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RESEARCH ARTICLE

Interaction of Liposomal and Polycationic Transfection Complexes with Pulmonary Surfactant

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Abstract

Background The delivery of genes to the airways holds promise for the treatment of lung diseases such as cystic fibrosis and asthma. Current non-viral gene delivery systems lack sufficient transfection efficiency. Pulmonary surfactant has been reported to be a barrier to gene transfer into the airways. Here we analyze the interaction of liposomal and polycationic transfection complexes with pulmonary surfactant.

Methods The efficiency of non-viral transfection of cultured human airway epithelial cells (16HBE14o−), COS7 cells and porcine primary airway epithelial cells was studied in the presence of various surfactant preparations in order to model the conditions prevailing in the airways during transfection.

Results The natural pulmonary surfactant, Alveofact, an extract from bovine lung lavage, was found to inhibit lipofection with lipofectAMINE for all cell lines investigated. Dendrimer meditated polyfection was unaffected for pulmonary cell lines and was weakly affected for COS7 cells. PEI-mediated polyfection was unaffected for all cell lines tested. The synthetic surfactant preparation Exosurf containing L-α-phosphatidylcholine-dipalmitoyl (DPPC) as the sole lipid ingredient had no statistically significant effect on polymer- and lipid-mediated transfection. The transfection efficiencies are related to structural changes in the DNA complexes as demonstrated by DNase-accessibility tests and fluorescence spectroscopy. In the presence of the phospholipid POPG, which is a constituent of Alveofact, DNA condensed in lipofectAMINE lipoplexes became accessible to DNaseI, while DNA condensed with PAMAM dendrimer or PEI was less accessible to DNase I as compared to lipoplexes. Consistently, the fluorescence of a DNA-intercalating dye increased after addition of Alveofact only in the case of lipoplexes.

Conclusions In contrast to lipofection, gene transfer with cationic polymers to airway epithelial cells is not inhibited by pulmonary surfactant *in vitro*. Depending on the surfactant concentration even an increase in polymer-mediated transfection can be seen. In conclusion, cationic polymers appear to be the more stable gene delivery systems for topical application into the airways. Copyright © 1999 John Wiley & Sons, Ltd.

Keywords gene therapy; gene transfer; lipofection; cystic fibrosis; dendrimer; polyfection; polyethylenimine; surfactant; DNase

Abbreviations ASF: airway surface fluid; BSA: bovine serum albumin; CF: cystic fibrosis; CVM: cytomegalovirus; DMSO: dimethylsulfoxide; DOPE: dioleoyl phosphatidyletyhanolamine; DOSPA: 2,3-dioleyloxy-*N*-(2-(spermine-carboxamido)ethyl)-*N*,*N*-dimethyl-1-propanaminium-trifluoroacetate; DPPC:

L-α-phosphatidylcholine-dipalmitoyl; DPPE: L-α-phosphatidylethanolamine-dipalmitoyl; DPPG: L-α-phosphatidyl-DL-glycerol-dipalmitoyl; DMEM: Dulbecco's modified Eagle medium; FCS: fetal calf serum; HBS: HEPES-buffered isotonic saline; MEM: Eagle's minimal essential medium; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PEI: polyethylenimine; PG: phosphatidylglycerol; POPC: L-α-phosphatidylcholine-β-oleoyl-γ-palmitoyl; POPG: L-α-phosphatidyl-DL-glycerol-β-oleoyl-γ-palmitoyl; POPE: L-α-phosphatidylethanolamine-β-oleoyl-γ-palmitoyl; PPAE: Porcine primary airway epithelial; RLU: relative light units; Opti-MEM I: serum reduced minimal essential medium; PAMAM: polyamidoamine; PBS: phosphate-buffered saline.

Introduction

Effective gene transfer into airway epithelial cells is a prerequisite for gene therapy of lung diseases such as cystic fibrosis (CF) and asthma. Viral as well as non-viral gene vectors have been applied to the airways by bronchoscopic instillation or aerosolization. Clinical trials directed at gene therapy of CF in humans have been swiftly initiated after the feasibility of the approach had been demonstrated in animals [1]. However, at present, non-viral delivery systems still suffer from the considerably low transfection rates in airway epithelial cells, the target tissue for gene therapy of CF. Animal studies showed that naked plasmid DNA transfected equally well upon instillation into the airways as did first generation lipoplexes [2]. In cell culture, however, lipoplexes perform orders of magnitude better than naked plasmid DNA. This discrepancy was found to be in part due to the interaction of lipoplexes with pulmonary surfactant [3]. Pulmonary surfactant is a composition of phospholipids, mostly phosphatidylcholine (PC) and phosphatidylglycerol (PG), and specific surfactant proteins SP-A, SP-B, SP-C and SP-D, which are synthesized and secreted by type II alveolar cells. Surfactant components are found in the airway surface fluid (ASF) of the respiratory epithelium and constitute a potential barrier of gene delivery to airway epithelial cells. DNA complexed to cationic liposomes is resistant to DNase. However, DNA is released from cationic liposomes in the presence of anionic lipids and acquires a DNase-sensitive conformation [4]. This phenomenon may account for the low efficiency of lipofection of the airways after topical application.

Polyplexes prepared from polycationic polymers, such as polyamidoamine (PAMAM) dendrimers or polyethyleneimine (PEI) and DNA are an alternative type of non-viral delivery system. These polymers display high transfection rates *in vitro* [5] and are promising vectors for *in vivo* applications.

In this study we compared the impact of surfactant preparations on the conformational state of lipofectamin lipoplexes, dendrimer polyplexes and PEI polyplexes by fluorescence spectroscopic methods and a DNaseI accessibility assay and correlate our findings with gene transfer efficiency data obtained *in vitro*.

Materials and methods

Chemicals

LipofectAMINE™, a 3 : 1 (w/w) mixture of the cationic lipid 2,3-dioleoyloxy-*N*-(2-(sperminecarboxamido)ethyl)-*N*, *N*-dimethyl-1-propanaminium-trifluoroacetate (DOSPA) and the neutral lipid dioleylphosphatidylethanolamine (DOPE) were purchased from GIBCO-BRL (Gaithersburg, MD, USA). Polyamidoamine (PAMAM) dendrimers, spheroidal polycationic polymers, were generously provided by Francis C. Szoka (University of California at San Francisco, CA, USA). Polyethylenimine (PEI) (average mol. wt 25 kDa) was obtained from Aldrich. Alveofact was purchased from Thomae, Germany. Alveofact is obtained from extraction of calf lungs and consists of 90% phospholipids. The most abundant phospholipids are zwitterionic PC (81.5% of lipid content) and the anionic PG (11.5% of lipid content) and about 1% by weight of protein, mostly surfactant proteins SP-B and SP-C (Dr von Seefeld, Dr Weller, Boehringer Ingelheim, Biberach, Germany, personal communication). The glycoproteins SP-A and SP-D are removed during the preparation of Alveofact. Exosurf (13.5 mg/ml DPPC, 1.5 mg/ml of hexadecanol, and 1 mg/ml tyloxapol) was purchased from Glaxo Wellcome Inc. (Research Triangle Park, NC, USA). Concentrations of Alveofact and Exosurf refer to the overall phospholipid and dipalmitoylphosphatidylcholine (DPPC) content of the preparations, respectively. The pure lipids L-α-phosphatidyl-DL-glycerol-dipalmitoyl (DPPG), L-α-phosphatidyl-DL-glycerol-β-oleoyl-γ-palmitoyl (POPG), L-α-phosphatidylcholine-dipalmitoyl (DPPC), L-α-phosphatidylcholine-β-oleoyl-γ-palmitoyl (POPC), L-α-phosphatidylethanolamine-dipalmitoyl (DPPE) and L-α-phosphatidylethanolamine-β-oleoyl-γ-palmitoyl (POPE), and DNaseI were purchased from Sigma-Aldrich (Deisenhofen, Germany).

Cell lines and primary cells

The cell line 16HBE14o− derived from human respiratory epithelial cells was generously provided by Dieter C. Gruenert (University of California at San Francisco, CA, USA). COS7 cells were purchased from the DSMZ-German Collection of Microorganisms and Cell Cultures, Dept of Human and Animal Cell Cultures. Porcine primary airway epithelial (PPAE) cells were prepared from trachea according to the procedure described by Yamaya *et al.* [6]. Briefly, after pulling off the surface epithelium, cells were dissociated by incubation with 0.4 mg/ml protease type XIV (Sigma-Aldrich, Deisenhofen, Germany) in phosphate-buffered saline (PBS) at 4°C overnight. Then, the enzyme was inhibited by addition of fetal calf serum (FCS) at a final concentration of 2.5%.

After removal of the denuded strips, the cells were pelleted ($200 \times g$, for 5 min), resuspended in a 1 : 1 mixture of Dulbecco's modified Eagle medium (DMEM) and Ham's F-12 containing 5% FCS or 2% Ultroser G serum substitute (Gibco/BRL, Eggenstein, Germany) and 50 μg/ml gentamycin as an additional antibiotic and plated on tissue culture dishes coated with 20 μg/cm^2 of calf skin collagen type I (Sigma-Aldrich, Deisenhofen, Germany). On the first day after plating, insulin (10 μg/ml), hydrocortisone (0.4 μg/ml), triiodothyronine (20 ng/ml), epidermal growth factor (25 ng/ml), endothelial cell growth supplement (7.5 μg/ml) and transferrin (5 μg/ml) were supplemented. All growth factors were obtained from Sigma-Aldrich (Deisenhofen, Germany) except transferrin, which was purchased from Gibco-BRL.

Cell culture

All cells were cultivated at 37°C in 5% CO_2 humidified atmosphere in media containing 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin if not otherwise stated. 16HBE14o− cells were cultivated in Eagle's minimal essential medium (MEM, Gibco BRL) and COS7 cells in DMEM (Gibco-BRL).

Plasmids

The plasmid pCMVluc containing the *Photinus pyralis* luciferase gene [7] under the control of the cytomegalovirus (CMV) promoter in the pCMVluc plasmid was used as a reporter. This plasmid was a generous gift from E. Wagner (Boehringer Ingelheim, Vienna, Austria). The plasmid was propagated in *E. coli* and purified using the Maxi Prep kit from Qiagen (Hilden, Germany).

The plasmid pEGFP-N1 was obtained from Clontech Laboratories (Palo Alto, CA, USA).

Lipids and lipid preparations

Stock solutions of DPPG, POPG, DPPC, POPC and POPE were prepared at a final concentration of 50 mM and DPPE at 100 mM. DPPG was suspended in HEPES-buffered isotonic saline (HBS). POPG, DPPC, POPC and POPE were dissolved in chloroform, evaporated *in vacuo* and rehydrated in HBS buffer. DPPE was diluted in dimethylsulfoxide (DMSO). Before use all lipids were homogenized by 14 pulses (40%) with a Branson sonifier W-250 (Level 4) using a microtip. Lipid preparations and individual lipids were added to preformed transfection complexes immediately prior to incubation with cells.

Transfection

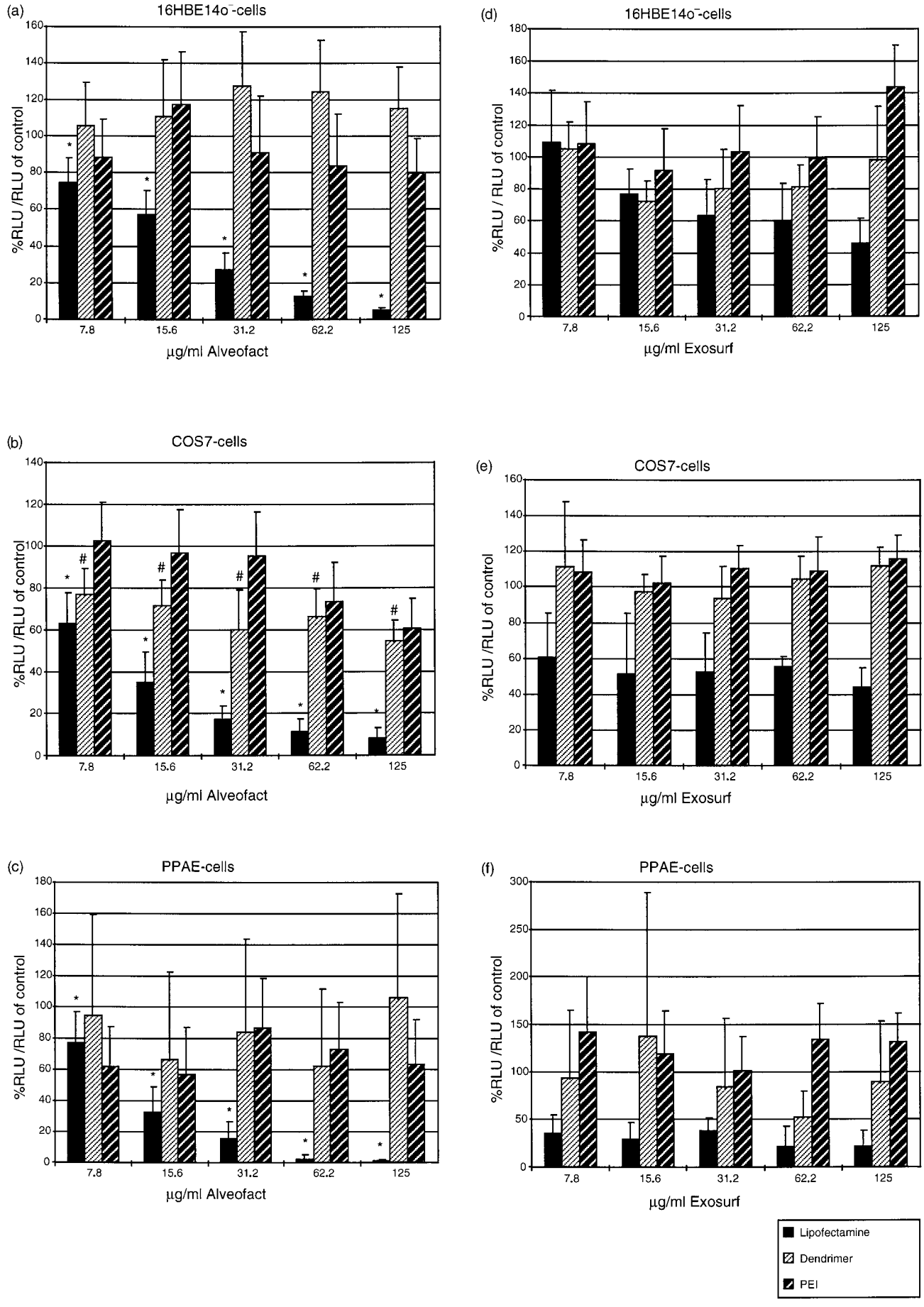
For transfection experiments, cells were seeded into 96-well tissue culture plates (30 000 cells/well) which is a density appropriate to yield 40–50% confluency on the next day. Prior to transfection, cells were washed twice with serum-free medium and the supernatant was removed. The cells were transfected with lipoplex or polyplex preparations at concentrations and ratios that had previously been determined to be optimal for transgene expression.

Lipoplexes were prepared by mixing 0.18 μg of DNA dissolved in 20 μl Opti-MEM I (GibcoBRL) with 1.92 μg of LipofectAMINE dissolved in 20 μl serum reduced minimal essential medium (Opti-MEM I) (DNA : lipid = 1 : 10.7, w/w). The mixture was incubated for 30 min at 37°C to allow formation of lipoplexes. Then, surfactant preparations or phospholipids in 20 μl of Opti-MEM I or Opti-MEM I alone were added. For transfection, 50 μl of the resulting mixture corresponding to 0.15 μg DNA and 1.6 μg of lipofectAMINE (DNA : lipid = 1 : 10.7 w/w) per well were added to the cells. Cells were incubated for 1 h at 37°C. Then, 200 μl of the appropriate growth media containing 10% FCS and antibiotics were added followed by incubation for 24 h.

Dendrimer–DNA polyplexes were prepared according to the method described by Haensler and Szoka [5]. Briefly, dendrimer polyplexes were formed by mixing 0.84 μg DNA in 20 μl HBS (150 mM NaCl, 10 mM HEPES/Na pH 7.3) and 1.68 μg of dendrimers in 20 μl HBS (1 : 2, w/w) and then incubated at room temperature for 20 min. PEI polyplexes were formed by mixing 1.2 μg DNA in 20 μl HBS and 0.84 μg of PEI in 20 μl HBS (1 : 0.7, w/w) and then incubated at room temperature for 20 min. Surfactant preparations or phospholipids in 20 μl of HBS or HBS alone were then added to the complex solutions. 50 μl of the resulting mixture corresponding to 0.7 μg of DNA and 1.4 μg of dendrimers (or 1.0 μg of DNA and 0.7 μg of PEI) were added per well containing cells and 150 μl of Opti-MEM I. The supernatant was removed 4 h after the addition of polyplexes and replaced by fresh medium containing 10% FCS. Cells were incubated for 24 h and then lysed by addition of 100 μl of lysis buffer (250 mM Tris, pH 7.8, 0.1% Triton X-100) and frozen at −70°C. Protein content and specific luciferase activity were determined using 10 μl of the lysates each. In some experiments Alveofact, Exosurf or individual phospholipids were added to the transfection medium in increasing concentrations to assess their effects on *in vitro* gene transfer. Luciferase activity was normalized to the control samples within each experiment. For 16 HBE14o− cells, the average control luciferase activity was $1.8 \pm 1.3 \times 10^8$ relative light units (RLU)/mg protein when using lipofectAMINE, $2.0 \pm 0.8 \times 10^9$ RLU/mg for dendrimer-mediated transfection and $4.1 \pm 1.2 \times 10^8$ RLU/mg protein when using PEI, for COS7 cells the results were $2.7 \pm 1.7 \times 10^9$, $3.8 \pm 1.7 \times 10^9$ and $2.9 \pm 1.9 \times 10^9$ RLU/mg respectively, and for PPAE were $1.2 \pm 0.8 \times 10^7$, $9.4 \pm 8.4 \times 10^6$ and $1.0 \pm 0.9 \times 10^7$ RLU/mg respectively.

Luciferase assay

Transfection efficiency was determined as luciferase expression using the Luciferase Assay System kit from



Promega (Heidelberg, Germany). Light units were determined with a Packard Topcount microplate scintillation counter (Canberra Packard, Dreieich, Germany). Protein concentration was measured according to Bradford [8] with bovine serum albumin (BSA) as the standard using the reagent from Biorad (Munich, Germany). Data were analyzed for statistical significance using the statistical analysis software Primer of Biostatistics by Stanton Glantz (University of California at San Francisco, CA, USA). For each data set one-way analysis of variance with correction for multiple comparison was performed. A difference is considered significant at $p < 0.05$.

DNase sensitivity assay

In the first experiment (Figure 3) restriction fragments of pEGFP-N1 DNA (GenBank Accession No. U55762, 4 and 0.7 kb generated by restriction with EcoRI and NotI) were complexed to polyamidoamine dendrimeres and lipofectAMINE at a ratio of 1 : 2 (w/w) and 1 : 6 (w/w), respectively, and exposed to DNaseI (2.2 ng or 0.05 U/μg DNA) at 37°C for 1 h in the absence or presence of POPG at the indicated concentrations. The reaction was terminated by the addition of sodiumdodecylsulfate to a final concentration of 5% and incubation for 15 min. After the addition of NaCl to a final concentration of 0.5 M, DNA was recovered from the aqueous phase of two subsequent phenol/chloroform extractions and a final extraction with chloroform, precipitated with two volumes of ethanol, separated by 1% agarose/TAE gel electrophoresis and visualized by ethidiumbromide staining. In the second experiment (Figure 4) restriction fragments of pEGFP DNA (GenBank Accession No. U55762, 4 and 0.7 kb generated by restriction with EcoRI and NotI) were complexed to polyamidoamine dendrimeres, PEI and lipofectAMINE at a ratio of 1 : 2 (w/w), 1 : 0.7 (w/w) and 1 : 10.7 (w/w), respectively, and exposed to DNaseI (670 ng or 15.2 U/μg DNA) at 37°C for 1 h in the absence or presence of POPG at the indicated concentrations.

Fluorescence assay

The fluorescent bisintercalator TOTO-1 was used to monitor the formation of complexes in the presence of cationic lipids or cationic polymers. The quenching of the strong fluorescence centered at 535 nm can be taken as a measure for complex formation and *vice versa* the recovery of the fluorescence as an indication for complex disassembly [9]. In a typical fluorescence assay a 2.5 μg/ml pCMVluc DNA solution in Opti-MEM I stained at a dye to basepair ratio (1 : 20) was used. The fluorescence was monitored in a Spex fluorimeter using a SUPASIL Hellmanex cuvette with 3-mm light path. The excitation wavelength was 490 ± 1 nm and the emission 535 ± 1 nm. The cationic agents were carefully pipetted and stirred into the DNA solution. After 30 min the relative fluorescence I/I_0 was monitored with I_0 being the fluorescence intensity of the initial DNA solution. Then Alveofact and Exosurf were added, respectively. The sample was allowed to equilibrate for 3 min before each fluorescence measurement.

Results

Effect of pulmonary surfactant and Exosurf on transfection mediated by lipofectAMINE, dendrimers and PEI

The effect of pulmonary surfactant (Alveofact) and Exosurf on the transfection efficiencies of lipofectAMINE–DNA, or dendrimer–DNA complexes or PEI–DNA complexes was assessed *in vitro* by adding increasing concentrations of Alveofact or Exosurf to the cell lines prior to incubation with the transfection solutions. Figure 1 summarizes the results. In each diagram the efficacy of lipid- and polymer-based transfection are compared. The study was carried out on three cell types. Alveofact inhibited the lipofectAMINE-mediated transfer of the luciferase gene to all three cell lines tested (Figure 1). Dendrimer-mediated transfection was not inhibited by Alveofact for pulmonary cell lines, while transfection of COS7 cells was inhibited to 55% of control at the highest concentration of Alveofact (125 μg/ml). Dendrimer-mediated transfection was unaffected in the presence of Exosurf for all three cell lines tested. PEI-mediated transfection was neither inhibited by Alveofact nor Exosurf. However, there was inhibition of lipofection in the presence of Exosurf in all three cell lines tested, but this did not reach statistical significance.

Figure 1. Inhibition of liposome, dendrimer- and PEI-mediated transfection by surfactant preparations. Alveofact (a, b, c) or Exosurf (d, e, f) were added to preformed complexes of pCMVLuc DNA and lipofectAMINE (solid bars), dendrimers (hatched bars) or PEI (solid hatched bars) at the indicated concentrations immediately prior to incubation with 16HBE14o− (a, d), COS7 (b, e) or porcine primary airway epithelial (PPAE) cells from trachea (c, f). Specific luciferase activities (RLU/mg total protein) of cell lysates were determined 24 h after transfection. Relative transfection efficiencies are given in % of control experiments performed in the absence of surfactant preparations. Microgram per milliliter concentrations of Alveofact and Exosurf refer to the overall lipid content and DPPC content, respectively. Bars represent the mean of independent experiments ±standard deviation (number of experiments = 5). Absolute specific luciferase activities determined in control experiments averaged up to $1.8 \pm 1.3 \times 10^8$, $2.0 \pm 0.8 \times 10^9$ and $4.1 \pm 1.2 \times 10^8$ RLU/mg (16HBE14o−), $2.7 \pm 1.7 \times 10^9$, $3.8 \pm 1.7 \times 10^9$ and $2.9 \pm 1.9 \times 10^9$ RLU/mg (COS7) and $1.2 \pm 0.8 \times 10^7$, $9.4 \pm 8.4 \times 10^6$ and $1.0 \pm 0.9 \times 10^7$ RLU/mg (primary epithelial cells) for lipofectAMINE, dendrimer-mediated and PEI-mediated transfections, respectively. *$p < 0.05$ vs RLU of control for lipofectAMINE-mediated gene transfer; #$p < 0.05$ vs RLU of control for dendrimer-mediated gene transfer

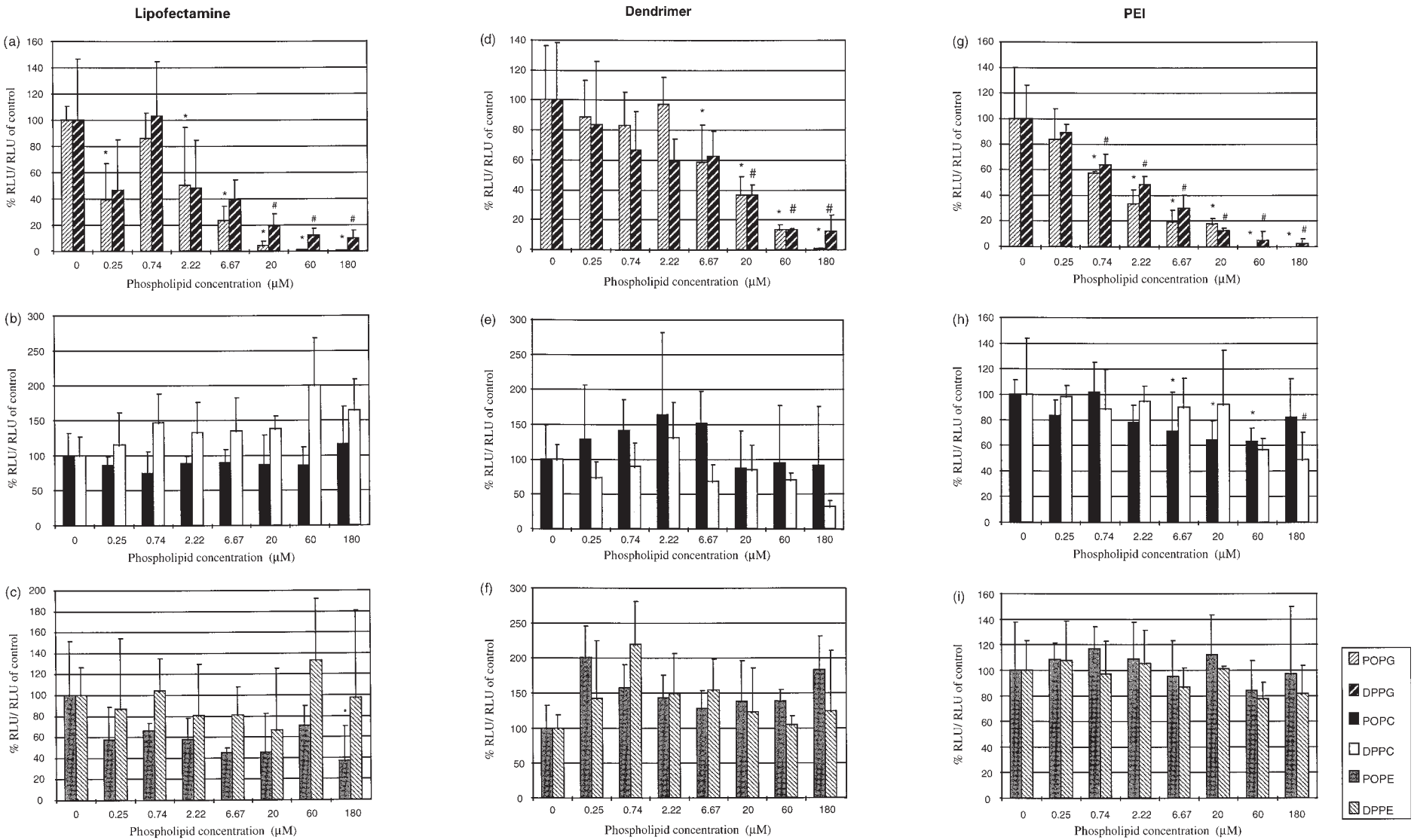


Figure 2. Inhibition of liposome, dendrimer-mediated or PEI-mediated transfection by anionic and neutral lipids. 16HBE14o− cells were transfected with pCMV-Luc complexed with lipofectAMINE (a)–(c) or dendrimers (d)–(f) or PEI (g)–(i) in the presence of DPPG, POPG, DPPC, POPC, DPPE or POPE at the indicated concentrations. Specific luciferase activity was determined after 24 h. Bars represent RLU/mg protein of one experiment (repeated twice with similar findings) relative to control transfections in the absence of lipids. (number of experiments = 5). *$p < 0.05$ vs RLU of control for lipofectAMINE-mediated gene transfer; #$p < 0.05$ vs RLU of control for dendrimer-mediated gene transfer

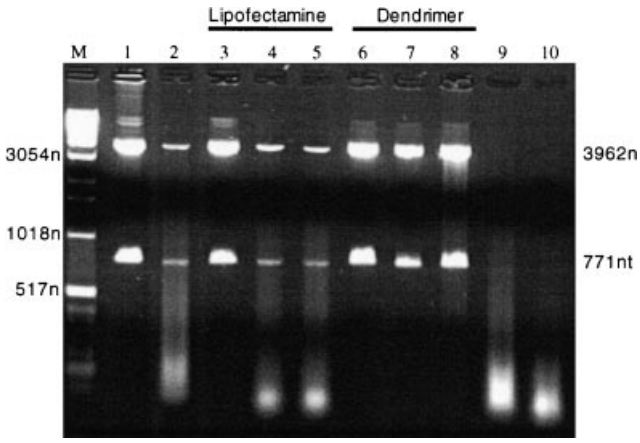


Figure 3. Digestion of restricted plasmid DNA (pEGFP-N1) by DNaseI (2.2 ng or 0.05 U/μg DNA) in complexes treated with various POPG-concentrations (1.7 mM POPG and 8.3 mM POPG). Preformed dendrimer–DNA and lipofectAMINE-DNA complexes (formed at a 2/1 dendrimer/DNA w/w ratio and 6/1 lipofectAMINE/DNA w/w ratio) were mixed with POPG and then subjected to DNaseI digestion for 4 h.

M: molecular marker
1: pEGFP-N1 (restricted with EcoRI and NotI)
2: pEGFP-N1 + DNaseI
3: pEFGP-N1 complexed with LipofectAMINE + DNaseI
4: pEFGP-N1 complexed with LipofectAMINE + 1.7 mMPOPG + DNaseI
5: pEFGP-N1 complexed with LipofectAMINE + 8.3 mMPOPG + DNaseI
6: pEFGP-N1 complexed with dendrimer + DNaseI
7: pEFGP-N1 complexed with dendrimer + 1.7 mMPOPG + DNaseI
8: pEFGP-N1 complexed with dendrimer + 8.3 mMPOPG + DNaseI
9: pEGFP-N1 + 1.7 mMPOPG + DNaseI
10: pEGFP-N1 + 8.3 mMPOPG + DNaseI

Effect of neutral and anionic lipids on transfection efficiency

Figure 2 summarizes the results for the transfection efficiencies of lipofectAMINE–DNA, dendrimer–DNA or PEI–DNA complexes in the presence of saturated and unsaturated lipids. In each diagram the influence of saturated and unsaturated lipids is compared. The study was carried out on three types of lipid head groups, i.e. PG, PC and PE. Neutral lipids at concentrations of up to 180 μM had little or no effect on lipofection (Figure 2b and c). Dendrimer-mediated gene transfer was again not inhibited in the concentration range tested (Figure 2e and f). PEI-mediated gene transfer showed a weak inhibition at the higher concentrations of PCs. In contrast to the neutral lipids there was a strong inhibition of liposomal and polymer-mediated transfection with PGs (Figure 2a, d and g).

However, in the presence of Alveofact at concentrations corresponding to PG concentrations of 20 μM (125 μg/ml) dendrimer-mediated and PEI-mediated gene transfer was largely unaffected (Figure 1a). Obviously, the inhibitory effect of pure PGs on dendrimer-mediated and PEI-mediated polyfection was neutralized or compensated by other constituents of Alveofact.

DNase susceptibility of DNA complexed with polymeric cations in the presence of POPG

The release of DNA from lipofectAMINE lipoplexes, PEI-complexes and polyamidoamine–dendrimer com-

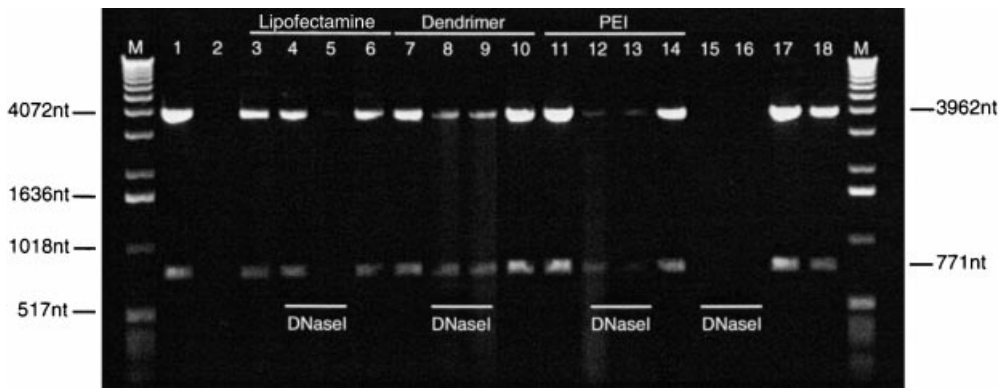


Figure 4. Digestion of restricted plasmid DNA (pEGFP-N1) by DNaseI (670 ng or 15.2 U/μg DNA) in complexes treated with a 0.9 mM POPG-concentration. Preformed dendrimer–DNA complexes (formed at a 2/1 dendrimer/DNA w/w ratio), PEI-DNA complexes (1 : 0.7 w/w ratio) and lipofectAMINE–DNA (1 : 10.7 w : w) were mixed with POPG and then subjected to DNAseI digestion for 4 h.

M: molecular marker
1: pEGFP-N1 (restricted with EcoRI and NotI)
2: pEGFP-N1 + DNaseI
3: pEGFP-N1 + LipofectAMINE
4: pEGFP-N1 + LipofectAMINE + DNaseI
5: pEGFP-N1 + LipofectAMINE + POPG + DNaseI
6: pEGFP-N1 + LipofectAMINE + Alveofact (125 μg/ml)
7: pEGFP-N1 + Dendrimer
8: pEGFP-N1 + Dendrimer + DNaseI
9: pEGFP-N1 + Dendrimer + POPG + DNaseI
10: pEGFP-N1 + Dendrimer + Alveofact
11: pEGFP-N1 + PEI
12: pEGFP-N1 + PEI + DNaseI
13: pEGFP-N1 + PEI + POPG + DNaseI
14: pEGFP-N1 + PEI + Alveofact
15: pEGFP-N1 + POPG + DNaseI
16: pEGFP-N1 + Alveofact + DNaseI
17: pEGFP-N1 + POPG
18: pEGFP-N1 + Alveofact

plexes in the presence of anionic lipid (POPG) was assayed in terms of accessibility to DNase attack. The integrity of restricted pEGFP plasmid DNA complexed to lipofectAMINE, dendrimer or PEI after exposure to DNaseI in the presence of the anionic lipid is shown in Figures 3 and 4. In control experiments, naked plasmid DNA exposed to the same concentrations of DNase was degraded, whereas DNA condensed to lipofectAMINE was not (Figure 3 and 4). DNA complexed to dendrimers or PEI showed some degradation at a high DNase concentration (Figure 4). In the presence of POPG, DNA complexed with lipofectAMINE was completely degraded in contrast to DNA complexed with dendrimers or PEI.

Effect of Alveofact and Exosurf on the release of DNA from its complexed state

The fluorescence of the DNA-binding fluorophore TOTO is quenched if DNA is complexed with cationic transfection agents. In Figure 5 the relative fluorescence intensity ratio is plotted with respect to the fluorescence of DNA–TOTO before condensation. It can be seen that lipofectAMINE and PEI quench 90% of the fluorescence. In contrast, dendrimers reduce the fluorescence to only 45%, indicating that the DNA is more loosely packed. The addition of lung surfactant alters the fluorescence of the intercalated fluorophore TOTO, if DNA packing is affected. This is the case for Alveofact added to lipofectAMINE–DNA complexes. The critical Alveofact concentration of about 60 μg/ml coincides with the critical concentration found to inhibit transfection. We have to assume that Alveofact reacts with lipofectAMINE complexes in a way which relaxes the DNA in the lipid condensed state or even releases the DNA entirely. DNA–PEI and DNA–dendrimer complexes exhibit unchanged fluorescence quenching up to Alveofact concentrations as high as 375 mg/ml.

Exosurf was found to have no effect on fluorescence quenching for all three types of complexes investigated. We explain this remarkable difference between Alveofact and Exosurf by the absence of anionic lipid in the latter while Alveofact contains about 10% PG lipids. The fact that anionic lipid shows the same effect as Alveofact on cationic lipid–DNA complexes is demonstrated using POPG liposomes under equivalent buffer conditions (Figure 5c).

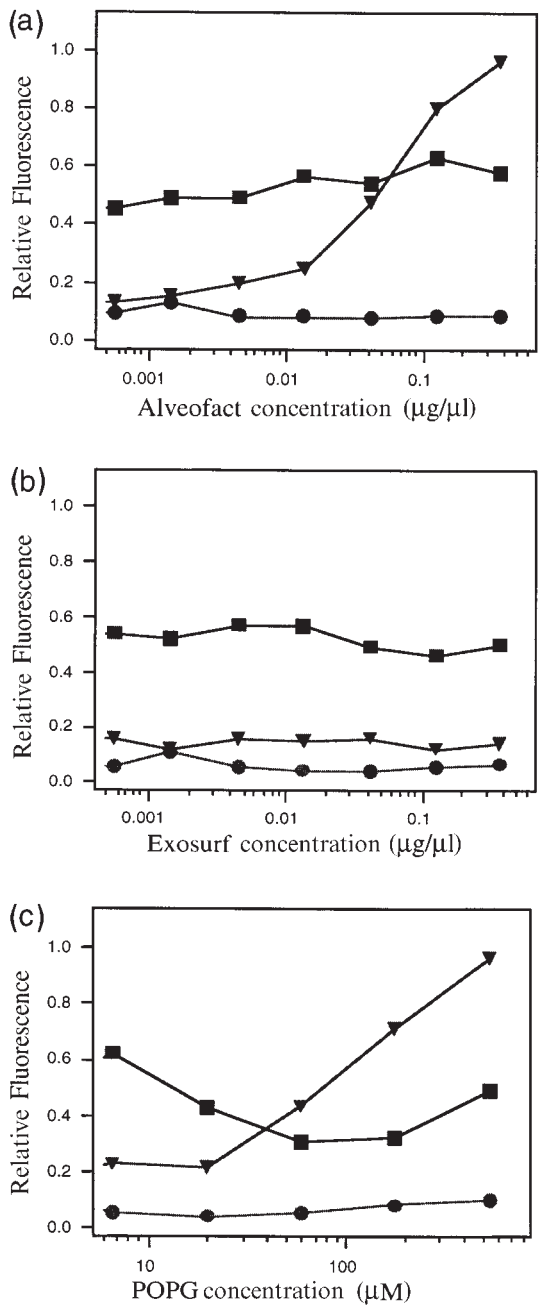


Figure 5. Effect of (a) Alveofact and (b) Exosurf on the relative fluorescence of DNA complexed with LipofectAMINE (▼), dendrimer (■) and PEI (●). The DNA was labeled with the fluorophore TOTO prior to aggregation. LipofectAMINE and PEI quench 90% of the DNA–TOTO fluorescence, dendrimers only 55%. The addition of Alveofact to LipofectAMINE–DNA complexes restores the fluorescence indicating the release of free DNA. In all other cases the fluorescence is uneffected by the lung surfactants

Discussion

Gene therapy of the pulmonary manifestations of CF requires targeting of gene vectors to the lung. The application modality via the airways allows the most direct access to the target cells which are airway epithelial cells while for other lung diseases access via the blood stream may be preferable. There is abundant literature on the application of viral and non-viral vectors to the lung, the best examined non-viral vectors being lipoplexes. First generation lipid vectors turned out to be not significantly more active in transfection of the lung than naked plasmid DNA. By mostly empirical methods, e.g. screening vast arrays of chemically different lipids and lipid formulations, lipoplexes have been identified with superior transfection efficiency in the lung upon topical application compared to naked plasmid DNA [10]. Despite this considerable progress with non-viral vectors

it is still an open question, among other unknowns, whether the comparatively low transfection efficiencies achievable with optimized lipoplexes will be sufficient to obtain a positive clinical outcome in gene therapy of CF.

Besides inefficient vectors, the actual major barrier to successful gene transfer to the lung is the lung physiology. In addition to the cellular obstacles such as the plasma and nuclear membranes, gene vectors have to pass the mucus layer and have to maintain their active structure upon interaction with the airway surface fluid in this organ. In this study we examined the interaction of a lipoplex (lipofectAMINE–DNA) and polyplexes (dendrimer–DNA or PEI–DNA) with two commercial preparations of lung surfactant, Alveofact and Exosurf, and their impact on vector efficiency in gene transfer to model cell cultures. We chose Alveofact as a model for lung surface fluid as it contains most of the natural phospholipids of the latter. Exosurf as a synthetic preparation contains only DPPC as the sole lipid ingredient and has been shown to enhance adenoviral transduction of the airways [11].

Based on previous work by others [3,4,12,13] we expected a transfection-limiting interaction of the surfactant preparations with the lipoplex. The mentioned authors had demonstrated that naturally-derived surfactant preparations [3] and Exosurf markedly inhibited liposome-mediated gene transfer [12]. Furthermore, it is known that anionic lipids are able to disassemble cationic lipid–DNA lipoplexes [4]. No such investigations were available for polyplexes.

In order to characterize more precisely the nature of the interactions of surfactant preparations with the gene vectors we performed a fluorescence quenching assay which monitors the ability of the fluorescent dye TOTO-1 to intercalate into DNA. As long as a lipoplex or polyplex remains tightly condensed no intercalation can take place and fluorescence is abolished. The addition of Alveofact to lipofectAMINE lipoplexes allowed the dye to intercalate which indicates a relaxation or even dissociation of the lipoplex in agreement with published results [14]. No such effect was observed for dendrimer or PEI polyplexes reflecting that DNA condensation is maintained upon incubation with Alveofact. No fluorescence increase was observed upon addition of Exosurf for both types of DNA complexes which was expected because Exosurf does not contain anionic lipids. In contrast, our results show that both interaction of lipoplexes as well as polyplexes with anionic phospholipids such as POPG, leads to a decreased transfection efficiency. One reason for this observation may be a change in the conformation of the complexes leading to different particle size and in consequence to decreased transfection rate. This might be the case for polyplexes. In addition, another possibility can be an increased susceptibility of the DNA moiety in lipoplexes to DNase degradation. DNA complexed to lipofectAMINE showed no degradation in the presence of even high amounts of DNase, but after adding, POPG degradation was observed even at low levels of DNase (Figure 3) and was complete at high levels of DNase (Figure 4). DNA complexed with polyplexes showed some degree of degradation in the presence of high DNase concentration, but adding POPG did not lead to an increase of degradation suggesting no additional relase of DNA from the polymer complexes. At the low DNase dose dendrimer–DNA was stable with and without POPG added.

From the biophysical studies it was to be expected that lipofection is inhibited by Alveofact but not or to a lesser extent by Exosurf while dendrimer and PEI polyfection should be less sensitive or even unaffected by both preparations. In fact, these were the observed trends as can be seen in Figure 1. The presence of Alveofact strongly inhibited lipofection in a dose-dependent manner while Exosurf had only a slightly inhibitory effect in one cell line examined (16HBE 14o−). Tsan *et al.* [12] examined the inhibitory effect of the surfactant preparation Survanta on the lipofection of lung cell lines. Our results with Alveofact, the composition of which is similar to Survanta, are in agreement with their observations while these researchers observed a strong inhibition of DDAB (dimethyldioctadecyl ammonium bromide) lipofection of rat type II alveolar epithelial cells [12] by Exosurf. Obviously, the impact of Exosurf on lipofection is dependent on the type of lipoplex used and the cell line examined. In this context it is interesting to note that Raczka *et al.* [15] found Exosurf to inhibit transfection of the airways by naked plasmid DNA which, based on its chemical composition, is unexpected.

Alveofact and Exosurf did not inhibit or slightly inhibited the dendrimer as well as PEI polyfection which indicates that their transfection-promoting structural characteristics are unaffected. It was a consistent observation that Alveofact even promoted transfection at certain Alveofact–polyplex ratios (column 3 in Figure 1a), an effect which can be the basis for optimizing dendrimer polyfection *in vivo*. To further characterize which components of Alveofact are responsible for the inhibitory effect, we examined the impact of individual lipids differing in headgroups and side chains on transfection. As expected from the DNAse sensitivity assay (Figure 3) and from published work by Xu *et al.* [4] and Zelphati *et al.* [13] anionic lipids strongly inhibited lipofection. With these lipids also polyfection was inhibited which can be explained by charge neutralization of polyplexes rather than by their disassembly. Non-viral gene vectors are believed to bind to cell surfaces primarily by charge interactions (except in receptor-mediated gene delivery). Interestingly, the inhibitory effect of the pure anionic lipids on polyfection is not observed when the same lipids at the same concentrations are presented to the complexes in the Alveofact matrix. Further studies are required to clarify this observation.

Zwitterionic lipids (DPPC, POPC, DPPE, POPE) had no statistically significant inhibitory impact on either lipofection or polyfection. The reduction in transfection efficiencies at the highest lipid concentrations can rather be attributed to toxic effects which have been observed visually. Phosphatidylethanolamines even

slightly enhanced polyfection but not to a statistically significant extent.

In summary, lipoplexes are inhibited in their potency to deliver genes to airway epithelial cells upon interaction with lung surfactant preparations and with lung surface fluid. DNA in lipoplexes is relaxed or released to such an extent that it becomes accessible to the action of nucleases similar to naked plasmid DNA. As one possible consequence, first generation lipoplexes may be as fragile as naked DNA in an *in vivo* surrounding that confronts them with free anionic lipids as is the case at the lung surface. In contrast, dendrimer and PEI polyplexes do not disintegrate upon surfactant interaction and maintain a more nuclease-resistant conformation. The inhibitory effect of anionic lipids on these vectors can probably be attributed to charge neutralization and the concomittant loss of binding capacity to cell surfaces while the other transfection-relevant biophysical characteristics should remain unchanged. Polyplexes offer a variety of possiblities to shield their surfaces from unwanted interactions such as modification with PEGs and similar compounds. From this we conclude that the basic core structure of a polyplex or also of lipo-polyplexes will ultimately turn out to be superior to pure lipoplexes in gene delivery to the airways.

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