Characterization of cationic lipid DNA transfection complexes differing in susceptibility to serum inhibition

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Abstract

Background: Cationic lipid DNA complexes based on DOTAP (1,2-dioleoyl-3-(trimethylammonium) propane) and mixtures of DOTAP and cholesterol (DC) have been previously optimized for transfection efficiency in the absence of serum and used as a non-viral gene delivery system. To determine whether DOTAP and DC lipid DNA complexes could be obtained with increased transfection efficiency in the presence of high serum concentrations, the composition of the complexes was varied systematically and a total of 162 different complexes were analyzed for transfection efficiency in the presence and absence of high serum concentrations.

Results: Increasing the ratio of DOTAP or DC to DNA led to a dose dependent enhancement of transfection efficiency in the presence of high serum concentrations up to a ratio of approximately 128 nmol lipid/µg DNA. Transfection efficiency could be further increased for all ratios of DOTAP and DC to DNA by addition of the DNA condensing agent protamine sulfate (PS). For DOTAP DNA complexes with ratios of ≤ 32 nmol/µg DNA, peak transfection efficiencies were obtained with 4 µg PS/µg DNA. In contrast, increasing the amount of PS of DC complexes above 0.5 µg PS/µg DNA did not lead to significant further increases in transfection efficiency in the presence of high serum concentrations. Four complexes, which had a similar high transfection efficiency in cell culture in the presence of low serum concentrations but which differed largely in the lipid to DNA ratio and the amount of PS were selected for further analysis. Intravenous injection of the selected complexes led to 22-fold differences in transduction efficiency, which correlated with transfection efficiency in the presence of high serum concentrations. The complex with the highest transfection efficiency in vivo consisted of 64 nmol DC/ 16 µg PS/ µg DNA. Physical analysis revealed a predicted size of 440 nm and the highest zeta potential of the complexes analyzed.

Conclusions: Optimization of cationic lipid DNA complexes for transfection efficiency in the presence of high concentrations of serum led to the identification of a DC complex with high transduction efficiency in mice. This complex differs from previously described ones by higher lipid to DNA and PS to DNA ratios. The stability of this complex in the presence of high concentrations of serum and its high transduction efficiency in mice suggests that it is a promising candidate vehicle for in vivo gene delivery.
Background

Cationic lipid DNA complexes have been used as a non-viral gene delivery system in cell culture [1–5] and in several animal models [6–11]. Apart from their relative ease of production and simple complexing protocols, risks attributed to viral vectors has prompted further interest in cationic lipid DNA gene delivery systems.

Only a few cationic lipid DNA complexes have been shown to work in the presence of biological fluids such as serum [12–14] and most in vitro transfection protocols routinely require that the target cells are exposed to the cationic lipid DNA complex in the absence of serum [3,15,16]. Human gene therapy phase 1 trials with cationic lipid DNA complexes for treatment of cancer and cystic fibrosis only led to low and transient expression of the therapeutic gene [10]. Thus, there is an intense effort to optimized cationic lipid DNA complexes in order to increase their gene transfer activity in the presence of physiological fluids such as serum. Current efforts in improving cationic lipid DNA complexes are mainly based upon a better understanding of the barriers for transfection [17–19] and the relationship between structure and function [20–27]. Amongst the barriers for transfection, serum has been reported to exhibit its inhibitory effect either by diminishing the amount of lipid DNA complexes associating with the target cells [3] or via serum proteins binding to lipid DNA complexes thereby diminishing their ability to deliver the transgene [28]. Lipoproteins have recently been incriminated as the major component responsible for the inhibitory effect of serum [29].

DOTAP (1,2-dioleoyl-3-(trimethylammonium) propane) and an equimolar mixture of DOTAP and cholesterol (DC) were selected as lipids in this study, since they are readily available and have already been extensively characterized. Initially, DOTAP was shown to give good in vivo transduction efficiency at a ratio of 35 nmol/µg DNA [30]. Addition of the helper lipid cholesterol enhanced in vivo transduction efficiency [31,32]. In another study, protamine sulfate was also shown to enhance transduction efficiency of DC-chol (3beta-[N',N'-dimethylaminoethane] - carbamoyl-cholesterol) / DOPE (dioleoylphosphatidyl-ethanolamine) and DOTAP / DOPE complexes [33]. In these studies, complexes were first optimized for transfection efficiency in the cell culture in the presence of low concentrations of serum. A few selected complexes were subsequently analyzed for transduction efficiency in vivo. Since the requirements for good transfection efficiency in cell culture might differ from the ones in vivo, the most efficient complexes in vivo might have been missed. Serum, which has been shown to inhibit transfection efficiency of cationic lipid DNA complexes [3,12,28], seems to be an important barrier to in vivo transfection efficiency. Therefore, complexes spanning a wide range of ratios of lipid to DNA to protamine sulfate were systematically analyzed in the present study for transfection efficiency in the presence of high concentrations of serum. To determine the relevance of these cell culture experiments for in vivo gene transfer, transduction efficiencies of selected complexes were also determined after intravenous injection of lipid DNA complexes in mice. The complex with the highest transfection efficiency in the presence of high serum concentrations in cell culture also gave the highest transduction efficiency after intravenous injection into mice. The optimized complex differed from the previously identified ones by higher lipid to DNA and protamine sulfate to DNA ratios. Since inhibition of transfection in cell culture by high serum concentrations correlated well with in vivo transduction efficiency, the effect of serum on biophysical properties of the complexes was also analyzed.

Results

Transfection efficiency of lipid DNA complexes in vitro

The lipids DOTAP and DOTAP-cholesterol (DC) were chosen for the preparation of cationic lipid DNA complexes since they gave good transfection efficiency in cell culture and in animal models [12,30,34–36]. To determine the optimal ratio of lipid to DNA, one µg of a plasmid encoding the luciferase reporter gene was complexed with increasing amounts of DOTAP (Fig. 1A) or DC (Fig. 1B). The functionality of the complexes were analyzed by transfection of 293A target cells and subsequent measurement of the luciferase activity in the cell extracts of the transfected cells. For both lipids a dose dependent increase in transfection efficiency was observed. Since protamine sulphate was reported to enhance transfection efficiency of cationic lipid DNA complexes [33], it was added in increasing concentrations to 1 µg of plasmid DNA prior to the addition of the lipids at all the previously analyzed lipid to DNA ratios. Protamine sulphate enhanced transfection efficiency by a factor of 10 to 100 at all lipid to DNA ratios tested. The strongest increase was observed at low lipid to DNA ratios. For DOTAP, the highest luciferase activities were obtained with 4 to 8 µg protamine sulphate at high DOTAP to DNA ratios (Fig. 1A). For high DC to DNA ratios, the transfection efficiency did not vary considerably in the presence of 0.5 to 16 µg protamine sulphate indicating that a wide range of protamine sulphate concentration could be used for complex formation. Similar experiments with a plasmid encoding the GFP reporter gene revealed that at the optimal ratios of lipid to DNA to protamine sulfate about 30–40% of the cells were transfected in the presence of 0.5% fetal bovine serum and up to 70% of the cells were transfected when transfection was done in the absence of serum (data not shown). Similar high transfection efficiencies were obtained with selected complexes in other immortalized cell lines (data not shown).
Toxicity of lipid DNA transfection complexes

Since toxicity of the lipid DNA complexes could limit their use, cell viability was analyzed after transfection with various ratios of lipid to DNA to protamine sulphate. For ratios of up to 64 nmol DOTAP/µg DNA cell viability was higher than 80% of the viability of untransfected cells independent of the amount of protamine sulfate added (Fig. 2A). At higher DOTAP to DNA ratios (> 64 nmol DOTAP/µg DNA), toxicity peaked at 8 µg protamine sulfate/µg DNA. High cell viabilities were observed for complexes containing DC at ratios ≤ 64 nmol lipid/µg DNA with limited correlation of the amount of protamine sulfate added to the complexes.

Transfection efficiency of lipid DNA complexes at high serum concentrations

Serum has been previously reported to interfere with transfection efficiency of lipid DNA complexes [12,28,36]. In these studies, lipid DNA complexes were usually optimized for transfection efficiency in the absence of serum, and small number of complexes were subsequently analyzed for transfection efficiency in the presence of high serum concentrations. To determine, whether DOTAP and DC lipid DNA complexes could be obtained with increased transfection efficiency in the presence of high serum concentrations, the 162 different complexes analyzed above were also studied for transfection efficiency after incubation with 66% serum. Lipid DNA complexes composed of ≤ 16 nmol lipid/µg DNA and ≤ 2 µg protamine sulfate/µg DNA were strongly inhibited by high serum concentrations (compare Fig. 1A and 3A), while complexes with higher amounts of DOTAP were inhibited to lesser extent or not at all. Peak transfection efficiencies of DOTAP complexes with less 16 nmol lipid/µg DNA in the presence of high concentrations of serum were obtained with 4 µg protamine sulfate/µg DNA (Fig. 3A). At higher DOTAP concentrations protamine sulfate affected transfection efficiency in the presence of high serum concentrations to a lesser extent. Transfection efficiencies of DC DNA complexes were only inhibited by high serum concentrations at high ratios of protamine sulfate to DNA (Fig. 1B versus 3B). Surprisingly, high serum concentrations increased transfection efficiency of DC complexes in the absence of protamine sulfate. Transfection efficiencies of complexes containing ≤ 4 nmol DC/µg DNA were also increased by high serum concentrations at low amounts of protamine sulfate (0.5 to 4 µg protamine sulfate/µg DNA). However, the highest transfection levels in the presence of high serum concentrations were obtained with DC complexes composed of 64 nmol lipid/µg DNA in the presence of a wide range of protamine sulfate to DNA ratios (0.5 to 64 µg protamine sulfate/µg DNA).

On the basis of these findings, two ratios of lipid to DNA to protamine sulphate, which seem to cover the range of efficient lipid DNA complexes, were chosen for each lipid from the 162 different complexes analyzed for further characterization of the effect of serum on transfection efficiency. Complexes with low lipid to DNA ratios consisted of 16 nmol DOTAP (DOTAP-low) or DC (DC-low)/1 µg DNA with 1 µg protamine sulphate, while complexes with a high lipid to DNA ratio were formed with 64 nmol DOTAP (DOTAP-high) or DC (DC-high)/1 µg DNA with 16 µg of protamine sulphate. The in vitro transfection efficiencies at low serum concentrations were very similar for DOTAP-low, DOTAP-high, DC-low, and DC-high, ranging from approximately 1 to 2 × 10^7 RLU/µg protein extract (Fig. 4) and did not change notably during preincubation of the complexes for up to four hours prior
to their addition to the cells (data not shown). However, mixing of the four selected complexes with 2 volumes of serum for less than 10 minutes, for 1 hour, or for 2 hours prior to transfection and subsequent determination of luciferase activities revealed striking differences. The DC-high complex was only marginally affected by 2 volumes of serum (Fig. 4). Preincubation with serum for two hours inhibited transfection with DC-low and DOTAP-high complexes by approximately 10- and 20-fold, respectively. Transfection with DOTAP-low complexes was almost completely blocked by preincubation with serum (Fig. 4).

**In vivo transfection efficiency of lipid DNA complexes**

To determine, the transfection efficiency of the four selected complexes *in vivo*, 25 µg luciferase expression plasmid were mixed with the lipids and protamine sulfate at the indicated ratios and injected into the tail vein of mice. Twenty four hours later the luciferase activity was determined in the lung extracts, the organ with the highest reporter gene activity following intravenous injection of cationic lipid DNA complexes ([34,37]; data not shown). The highest luciferase activities were obtained with the DC-high complex (Fig. 5), which was also least inhibited by high serum concentrations (Fig. 4). Intermediate levels of transfection efficiencies were obtained with DC-low and DOTAP-high, both of which also showed intermediate levels of inhibition by high serum concentrations. DOTAP-low transfection complexes, which were strongly inhibited at high serum concentrations, only gave low luciferase activities *in vivo*. Thus, the *in vivo* transfection efficiency seems to correlate well with the transfection efficiency in cell culture in the presence of high serum concentrations.

**Physical properties of lipid-DNA transfection complexes**

The size and the charge of the four selected lipid DNA complexes was examined by light scattering and laser electrophoresis to determine the importance of these parameters for *in vivo* transfection efficiency and serum inhibition. The four selected complexes were positively charged with charges ranging from 13.1 mV for DOTAP-low to 19.6 mV for DC-high (Table 1). With the exception of DC-low complexes, lower zeta potentials were observed when the complexes were formed in the absence of protamine sulfate (data not shown). The largest size with 0.44 µm was predicted for DC-high complexes (Table 1), while the other three complexes had a predicted size of 0.12 to 0.19 µm. For these three complexes, protamine sulfate reduced the size of complexes since control complexes formed at the same lipid to DNA ratios in the absence of protamine sulfate had sizes ranging from 0.42 µm to 0.45 µm (data not shown), confirming the previously reported condensing effect of protamine sulfate [31,33]. However, DC-high complexes were not condensed by addition of protamine sulfate, suggesting that a small size of the complexes is not required for good transfection efficiency *in vivo* and in the presence of high serum concentrations.

**Turbidity of lipid-DNA complexes following exposure to fetal bovine serum**

The sensitivity of the complexes to serum seems to be an important parameter for *in vivo* transfection efficiency. To directly assess the effect of serum on the structure of the li-

| Table 1: Physical properties of the lipid DNA complexes |
|-----------------|----------|-----------------|
| Complex         | Size (µm) | Zeta potential (mV) |
| DOTAP-low       | 0.19 ± 0.084 | 13.1 ± 5.6 |
| DOTAP-high      | 0.14 ± 0.038 | 16.7 ± 1.8 |
| DC-low          | 0.12 ± 0.024 | 15.9 ± 2.1 |
| DC-high         | 0.44 ± 0.088 | 19.6 ± 3.9 |

The mean size and the standard deviation of the major population of the different lipid DNA complexes are indicated.
pid-DNA complexes, the turbidity of the complexes was determined before and after addition of two volumes of serum. After addition of serum the turbidity increased within 10 minutes but remained rather constant for the rest of the observation period in case of DOTAP-low, DC-low and DC-high complexes (Figure 6). This might indicate formation of rather stable complexes with little tendency to additional aggregation. For DOTAP-high complexes we observed a continuous increase in turbidity after addition of serum for at least 150 minutes, indicating aggregation of the complexes. Since the transfection efficiency of DOTAP-low complexes was inhibited to a larger extent by high serum concentrations than the transfection efficiency of DOTAP-high complexes (Fig. 4), stable turbidity values after addition of serum were not predictive for resistance to inhibition of transfection efficiency by high serum concentrations.

**Effect of serum on interaction of the lipids**

A second approach, based on fluorescence energy transfer (FRET) was used to study lipid interactions during complex formation and to assess the direct effect of serum on the interaction between the lipids. During preparation of the liposomes, the fluorescent molecules N-NBD-PE (donor) and RH-PE (acceptor) are incorporated at a low molar ratio (1% mol/mol of both NBD-PE and RH-PE) in the vesicle bilayer. Under steady state conditions with appropriate ratios of NBD-PE to RH-PE, the fluorescence emitted by NBD-PE is reduced because the emission wavelength of NBD-PE overlaps with the excitation wavelength of RH-PE. When a perturbation of the lipid bilayer occurs as a result of interaction with either DNA or serum components a decrease in the surface densities of RH-PE and NBD-PE occurs resulting in an increase in NBD-PE fluorescence which was measured in this experiment. An increase in NBD-PE emission intensity therefore indicates a reduction in FRET probably due to changes in the conformation of the liposomes or complexes.

For DC-low, addition of DNA and protamine sulphate to the labelled liposomes led to a >10% increase in the emis-
sion intensity of NBD-PE, while the increase was less striking during formation of DC-high, DOTAP-low, and DOTAP-high complexes (Fig. 7). Fluorescence intensity increased after addition of serum for all complexes (> 40%) with minor differences between different protocols (Fig. 7). To disrupt the complexes completely, Triton X-100 was added, which led to a further increase in fluorescence intensities. The highest levels of fluorescence intensity obtained after addition of Triton X-100 was very similar for all the complexes (data not shown).

Discussion and Conclusions
Screening of a large number of DOTAP or DOTAP cholesterol DNA complexes spanning a wide range of ratios of lipid to DNA in the presence or varying amounts of protamine sulfate for transfection efficiency in the presence of high serum concentrations led to the identification of complexes highly resistant to the inhibitory effects of serum. The transfection efficiency of complexes containing low amounts of DC and protamine sulfate could even be increased by addition of high serum concentrations. This might be due to an enhanced binding of DC based complexes to the target cells with increasing serum concentrations [12] in the absence of destabilizing effects of serum on these complexes. Why a similar effect is not noticed with complexes containing high amounts of DC may be due to saturation resulting from a larger amount of DC associating with the cells. Alternatively, serum components might associate with complexes containing low amounts of DC more efficiently. However, the highest transfection efficiencies in the presence of high serum concentrations were obtained with complexes containing high amounts of DC (≥ 64 nmol lipid/µg DNA) in the presence of a wide range of protamine sulfate to DNA ratios (0.5 to 64 µg protamine sulfate/µg DNA). Interestingly, the transfection efficiency of four selected complexes (DOTAP-low, DOTAP-high, DC-low, DC-high) in the presence of high serum concentrations, but not at low serum concentrations, paralleled transduction efficiency in vivo after intravenous administration. This suggests that serum is a main barrier to in vivo gene transfer after intravenous injection of cationic lipid DNA complexes. However, it is important to note, that other routes of administration might differ in the requirements for good transduction efficiency [32,37].

The highest transduction efficiencies after intravenous injection were obtained with DC-high complexes composed of 64 nmol DC/µg DNA/16 µg protamine sulfate. This complex differs from previously used ones, which were similar in composition to our DC-low complexes, by a higher ratio of DC to DNA. Whether the higher ratio of protamine sulfate to DNA also contributes to higher transduction efficiency in vivo in comparison to DC-low like complexes remains to be determined, but similar transfection efficiencies of complexes containing 64 nmol DC/µg DNA/16 µg protamine sulfate were obtained with DC-high complexes and complexes containing high amounts of DOTAP.
DNA and varying amounts of protamine sulphate (ranging from 0.5 to 32 µg/µg DNA) in the presence of high concentrations of serum argue against it.

Our attempt to correlate physical properties of the complexes with high transfection efficiency in the presence of high serum concentrations was not successful. Neither size, charge, lipid interactions or lack of aggregation paralleled transfection efficiency in the presence of high serum concentrations. One of the reasons for this failure might be the fact that the structure of the complexes can only be determined for the dominating population of complexes. Since presumably only the DNA of a small percentage of complexes reaches the nucleus for transcription a minor population of complexes undetectable by biophysical methods might actually be the biologically active one leading to transfection. In this case, only physical separation of the complexes will allow the establishment of a firm correlation of physical parameters with transfection efficiency. At present, comparison of a large number of different complexes and selection of the most efficient ones for further analysis is still required. Transfection efficiency in the presence of high serum concentrations might be a good initial screening system prior to in vivo experiments.

By systematically varying the ratio of lipid to DNA to protamine sulfate we identified a lipid DNA complex (DC-high) composed of 64 nmol DC/µg DNA/16 µg protamine sulfate with a predicted size of 440 nm and zeta potential of 19.6 mV that is hardly inhibited by high serum concentrations and that gave the highest in vivo transduction efficiency after intravenous injection into mice. This DC-high complex differs from previously described DC complexes [38] by increased ratios of lipid and protamine sulfate to DNA. These parameters might contribute to the good transduction efficiency of DC-high complexes in vivo. Further characterization of the biological properties of this complex is required to determine its usefulness for gene therapy approaches.

Materials and Methods

Chemicals

1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP), cholesterol, NBD-PE (N-[7-nitrobenz-oxa-1,3-diazoyl-4-yl]-phosphatidylethanolamine) and Rh-PE (N-[lissamine-rhodamine-B-sulfonyl]-phosphatidylethanolamine) were purchased from Avanti Polar Lipid (Alabaster, AL, USA). Protamine sulphate and MTT (Dimethyl-thiazol-diphenyl-tetrazolium-bromide) were from Sigma (Darmstadt, Germany). The Luciferase assay kit was purchased from Promega (Manheim, Germany). All other chemicals were of reagent grade.

Plasmids

The reporter plasmid containing the firefly luciferase cDNA protein was cloned into the backbone of pcDNA3.1 plasmid (Invitrogen, Groningen, Netherlands). The expression of the reporter gene is under the control of Cytomegalovirus (CMV) immediate early promoter. The plasmid pEGFP-C1 expressing the enhanced green fluorescent protein cDNA driven by the CMV promoter was obtained from Clonetech (Heidelberg, Germany). Plasmid DNA was prepared with the Qiagen plasmid Giga kit (Valentia, CA, USA) according to the manufacturer’s instruction and dissolved in phosphate buffered saline (PBS, pH 7.4).

Preparation of liposomes

Cationic liposomes containing DOTAP or DOTAP and cholesterol in a 1:1 molar ratio were prepared using a previously described method [39]. The lipid was initially dried from chloroform, subsequently dispersed in PBS (pH 7.4) and shaken at a temperature above the gel-to-liquid-crystalline transition temperature of the lipid (50°C) for 10 min. The milky solution was sonicated for 10 min under temperature control using a Branson sonifier 250 (Branson Sci., USA) at minimum ultrasound power to avoid extensive bubble formation.
**Preparation of lipid DNA complexes**

The plasmid DNA was diluted to a concentration of 1 µg/µl in PBS (pH 7.4) prior to complex formulation. All reagents were sterile and were adjusted to room temperature before complex preparation. Protamine sulphate was added to DNA diluted in PBS at a weight ratio of 1 µg protamine sulphate per µg DNA (DC- and DOTAP-low) or 16 µg protamine sulphate per 1 µg DNA (DC- and DOTAP-high), the mixture was vortexed briefly and incubated at room temperature for 5 minutes. The respective amounts of DOTAP (DOTAP-high: 64 nmol/µg DNA; DOTAP-low: 16 nmol/µg DNA) or DC (DC-high: 64 nmol/µg DNA; DC-low: 16 nmol/µg DNA) were added to the solution at a final volume of 50 µl, the mixture was vortexed briefly and further incubated for ten minutes at room temperature. Stable fluorescence intensity values were obtained within minutes after addition of compounds (Fig. 7) indicating rapid completion of mixing.

**Laser electrophoresis and light scattering**

The electrophoretic mobilities of the complexes were measured using a Zetasizer 4 device (Malvern, U.K.). Lipid DNA complexes were formed with 4 µg DNA and the respective amounts of Lipid and protamine sulfate in a volume of 200 µl. The volume was adjusted to 4 ml by addition of PBS. The mobilities of the particles were measured using a modulation frequency of 1000 Hz, the electrode current was 5 mA. Light scattering at 90° was measured using the same sample. Multimodal analysis was used for the deconvolution of the data. The number distribution mode were used for the data analysis.

**Transfection of mammalian cells in vitro**

About 40000 293A cells (Quantum Biotechnology Inc., Montreal, Canada) were seeded into a 24 well plate and grown in a 37°C incubator in a 5% CO₂ in a final volume of 1 ml DMEM from GIBCO (Karlsruhe, Germany). The next day, the cells (60–70% confluent) were then transfected by adding 50 µl complex solution containing 1 µg of DNA to each well of the plate without removing the 10% fetal bovine serum containing medium from the cells. The cells were then incubated for 48 hours without removing the medium. When transfections were set up in the absence of serum, cells were incubated with the complexes for 4 hours in serum free medium before the medium was replaced with fresh medium containing 10% fetal bovine serum. To study serum inhibition, complexes formed as described above were incubated with two volumes of heat inactivated fetal calf serum (GIBCO; Karlsruhe, Germany) prior to addition to 293A cells. Two days after transfection cells were washed with PBS and lysed with 200 µl lysis buffer (0.05% Triton X-100, 2 mM EDTA, 0.1 M Tris, pH 7.8) through one freeze-thaw cycle. The cell lysate was then transferred to microcentrifuge tubes and centrifuged for 10 minutes at 14000 g at 4°C. The supernatant was diluted one to twenty and 20 µl of the diluted sample was used for the luciferase assay according to the manufacturer’s instructions. The luminescence was determined in a luminometer from Murex (Germany) The protein concentration was determined using a multiplate assay from Biorad (Hilden, Germany) according to the manufacturer’s instructions. For quantification, a standard containing known amounts of BSA in luciferase lysis buffer was included. Reporter gene expression was expressed as relative light units (RLU) per µg protein extract. Each data point represents the mean ± the standard deviation of triplicate experiments.

**Cytotoxicity assay**

293A cells transfected as described above were assayed for cell survival using the MTT reagent. The MTT reagent was diluted in PBS to a final concentration of 2.5 mg/ml then filtered using a 0.22 µm filter. 50 µl of the stock solution was then added to each well of the transfected plates and incubated for 1 hour at 37°C under 5% CO₂. After an hour the medium was removed and 200 µl of 0.04 N HCl was used to dissolve the resulting blue formazan crystals in living cells. The extract was then diluted 1:4 in 0.04 N HCl and the optical density was determined at 540 nm. Untransfected 293A cells were used as control.

**In vivo transfection**

The animal experiments were approved by the local authorities. Female Balb-C mice 6–8 weeks old were obtained from the experimental animal services of the University of Leipzig and housed in accordance with institutional guidelines. Individual mice in groups of five were injected via the lateral tail vein with 25 µg of DNA formulated in the complexes in a total volume of 300 µl. Twenty-four hours following i.v. injection, the mice were killed by cervical dislocation and the lungs were collected. The organ was then washed with PBS and homogenized in 500 µl of lysis buffer (Promega, Mannheim, Germany). After two cycles of freeze-thaw, the homogenates were centrifuged at 14000 g at 4°C for 10 minutes. The supernatant was analyzed for luciferase activity as described above.

**Turbidity assay**

The relative absorbance of the complexes was determined at 600 nm according to a method described [38]. Complexes were formed with 4 µg DNA and the respective amounts of lipids and protamine sulfate in a volume of 200 µl. After addition of 400 µl PBS the absorbance of the complexes was determined for 10 minutes in the absence of serum with PBS as the blank control. A volume of 400 µl serum was added and the kinetics of the turbidity was measured with stirring at 37°C for 5 hours. Serum in PBS was used as the blank control.
Multi-step lipid mixing assay

The multi-step lipid mixing assay was adapted from the methods described by Wasan [21]. Structural reorganization of the complexes after binding of DNA and serum was followed by the fluorescence resonance energy transfer method, using NBD-PE and Rh-PE. The liposomes were composed of DOTAP or DC and 1% (mol/mol) of both NBD-PE and Rh-PE. The liposomes with the incorporated fluorophores were suspended in 2 ml of PBS (pH 7.4). The fluorescence measurements were carried out at 25°C with an excitation wave length of 465 nm. The emission was recorded at a wavelength of 520 nm using a Perkin-Elmer LS50B spectrophotometer. The recording started after the system had almost reached equilibrium. Initially, fluorescence readings of the respective liposomes (DOTAP-low and DC-low: 64 nmol; DOTAP-high and DC-high: 256 nmol) in 2 ml PBS were taken continuously at 25°C for 5 minutes, 4 µg DNA mixed with protamine sulfate (4 µg for DOTAP-low and DC-low; 64 µg for DOTAP-high and DC-high) diluted in 500 µl of PBS (pH 7.4) were added and changes in the fluorescence emission of the complexes at 520 nm wave length of were monitored while stirring for another 5 minutes. To correct for the decrease in fluorescence by increasing the volume, the fluorescence intensities obtained after addition of PBS were subtracted from the values obtained after addition of PBS with DNA. The resulting change in fluorescence intensities was added to the fluorescence value obtained with liposomes alone. To determine the effect of serum, 800 µl of FBS was added and mixed briefly. Fluorescence values were monitored and corrected for dilution as before. Finally 66 µl of Triton X-100 (0.2% v/v) was mixed with the complexes to completely solubilize the vesicles. The mean of the fluorescence intensity values obtained after addition of Triton X-100 was set as 100% and all values were expressed as percentage of this mean.

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