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Research paper

Effect of cationic lipid and matrix lipid composition on solid lipid nanoparticle-mediated gene transfer

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Abstract

This investigation is focused on the enhancement of in vitro transfection activity by optimizing cationic lipid and matrix lipid composition of solid lipid nanoparticles (SLN). For this purpose SLN were formulated by using two different matrix lipids and six different cationic detergents. These 12 formulations were tested for physical parameters such as particle size, zeta potential and DNA-binding capacity, and also for their biological properties such as cytotoxicity and in vitro transfection efficiency. The SLN were produced by hot high-pressure homogenization, all formulations were physically stable and showed a highly positive surface charge (+34 to +45 mV). In vitro cytotoxicity measurements on COS-1 cells revealed that cytotoxicity is strongly dependent on the cationic lipid used. SLN made from one-tailed cationic detergents were highly cytotoxic. In contrast the two-tailed cationic lipids were all well tolerated. Transfection activity seems to be determined by both the cationic lipid and the matrix lipid used. Here, the combination of cetylpalmitate and *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride led to significantly higher transfection efficiencies than in all other tested combinations. These results indicate that well tolerated and highly efficient in vitro transfection could be achieved with SLN whenever selecting good combinations of two-tailed cationic lipids and matrix lipids.

Keywords: Solid lipid nanoparticles; Non-viral gene delivery; Non-viral DNA vectors; Cationic lipids; Matrix lipid; Cytotoxicity

1. Introduction

A large array of non-viral transfection agents have currently been investigated for in vitro and in vivo applications, including cationic peptides [1], dendrimers [2], polycationic polymers [3] and the most popular cationic lipids [4]. Gene delivery mediated by oil-in-water nanoemulsions formulated with cationic lipids has been described [5]. So far, minimal attention has been paid to the use of solid lipid nanoparticles (SLN) as DNA carriers, although these may offer a number of technological advantages. These include a better storage stability in comparison to liposomes, a relatively easy production without any organic solvent [6], the possibility of steam sterilization [7] and lyophilization [8], large scale production with qualified production lines [9] and the use of substances that are generally accepted as safe [10].

SLN were invented at the beginning of the 1990s, and were either produced by high-pressure homogenization [11] or by microemulsion technique [12]. From the point of view of production and regulatory aspects, high-pressure homogenization is considered as the method of choice. SLN consist of a solid matrix. SLN are comparable with parenteral emulsions with the difference that in SLN-formulations the liquid lipid (oil) is replaced by a solid lipid. Due to their solid particle matrix, they can protect incorporated ingredients against chemical degradation [13] and allow modification of release of active compounds [14].

Recent investigations in our laboratories have shown that some preparations of cationic SLN were able to transfect mammalian cells (COS-1) in vitro [15]. The aim of this study was the enhancement of transfection activity by optimizing the SLN composition. For this purpose, 12

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different SLN were formulated from six different cationic lipids and two matrix lipids, and were tested regarding formulation stability, plasmid immobilization, cytotoxicity and transfection efficiency.

2. Materials and methods

2.1. Materials

Compritol ATO 888 was provided by Gattefossé (Weil am Rhein, Germany). Cetylpalmitate (Cutina Cp) was a gift from Henkel (Düsseldorf, Germany), Tween 80 and Span 85 from ICI Surfactants (Eversberg, Belgium). The cationic surfactant *N*,*N*-di-(β-stearoylethyl)-*N*,*N*-dimethyl-ammonium chloride (Esterquat 1, EQ 1) was provided by Gerbu Biotechnik (Gaiberg, Germany). Benzalkonium chloride (BA), cetylpyridinium chloride (CPC), cetrimide (CTAB), *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTAP), chloroquine (CQ) phosphate and polyethylenimine (pEI, 25,000 kDa) were purchased from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany). Dimethyldioctadecylammonium bromide (DDAB) was obtained from Clariant GmbH (Frankfurt a. Main, Germany). Organic formulas of the matrix lipids and the cationic lipids are shown in Fig. 1.

The cell culture reagents were purchased from PromoCell (PromoCell GmbH, Heidelberg, Germany).

2.2. SLN production

The SLN were produced by hot high-pressure homogenization as described by Mehnert and Mäder [6]. Briefly, the solid lipids were heated to approximately 10 °C above their melting points. The mixture of molten lipid (4% w/w when referred to the final product) and the hot aqueous solution of surfactant and cationic lipid (2% w/w Tween 80 and Span 85 in a 7:3 ratio and 1% w/w cationic lipid) formed a pre-emulsion after stirring for 1 min. All applied homogenizers are piston-gap homogenizers, which mostly differ in the processable product volume, which may lead to slightly different particle sizes and distributions.

The 1.5 kg batches of SLN containing cationic lipids CPC, CTAB, BA, DDAB or EQ1 were produced using a specially modified LAB 60 homogenizer (APV-Gaulin, Lübeck, Germany) [9], which worked continuously. Formation of the pre-emulsion was performed in the feeding vessel of the homogenizer using a built-in stirring unit. The dispersions were processed in circulation for 30 min at 85 °C and were subsequently passed through two homogenization valves: a first main homogenization valve (500 bar), and a second valve (50 bar) that created a certain reverse pressure and which was also in charge of redispersing coalesced droplets. In general, the combination of two gaps leads to very homogeneous particle distributions.

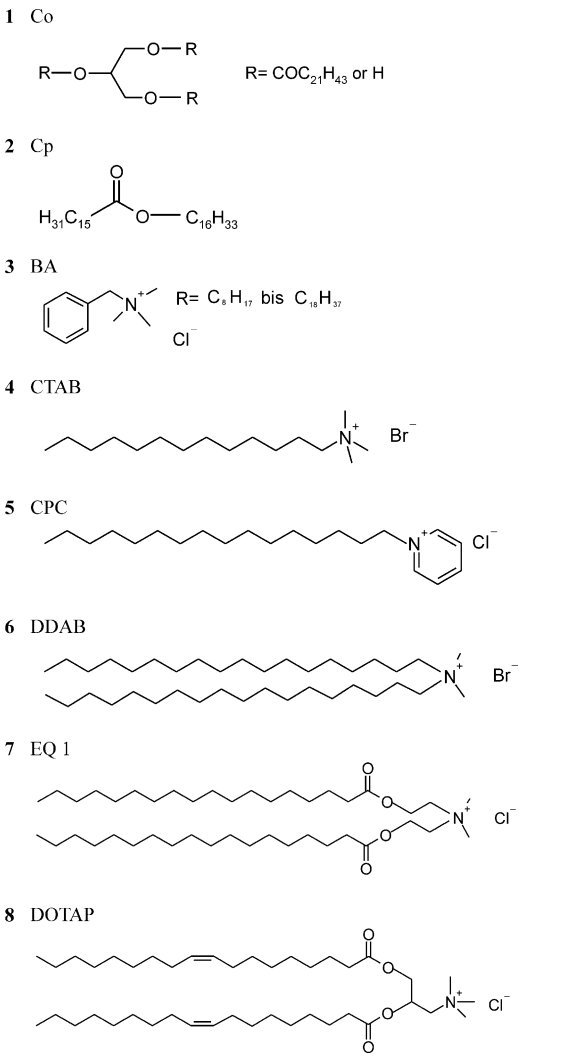

Fig. 1. Structures of the employed matrix lipids and cationic lipids. **1**, Compritol ATO 888 (Co) a mixture of mono-, di- and triglycerides of behenic acid (C_{22}); **2**, cetylpalmitate (Cp); **3**, benzalkonium chloride (alkyldimethylbenzylammonium chloride, BA); **4**, cetrimide (tetradecyltrimethylammonium bromide, CTAB); **5**, cetylpyridinium chloride (hexadecylpryridinium chloride, CPC); **6**, dimethyldioctadecylammonium bromide (DDAB); **7**, *N*,*N*-di-(β-stearoylethyl)-*N*,*N*-dimethyl-ammonium chloride (Esterquat 1, EQ 1); **8**, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTAP).

The 3.5 g batches of SLN containing cationic lipid DOTAP were homogenized using the heatable Emulsi-Flex®-B3 (Avestin Inc., Ottawa, Canada), which worked discontinuously. The pre-emulsion was obtained using a high-speed stirrer (Ultra Turrax T25, Jahnke and Kunkel, Germany) and homogenized at 85 °C whilst applying a pressure of 480 bar and four homogenization cycles.

In a second cycle 25 g batches of SLN containing DOTAP were produced using the heatable, discontinuously working LAB 40 homogenizer (APV-Gaulin, Lübeck, Germany). Here the pre-emulsion (also obtained applying a high-speed stirrer) was homogenized for three cycles at 500 bar and 85 °C.

2.3. Size and zeta potential measurement

Particle size was analysed by photon correlation spectroscopy (PCS) using a Zetasizer 4 (Malvern Instruments, Herrenberg, Germany). PCS gives information about the mean diameter of the bulk population (so-called *z*-average) and the width of distribution via the polydispersity index (PI). The surface charge was determined using the same equipment. Zeta potential was measured in water at pH 7.4 (adjusted with sodium hydroxide) containing sodium chloride to adjust the conductivity to 50 μS/cm. Measurements were performed in the large bore cell at 20 V/cm field strength. The electrostatic mobility was converted to the zeta potential using the Helmholtz–Smoluchowski equation.

2.4. Agarose gel electrophoresis

SLN–DNA complexes were prepared by mixing in 25 mM HEPES (pH 7.4) at a final plasmid concentration of 10 μg/ml and subjected to an agarose gel (0.7% ethidium bromide included for visualization) to electrophoresis for 2 h at 5 V/cm. Images were obtained using a UV transluminator and a Geldoc 2000 gel documentation system (Biorad, Munich, Germany), with the settings adjusted to avoid signal saturation. Band integration and background correction were obtained using Molecular Analyst software Version 1.1 (Biorad). The fraction of mobile DNA was subsequently calculated from its difference to free DNA (without SLN).

2.5. Cell culture

The African green monkey kidney fibroblast-like cell line COS-1 was obtained from DSMZ (Braunschweig, Germany) and maintained in Dulbecco's Modified Eagles' Medium (DMEM), supplemented with 10% fetal calf serum. Cells were incubated at 37 °C with 5% CO_2 in air and subcultured every 2–3 days using trypsin/EDTA.

2.6. Cytotoxicity

Cells were seeded on 96 well plates at a density of 10,000 cells per well and allowed to adhere overnight. The SLN were diluted in distilled water and then added to the same volume of 2 × concentrated DMEM. The medium was removed from the semi-confluent cells. Cells were incubated with 200 μl of the SLN dilutions in DMEM, and after incubation for 4 h at 37 °C the amount of lactate dehydrogenase (LDH) was determined in 100 μl of the supernatant. The quantity of LDH correlates with the extent of membrane damage and was related to cells incubated with 2% TritonX in medium (0% viability) and cells incubated in DMEM (100% viability). The assay system was obtained from Boehringer Mannheim (Germany), and used according to the manufacturer's instructions. The assay was performed in six replicates.

2.7. Plasmid

The β-galactosidase expression plasmid *pCMVβ* was purchased from ATCC (Manassas, VA) and transformed into *E. coli* DH5α. A Gigaprep from 2500 ml of overnight culture was performed according to the manufacturer's instructions (Endofree Giga Prep, QIAGEN, Hilden, Germany). The DNA was precipitated in 70% ethanol and reconstituted in 5 ml of 10 mM Tris–HCl (pH 8.5). The DNA concentration was determined using Hoechst 33258 dye.

2.8. Preparation of SLN–DNA complexes

The SLN or pEI were diluted in distilled water. Three hundred and twenty-five microlitres of plasmid (20 μg/ml in 25 mM Hepes) and 325 μl SLN or pEI (5–80 μg/ml) dilution were mixed in an Eppendorf tube. The concentrations of SLN dilutions were optimized by agarose gel electrophoresis for each particle. The employed cationic lipid:DNA ratio (w/w) is indicated in the caption for each figure. After 20 min of equilibration at room temperature, the complexes were diluted with either 650 μl of 2 × concentrated DMEM, 2 × DMEM supplemented with 10% FCS, 2 × DMEM supplemented with 100 μM CQ or 2 × DMEM supplemented with FCS and CQ to a final volume of 1300 μl.

2.9. Transfection procedure and β-galactosidase measurement

For transfection COS-1 cells were seeded on 96 well plates at a density of 10,000 cells per well and allowed to adhere overnight. The medium was removed from the 50–70% confluent monolayers and 200 μl of the complex dilution was added. Cells were incubated with the complexes for 4 h at 37 °C. The cells were washed once with PBS and allowed to grow for a further 48 h in DMEM containing 10% FCS.

Average β-galactosidase activities per well were determined using the luminescence coupled Galacto-Star® assay system (Applied Biosystems). Wells were washed twice with PBS and lysed with 100 μl of mammalian cell lysis buffer per well for 15 min at 4 °C. Fifty microlitres of lysate was transferred to black isoplates, mixed with 100 μl of freshly prepared reaction solution and incubated for 90 min at room temperature to reach the plateau of light emission. Luminescence was measured in a Wallac 1450 Microbeta Trilux (PE Biosystems) over an integration time of 1 s. Total protein content was determined from 25 μl lysate using the BCA assay (Pierce). All assays were performed in six replicates. Transfection efficiency is quantified in Relative Light Units (RLU)/mg protein.

Table 1
Composition of SLN formulations, their production method and physicochemical characterization

Formulation	Matrix lipid	Cationic lipid	Homogenizer	Size (nm)	Polydispersity index	Zeta potential (mV)
Co_CTAB	Compritol 4%	CTAB 1%	LAB 60	148	0.194	39.4
Co_CPC	Compritol 4%	CPC 1%	LAB 60	154	0.241	44.3
Co_BA	Compritol 4%	BA 1%	LAB 60	134	0.199	39.5
Co_DDAB	Compritol 4%	DDAB 1%	LAB 60	158	0.241	41.8
Co_EQ1	Compritol 4%	EQ1 1%	LAB 60	144	0.239	39.9
Co_DOTAP	Compritol 4%	DOTAP 1%	EmulsiFlex®-B3	327	0.464	39.0
Cp_CTAB	Cetylpalmitate 4%	CTAB 1%	LAB 60	131	0.190	37.0
Cp_CPC	Cetylpalmitate 4%	CPC 1%	LAB 60	113	0.201	38.6
Cp_BA	Cetylpalmitate 4%	BA 1%	LAB 60	127	0.225	37.7
Cp_DDAB	Cetylpalmitate 4%	DDAB 1%	LAB 60	113	0.201	36.7
Cp_EQ1	Cetylpalmitate 4%	EQ1 1%	LAB 60	110	0.136	34.1
Cp_DOTAP	Cetylpalmitate 4%	DOTAP 1%	EmulsiFlex®-B3	244	0.322	44.8

All formulations were stabilized with 2% of a mixture from Tween 80 and Span 85 (w/w ratio 7:3).

2.10. Statistical analysis

Results are reported as means ± standard deviation (SD). The statistical analysis between different groups has been determined with a non-paired *t*-test. Differences were considered statistically significant if $P < 0.05$.

3. Results

3.1. SLN production

To investigate the influence of cationic lipid and matrix lipid composition on cytotoxicity, plasmid binding and transfection efficiency, 12 different SLN-formulations were produced by high-pressure homogenization (Table 1).

The matrix lipid was either Compritol (Co, a mixture of mono-, di- and triglycerides of behenic acid) or the wax cetylpalmitate (Cp). In order to obtain a positive surface charge six different cationic lipids/detergents were employed.

3.2. Size and zeta potential measurement

Results of size and zeta potential measurements are shown in Table 1. The surface charge as determined by zeta potential measurement was highly positive (+34 to +45 V) for all formulations. The particle sizes of SLN produced at the LAB 60 were around 110–160 nm. The DOTAP-SLN which were produced using the EmulsiFlex®-B3 showed larger particle diameters (244/327 nm) and a broader size distribution (higher PI).

The formulations showed excellent storage stability, the particle diameters and PIs did not change or only negligibly by a few nanometres during 250 days (data not shown). Formulations were stored in the dark and at room temperature (with the exception of the DOTAP-SLN, which were stored at 4–8 °C due to the oxidation-sensitive double bonds).

3.3. Agarose gel electrophoresis

The binding of the cationic SLN to the polyanionic DNA was studied using analysis of the electrophoretic mobility of the DNA within an agarose gel (the so-called electrophoretic mobility shift assay, EMSA). Efficient complexation of *pCMVβ* by cationic SLN led to immobilization and prevention of ethidium bromide intercalation with DNA.

All SLN-formulations were able to immobilize *pCMVβ* in the EMSA (Fig. 2). DDAB–Compritol- and the EQ1-SLN immobilized DNA in a cationic lipid to DNA ratio (w/w) of only 0.5–1.0:1. To bind DNA with

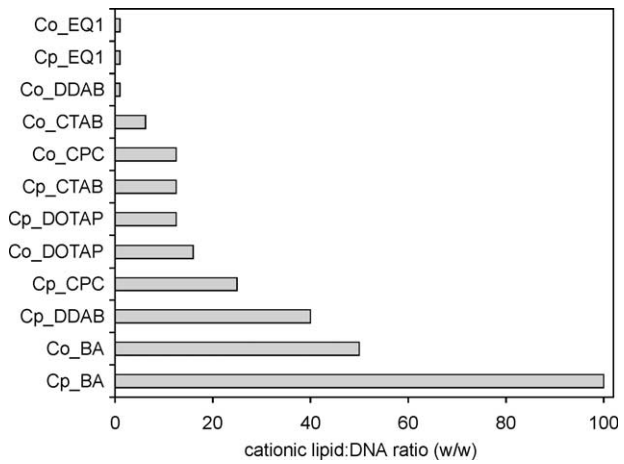

Fig. 2. DNA immobilization by cationic SLN. For every SLN formulation, SLN–DNA complexes with increasing amounts of SLN were prepared and analysed for DNA immobilization ability. The amounts of free DNA were related to uncomplexed DNA (100% mobile) run on the same gel. To quantify the DNA-immobilization ability, the cationic lipid:DNA ratios (w/w) required for at least 95% immobilization are compared.

DDAB–cetylpalmitate-, DOTAP-, CPC- or CTAB-SLN a higher excess of cationic lipid was necessary. A huge excess of BA-SLN was required to immobilize the plasmid. Primarily, the nature of cationic lipid determines the ability to bind DNA, although the binding-properties are also influenced by the kind of matrix lipid used. For example, the DDAB-SLN made from Compritol immobilized DNA in a much smaller excess of cationic lipid (0.5–1.0:1) than DDAB-SLN made from cetylpalmitate (40:1). The same behaviour could also be observed for the CPC-SLN. Because of their poor DNA binding-properties BA-SLN were excluded from further tests.

3.4. Cytotoxicity

In vitro cytotoxicity was tested on COS-1 cells by determining the LDH release. Measuring LDH release (Fig. 3) assessed the initial perturbation of cell integrity during the 4 h incubation.

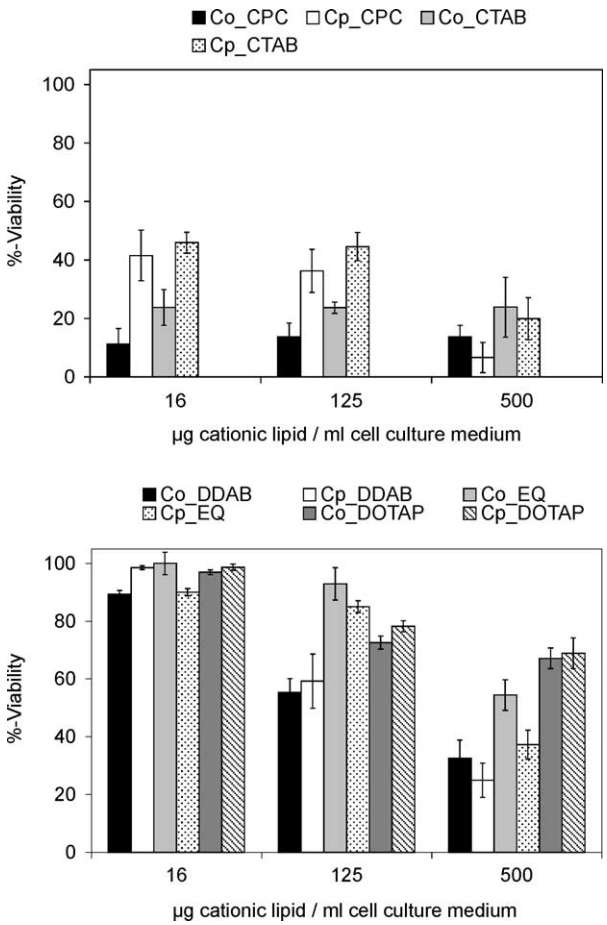

Fig. 3. Cytotoxicity studies. COS-1 cells were incubated with different SLN formulations in various concentrations. LDH release was determined after 4 h. Upper, SLN made from one-tailed cationic lipids showed even in low concentrations significant cytotoxicity. Lower, SLN made form two-tailed cationic lipids are well tolerated in transfection efficient concentrations (e.g. the Cp_DOTAP–DNA complexes were most efficient in a 2.5:1 cationic lipid:DNA ratio corresponding to 12.5 µl DOTAP/ml incubation medium).

The one-tailed cationic lipids (CPC and CTAP, see Fig. 1) showed significant toxicity in concentrations required for efficient DNA-immobilization. In contrast, the two-tailed cationic lipids (DDAB, EQ1, DOTAP, see Fig. 1) were all well tolerated in transfection-efficient concentrations (e.g. the Cp_DOTAP–DNA complexes were most efficient in a 2.5:1 cationic lipid:DNA ratio corresponding to 12.5 µl DOTAP/ml incubation medium).

3.5. Transfection

The transfection assay was only performed using the non-toxic SLN made from the two-tailed cationic lipids (DDAB, EQ1 and DOTAP). All formulations were tested using DMEM in a variety of ways. Pure DMEM, DMEM with 100 µM CQ added, DMEM supplemented with 10% foetal bovine serum (FCS) and DMEM with CQ and FCS were put to the test. These four different media were employed to determine the effect of serum and lysosomotropic substances like CQ on the transfection efficiency. The results of this are shown in Fig. 4.

All formulations significantly increased transfection compared to naked DNA, and the activities of DDAB- and EQ1-SLN and the DOTAP–Compritol-SLN were much the same. The transfection efficiency of the DOTAP–cetylpalmitate-SLN was statistically significantly higher (about 10 ×) and almost comparable to pEI (Fig. 5). pEI is one of the most effective cationic polymers for gene delivery currently available [16].

The efficiency of all formulations increased in the presence of CQ.

The addition of serum reduced the activity to different extents. The efficiency of DOTAP-SLN decreased by only

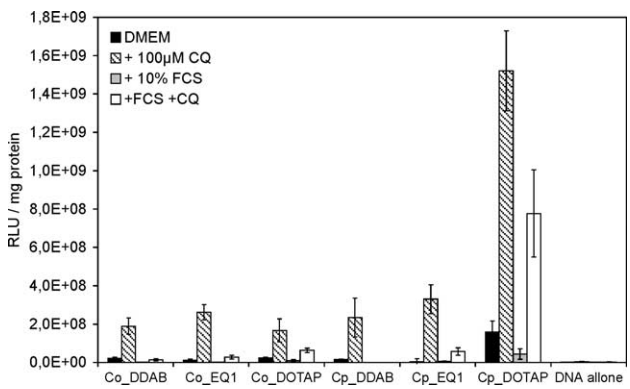

Fig. 4. Transfection efficiency (RLU/mg protein) of different SLN–DNA complexes in varying incubation media (DMEM, DMEM + 100 µM chloroquine, DMEM + 10% FCS, DMEM + CQ + FCS). The complexes of all six SLN formulations were tested in five different cationic lipid to DNA ratios that were arranged around the ratio of total DNA immobilization determined by agarose gel electrophoresis. The efficiencies of the best cationic lipid:DNA ratios w/w (2:1 for Co_DDAB and Co_EQ, 2.5:1 for Co_DOTAP, Cp_DDAB and Cp_DOTAP and 4:1 for Cp_EQ) are compared here.

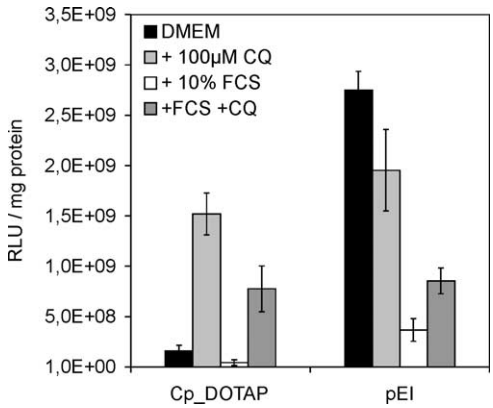

Fig. 5. Transfection efficiency of Cp_DOTAP-(cationic lipid:DNA w/w 2.5:1) and pEI-DNA (pEI:DNA w/w 4:1) complexes in different incubation media (DMEM, DMEM + 100 μM chloroquine (CQ), DMEM + 10% FCS, DMEM + CQ + FCS).

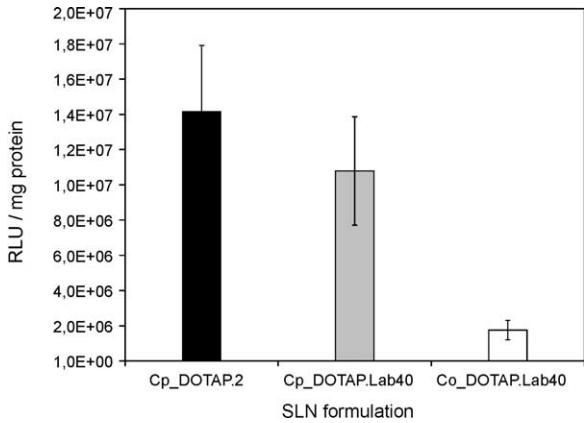

Fig. 6. Transfection efficiency of SLN formulated from different matrix lipids. SLN were composed of 0.5% DOTAP, 2% Tween80/Span85 and 4% Compritol (Co_DOTAP.Lab40) or cetylpalmitate (Cp_DOTAP.2 and Cp_DOTAP.Lab40) and were applied in a DOTAP:DNA weight ratio of 2.5:1. The SLN were produced with different homogenizers: Cp_DOTAP.Lab40 and Co_DOTAP.Lab40 using the LAB 40 homogenizer and Cp_DOTAP.2 with the EmulsiFlex®-B3.

around 50%, whilst the activity of the other SLN was more affected by serum supplement.

DOTAP–cetylpalmitate-SLN showed significantly higher transfection efficiency compared to the other formulations tested. They were peculiarly even more active than the DOTAP–Compritol-SLN (Fig. 4). To investigate whether the superiority of the DOTAP–cetylpalmitate-SLN was reproducible and if the effectiveness had been influenced by the production method, we tested three additional formulations of DOTAP-SLN produced with different homogenizers and two different matrix lipids. All three were formulated from 4% matrix lipid, 2% Tween/Span mixture and 0.5% DOTAP. The amount of DOTAP was decreased from 1 to 0.5% because it was found, that the transfection efficiency did not change statistically significantly (data not shown) when reducing the DOTAP amount from 1 to 0.5%. The first (Cp_DOTAP.2) was formulated from the wax cetylpalmitate and produced in the same way as Cp_DOTAP, by homogenization in the EmulsiFlex®-B3. The second formulation (Cp_DOTAP.Lab40) was also made from cetylpalmitate, but produced in the LAB 40 homogenizer in 25 ml batches, which led to smaller particle sizes (PCS mean diameter: 170.2 nm) and a narrower distribution (p*I* 0.170). The third formulation (Co_DOTAP.Lab40) was formulated from the glyceride Compritol and produced in the LAB 40. In utilizing this homogenizer type the particle size was also smaller (188.2 nm, p*I* 0.305) and comparable to the previous formulations homogenized at the LAB 60.

Fig. 6 presents the results of the transfection experiment on COS-1 cells in DMEM.

SLN composed of DOTAP and cetylpalmitate showed repeatedly higher transfection activities than DOTAP–Compritol-SLN. This superiority was statistically significant. It can be argued that the matrix lipid strongly influences the transfection effect, though no statistically significant difference in effectiveness could be observed between the similarly composed formulations produced with different homogenizers.

4. Discussion

Olbrich et al. [15] showed that some preparations of cationic SLN could transfect mammalian cells in vitro. The aim of this study was to improve the SLN-formulations with regard to transfection efficiency using six different cationic detergents/lipids and two matrix lipids.

Production by hot high-pressure homogenization led to stable nanoparticles of about 110–160 nm (LAB 60) or 220 and 330 nm (EmulsiFlex®-B3) size. Applying the EmulsiFlex®-B3 the particle size was strongly influenced by the matrix lipid used. In general, the hot high-pressure homogenization of SLN becomes easier by scaling up [9]. The incorporation of 1% cationic lipid/detergent induced a highly positive zeta potential of about +35 to +45 mV.

All 12 formulations tested were able to immobilize *pCMVβ* in EMSA. The excess of cationic lipid required for DNA complexation ranges from 0.5 to 100 (w/w) and was strongly determined by the cationic lipid used. SLN made from EQ1 immobilized DNA in a ratio of 0.5–1.0:1 (w/w), and SLN from DOTAP in a ratio of 7.5:1–10:1 (w/w) whilst SLN from BA needed an excess of cationic lipid to DNA of 50–100:1 (w/w). However, the amount of SLN required for efficient complexation was influenced not only by the cationic lipid, but also by the matrix lipid. The DDAB-SLN and the CPC-SLN made from Compritol complexed DNA in smaller amounts than the counterparts made from cetylpalmitate.

The cytotoxicity was determined by measuring the LDH release. The tested one-tailed cationic lipids/detergents

(CPC and CTAB) were too toxic to be used in cationic SLN for transfection. These results correlate to the findings for liposomes made from one-tailed cationic lipids, which also showed high cytotoxicity [17]. In contrast, the two-tailed cationic lipids (DDAB, EQ1 and DOTAP) were very well tolerated in transfection-efficient concentrations. For the production of cationic SLN the use of cost-effective but cytotoxic cationic detergents is apparently no alternative to the more expensive but well tolerated cationic lipids. Cytotoxicity seems to be not only determined by the number of aliphatic tails in the cationic lipid molecule, but also by its biodegradability. It is reported that easily metabolizeable cationic lipids are less cytotoxic than less biodegradable ones [18]. DOTAP and EQ1 have ester bonds; in CPC, CTAB and DDAB the aliphatic chain is directly linked to the amine. These results correlate with our findings, in that DDAB was found to be a little less tolerant than EQ1 and DOTAP.

Due to the high toxicity of the one-tailed detergents, only the SLN made from two-tailed cationic lipids were tested for transfection efficiency. The transfection assay was performed on COS-1 cells in different incubation media. All tested SLN increased transfection rates significantly compared to the free DNA in each medium. The DDAB- and EQ1-SLN and the DOTAP–Compritol-SLN showed moderate activities of the same range. The SLN made from DOTAP and cetylpalmitate displayed very effective transfection, almost comparable to pEI. In analysing the first step of formulation optimization these results are of considerable importance.

The addition of 100 μM CQ to the incubation medium increased the efficiency. CQ is a so-called lysosomotrophic agent, which reduces lysosomal degradation and enhances endosomal escape [19,20]. This suggests that the DNA–SLN complexes are internalized by endocytosis via some endolysosomal pathway [21].

In the presence of serum the activity of non-viral transfection agents, such as liposomes, decreases [4,22]. Supplementing the medium with serum decreased the activity of SLN to different extents. The DDAB- and the EQ1-SLN were much more affected than those from DOTAP. In the presence of 10% FCS, DOTAP-SLN were only reduced to 50% of their efficiency without FCS.

No correlation between the ability of plasmid immobilization and effective transfection could be found. The SLN which were able to immobilize the DNA in the lowest SLN:DNA ratios (Co_EQ, Cp_EQ, Co_DDAB) were not those which transfected best. For these formulations, it is possible that the binding is too tight, leading to insufficient dissociation after internalization.

The excellent activity and superiority of DOTAP–cetylpalmitate-SLN was reproducible. In a second set of experiments three formulations made from DOTAP were compared. Two were made from cetylpalmitate but produced with different homogenizers in varying volumes, which subsequently led to different particle sizes and distributions. Both formulations showed comparable high transfection efficiencies. DOTAP–cetylpalmitate-SLN again showed statistically significant higher activities than DOTAP–Compritol-SLN. In cationic emulsions for transfection the choice of the oil component is an important factor in controlling the transfection efficiency [23]. The same could be stated for the matrix lipid in cationic SLN for transfection.

The transfection efficiency of cationic SLN was influenced not only by the cationic lipid composition, but could also be modulated by the matrix lipid used. It has been proven that good combinations of cationic lipid and matrix lipid could achieve high transfection activities. The type of homogenizer employed for production (which influences the particle size and distribution) showed no significant effect on transfection activity.

SLN have several technological advantages compared to liposomes or polymeric nanoparticles. The production by hot high-pressure homogenization is easy and no organic solvents are required [10]. Scaling up is standardized up to 50 kg batches [9] and steam sterilization is possible [7].

DOTAP–cetylpalmitate-SLN (Cp_DOTAP) showed high tolerance and good transfection efficiency even in the presence of serum. Cp_DOTAP demonstrated excellent storage stability even after steam sterilization (data not shown). Such properties present the SLN–DNA complexes themselves as a very promising platform for the further development of non-viral transfection agents or even in vivo gene medicines. Necessary optimizing steps will include the investigation of formulations with different particle sizes and distributions [24], extensive investigations with different matrix lipids, additional helper-lipids (e.g. Dope) for enhancing endosomal escape [25,26] or the incorporation of nuclear localization signals (NLCs) [27].

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