed for 4 h at 37°C in a humidified atmosphere of 95% air–5% CO_2. Following transfection, media containing liposome/DNA complexes were removed and replaced with the appropriate growth media.

2.6. β-Galactosidase assay

β-Galactosidase cell activity was evaluated using a photometric assay [5], 48 h post-transfection. After medium removal, cells were lysed by the addition of 50 μl of lysis buffer (0.1% Triton X-100, 250 mM

Tris-HCl pH 8.0) per well, and maintained at 0°C for 30 min. The total protein amount present in each lysate was calculated using the Bradford method [26]. The same amount of total proteins (10 μg/well) in a final volume of 100 μl sample buffer (0.5% BSA in phosphate-buffered saline) was added to each well, to minimize the effect of cell number and growth. One hundred and fifty microliters of substrate (1 mg/ml of chlorophenol red galactopyranoside, CPRG) in β-galactosidase buffer (60 mM Na_2HPO_4, pH 8.0, 1 mM $MgSO_4$, 10 mM KCl, 50 mM β-mercaptoethanol) was added to each well, and the plate analyzed at 580 nm in a microtiter plate reader after the color developed (15 min to 24 h). A standard curve of β-galactosidase activity (range 5000 to 78.1 pg of standard Sigma enzyme) was used to convert the absorption readings to pg of β-galactosidase, after subtraction of background values. Data were expressed as pg of β-gal/μg of protein.

2.7. Analysis of intracellular distribution of complexes by CLSM

Analysis of the intracellular distribution of DC-Chol/DOPE (3:2 molar ratio)/DNA complexes was carried-out on transfected cells. The plasmid was labeled using SYBR Green I (Molecular Probes, Eugene, USA) before adding liposomes preparations. Cells, grown on 24×24 mm coverslips at density of 7×10^4 cells/cm^2, were transfected replacing their normal growth medium with 2 ml/dishes of serum-free culture medium containing 10% of transfection mixture. Controls were performed replacing normal medium with serum-free media containing SYBR Green I at working dilution of 1:10 000. Following different times of transfection, ranging from 3 min to 4 h, media containing liposomes/DNA complexes or fluorescent probes for the controls were removed, cells washed three times in PBS and processed for CLSM observation. Transfected cells were fixed with 2% paraformaldehyde in PBS (pH 7.4) for 10 min at room temperature. After washing in PBS, coverslips were mounted on glass microscope slides, in presence of glycerol/PBS in the ratio 4:1 and observed on a LEICA TCS 4D CLSM, supplemented with Argon/Kripton laser and equipped with 40×1.00–0.5 and 100×1.3–0.6 oil immersion lenses. The excitation and the emission wavelengths employed were 488 and 510 nm, respectively. The acquisitions were recorded, employing the pseudo-color representation.

3. Results

3.1. Transfection efficiency as function of liposome to DNA concentration ratios and incubation time

Fig. 1 reports the level of transgene expression as function of liposome to DNA concentration ratios. The concentration of DNA has been kept constant, whereas that of liposome has been varied. Each liposome formulation had a different charge ratio value, at which the transfection efficiency reaches the highest value. Optimal transfection efficiency varied between 1000 and 4000 pg β-gal/μg protein. DC-Chol–DOPE resulted to be the most effective vehicle of DNA in this set of experiments. A drastic change of transfection efficiency with a slight increment of the liposome/DNA concentration ratio was observed

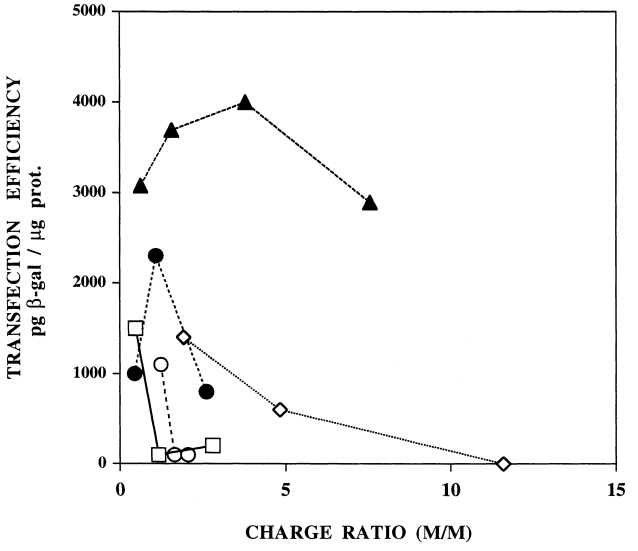

Fig. 1. Transfection efficiency of different liposome/DNA complexes into tracheal epithelial cells reported as function of liposome/DNA concentration ratio. The transfection efficiency has been evaluated by the β-gal assay and expressed as pg β-gal/μg protein. Liposome and DNA concentrations are expressed as molarity. The plasmid complexed with liposomes is pCMV–β-gal. Transfected cells are tracheal epithelial 56FHTE8o$^-$. ▲, DC-Chol–DOPE/DNA; ●, DOTAP–DOPE/DNA; □, lipofectin/DNA; ◇, lipofectamine/DNA; ○, LipoTAXI.

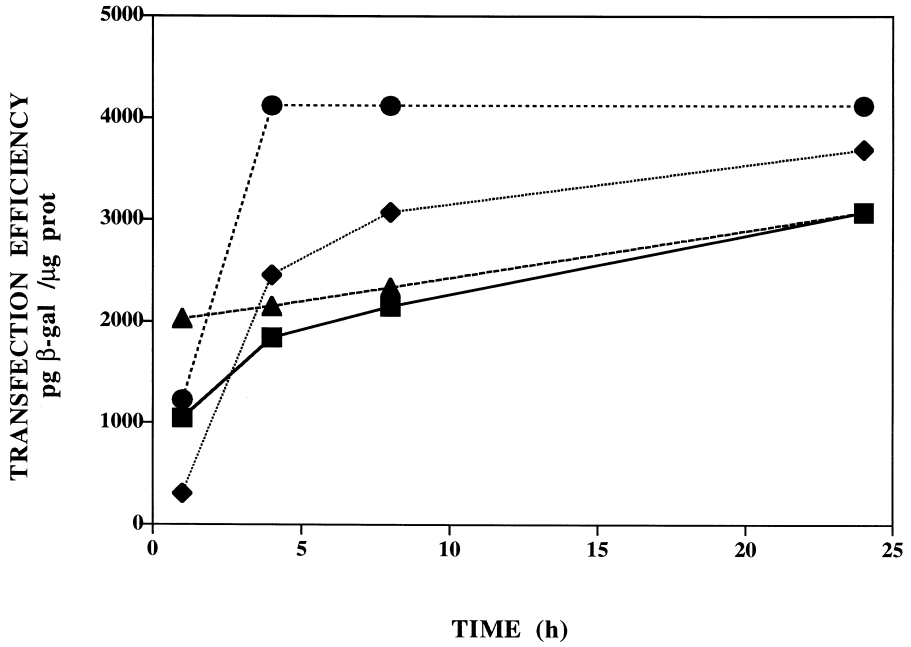

Fig. 2. Kinetics of transfection efficiency using different DC-Chol–DOPE/DNA complexes. The transfection efficiency has been evaluated by the β-gal assay and expressed as pg β-gal/μg protein. The time is expressed as hours. Concentrations are expressed as molarity. The plasmid complexed with DC-Chol–DOPE liposomes is pCMV–β-gal. Transfected cells are tracheal epithelial 56FHTE8o$^-$ cells. Different DC-Chol–DOPE/DNA complexes have different liposome/DNA concentration ratios. ■, DC-Chol–DOPE/DNA = 0.6; ◆, DC-Chol–DOPE/DNA = 1.6; ●, DC-Chol–DOPE/DNA = 3.8; ▲, DC-Chol–DOPE/DNA = 7.6.

for DOTAP–DOPE/DNA complexes, being 2–2.5-fold more effective than lipofectamine and 10–15-fold more effective than LipoTAXI and lipofectin (Fig. 1). At high values of charge ratio, i.e. at high values of liposome concentration, little or no gene expression was observed for lipofectin, lipofectamine and lipoTAXI.

This is probably due to cell toxicity, and is most

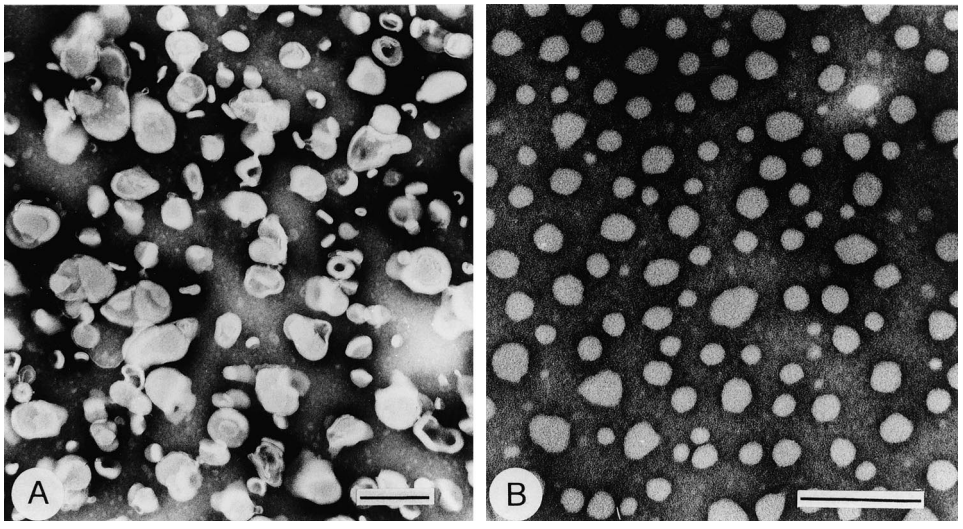

Fig. 3. Transmission electron micrographs of DC-Chol–DOPE liposomes. TEM micrographs of negative stained DC-Chol–DOPE liposomes, prepared from a lipid solution of 1.2 mg/ml (A) and 3.6 mg/ml (B). Scale bars: A, 200 nm; B, 150 nm.

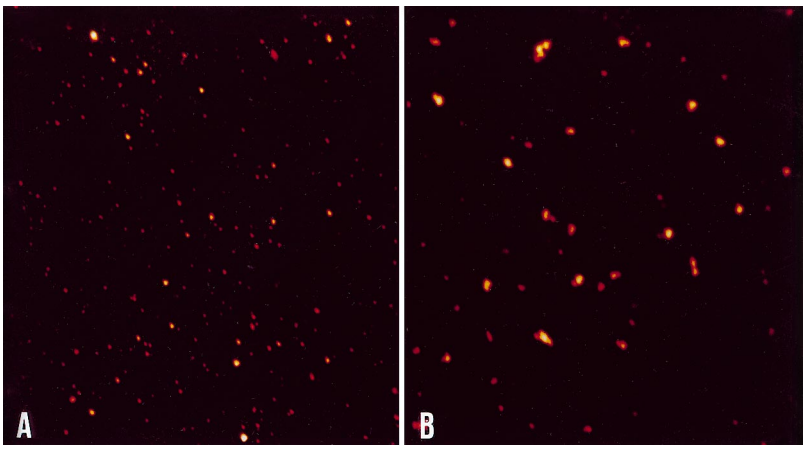

Fig. 4. Confocal laser scanning images of DC-Chol–DOPE liposomes complexed with labeled DNA. CLSM photomicrographs A and B refer to DC-Chol–DOPE/DNA complexes, prepared from DC-Chol–DOPE lipid solutions of 1.2 and 3.6 mg/ml concentration, respectively. Liposome/DNA concentration ratio = 5:1 (M/M). DNA was labeled with SYBR Green dye. ×70.

likely to be related to the lipid component of the complexes. In fact, direct microscopic examination of exposed cells showed evidence of variable degree of cell damage (data not shown).

In order to examine thoroughly the activity of DC-Chol/DOPE liposomes, we evaluated the kinetics of cell transfection at different liposome to DNA concentration ratios (Fig. 2). In this case, also the DNA

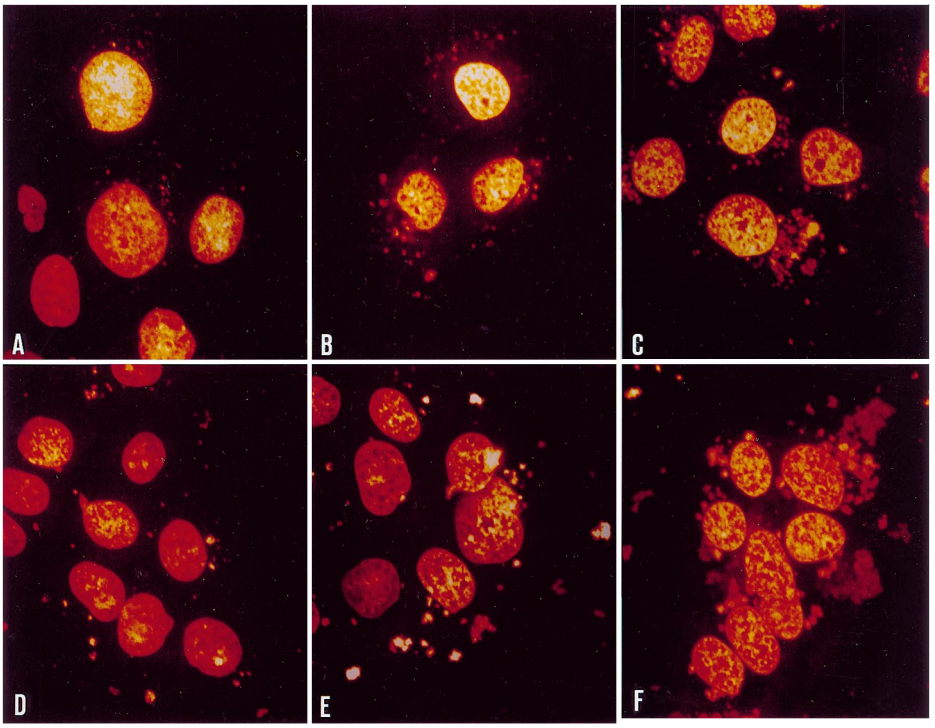

Fig. 5. Confocal laser scanning images, taken at different times, of tracheal epithelial cells transfected with DC-Chol–DOPE/DNA complexes. Liposome have been prepared from DC-Chol–DOPE lipid solutions of 1.2 mg/ml (A–C) and 3.6 mg/ml (D–F) concentrations. Liposome/DNA concentration ratio = 5:1 (M/M). The plasmid complexed with liposomes is pCMV–β-gal and it has been labeled with SYBR Green dye. Transfected cells are tracheal epithelial 56FHTE8o$^-$ cells. Micrographs have been taken at 0.5 h (A,D), 1.5 h (B,E), and 3.0 h (C,F), after addition of liposomes/DNA complexes to the culture medium. ×70.

concentration was kept constant, while that of liposome was varied. At low liposome/DNA ratios (0.6 and 1.6), the level of β-gal expression showed a steady increase with time. In contrast, at liposome/DNA ratio of 3.8, the maximum transfection efficiency was reached after 4 h, and then it remained constant. At a higher liposome/DNA ratio (7.6), a decrease of the β-gal activity with time was detected. This reduced expression most likely reflects toxicity, which is increasing in cells exposed to higher liposome concentrations, confirming results shown in Fig. 1. Briefly, the transfection rate increases, as the liposome/DNA concentration ratio tends to increase. However, the transfection efficiency tends to the same value for all the liposome/DNA concentration ratios with increasing time.

3.2. *Structural analysis of DC-Chol–DOPE liposomes by TEM, of DC-Chol–DOPE/DNA complexes by CLSM, and study of the intracellular distribution by CLSM of complexes*

To acquire further insights into the relationship between lipid solution concentration and transfection activity, we examined the morphology of DC-Chol–DOPE liposomes, prepared from two different lipid concentrations, namely 1.2 and 3.6 mg/ml, and the intracellular distribution of DC-Chol–DOPE/DNA complexes, prepared from the two different liposome populations. TEM analysis has shown differences both in shape and in size between examined preparations; in particular, liposomes prepared from the lipid solution of 1.2 mg/ml have an heterogeneous size distribution of 50–200 nm (Fig. 3A), while vesicles prepared from the lipid solution of 3.6 mg/ml displayed a more homogeneous 50 nm average size distribution (Fig. 3B).

Fig. 4 shows structures of DC-Chol–DOPE/DNA complexes observed by CLSM. Lipoplexes prepared from liposomes (1.2 mg/ml) are small and homogeneous (Fig. 4A), while lipoplexes prepared from liposomes (3.6 mg/ml) are larger with a heterogeneous size distribution (Fig. 4B). Both complexes revealed fluorescence in 56FHTE8o$^-$ cells after 30 min of transfection, with a maximum of signal intensity up to 3 h (Fig. 5). However, fluorescent spots were predominantly distributed around the perinuclear region when liposomes prepared from lipid concentration of 1.2 mg/ml were used for transfection, while a random aggregation was observed when liposomes prepared from lipid concentration of 3.6 mg/ml were used. Interestingly, only cells transfected with liposomes prepared from lipid concentration of 1.2 mg/ml showed a progressive increase of nuclear fluorescence intensity (Fig. 5A–C). No signs of nuclear fragmentation were detected at both concentrations used (Fig. 5).

4. Discussion

Cationic liposome-based gene delivery is at present one of the most effective and least toxic means of non-viral gene transfer [16,17]. Cationic liposomes are non-immunogenic, non-inflammatory and quite applicable in a variety of gene therapy protocols [18,19]. Evaluation of cationic lipids and liposomes usually involves comparison of DNA transport properties of different lipids and liposome formulations. In fact, intrinsic physicochemical properties of liposome/DNA complexes and cell-type-specific factors influence DNA uptake or gene expression [20–24]. In this respect, complexation process was shown to be thermodynamically as well as kinetically controlled [25]. The activity of individual liposome formulations is very different throughout gene transfer protocols, depending on their structure and charge, on different properties of DNA complexation and microenvironment encountered by the liposome/DNA complexes during transfection [20–24]. The charge ratio is considered fundamental for optimizing in vitro transfection protocols [4]. This is confirmed in this study by the behavior of DOTAP–DOPE/DNA complexes, which show a correlation between increase of the lipid (L) to DNA (D) ratio and transfection efficiency (1000 pg β-gal/μg protein vs. 2300 pg, β-gal/μg protein for L/D ratios of 0.4 and 1.09, respectively). A further increase of this ratio results in decreased transfection efficiency, probably due to the high instability of complexes with respect to size at cationic liposome/DNA molar ratios between 1.3 and 5.0 [26]. This effect appears less perceptible for DC-Chol–DOPE/DNA complexes. In this case, when L/D ratio increases from L/D = 0.6 to

L/D = 3.8, a continuous increase from 3070 to 4000 pg β-gal/μg protein is detected. The different behavior of DC-Chol–DOPE/DNA complexes could be explained according to considerations of Safinya et al. [27]. These authors investigate the correlation between the stoichiometric charge neutrality of lipid and DNA and the structure of liposome/DNA complexes. In fact, when the liposome/DNA ratio is positive, the complexes are positive and are observed to strongly repel each other, thus remaining as individual or a few linked globules. On the other hand, when the complexes are near the isoelectric point, the individual globules tend to stìck when they collide, due to van der Waals' attractions overcoming weak electrostatic repulsion, leading to larger aggregates of globules. However, at a high liposome/DNA concentration ratio, even DC-Chol/DOPE shows a progressive reduction of expression, which probably reflects lipid toxicity since cells are exposed to high complex concentrations for longer period of time. Cytotoxic effects detected after long incubation times could not be pivotal in vivo since it is likely that any target cell is exposed to the complexes only for a short time. Therefore there is a demand to maximize the chances of transfection during this time [4]. However, we noted an inhibition of gene expression at high liposome concentration without obvious cell damage, suggesting an intrinsic failure of high concentrated liposome solution to efficiently transfect 56FHTE8o$^-$ epithelial cells. It has been suggested that a high liposome concentration induce the formation of non-transfectable aggregates of liposome/DNA complex [4]. In this study, we documented that lipid concentration induces morphologic changes of liposomes, with important consequences on their transport properties. The apparent reversal of the size and uniformity of the particles in Fig. 3 compared to Fig. 4 is of interest and merits investigation. However, the literature reports data on the variation on liposome dimension, due to interaction with macromolecules. For example, the interaction of DPPC-Chol vesicles with ascorbate oxidase reduced the mean diameter of the vesicles by about 50%, perhaps through a change in surface tension [15,31]. Complexes prepared from liposomes obtained by a solution of DC-Chol–DOPE lipid of 1.2 mg/ml concentration appear small and homogeneous in size, leading to a transfection efficiency of 4000 pg of β-gal/μg protein, whereas complexes prepared from liposomes obtained by the more concentrated lipid solution (3.6 mg/ml), show a heterogeneous size distribution and are about 4-fold less active. The greater transfection efficiency achieved with the smaller sized and more homogeneously distributed liposome/DNA complexes probably reflects a more facilitated entry of the complexes into the epithelial cells by endocytosis process [1]. This hypothesis is confirmed by the intracellular distribution of the small and homogeneous complexes, which are localized around the perinuclear region, as previously reported [28–30]. In contrast, large and heterogeneous complexes are essentially restricted to cytoplasm.

We have demonstrated that DC-Chol–DOPE/DNA complexes efficiently transfect human tracheal epithelial cells, which are targets for a number of current gene therapy protocols. Moreover, we have demonstrated that the transfection efficiency of DC-Chol–DOPE/DNA complexes dramatically depends on the liposome to DNA concentration ratios. Although it is difficult to extrapolate conditions and formulations from in vitro experiments to in vivo setting of gene therapy protocols, our results provide important insights into the delivery of DNA to human epithelial cells.

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