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Biophysical and lipofection studies of DOTAP analogs

Anne E. Regelin [a], Stefan Fankhaenel [a], Laura Gürtesch [a,b], Claudia Prinz [a], Günter von Kiedrowski [b], Ulrich Massing [a,*]

[a] *Tumor Biology Center, Department of Clinical Research, Breisacher Straße 117, D-79106 Freiburg, Germany*
[b] *Department of Bioorganic Chemistry, University of Bochum, Universitätsstraße 150, NC 2/173, D-44780 Bochum, Germany*

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Abstract

In order to investigate the relationship between lipid structure and liposome-mediated gene transfer, we have studied biophysical parameters and transfection properties of monocationic DOTAP analogs, systematically modified in their non-polar hydrocarbon chains. Stability, size and (by means of anisotropy profiles) membrane fluidity of liposomes and lipoplexes were determined, and lipofection efficiency was tested in a luciferase reporter gene assay. DOTAP analogs were used as single components or combined with a helper lipid, either DOPE or cholesterol. Stability of liposomes was a precondition for formation of temporarily stable lipoplexes. Addition of DOPE or cholesterol improved liposome and lipoplex stability. Transfection efficiencies of lipoplexes based on pure DOTAP analogs could be correlated with stability data and membrane fluidity at transfection temperature. Inclusion of DOPE led to rather uniform transfection and anisotropy profiles, corresponding to lipoplex stability. Cholesterol-containing lipoplexes were generally stable, showing high transfection efficiency at low relative fluidity. Our results demonstrate that the efficiency of gene transfer mediated by monocationic lipids is greatly influenced by lipoplex biophysics due to lipid composition. The measurement of fluorescence anisotropy is an appropriate method to characterize membrane fluidity within a defined system of liposomes or lipoplexes and may be helpful to elucidate structure–activity relationships.

Keywords: DOTAP; Analog; Lipofection; Transfection; Fluorescence anisotropy; Helper lipid

1. Introduction

Since cationic lipids were shown to be potent candidates for liposome-mediated gene transfer [1], the development of new and more efficient transfection lipids has become one of the main targets of liposome research.

Lipoplexes, i.e. complexes composed of (cationic)

Abbreviations: CHOL, cholesterol; DLTAP, *N*-[1-(2,3-dilauroyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate; DMRIE, *N*-[1-(2,3-dimyristyloxy)propyl]-*N*,*N*-dimethyl-*N*-(2-hydroxyethyl) ammonium bromide; DMTAP, *N*-[1-(2,3-dimyristoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate; DPH, 1,6-diphenyl-hexa-1,3,5-triene; DPTAP, *N*-[1-(2,3-dipalmitoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate; DSC, differential scanning calorimetry; DSTAP, *N*-[1-(2,3-distearoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate; FCS, fetal calf serum; LPTAP, *N*-[1-(2-palmitoyloxy-3-lauroyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate; HBS, HEPES-buffered saline; HPTLC, high-performance thin-layer chromatography; QELS, quasi-elastic light scattering; rlu, relative light units; RT, room temperature; T_c, phase transition temperature

* Corresponding author. Fax: +49-761-206-2174; E-mail: umas@tumorbio.uni-freiburg.de

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liposomes and DNA [2], serve as vehicles to deliver DNA into cells and tissues. Although there have been observed several types of lipoplex structures [3–6], the discussion about which type is most effective is still going on [7]. It has not yet been possible to predict lipofection results from biophysical data on liposomes and the respective lipoplexes [8], because there is a variety of influencing parameters concerning not only lipids and lipoplex formation, but also reporter plasmids, cell lines, etc.

Accordingly, it is very useful to have a model system which allows the successive variation of single parameters. Therefore, we have used a model system of constant cell line and plasmid, and similar protocols for liposome preparation, lipofection and biophysical measurements. In particular, on the basis of lipid structure, the influence of structural elements, such as the cationic head group or the hydrophobic part, on biophysical properties as well as on lipofection can be examined without changing experimental conditions. In order to investigate the influence of the hydrocarbon chain of bilayer-forming cationic lipids on lipofection, we have used systematically varied analogs of the monocationic lipid DOTAP (*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate). DOTAP, which is widely known as transfection lipid, consists of a monocationic trimethylammonium head group and two unsaturated hydrocarbon chains, derived of oleic acid. The DOTAP analogs used in the current study differ from DOTAP only in the non-polar molecule part: they have saturated hydrocarbon chains, based on different fatty acids consisting of 12 (lauroyl-residue), 14 (myristoyl-), 16 (palmitoyl-), or 18 (stearoyl-) C-atoms. According to the structural similarity to DOTAP (with its trivial name dioleoyloxytrimethylammonium propane) they are called DLTAP, DMTAP, DPTAP, DSTAP, respectively, or, when consisting of two different fatty acid residues LPTAP (Fig. 1).

Since helper lipids are often used in combination with transfection lipids and can possibly enhance lipofection efficiency [9–12], we have also examined the influence of the helper lipids DOPE (1,2-di-[*cis*-9-octadecenoyl]-*sn*-glycero-3-phosphoethanolamine) and cholesterol on biophysical parameters and transfection properties of DOTAP analogs. Thus we have determined parameters, such as stability and size of

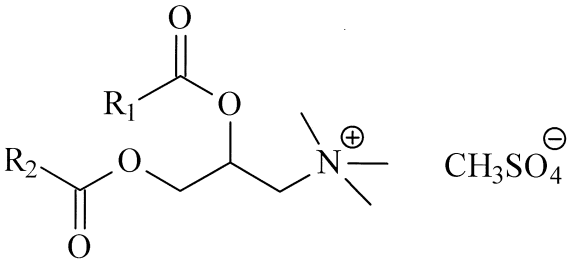

Fig. 1. Structure of the DOTAP analogs (*N*-[1-(2-R_1-oxy,3-R_2-oxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate) used in the current study. DOTAP, R_1, R_2 = oleoyl (18:1); DSTAP, R_1, R_2 = stearoyl (18:0); DPTAP, R_1, R_2 = palmitoyl (16:0); DMTAP, R_1, R_2 = myristoyl (14:0); LPTAP, R_1 = palmitoyl (16:0), R_2 = lauroyl (12:0); DLTAP, R_1, R_2 = lauroyl (12:0).

liposomes and lipoplexes, phase transition behavior, fluorescence anisotropy, and lipofection efficiency in order to enlighten the (cor-) relation between these biophysical data and lipofection efficiency.

2. Materials and methods

2.1. Preparation of liposomes

The cationic lipids DOTAP, DSTAP, DPTAP, DMTAP, LPTAP, and DLTAP were synthesized in our laboratory (paper submitted). DOPE and cholesterol (purity 99%) were purchased from Sigma-Aldrich, Deisenhofen, Germany. Purity of the lipids was checked by HPTLC. Lipids and mixtures with the helper lipids DOPE (in a molar ratio of 1:1) or cholesterol (molar ratio of 2:1) were dissolved in chloroform/methanol (2/1, v/v). Dried lipid films were prepared by removing the solvent under vacuum evaporation. Then the films were hydrated in HBS (20 mM HEPES, 130 mM NaCl, pH 7.4) and sonicated in a water bath sonicator (Bandelin Sonorex RK 100H; Berlin, Germany) for 20 min at the respective temperature (above T_c) to form liposomes. For each preparation, the size distribution of liposomes was determined by QELS using a Nicomp Submicron Particle Sizer (Model 370, Santa Barbara, CA, USA). For stability measurements, liposomal size was determined at preparation temperature; then temperature was decreased stepwise (5°C per step) monitoring the size distribution over 15 min, respectively.

Instability of a liposomal dispersion was defined as a rapid increase of vesicle size during the 15 min period along with the visual detection of large/macroscopic aggregates (at the bottom of the cuvette).

T_c of the lipids was determined by DSC (DSC-7, Perkin Elmer, Überlingen, Germany). Phase transition profiles of the lipids were derived from anisotropy curves.

2.2. Formation of lipoplexes

Plasmid DNA (pCMXluc (a generous gift from R. Schüle) containing a firefly luciferase gene driven by a CMV promoter) was amplified in *Escherichia coli* and purified using a Qiagen Plasmid Maxi Kit (Qiagen, Hilden, Germany). Purity and concentration of the DNA were determined by absorbance at 260 and 280 nm and by gel electrophoresis on a 1% agarose gel. DNA–lipid complexes (lipoplexes) were prepared by adding plasmid DNA in HBS to the liposome formulation at a charge ratio of 2.5:1 (cationic lipid/DNA) at a final concentration of 125 ng DNA and 1 nmol lipid per 96-well amount. The complexes were then allowed to form for a period of 15 min (at room temperature or 37°C). The size distribution of the lipoplexes was monitored by QELS at the respective preparation temperature (T_{prep}; Table 2), transfection temperature (37°C) and room temperature RT (23°C).

2.3. Anisotropy measurements of liposomes and lipoplexes

Cationic lipids (and helper lipids, if required) were dissolved in chloroform/methanol (2/1, v/v). DPH (Sigma–Aldrich) dissolved in tetrahydrofuran (2 mM) was then injected to yield a molar ratio of 200:1 of total lipid:DPH. Lipid films were prepared as described above by removing the solvent by vacuum evaporation. The films were hydrated in HBS to give a final lipid concentration of 200 μM. The dispersion was sonicated for 15 min at the respective temperature (10°C above the T_c of the respective lipid) to form liposomes. Afterwards, plasmid DNA was added to DPH-labeled liposomes to yield a lipid/DNA charge ratio of 2.5:1. Lipoplexes were then allowed to form for a period of 30 min. Anisotropy measurements were performed in a Perkin Elmer Luminescence Spectrometer LS50B (Perkin Elmer, Überlingen, Germany) at 360 nm (excitation wave length) and 430 nm (emission). Slit widths were 2.5 nm in both cases. The fluorescence intensities of the emitted light polarized parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the excited light were recorded at various temperatures. The fluorescence anisotropy (r) values were calculated from the equation $r=(I_{\parallel}-GI_{\perp})/(I_{\parallel}+2GI_{\perp})$, where G is the instrumental grating factor.

2.4. Cell transfection

COS-7 cells (ATCC CRL-1651) were maintained in EMEM/10% FCS/1% penicillin–streptomycin (BioWhittaker, Verviers, Belgium) at 37°C/5% CO_2 in a humidified atmosphere. Twenty-four hours prior to cell transfection, 10 000 cells per well were seeded in 96-well microtiter plates (Greiner, Frickenhausen, Germany) in 200 μl medium. Shortly prior to transfection, medium was removed from each well and replaced by 100 μl of FCS-free medium. Then 90 μl of the lipoplex–HBS formulation were added. All experiments were performed in 6-fold determination (6 wells per lipoplex type) with the exception of DOTAP (in quadruplicate).

The plate was centrifuged at $280\times g$ for 1 min and incubated for 6 h at 37°C. Medium was then removed, and 200 μl of fresh, FCS-containing medium was added. The cells were further incubated for 42 h.

2.5. Luciferase assay and protein determination

Medium was aspirated from the wells and the cell layer was washed with 0.9% NaCl. The cells of each well were lysed in 50 μl lysis buffer (Boehringer Mannheim, Germany) at room temperature for 15 min. After lysis, 30 μl of 0.9% NaCl was added to increase the volume; equal amounts (20 μl) of the cell suspension were used for the determination of luciferase activity and determination of cellular protein, respectively.

In the luciferase assay, 80 μl of luciferase reagent (25 mM glycylglycine, 5 mM ATP, 0.2 mM luciferin) was added to 20 μl of the homogenized cell suspension in a white 96-well microplate (Corning Costar, Bodenheim, Germany), and the enzymatic activity

was measured over a period of 10 s by means of a Lumistar microplate luminometer (BMG, Offenburg, Germany). The amount of cellular protein was quantified in a BCA assay (Pierce, Rockford, IL, USA) using 20 μl of the cell lysate or bovine serum albumin as internal calibration standard and adding 200 μl of BCA reagent (according to the supplier's protocol). Following an incubation period of 2 h at room temperature under protection from light, the colorimetric measurement was performed at 550 nm by means of a microplate reader (type 'spectra', SLT-Tecan, Crailsheim, Germany). Luciferase activity was expressed as RLU/μg of protein, and transfection efficiency as luciferase activity of the respective lipoplex compared to that of DOTAP lipoplex as standard (set 100%).

3. Results

3.1. Liposomes

3.1.1. Stability

Apart from DOTAP, all liposomes made of DOTAP analogs were unstable at RT and large lipid aggregates precipitated within a few minutes after liposome formation (Table 1). In contrast, when liposomes were prepared at temperatures above the phase transition temperature (T_c) of the respective DOTAP analog, which was higher than RT, no aggregation could be observed. Subsequently reducing the temperature of the liposome dispersions resulted again in the formation of macroscopic lipid aggregates.

When helper lipids, DOPE or cholesterol, were incorporated, stability of the resulting liposomes increased. In most cases, mixtures of one DOTAP analog and DOPE led to stable liposomes at RT with the exception of DSTAP/DOPE and DPTAP/DOPE which were stable at temperatures above 50 or 35°C, respectively (Table 1). In contrast, all liposomes consisting of DOTAP analog plus cholesterol were stable at RT.

3.1.2. Size

Note: In our study, QELS was used in short time measurements. Since this method is not suitable to describe the size of liposomes (or lipoplexes, see below) very precisely – in particular in short time measurements – the measurements were used to indicate relative sizes of liposomes formed from different analogs and to document liposome (in-) stability.

Liposomes of the different lipid compositions generally showed mean diameters smaller than 100 nm. QELS measurements of liposomes composed of pure cationic lipids without helper lipids resulted in diameters between 23 (LPTAP) and 58 nm (DSTAP). When liposomes consisted of DOTAP analogs and the helper lipid DOPE, a broader size distribution was observed, ranging from approximately 20 nm for LPTAP/DOPE liposomes to approximately 160 nm for DSTAP/DOPE liposomes. In contrast, mean diameters of liposomes containing DOTAP analogs and cholesterol displayed a smaller range from 53 nm (LPTAP/CHOL) to 95 nm (DSTAP/CHOL). No correlation between liposome size and acyl chain length could be observed.

3.2. Lipoplexes

3.2.1. Stability

Lipoplexes were defined as stable when the size distribution did not change significantly over the period of at least 1 h after formation and no large aggregates occurred.

With the exception of DOTAP, all lipoplexes consisting of pure DOTAP analogs were unstable at RT (Table 2). In contrast, when formed at the temperature required for preparation of stable liposomes (Table 1), lipoplexes were stable, but subsequent reduction of the temperature led to rapid formation of large aggregates in the lipoplex dispersion. Regarding lipoplex stability at the transfection temperature of 37°C, lipoplexes made of the lipids DOTAP, DMTAP and LPTAP were found to be stable (Table 2).

When DOPE was included in lipoplexes, only DSTAP/DOPE and DPTAP/DOPE lipoplexes were unstable at RT, whereas inclusion of cholesterol generally led to stable lipoplexes at RT. Lipoplexes containing either of the helper lipids also showed stability at 37°C (Table 2).

Comparing Tables 1 and 2, it is obvious that lipoplexes show nearly the same stability profile as the liposomes they are based on. Apart from DLTAP lipoplexes which were unstable at 37°C although

Table 1
Minimum temperature required for formation of temporarily stable liposomes containing DOTAP or DOTAP analogs[a]

Lipid	T_c [b]	Without helper lipid	With DOPE (1:1)	With cholesterol (2:1)
DOTAP	< 5°C	RT	RT	RT
DSTAP	62.9°C	55°C	50°C	RT
DPTAP	52.8°C	50°C	35°C	RT
DMTAP	39.1°C	40°C	RT	RT
LPTAP	43.1°C	35°C	RT	RT
DLTAP	24.9°C	25°C	RT	RT

[a]Liposomes were defined as stable according to the size determination by QELS (see Section 2 for further details) and in absence of large/macroscopic aggregates.
[b]T_c was determined by differential scanning calorimetry.

DLTAP liposomes were stable even at 25°C, lipoplex stability at 25 or 37°C agreed with stability of the corresponding liposomes below or about 25 or 37°C, respectively.

3.2.2. Size

As to the size distributions of lipoplexes, QELS measurements showed diameters of lipoplexes made of pure cationic lipids ranging from 145 nm (LPTAP) to 475 nm (DOTAP). Lipoplexes containing the helper lipid DOPE also showed a heterogenous size distribution from 109 nm (LPTAP/DOPE) to 446 (DSTAP/DOPE), while cholesterol-containing lipoplexes displayed a rather homogenous distribution from 171 nm (LPTAP/CHOL) to 303 nm (DOTAP/CHOL). The distributions seemed to be independent of the acyl chain length of the cationic lipid (data not shown).

3.3. Fluorescence anisotropy measurements

3.3.1. Liposomes

Anisotropy curves were monitored over a temperature range from 20 to 40°C or 50°C. Only in cases where lipid components with long hydrocarbon chains were used, e.g. DPTAP and DSTAP, this range was extended until 60 or 70°C, respectively.

Generally, there could be found two different types of anisotropy profiles (Fig. 2A): DPTAP, DMTAP and LPTAP liposomes each displayed a very distinct phase transition, where anisotropy dropped from a high anisotropy level to a lower level (with a difference of about 0.2) within a small temperature range. In the case of DPTAP liposomes, this sharp decrease could be seen at about 40°C, as for DMTAP and LPTAP about 30°C, though the decrease took a slightly broader temperature range. The curve profile

Table 2
Stability of lipoplexes containing DOTAP or DOTAP analogs

Lipid	Without helper lipid			With DOPE (1:1)			With cholesterol (2:1)		
	RT	37°C	T_{prep} [a]	RT	37°C	T_{prep}	RT	37°C	T_{prep}
DOTAP	+[b]	+	RT	+	+	RT	+	+	RT
DSTAP	−[c]	−	70°C	−	+	50°C	+	+	RT
DPTAP	−	−	60°C	−	+	35°C	+	+	RT
DMTAP	−	+	50°C	+	+	RT	+	+	RT
LPTAP	−	+	50°C	+	+	RT	+	+	RT
DLTAP	−	−	40°C	+	+	RT	+	+	RT

[a]Preparation temperature of the liposomes prior to formation of lipoplexes. DNA was added to the liposomes immediately after preparation; lipoplexes were then allowed to form either at RT (when liposomes were stable at RT) or at 37°C (liposomes stable at temperatures above RT).
[b]Stability of lipoplexes was assumed when vesicle size was nearly constant during the size determination by QELS and no macroscopic aggregates were observed.
[c]Lipoplexes were defined as unstable when vesicle size increased rapidly during the size measurement and/or macroscopic aggregates were visible in the cuvette.

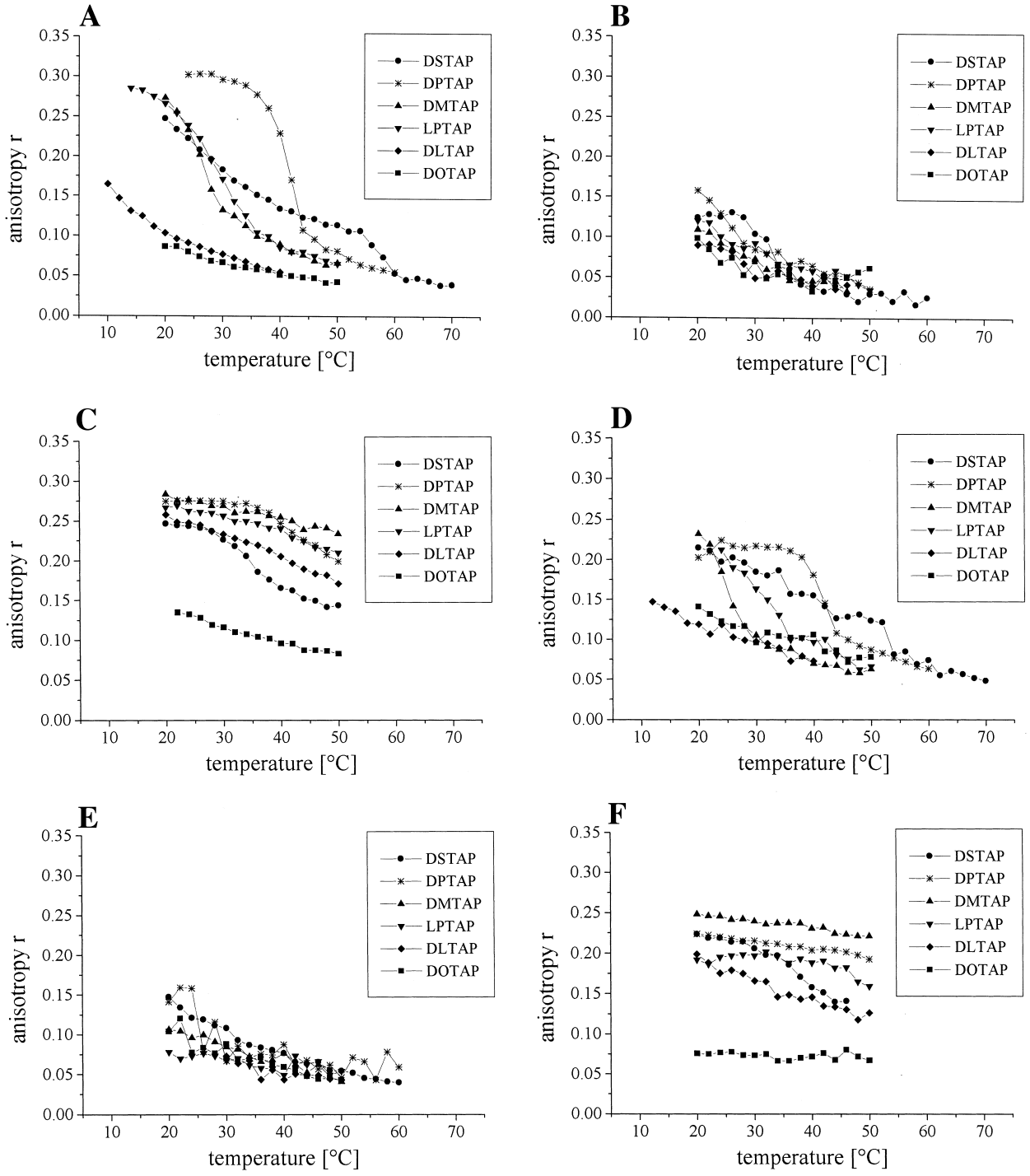

Fig. 2. Fluorescence anisotropy measurements of DPH-labeled liposomes and lipoplexes containing monocationic DOTAP analogs. Liposomes (containing 0.5 mol% of the fluorophore DPH) were composed of (A) pure DOTAP analogs or (B) DOTAP analogs plus 100 mol% of DOPE or (C) DOTAP analogs plus 50 mol% of cholesterol. Lipoplexes were prepared by adding plasmid DNA to the respective DPH-labeled liposomes leading to a lipid/DNA charge ratio of 2.5:1. Lipoplexes contained either (D) pure DOTAP analogs or (E) DOTAP analogs and DOPE or (F) DOTAP analogs and cholesterol. In each case, fluorescence anisotropy r was determined at different temperatures, represented by the data points.

of DSTAP liposomes showed no distinct phase transition. Instead a less sharp, rather monotonous decrease occurred below 55°C, followed by a smaller and less distinct reduction of anisotropy. In contrast, the curve profile of DLTAP liposomes was monotonous with obviously lower anisotropy levels, not displaying any form of sharp decrease in anisotropy at all. DOTAP liposomes showed a nearly similar profile.

Inclusion of helper lipids had a very striking effect on anisotropy profiles: Anisotropy curves of the different DOPE-containing liposomes were nearly similar, in shape as well as in anisotropy levels (Fig. 2B). Most curves showed a more or less monotonous and slow decrease of anisotropy at much lower anisotropy levels compared to the corresponding liposomes without helper lipids (Fig. 2A). Only the profiles of DLTAP/DOPE and DOTAP/DOPE liposomes were almost similar to those of the DOPE-free liposomes. Distinct phase transitions were not observed in any case. Only DSTAP/DOPE and DPTAP/DOPE liposomes displayed a slightly sharper decrease of anisotropy.

When cholesterol was used as additional liposome component, anisotropy profiles differed more from each other (Fig. 2C): typical phase transition was never seen, instead most curves displayed a monotonous profile with slowly decreasing anisotropy. DPTAP/CHOL and DSTAP/CHOL liposomes showed a somewhat steeper decrease, though at different anisotropy levels and temperatures, respectively (Fig. 2C). DMTAP/CHOL and LPTAP/CHOL liposomes had identical, rather monotonous profiles which decreased rather slowly. The anisotropy profile of DLTAP/CHOL liposomes was parallel to that of LPTAP/CHOL, but at slightly lower anisotropy level. Liposomes consisting of DOTAP/CHOL displayed a special curve profile with the lowest anisotropy level compared to the other analogs. They also showed the smallest decrease in anisotropy (approximately 0.05) over the whole temperature range. The curve was quite similar to the curves of DOTAP or DOTAP/DOPE liposomes.

3.3.2. Lipoplexes

When anisotropy profiles of lipoplexes consisting of pure cationic lipids and DNA were determined (Fig. 2D), the curve distribution resembled that of the corresponding liposomes (Fig. 2A). Nevertheless, initial anisotropy levels at low temperatures were more or less reduced. Apart from that, the anisotropy curves of DPTAP-, DMTAP- and LPTAP-containing lipoplexes displayed distinct phase transitions (Fig. 2D). In contrast, lipoplexes composed of DSTAP as lipid component showed a smaller drop at about 50°C and a rather monotonous curve at lower temperatures. Again, DLTAP and DOTAP had monotonous profiles at the lowest anisotropy levels.

Inclusion of DOPE in lipoplexes led to a similar, striking change in the resulting curve profiles as observed on DOPE-containing liposomes: no distinct difference between both curve distributions could be observed. Regarding the temperature range from 20 to 50°C, anisotropy curves of DOPE-containing lipoplexes decreased monotonously (Fig. 2E). There was hardly any difference between the individual curves, neither in anisotropy level nor in curve shape.

Lipoplexes containing the helper lipid cholesterol generally showed monotonous profiles with decreased anisotropy levels compared to the anisotropy values of the corresponding liposomes. With the exception of DSTAP/CHOL, profiles were more or less monotonous with only small changes in anisotropy over the whole temperature range (Fig. 2F). Nevertheless, each type of lipoplexes had its individual anisotropy level. DOTAP/CHOL lipoplexes displayed the lowest anisotropy level which remained constant over the whole temperature range.

3.4. Lipofection results

Lipofection results were expressed as lipofection efficiency standardized on DOTAP (set 100%) in order to simplify the comparison between different experiments or plates. The DOTAP standard was defined as mean of five DOTAP standards (each consisting of four to six wells) from five different experiments (i.e. five different plates; $n = 22$ wells).

3.4.1. DOTAP analogs

Lipofection mediated by the monocationic lipids LPTAP and DMTAP displayed the highest transfection efficiencies of all DOTAP analogs (Fig. 3). In the case of LPTAP, lipofection efficiency reached 214% of the DOTAP standard, whereas DMTAP

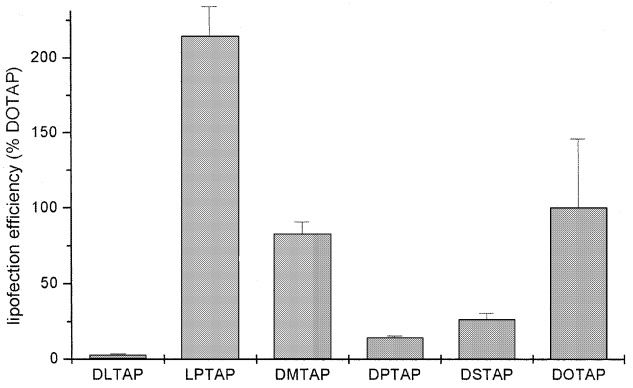

Fig. 3. Transfection efficiency of lipoplexes consisting of DOTAP analogs and pCMXluc in COS-7 cells. The charge ratio of cationic lipid to DNA was 2.5:1. Transfection efficiency was expressed as rlu per μg of cellular protein, for a better comparison standardized on the lipofection efficiency of DOTAP lipoplexes (set 100%). Each bar represents the mean (±S.D.) of six wells of a 96-well microtiter plate with the exception of the DOTAP standard (in quadruplicate).

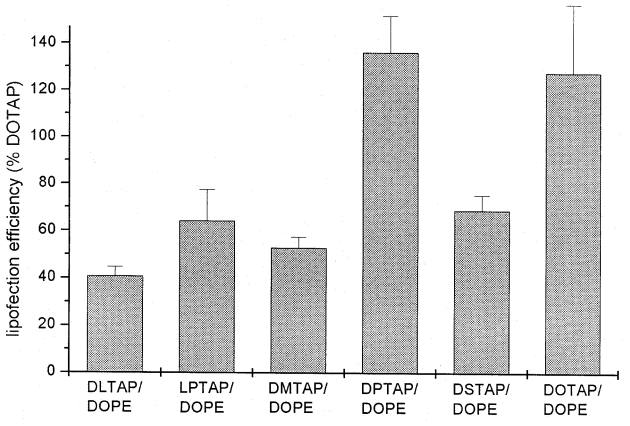

Fig. 4. Lipofection mediated by DOTAP analogs combined with 100 mol% of DOPE. Lipofection was performed using lipoplexes of the vector pCMXluc and the respective lipids. All lipofection results (rlu/μg of protein) were given as ratio of the transfection efficiency of the respective lipid to that of the DOTAP standard (set 100%). Experimental conditions were as described in Fig. 3.

showed about 80% of DOTAP. In contrast, DSTAP and DPTAP remained at transfection efficiencies below 25%. DLTAP lipoplexes led to very low transfection efficiencies, only slightly above background level.

3.4.2. DOTAP analogs and DOPE

Lipoplexes based on the respective DOTAP analog plus an additional 100 mol% of the helper lipid DOPE were tested in a similar set of transfection experiments. Transfection efficiencies ranged from 40 to 136% of the DOTAP standard (Fig. 4).

Comparing the different lipids, the mixture containing DLTAP led again to the lowest lipofection efficiency (about 40% of the standard). Lipoplexes composed of the lipid combination DSTAP/DOPE, DMTAP/DOPE, or LPTAP/DOPE were quite similar in their transfection efficiencies, being around 60% of the standard, whereas DPTAP/DOPE and DOTAP/DOPE displayed transfection efficiencies higher than 100%. To summarize, lipofection results mediated by the different DOTAP analog/DOPE lipoplexes displayed a somewhat more homogenous picture compared to lipoplexes consisting of pure cationic lipids.

3.4.3. DOTAP analogs and cholesterol

Inclusion of an additional 50 mol% of cholesterol into lipoplexes (finally containing 33 mol% of cholesterol) resulted in a different profile of lipid-mediated transfection: whereas DLTAP/CHOL and DSTAP/CHOL showed low transfection efficiencies (about 20% of the standard), DPTAP/CHOL, LPTAP/CHOL, and DOTAP/CHOL led to efficiencies around the 100% DOTAP standard, ranging from

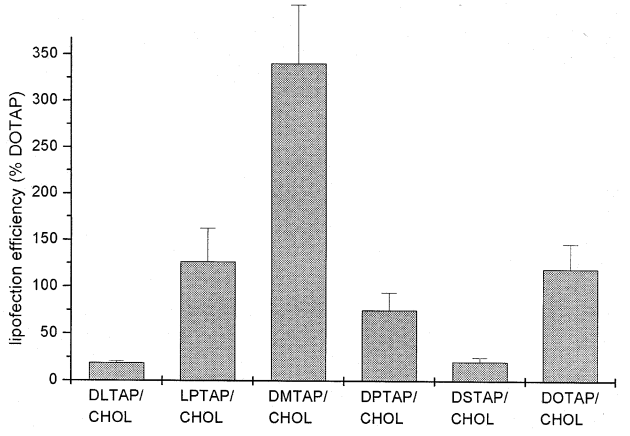

Fig. 5. Lipofection results of cholesterol-containing lipoplexes of DOTAP analogs (in a molar ratio of 1:2 of cholesterol: cationic lipid) and pCMXluc. Lipofection efficiencies (expressed in rlu/μg of cellular protein) of the lipids were given as percentage of the DOTAP standard (set 100%). Experimental conditions were similar to other lipofection experiments (Figs. 3 and 4).

75 to 126% (Fig. 5). The only exception was seen in the case of lipoplexes containing DMTAP/CHOL which reached an efficiency of even more than 300% of the standard.

4. Discussion

4.1. Stability of liposomes

Apart from DOTAP, none of the liposomes composed of pure DOTAP analogs form stable liposomes at RT. This instability can be attributed to the effect of the phase transition temperature (T_c) of the cationic lipids which also influences the thermal behavior of the liposomal bilayer. Accordingly, DOTAP is the only lipid in this series having a T_c (< 5°C (Table 1); -11.9°C [13]) minor to RT due to its unsaturated fatty acid chains. As to the DOTAP analogs which all bear saturated fatty acid chains, an increase in hydrocarbon chain length corresponds with increasing T_c [14], explaining why the temperature required for formation of stable liposomes increases from DLTAP to DSTAP (Table 1).

Other liposome-forming lipids, such as lecithins, do not show liposome instability at temperatures below T_c. The only difference between both families of lipids consists in the structure of the head group, the amphiphilic, neutral phosphocholine versus the cationic trimethylammonium group. Since a decrease in head group size favors the transition into an inverted hexagonal (H_{II}) phase, the formation of stable liposomes can be explained by the preference of the lamellar to the H_{II} phase. This is evident in the case of lecithin liposomes which do not show H_{II} phase transition below 85°C [15]. As to the DOTAP analogs, transition into the H_{II} phase might be responsible for the observed instability of the liposomes. We suggest that this could initially be caused by the formation of narrow ion pairs between the cationic head groups (soft character) and the soft anionic counter ions at and below their T_c. Thus, electrostatic repulsion between the cationic head groups could be reduced, leading to a decrease in the effective head group size and finally to a transition into the H_{II} phase. A simple model depicted in Fig. 6 illustrates this theory.

In cases where the helper lipids DOPE or cholesterol are included in liposomes, stable liposomes can be formed at RT. The only exceptions are DSTAP/DOPE and DPTAP/DOPE needing higher temperatures for formation of stable liposomes. The increase in liposome stability can be explained, on the one hand, by the low T_c of DOPE (-16°C [13]), reducing the phase transition temperature of the respective lipid mixture below RT. Thus, when included in liposomes in an appropriate molar ratio, e.g. 1:1, DOPE can improve liposomal stability [9] as also demonstrated in our experiments. On the other hand, DOPE is a cone-shaped molecule with a tendency to form hexagonal H_{II} phases [15,16]. This probably accounts for the instability of DSTAP/DOPE and DPTAP/DOPE liposomes with regard to the nonpolar part of the cationic lipids, both of which bearing the longest chains relative to the other analogs. The longer the hydrophobic tails of the lipid molecules, the more space they are supposed to require.

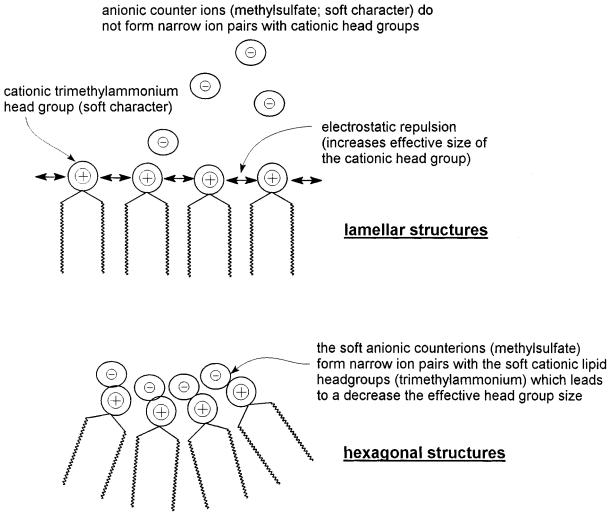

Fig. 6. Model describing the interaction between DOTAP analogs in a lipid bilayer. (Note: the drawing is strongly simplified: only the upper monolayer of the bilayer is shown.) The cationic head groups of the lipids interact differently with each other and with their counter ions, either leading to lamellar bilayer structures (top) or to hexagonal structures (bottom). Top: at temperatures above the phase transition temperature, high electrostatic repulsion between the positively charged head groups leads to an increase in their relative size, resulting in the formation of a lamellar bilayer. Bottom: when the anionic counterions interact with the positively charged lipid head groups (at lower temperatures), the actual head group size is reduced. Hexagonal membrane structures can be formed, possibly resulting in unstable liposomes.

When combined with the cone-like DOPE molecule [15], H_{II} phases could be formed, resulting in large aggregates. When packing parameters are decreased at higher temperatures [14], lamellar phases can be formed (cf. Fig. 6) leading to stable liposomes.

As cholesterol-containing liposomes are stable, even at RT, cholesterol seems to have a positive influence on bilayer stability. This can be attributed to interactions between the rigid cholesterol molecule and the hydrophobic part of the more flexible cationic lipid, thus preventing crystallization of the lipid membrane [14] which remains flexible ('fluid'). This will be discussed in more detail in the context of membrane fluidity.

4.2. *Fluorescence anisotropy correlated with stability of liposomes*

Fluidity is an important biophysical parameter characterizing a lipid bilayer. Thus, the ability of a lipid to form stable liposomes (and stable lipoplexes) ought to be regarded along with anisotropy measurements. Fluorescence anisotropy (r) serves as a measure of membrane fluidity: The higher the (re-) orientation of an excited fluorophore in a lipid bilayer (i.e. with increasing fluidity), the smaller gets the anisotropy [18]. In our experiments we used the nonpolar fluorophore DPH (1,6-diphenylhexa-1,3,5-triene) which is completely embedded in the hydrophobic part of the bilayer [18]. Thus, changes in the anisotropy level are only recorded when they are caused by changes of fluidity (of DPH) in the nonpolar region of the bilayer.

Liposomes composed of pure DOTAP analogs display characteristic temperature–anisotropy curves (or profiles) and in all cases, except for DSTAP, typical phase transition can be seen. When anisotropy levels are high ($r > 0.2$; Fig. 2A), i.e. membranes show rigid character, there can also be found unstable liposomes. When phase transition to the fluid state is completed at smaller r, as for DLTAP and DOTAP, liposomes are supposed to be stable. Yet, although DOTAP and DLTAP liposomes display nearly similar anisotropy profiles, only DOTAP forms stable liposomes at RT. This can be explained by the shortness of the hydrocarbon chains of the DLTAP molecule. Considering the fact that DCTAP, the DOTAP analog with hydrocarbon chains of 10 C-atoms, does not form liposomes at all (data not shown), a chain length of 12 C-atoms in the case of DLTAP could be too short to allow formation of stable liposomes.

When anisotropy curves are compared with each other, it is obvious that the profiles of DMTAP and LPTAP liposomes are nearly similar. This similarity can be explained on account of the mean length of the acyl chains, since DMTAP consists of two chains of 14 C-atoms and LPTAP of two different chains of 12 and 16 C-atoms, respectively.

In the liposomal system of monocationic lipids combined with DOPE, anisotropy curves are very similar showing relatively low anisotropy values. Distinct phase transitions can no longer be seen in contrast to the curves of pure DOTAP analogs. This observation differs from the results of Balasubramaniam et al. [19] who investigated a comparable system of monocationic lipids derived from DMRIE (*N*-[1-(2,3-dimyristyloxy)propyl]-*N*,*N*-dimethyl-*N*-(2-hydroxyethyl) ammonium bromide) consisting of a hydroxyethyl-derivatized ammonium head group and hydrophobic chains varying from 8 to 18 C-atoms. After combination of some of these lipids with DOPE, distinct phase transitions were still observed, and T_c was only slightly reduced [19]. This contrary behavior may be due to the different head groups used in both systems resulting in different phase transition behavior of the lipids combined with DOPE.

When cholesterol is used as helper lipid, no distinct phase transitions in anisotropy curves can be seen, but the curves describe individual, differing fluidity levels. This observation is consistent with anisotropy measurements of a monocationic tetraalkylamine (*N*,*N*-dioctadecyl-*N*,*N*-dimethylammonium bromide) and its diester analog (*N*,*N*-di-[(2-palmitoyloxy)ethyl]-*N*,*N*-dimethyl ammonium bromide) showing a broadening of the phase transition range with increasing cholesterol content: higher percentage of cholesterol ($> 10\%$) included in liposomes resulted not only in a broader melting profile, but also abolished the sharp curve profile [20]. Cholesterol, a component of eukaryotic cell membranes, is known to make membranes more rigid at high concentration. It prevents membrane crystallization, although it does not inhibit lateral mobility within the membrane [17]. Accordingly, we could observe a stabilizing effect on liposomes as seen on the stability data

of cholesterol-containing liposomes at RT and 37°C (Table 1). When compared with the respective liposomes made of pure DOTAP analogs, anisotropy values of both liposome types are almost similar at 20°C in most cases. At higher temperatures (above T_c of the pure DOTAP analogs), increased anisotropy due to incorporation of cholesterol can be attributed to an increase in chain order [21], finally leading to higher liposome stability. This might be explained by a reduction of electrostatic interactions between the head groups, because the cationic lipids are separated by the cholesterol molecule (cf. the model in Fig. 6). But since the DOTAP analogs we used bear the same head group, the differences in their anisotropy profiles upon addition of cholesterol into liposomes can rather be attributed to interactions between the hydrophobic molecule part of cholesterol and the hydrocarbon chains of the respective cationic lipid [20,21].

Taken together, within the three groups of liposomes containing DOTAP analogs, fluidity seems to be a prerequisite for stability of the respective liposomes. Exceptions, i.e. unstable liposomes in spite of 'apparent' fluidity, may be found when packing parameters of the lipids are unsuitable for formation of stable liposomes.

4.3. Stability of lipoplexes

The data concerning the stability of liposomes and lipoplexes reveal a strong correlation between the formation of stable liposomes and the formation of stable lipoplexes, made of the corresponding lipid or lipid mixture, at a specific temperature. As seen on liposomes, the addition of the helper lipids DOPE or cholesterol also improves lipoplex stability.

4.4. Correlation of lipoplex stability and fluorescence anisotropy

Regarding the anisotropy curves of lipoplexes containing pure DOTAP analogs at the transfection temperature, there can be observed two groups of profiles (Fig. 2D): in cases where distinct phase transitions occur, as for DPTAP, DMTAP, and LPTAP lipoplexes, respectively, anisotropy levels at 37°C can be found either at high anisotropy values, describing 'rigidity', or at small anisotropy values (following phase transition), characterizing the 'fluid' state. Anisotropy levels of DPTAP lipoplexes at 37°C can be interpreted as rigid which is consistent with their not forming stable lipoplexes at 37°C (Table 2), whereas DMTAP and LPTAP lipoplexes show fluidity, corresponding with lipoplex stability.

In the case of lipoplexes consisting of pure DOTAP analogs which cannot be characterized by a distinct phase transition at the regarded temperature range, the difference between 'fluid' and 'rigid' bilayer behavior is less obvious. For instance, anisotropy values of DSTAP lipoplexes indicate rigid behavior at 37°C (Fig. 2D), corresponding with lipoplex instability. Since anisotropy values of DLTAP lipoplexes at 37°C lie in the lower part of the roughly monotonous curve, high membrane fluidity and hence lipoplex stability could be expected. Instead DLTAP leads to unstable lipoplexes, which corresponds with liposome instability at 37°C, most probably due to its short acyl chains, as discussed above. DOTAP lipoplexes show an almost constant curve profile of low anisotropy, correspondent with lipoplex stability.

When the anisotropy curves of the lipoplexes composed of pure DOTAP analogs are compared to those of the corresponding liposomes, addition of DNA to liposomes seems to increase fluidity at temperatures below T_c, in the 'rigid' state. Below T_c interaction of the negatively charged DNA backbone with the monocationic head groups could lead to a less tightly packed organization, increasing the distance between the lipid molecules in the membrane.

Above T_c, in the 'fluid' state, addition of DNA more or less tends to reduce fluidity. The interaction of the positively charged head groups with the polyanion DNA seems to decrease lateral diffusion of the lipids in the membrane. To test this hypothesis, a higher amount of DNA was used for complexation, leading to a decreased charge ratio of 1:1 instead of 2.5:1. There could be observed a parallel shift of the anisotropy curve to higher anisotropy levels (data not shown), i.e. decreased fluidity. Our data are supported by [22], observing a small enhancement of anisotropy of DOTAP lipoplexes relative to liposomes at a charge ratio of 1:1.

In general, the shape of the phase transition profiles and T_c of the lipoplexes resemble those of the respective liposomes. Corresponding results were ob-

tained by Bhattacharya and Mandal who did not observe significant differences in the general phase transition behavior and the T_c value between DPTAP alone and DPTAP–DNA complexes [22].

Since anisotropy profiles of lipoplexes containing DOTAP analogs and the helper lipid DOPE are almost similar, lipoplex stability at 37°C corresponds with nearly identical anisotropy levels, i.e. similar membrane fluidities. In this case, complexation of DNA does not seem to influence bilayer fluidity very strongly, in particular at 37°C. The membrane system rather seems to be controlled by high amounts of the amphiphilic DOPE molecule.

Considering the fact that cholesterol-containing lipoplexes are stable even at RT, the corresponding, almost constant anisotropy profiles correlate with lipoplex stability. Although individual anisotropy levels differ from each other, none of the curves show phase transition. As in the case of cholesterol-containing liposomes, differing anisotropy levels do not reflect differences in stability of the lipoplexes. Since anisotropy levels of lipoplexes with cholesterol incorporated are slightly lower than those of the corresponding liposomes in nearly all cases (with the exception of DSTAP-containing lipoplexes), the addition of DNA to liposomes, i.e. the formation of lipoplexes, seems to increase bilayer fluidity. This, hence, leads to a decrease in the packing order of the cationic lipid–cholesterol system reflected in a higher mobility of the DPH-label in the membrane.

In summary, as characteristic differences between liposome and lipoplex anisotropy, respectively, can be seen on some, but not all types of lipids and lipid mixtures, the influence of DNA on bilayer fluidity and stability seems to be less strong than the influence of (helper) lipid composition. With some exceptions as already discussed for liposomes, the stability of lipoplexes at a certain temperature can be derived from the phase transition behavior which is reflected in anisotropy data. Since actual anisotropy levels can differ between the three lipid systems, only data within the related lipid systems can be compared directly.

4.5. *Can lipofection results be correlated with lipoplex stability and/or fluidity?*

Lipofection efficiencies of the three types of lipoplexes containing DOTAP analogs are studied under similar conditions in COS-7 cells using a serum-free in vitro system. When lipofection efficiencies of lipoplexes composed of pure DOTAP analogs are examined, there is a strong correlation with liposome and lipoplex stability as well as with bilayer fluidity. In the case of DSTAP, and even more DPTAP, low lipoplex fluidity (Fig. 2D) at 37°C correlates with lipoplex instability and low transfection efficiency (Fig. 3). DMTAP and LPTAP, lipids which are nearly similar in their biophysical properties as discussed above, show the highest, though not equal, lipofection efficiencies apart from DOTAP. Since both lipids are able to form stable liposomes and lipoplexes at 37°C corresponding with high and quite similar fluidity, suitable preconditions for high lipofection efficiency are given. Nevertheless, anisotropy profiles show only small differences, which makes it difficult to explain why LPTAP leads to higher lipofection efficiency than its 'counterpart'. By contrast, DLTAP lipoplexes which show high fluidity, but are not stable at 37°C, display very low transfection efficiency. This can be put down to an unsuitable chain length, as discussed above, resulting in low lipofection efficiency. Thus, though lipoplex stability can differ from anisotropy profiles in some cases, formation of lipoplexes stable at transfection temperature seems to be an important precondition for efficient lipofection.

When the amphiphilic helper lipid DOPE is involved, lipofection efficiencies correspond to anisotropy measurements showing rather uniform distributions independent of the different lengths of hydrophobic residues. This may be due to the strong influence of DOPE on liposomal bilayers (see above). In the context of lipofection, DOPE, on the contrary, is known to destabilize endosomal membranes and to play an important role in the resulting membrane fusion process [10,23,24].

Since lipofection efficiency is enhanced only in cases where lipofection efficiency is low without DOPE, the extent of its effect seems to depend on the lipid used. By contrast, the transfection efficiency of LPTAP lipoplexes is even decreased by the addition of DOPE. Consistent with our data, different degrees of enhancement by DOPE were also found in a related system of cationic diester lipids (analogs of DORI, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*-[1-(2-hy-

droxy)ethyl]-*N*,*N*-dimethyl ammonium iodide) containing acyl chains of varied length (C14:0 to C18:0) [19]. In the current study, highest lipofection efficiencies in the DOPE-containing system do not lead to lipofection efficiencies much higher than the DOTAP standard (DPTAP/DOPE and DOTAP/DOPE reaching no more than 130%, Fig. 4). So from our results, DOPE seems to equalize not only the transfection capabilities, but also the biophysical properties of the different lipids and liposomes in general as seen on the anisotropy profiles.

Although cholesterol has a stabilizing effect on liposomes and lipoplexes leading to stable lipoplexes with similar size distributions at 37°C, anisotropy curves (Fig. 2F) and lipofection results (Fig. 5) reveal differences between the analogs: DMTAP/CHOL lipoplexes, for instance, displaying the highest anisotropy level, also lead to the highest transfection efficiency. In comparison, lipofection efficiencies of DPTAP/CHOL, LPTAP/CHOL and DSTAP/CHOL lipoplexes are much lower, but anisotropy values at 37°C are also smaller, though do not differ very much from each other. DLTAP/CHOL lipoplexes with the lowest anisotropy level of the DOTAP analogs show the lowest lipofection efficiency. Taking these data together, in the system of lipoplexes containing cholesterol and DOTAP analogs with saturated hydrocarbon chains, relatively high or increasing (DSTAP/CHOL) fluidity at 37°C coincides with low(er) transfection efficiency.

5. Summary

Taking our results together, lipofection efficiency is strongly influenced by stability and fluidity of the lipoplexes. As seen on the group of pure DOTAP analogs, fluid behavior of lipoplex membranes determines their stability which is a prerequisite for efficient lipofection. In the system of DOTAP analogs combined with DOPE this observation is confirmed. Yet similar fluidities of the lipoplexes make it difficult to explain differences in lipofection efficiency on account of membrane fluidity. In the system of lipoplexes containing DOTAP analogs and cholesterol, in contrast, individual relative fluidities parallel differences in transfection efficiency. When fluidity per se is given, higher anisotropy levels seem to improve lipofection efficiency, probably caused by differences in intracellular stability of the lipoplexes. Thus, DNA release into the cytoplasm might be optimized.

It ought to be noted that the actual anisotropy level should not serve to predict lipofection results when different systems of lipoplexes (containing or not containing different helper lipids) are compared. Nevertheless, anisotropy measurements represent a very helpful tool to characterize systems of transfection lipids on account of their relative membrane fluidity, though the direct comparison of the three systems regarded in this study may be very difficult to draw.

The current study investigates the biophysical properties of liposomes and lipoplexes outside cells (in a serum-free environment) and the 'result' of the gene transfer: the activity of the luciferase reporter protein. Accordingly, same questions remain: How and how efficiently do lipoplexes enter the cell? Also the kinetics of DNA release have to be studied, once the lipoplexes have been taken up by the cell. Finally, the fate of DNA on its pathway to the nucleus is very important for successful and efficient gene transfer [25,26]. Concerning these aspects, lipid composition and biophysical characteristics of lipoplexes determine interaction with endosomal membranes to permit DNA release into the cytosol [10,27,28]. Further investigation on the behavior of lipoplexes inside the cell will help to elucidate this involved mechanism.

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