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Preparation and characterisation of a new lipospermine for gene delivery into various cell-lines

Detlef Groth [a,*], Oliver Keil [b], Cathleen Lehmann [a], Manfred Schneider [b], Michael Rudolph [a], Regina Reszka [a]

[a] *Max Delbrück Center for Molecular Medicine, Berlin-Buch, Group Drug Targeting, Robert-Rössle-Strasse 10, D-13125 Berlin, Germany*

[b] *FB-9-Organische Chemie-Bergische Universität, GH-Wuppertal, D-42097 Wuppertal, Germany*

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Abstract

A novel cationic amphiphile consisting of a hydrophobic 1,3 dioleoylglycerol moiety and a positively charged spermine head group, 1,3-dioleoyloxy-2-(N^5-carbamoyl-spermine)-propane (DOCSPER), has been prepared. Based on naturally occurring materials, DOCSPER is susceptible to metabolic degradation and a promising agent for delivery of therapeutical genes in vivo. In aqueous solutions this lipospermine spontaneously forms liposomes with a size of about 20–100 nm. Liposomes composed of DOCSPER and the helper lipid dioleoylphosphatidylethanolamine (DOPE) were several hundred nm in size. DOCSPER liposomes were tested for their ability to transfect the rat glioblastoma cell-line F98, the rat colon carcinoma cell-line CC531, the T-cell line Jurkat and the human mamma tumour cell-lines MCF-7 and MaTu. Transfectability and toxicity of liposomes prepared with DOCSPER were compared with the Lipofectin reagent. Toxicity was significantly lower and transfection efficiencies were similar compared to Lipofectin. Lipid/DNA complex formation was controlled by turbidity measurements allowing easy determination of optimal lipid/DNA ratio for transfection.

Keywords: Transfection; Cationic liposome; Liposome; Gene therapy; DNA delivery

1. Introduction

Transfection of eukaryotic cells with DNA has become an important technique in molecular biology for studying gene function and in gene therapy. Among the commonly used methods, cationic lipid mediated gene transfer (cytofection, see Felgner et al., 1995; Gao and Huang, 1995) has several advantages in comparison to methods using viruses, DEAE-dextran or calcium phosphate as the formation of cationic lipid/DNA

* Corresponding author. Tel.: +49 30 94063212; fax: +49 30 9406213.

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complexes is easy to perform and the security requirements are low. Lipid and DNA are diluted separately, mixed, incubated for several minutes and the formed lipid/DNA complex is added to the cells. Cationic lipids have been recently used in the transfer of RNA (Malone et al., 1989) and proteins (Debs et al., 1990) into living cells as well.

The mechanism of transfection with DNA depends on the physicochemical properties of the lipid/DNA complex and the stability of this complex in biological fluids. Introduction of DNA into the cell is characterized by adsorption of the lipid/DNA complex on the cell surface, uptake by endocytosis/phagocytosis or membrane fusion, the release from the endosome and the targeting of the DNA to the nucleus. The most widely used cationic liposome formulation is Lipofectin which consists of an equimolar mixture of the monocationic double chain amphiphile *N*-(1-(2,3-dioleoxy)propyl)-*N*,*N*,*N*-trimethylammonium-chloride (DOTMA) and DOPE (Felgner et al., 1987). Lipofectin is effective in delivering DNA, RNA and negatively charged proteins, but limited by its toxicity and high costs for the reagent.

Many cationic amphiphiles require a common neutral phospholipid for liposomal stabilisation and gene delivery. In contrast, double chain amphiphiles such as dioctadecylamidoglycylspermine (DOGS; Loeffler and Behr, 1993) and 1,3-dioleoyloxy-2-(6-carboxy-spermyl)-propylamid (DOSPER; Buchberger et al., 1996) are able to deliver DNA without a neutral lipid component. These lipids contain the polyamine spermine, a natural counterion for DNA, mediating efficient complexation and protection of DNA. Nevertheless it was shown that transfection efficiency can be enhanced if these lipids are formulated with DOPE (Thierry et al., 1997). DOPE is a neutral phospholipid, which was shown to enhance transfection in vitro when added to cationic lipids (Pinnaduwage et al., 1989; Farhood et al., 1995). Spermine containing cationic double chain amphiphiles are promising agents for the delivery of pharmaceutical genes in gene therapy. However for in vivo approaches, such as continuous delivery (Zhu et al., 1996) or systemic applications (Thierry et al., 1997) of DNA, the selection of the optimal complex formation conditions and the stability of these lipid/DNA complexes are essential to gain in vivo appropiate levels of gene expression.

Here the synthesis of the lipospermine DOCSPER is described, containing a hydrophobic glycerol anchor group and a spermine group as the cationic head group. In contrast to DOSPER, DOCSPER has the naturally occurring glycerol instead of 2-amino-1,3-propandiol as backbone. The aim of this work was to characterise this lipospermination with regard to the requirement of a neutral helper lipid, complexing conditions and transfection properties. For this purpose the different physicochemical aspects by electron microscopy and turbiditiy measurements have been examined and the efficiency of in vitro gene delivery tested.

2. Materials and methods

2.1. Materials

All chemicals required for synthesis were purchased from Fluka Chemie AG (Buchs, Switzerland) in the highest available purity. All solvents were of distilled quality and products were purified by column chromatography on silica gel 60 (Merck, Darmstadt, Germany). N^1,N^{10},N^{14}-tris-(tert-butoxycarbonyl) spermine was synthesized according to the method of Nagarajan and Ganem (1985) by reaction of spermine with 3 equivalents 2-(-*t*-butoxycarbonyloxyimino)-2-phenylacetonitrile (BOC-ON). Uranyl acetate was purchased from Merck.

DOPE, β-galactosidase standard and *p*-nitrophenylphosphate (*p*NPP) were products of Fluka. Chlorophenol red galactopyranosid (CPRG) and DOSPER were purchased from Boehringer Mannheim (Mannheim, Germany). Triton X-100, MES (2-(*N*-morpholino)ethanesulfonic acid) and Hanks' balanced salt solution (HBSS) were purchased form Sigma (St. Louis, MO). Cell culture media, 100 × antibiotic-antimycotic solution and Lipofectin were obtained from Life Technologies,

(Gaithersburg, MD). DOTMA was a gift from R.J. Debs.

2.2. Plasmid DNA

The pUT651 plasmid carrying the *Escherichia coli* lacZ gene was obtained from Cayla (Toulouse, France). Supercoiled plasmid DNA was transformed into *E. coli* using a chemical method (Groth et al., 1996) and purified by alkaline lysis and column chromatography using Qiagen-tips from Qiagen (Hilden, Germany).

2.3. Synthesis of DOCSPER[1] cationic lipid

The cationic lipid DOCSPER (5) was synthesized as outlined in Fig. 1A. As starting material the suitably protected glycerol derivative 1,3-dibenzyloxy-2-propanediol was chosen (1) which was first functionalized in the 2-position using phosgene in toluene leading to the corresponding chloroformate (2.2) which was further reacted with the BOC-protected spermine N^1,N^{10},N^{14}-tris-(tert-butoxycarbonyl) spermine. Subsequent catalytic hydrogenation in the presence of Pd/C gave the building block (3) with two free hydroxy groups. The essential oleoyl groups were introduced by esterification of (3) with oleic acid using *N*,*N*-dicyclohexylcarbodiimide (DCC) and catalytic amounts of 4-*N*,*N*-dimethylaminopyridine (DMAP). The thus obtained fully protected intermediate (4) was purified to homogeneity by column chromatography. Final deprotection of (4) with trifluoroacetic acid afforded pure DOCSPER (5) as tris-trifluoroacetate.

2.4. Preparation of liposomes

Chloroformic solutions of the lipids were mixed in a sterile glass vial and the solvent was removed by evaporation and several hours drying under vacuum. Liposomes were prepared by addition of deionized water to a final lipid concentration of 1 mg/ml under shaking for several hours to form multilamellar vesicles.

[1] Schneider et al., Newly cationic amphiphiles for the gene transfer. German patent application (Nr. 19631 189.6).

2.5. Electron microscopy

For transmission electron microscopy the negative staining technique was used. The samples were dropped on a formvar/carbon coated copper grid for 15 min, replaced with 2% aqueous uranyl acetate and stained for 20 min. Finally the fluid was removed and the grids were dried. Samples were examined in a JEM 100 CX transmission electron microscope.

2.6. Turbidity measurements

Lipid/DNA complexes were prepared by mixing of separately diluted lipid and DNA in a 96-well plate, according to Felgner et al. (1994). Briefly, the cationic lipid formulation was six times diluted 2-fold, horizontal, from 30 to about 1 μg per well in 150 μl serum-free DMEM/25 mM HEPES. The DNA was diluted seven times down on a separate plate 2-fold from 6 to approximately 0.2 μg per well in an equal volume of serum-free DMEM/25 mM HEPES; in the eight row only medium was given. The DNA solution was added to the lipid and plates were incubated for 30 min. The turbidity was measured in a microplate reader at 690 nm before complexes were added to the cells.

2.7. Cell culture and transfection

MaTu, MCF-7, CC531 and Jurkat cells were maintained in RPMI 1640 medium, F98 cells were grown in Dulbeccos modified Eagle medium (DMEM). Media were supplemented with 10% fetal calf serum and antibiotic-100 U/ml penicillin G, 100 μg/ml streptomycin sulfate and 0.25 μg/ml amphotericin. Cells were cultivated in 5% CO_2 at 37°C. For gene transfer studies flat bottom 96-well plates were used. About 10 000–20 000 cells which were in 100 μl DMEM medium, containing 10% fetal calf serum per well were plated 24 h before transfection. The following day lipid/DNA mixtures were prepared by mixing of serial diluted DNA and lipid in a 96-well plate as decribed above (2.6). For formation of DNA/lipid complexes the mixture was incubated for 30 min at room temperature. Thereafter 100 μl of lipid/

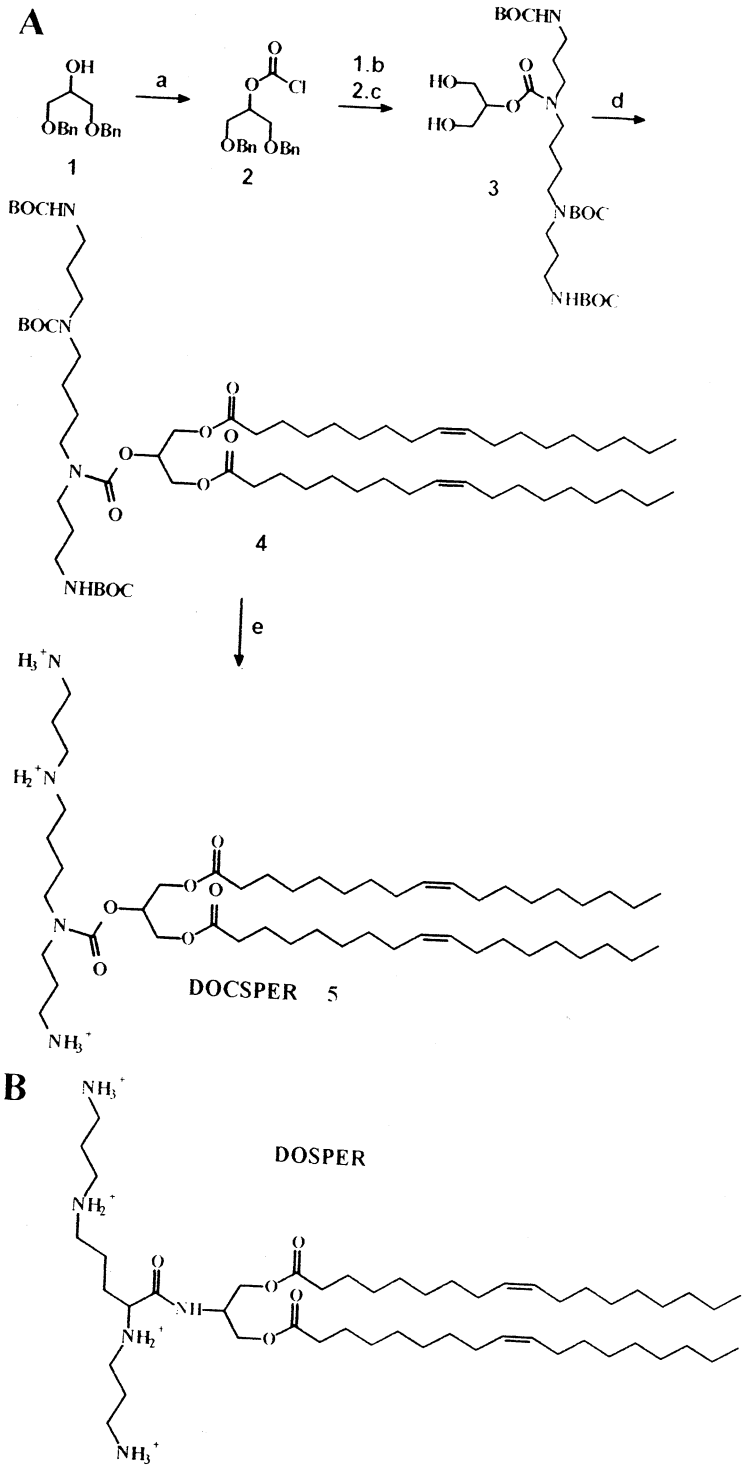

Fig. 1. A. Synthesis scheme of 1,3-dioleoyloxy-2-(N^5-carbamoyl-spermine)-propane (DOCSPER) and (a) 20% phosgene in toluene, 24 h, rt; (b) N^1,N^{10},N^{14}-tris-(tert-butoxycarbonyl)-spermine, NEt_3, 4 h; (c) 5% Pd/C, H_2; (d) DCC, DMAP, oleic acid, 24 h, rt; and (e) TFA, CH_2Cl_2, 1 h. B. Structure of the related product DOSPER.

DNA complex from the mixing plate was added to the cells. The resulting serum concentration was 5%. After incubation for 3–5 h the medium was supplemented with 20% fetal calf serum containing DMEM medium, to achieve a final serum concentration of 10%. The cells were further incubated for 24–48 h for expression of the reporter gene. For the complex formation kinetic studies, the incubation time was increased from 10 to 120 min. Then 50 μl of lipid/DNA mixture was added to the cells and cells were incubated overnight. After 16 h, medium was removed and fresh DMEM containing 10% fetal calf serum was added. Cells were assayed for β-galactosidase expression after a further 24 h.

2.8. Toxicity and reporter gene assay

For dual determination of toxicity and reporter gene expression a modified acidic phosphatase assay was combined with a colorimetric β-galactosidase assay (manuscript in preparation). Briefly, 24–48 h after the transfection the medium was removed and the cells were washed once with phosphate buffered saline, pH 7.4. Then 80 μl buffer containing 10 mM pNPP, 0.02 M MES (pH 5.5) and 0.1% Triton X-100 were added to each well and the plates were placed in an incubator at 37°C, 5% CO_2 for 20–30 min. Thereafter reaction was completed by addition of 20 μl 0.5 M Tris–HCl (pH 8.0). Then the optical density was measured at 405 nm (reference filter 690 nm) and cell viability was calculated in comparison with untreated control cells. In order to measure reporter gene expression thereafter 150 μl of β-galactosidase substrate solution (1 mg/ml chlorophenol red galactopyranosid in HBSS) was added to the same plate. After 2 min to 24 h, depending on the level of gene expression, the optical density was measured on a microplate reader at 540 nm (reference filter 690 nm). The level of expression was calculated and expressed as absolute amount of β-galactosidase enzyme with the aid of a calibration curve. The dilutions of β-galactosidase used for the calibration curve were treated in the same way as the cells.

In order to remove medium from non-adherent Jurkat cells, the 96-well plates were centrifuged at 3000 rpm for 10 min (Heraeus Megafuge 1.0 R). Because of their lower gene expression, another β-galactosidase assay, according to Lim and Chae (1989), was used. Using 100 μl of buffer containing 1.5 mg/ml 2-nitrophenyl-β-D-galactopyranoside (ONPG), 0.1% Triton X-100 in HBSS was added to each well and the cells were incubated at room temperature for up to 16 h until color developed. Optical density was determined on a microplate reader at 405 nm (reference filter 690 nm).

3. Results

3.1. Synthesis of the cationic glycerol derivative

A cationic derivative of glycerol was synthesised, containing a spermine head group. For the detailed structure and synthesis see Fig. 1A. In contrast to DOSPER, (compare Fig. 1B) which contains two primary and two secondary amino groups, the glycerol derivative DOCSPER contains two primary amino groups and only one secondary amino group. The positive charge of DOCSPER is lower, due to reduced electronegativity of the other former secondary nitrogen, it is coupled to the electron withdrawing biodegradable carbamoyl linkage. The charge of the primary amino group is pH dependent. At the physiological pH of 7.4, based on pK_a values reported for ethylendiamine (Gordon and Ford, 1972) DOSPER has a higher net positive charge (about $+3$ versus $+2$) as compared to DOCSPER. However both lipids contain two primary amino groups, thereby permitting buffering of the pH in the lysosome.

3.2. Preparation of cationic liposome formulations

DOCSPER/DOPE liposomes with 10, 25, 50, 75 and 100% (w/w) DOCSPER and DOTMA/DOPE liposomes with 50 and 75% (w/w) DOTMA were prepared. The exact compositions and designations are shown in Table 1. Liposomes composed of 25% (w/w) DOTMA and 75% DOPE were not stable. Comparison of the DOTMA 50 liposomes with the commercially

available Lipofectin reagent showed similar physicochemical and gene transfer properties (data not shown).

3.3. Effect of DOPE content on liposomal size

To determine the structure and size of the liposomes a negative staining technique was performed for electron microscopy. As shown in Fig. 2, DOCSPER liposomes without DOPE (DOCSPER 100) had a size distribution between 20 and 100 nm. DOCSPER liposomes containing 50% (w/w) DOPE (DOCSPER 50) were multi-lamellar and their size ranged from 200 to 500 nm. DOCSPER formulations with 90% (w/w) DOPE were very heterogenous and formed non-regular structures (not shown).

3.4. Characterisation of cationic lipid/DNA complex formation by turbidity

In order to investigate the relationship between turbidity and transfectability of lipid/DNA complexes MCF-7 cells were transfected with a constant lipid and various DNA amounts. After addition of DNA to diluted liposomes, the turbidity of the mixture was increased indicating the formation of lipid/DNA complexes. This increase depends on the amount of DNA added. Keeping the amount of lipid constant the turbidity of the mixture increased up to a certain amount of DNA, with higher DNA amounts turbidity again decreased. (Fig. 3). The observed turbidity peak correlated with the optimal lipid/DNA mixture, required for transfection of cells. In the case of liposomes with a higher amount of DOPE the range of optimal DNA amounts was broader and the minimal amount of DNA was lower, thereby permitting transfection with less DNA.

Table 1
The compostion of the six liposomal formulations used in this work

Formulation	Cationic lipid (weight %)	DOPE (weight %)
DOCSPER 100	100	0
DOCSPER 75	75	25
DOCSPER 50	50	50
DOCSPER 25	25	75
DOTMA 75	75	25
DOTMA 50	50	50

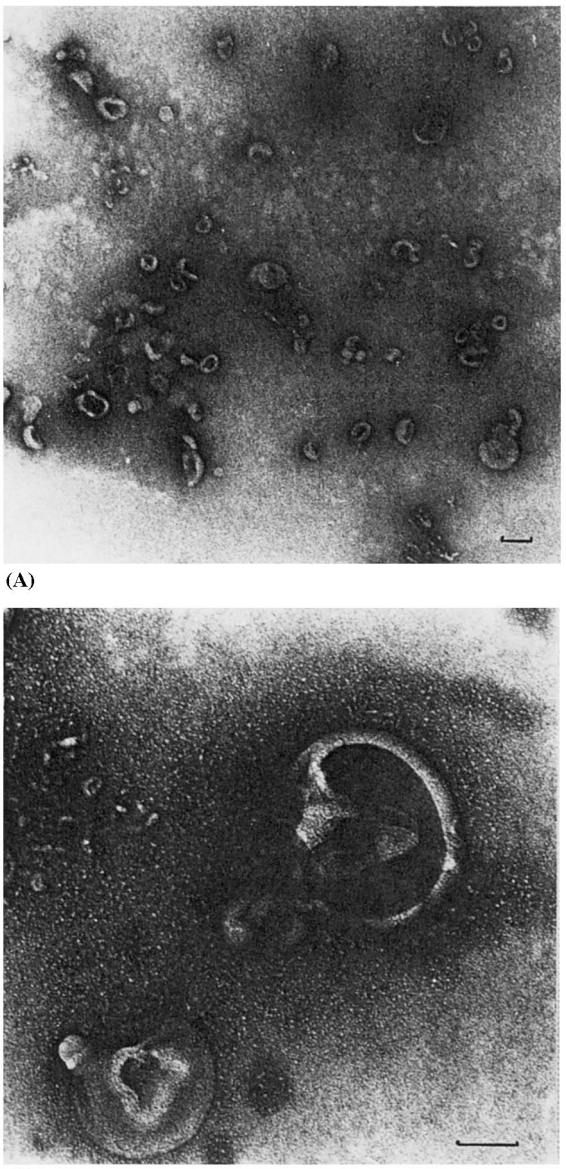

Fig. 2. Electron micrographs of DOCSPER 100 (A) and DOCSPER 50 (B) liposomes, negatively stained with uranyl acetate. Magnifications were 49 000 (A) and 97 000 (B). Bar indicates 100 nm.

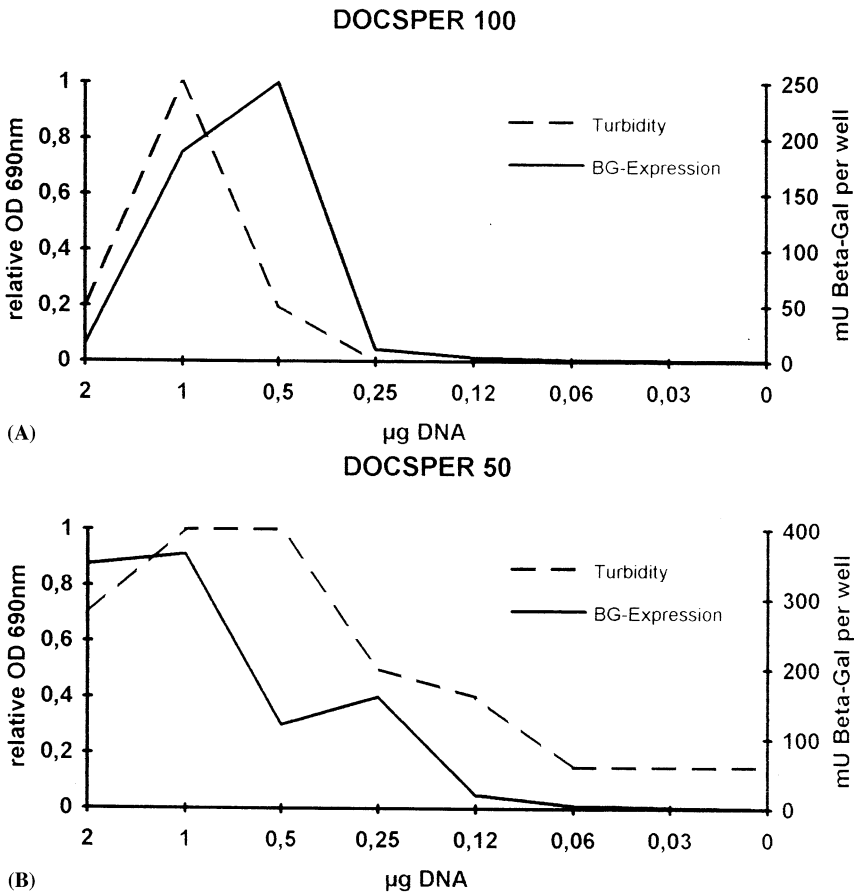

Fig. 3. Light scattering of lipid/DNA complexes and gene expression on MCF-7 cells. Various amounts of DNA were complexed with 5 μg of DOCSPER 100 (A) and DOCSPER 50 (B) liposomes for 30 min in 100 μl serum-free DMEM/25 mM HEPES medium. Then the turbidity was measured with a microplate reader at 690 nm. The highest turbidity value was set at 1 for each DOCSPER formulation. After measurement of turbidity 100 μl of lipid/DNA mixture were given to MCF-7 cells on a 96-well plate and assayed for β-galactosidase expression as described under material and methods. The mean expression values for two different wells are shown.

3.5. Time-course of lipid/DNA complex formation

A time-course for optimal complex formation was determined by transfection of MaTu cells. As shown in Fig. 4, complex formation for DOCSPER 50 was very fast and effective with high concentrations of lipid and DNA. Longer incubation times with more concentrated lipid/DNA mixtures resulted in the formation of larger particles; which were visible in the light microscope and less effective in transfection. At low concentrations of lipid and DNA, longer incubation times were required for optimal transfection. Similar results were obtained with other lipid/DNA (L/D) ratios. In case of DOCSPER 100 the efficiency increased over time for all L/D ratios and concentrations tested, indicating slower complex formation velocity. In further studies for complex formation incubation times of 30 min were used.

3.6. Effect of helper lipid on DNA delivery and toxicity

To determine the optimal lipid composition, liposome formulations containing different helper lipid and DOCSPER amounts were prepared and

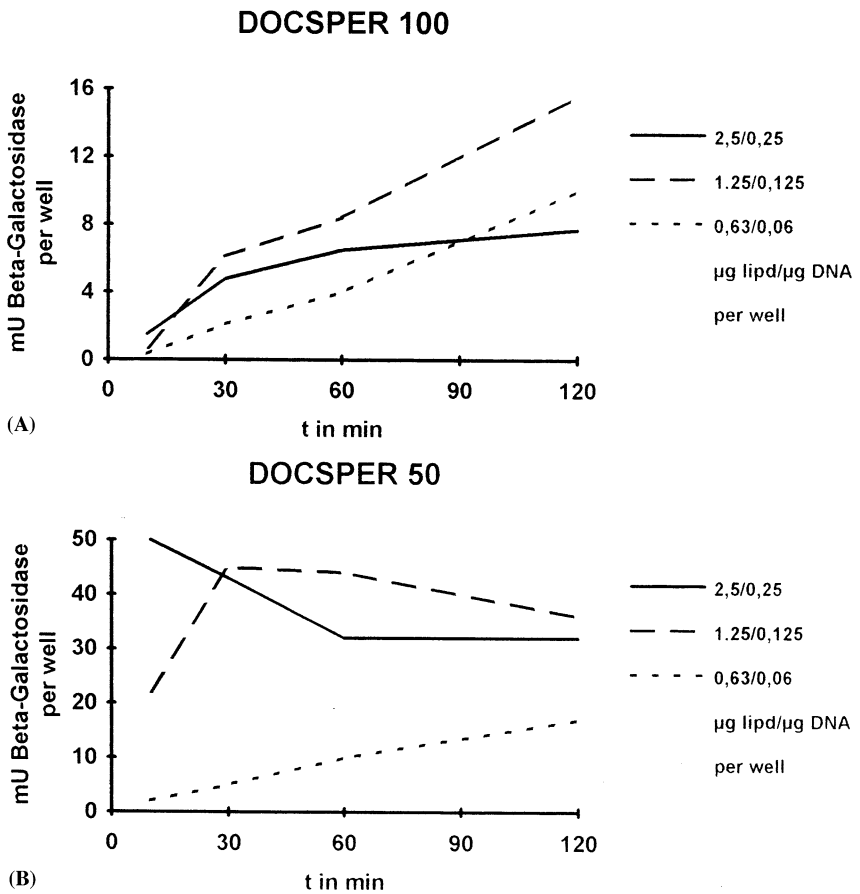

Fig. 4. Time dependence of lipid/DNA complex formation. Complexes were prepared in 300 μl serum-free medium by separate dilution pUT651 and DOCSPER 100 (A) or DOCSPER 50 (B) liposomes in serum-free DMEM/25 mM HEPES medium. After mixing and incubation for 10, 30, 60 and 120 min 50 μl of lipid/DNA mixture were added to MaTu cells in 96-well plates. After 16 h medium was replaced and after 48 h β-galactosidase assay was performed. The experiment was repeated twice with similar results.

tested with regard to their transfection efficiencies on several tumor cell-lines. Efficiencies and toxicities were compared with liposomes prepared from DOTMA and DOPE. The transfection efficiency and the toxicity for different lipid and DNA concentrations was tested on MCF-7, MaTu, CC531, Jurkat and F98 cells (Figs. 5 and 6). In general, gene expression was quite different for the various cell-lines tested. The reporter gene expression increased with increasing DNA amount until a saturation was achieved. In case of DOCSPER, liposomes complexed with higher DNA amounts were even inhibitory for transfection. How much DNA was necessary for maximal expression depends on cell confluency and the cell-line used. The optimal L/D ratio required for transfection depends on the cell-line as well. For example, for F98 and MaTu cells, the L/D ratio for optimal transfection using DOCSPER 50 was 10 and for MCF-7 cells between 20 and 2.5 (Fig. 5A–C). DOCSPER liposomes with increased amounts of helper lipid had a broader range of effective L/D ratios. Low DNA amounts could be delivered most efficiently with DOCSPER 25 liposomes. These observations were in agreement with the previously performed turbidity measurements. Higher lipid amounts were in most cases less

(A) MCF-7 Cell Viability

DOTMA 50

	10	5	2,5	1,25	0,63	0,31
2	0,00	0,03	0,09	0,27	0,58	0,76
1	0,06	0,01	0,04	0,17	0,65	0,84
0,5	0,05	0,02	0,02	0,16	0,57	0,59
0,25	0,21	0,03	0,02	0,09	0,39	0,78
0,125	0,39	0,17	0,03	0,03	0,27	0,75
0,063	0,63	0,62	0,29	0,10	0,38	0,64
0,031	0,57	0,80	0,53	0,18	0,37	0,71
0	0,74	1,01	1,06	1,07	0,74	0,98

DOTMA 75

	10	5	2,5	1,25	0,63	0,31
2	0,02	0,02	0,16	0,32	0,72	0,85
1	0,02	0,01	0,08	0,30	0,88	0,86
0,5	0,02	0,03	0,01	0,11	0,61	0,95
0,25	0,28	0,02	0,02	0,05	0,55	0,90
0,125	0,74	0,60	0,04	0,04	0,38	0,93
0,063	0,86	0,98	0,69	0,17	0,16	0,85
0,031	0,85	0,96	0,91	0,43	0,44	0,55
0	0,80	1,03	0,97	0,98	0,99	1,10

DOCSPER 100

	10	5	2,5	1,25	0,63	0,31
2	0,49	1,12	1,17	1,11	1,14	1,11
1	0,16	0,76	1,15	1,16	1,15	1,10
0,5	0,65	0,80	0,56	1,10	1,15	1,13
0,25	0,69	1,11	0,86	0,99	1,12	1,09
0,125	0,55	1,15	1,17	0,95	1,08	1,10
0,063	0,75	1,09	1,15	1,07	1,09	1,11
0,031	0,70	0,95	1,10	1,08	1,04	1,03
0	0,72	1,11	1,13	1,07	1,05	1,03

DOCSPER 75

	10	5	2,5	1,25	0,63	0,31
2	0,21	0,74	0,84	0,97	1,08	1,18
1	0,05	0,32	0,74	0,99	1,08	1,17
0,5	0,20	0,08	0,30	0,99	1,06	1,16
0,25	0,78	0,80	0,18	0,84	1,05	1,14
0,125	0,92	1,11	0,82	0,48	1,09	1,11
0,063	0,79	1,09	1,10	0,90	0,87	1,12
0,031	0,85	1,06	1,05	0,89	0,85	1,07
0	0,79	0,95	1,01	1,00	1,04	1,13

DOCSPER 50

	10	5	2,5	1,25	0,63	0,31
2	0,06	0,57	0,80	1,03	1,04	1,07
1	0,06	0,26	0,80	1,03	1,00	1,10
0,5	0,06	0,04	0,38	1,03	1,02	1,07
0,25	0,48	0,09	0,04	0,67	0,97	1,07
0,125	0,77	1,01	0,12	0,11	0,91	1,04
0,063	0,99	1,12	1,06	0,66	0,66	1,07
0,031	0,97	1,10	0,95	0,85	0,66	1,03
0	1,08	1,16	1,09	1,08	1,07	1,14

DOCSPER 25

	10	5	2,5	1,25	0,63	0,31
2	0,43	0,66	0,88	0,90	1,03	1,18
1	0,43	0,43	0,80	0,94	0,99	1,14
0,5	0,14	0,08	0,34	0,77	1,00	1,07
0,25	0,56	0,07	0,11	0,72	1,00	1,07
0,125	0,89	0,46	0,10	0,38	1,02	1,11
0,063	1,01	0,91	0,35	0,27	0,70	1,06
0,031	0,98	0,94	0,68	0,37	0,63	1,10
0	1,04	1,17	1,00	1,12	1,09	1,12

MCF-7 BG-Expression

DOTMA 50

	10	5	2,5	1,25	0,63	0,31
2	28	139	336	399	268	147
1	82	107	295	412	233	131
0,5	62	127	237	442	251	162
0,25	48	73	187	313	255	180
0,125	16	72	149	225	293	134
0,063	8	31	93	178	195	116
0,031	7	20	47	106	142	123
0	2	0	1	0	1	1

DOTMA 75

	10	5	2,5	1,25	0,63	0,31
2	21	63	184	175	125	72
1	16	29	168	285	123	63
0,5	23	43	93	198	136	95
0,25	29	63	150	227	114	126
0,125	11	49	125	231	279	108
0,063	4	7	33	133	203	122
0,031	4	6	26	67	130	184
0	0	1	1	0	0	0

DOCSPER 100

	10	5	2,5	1,25	0,63	0,31
2	22	3	9	9	10	0
1	75	108	8	10	12	10
0,5	55	87	256	8	12	1
0,25	10	14	234	67	6	1
0,125	5	9	3	227	12	3
0,063	3	3	2	8	17	4
0,031	-2	0	1	5	16	0
0	1	0	-1	2	-2	1

DOCSPER 75

	10	5	2,5	1,25	0,63	0,31
2	44	21	62	83	70	44
1	125	59	55	63	80	55
0,5	222	353	108	47	78	48
0,25	29	120	342	94	89	70
0,125	9	18	200	233	104	75
0,063	7	8	10	33	62	28
0,031	8	10	9	15	71	10
0	0	2	1	1	0	2

DOCSPER 50

	10	5	2,5	1,25	0,63	0,31
2	112	334	131	63	27	9
1	92	306	200	83	37	13
0,5	170	130	291	90	28	18
0,25	75	257	196	216	51	27
0,125	12	16	321	316	103	37
0,063	2	1	8	138	167	23
0,031	1	3	8	22	159	44
0	-1	-1	-1	-1	1	0

DOCSPER 25

	10	5	2,5	1,25	0,63	0,31
2	390	520	361	200	112	47
1	315	545	372	179	102	36
0,5	131	250	427	316	142	65
0,25	103	196	370	285	146	56
0,125	26	178	301	457	107	32
0,063	5	35	149	275	229	41
0,031	1	16	71	187	197	39
0	1	0	-1	1	0	0

Fig. 5. (*Continued*)

(B) MaTu Cell Viability

DOTMA 50

	10	5	2,5	1,25	0,63	0,31
2	0,23	0,20	0,27	0,40	0,61	0,69
1	0,18	0,17	0,25	0,32	0,51	0,68
0,5	0,16	0,17	0,16	0,39	0,66	0,89
0,25	0,27	0,22	0,18	0,23	0,58	0,84
0,125	0,30	0,23	0,19	0,18	0,70	0,96
0,063	0,39	0,27	0,25	0,33	0,48	1,01
0,031	0,38	0,49	0,41	0,40	0,73	0,88
0	0,48	0,63	0,68	0,68	0,87	1,03

DOTMA 75

	10	5	2,5	1,25	0,63	0,31
2	0,23	0,26	0,39	0,77	1,01	1,10
1	0,18	0,17	0,35	0,61	0,99	1,08
0,5	0,17	0,18	0,17	0,75	0,99	1,11
0,25	0,35	0,14	0,14	0,38	0,94	1,26
0,125	0,59	0,39	0,30	0,22	1,02	1,19
0,063	0,61	0,66	0,54	0,59	0,69	0,93
0,031	0,78	0,95	0,72	0,60	0,69	1,12
0	0,73	0,77	1,08	1,05	1,04	1,12

DOCSPER 100

	10	5	2,5	1,25	0,63	0,31
2	0,16	0,66	0,81	0,90	0,92	0,85
1	0,17	0,59	0,85	0,84	0,80	0,90
0,5	0,23	0,77	0,90	0,83	0,92	1,01
0,25	0,12	0,67	0,93	0,97	0,92	0,96
0,125	0,23	0,62	0,95	1,00	0,92	0,94
0,063	0,26	0,80	1,00	0,98	1,06	0,94
0,031	0,15	0,48	0,86	1,00	1,00	0,99
0	0,10	0,66	0,91	0,84	0,89	1,12

DOCSPER 75

	10	5	2,5	1,25	0,63	0,31
2	0,56	0,62	0,72	0,84	0,94	1,00
1	0,47	0,50	0,78	0,77	0,86	0,95
0,5	0,70	0,45	0,68	0,85	0,91	0,97
0,25	0,69	0,83	0,75	0,84	0,92	0,91
0,125	0,63	0,83	0,85	0,89	0,85	1,01
0,063	0,57	0,83	0,89	0,92	0,90	0,97
0,031	0,56	0,86	0,80	0,94	0,93	0,92
0	0,62	0,76	0,90	1,01	0,86	1,01

DOCSPER 50

	10	5	2,5	1,25	0,63	0,31
2	0,81	0,95	0,86	0,84	0,89	0,72
1	0,55	0,79	1,05	0,92	0,80	0,82
0,5	0,53	0,50	0,82	0,99	0,88	0,84
0,25	0,82	0,77	0,80	0,82	0,93	0,81
0,125	0,73	0,90	1,00	0,97	0,91	0,94
0,063	0,86	1,08	1,07	1,04	1,05	0,95
0,031	0,75	0,99	1,04	1,08	0,95	1,05
0	0,92	0,91	1,05	0,98	0,94	1,08

DOCSPER 25

	10	5	2,5	1,25	0,63	0,31
2	0,81	0,83	0,90	0,90	0,92	0,96
1	0,71	0,82	0,86	0,94	0,93	0,98
0,5	0,60	0,71	1,01	1,04	1,01	0,95
0,25	0,82	0,47	0,82	1,04	0,99	0,94
0,125	0,80	0,85	0,89	1,02	1,16	0,97
0,063	0,96	0,88	1,03	1,05	1,15	0,96
0,031	0,90	0,76	0,86	1,04	1,11	1,00
0	0,90	1,08	0,91	0,97	1,08	1,20

MaTu BG-Expression

DOTMA 50

	10	5	2,5	1,25	0,63	0,31
2	13	24	63	89	67	26
1	7	13	58	92	79	25
0,5	1	7	35	124	90	35
0,25	15	6	9	93	112	39
0,125	3	7	8	30	105	32
0,063	0	5	14	23	95	28
0,031	1	6	10	25	63	20
0	0	0	0	0	0	0

DOTMA 75

	10	5	2,5	1,25	0,63	0,31
2	4	54	55	57	19	9
1	1	6	79	65	23	8
0,5	3	2	9	109	31	10
0,25	8	2	4	91	46	11
0,125	1	10	4	42	73	10
0,063	0	6	10	26	55	14
0,031	0	1	8	8	32	19
0	0	0	0	0	0	0

DOCSPER 100

	10	5	2,5	1,25	0,63	0,31
2	0	0	0	1	0	0
1	1	1	1	0	0	0
0,5	0	1	1	0	1	0
0,25	0	0	4	0	0	0
0,125	0	0	1	1	0	0
0,063	0	0	0	1	0	0
0,031	0	0	0	0	0	0
0	0	0	0	0	0	0

DOCSPER 75

	10	5	2,5	1,25	0,63	0,31
2	9	2	6	4	2	0
1	13	13	9	5	2	0
0,5	8	27	17	4	2	0
0,25	1	7	26	3	1	0
0,125	0	1	5	4	0	0
0,063	0	0	2	1	0	0
0,031	0	0	2	1	0	0
0	0	0	0	0	0	0

DOCSPER 50

	10	5	2,5	1,25	0,63	0,31
2	18	3	1	0	0	0
1	34	8	1	0	0	0
0,5	88	65	5	1	0	0
0,25	11	92	66	1	0	0
0,125	0	5	16	14	0	0
0,063	0	1	1	2	1	0
0,031	0	0	0	0	0	0
0	0	0	0	0	0	0

DOCSPER 25

	10	5	2,5	1,25	0,63	0,31
2	5	4	5	2	1	0
1	14	6	5	2	1	0
0,5	61	33	12	2	1	1
0,25	15	109	20	3	2	0
0,125	2	25	52	7	0	0
0,063	0	5	21	10	2	1
0,031	0	5	18	6	5	1
0	0	0	0	0	0	0

Fig. 5. (*Continued*)

(C) F98 Cell Viability

DOTMA 50

	10	5	2,5	1,25	0,63	0,31
2	0,49	0,79	0,95	0,81	1,11	0,92
1	0,26	0,75	1,08	1,04	1,00	1,15
0,5	0,28	0,44	1,02	1,04	0,97	0,98
0,25	0,36	0,49	0,77	1,08	1,18	1,05
0,125	0,37	0,57	0,73	0,89	0,99	1,01
0,063	0,28	0,68	0,73	0,73	0,86	0,78
0,031	0,28	0,74	0,82	0,80	0,76	1,12
0	0,37	0,71	0,87	0,84	0,82	0,57

DOTMA 75

	10	5	2,5	1,25	0,63	0,31
2	0,13	0,89	0,95	0,95	0,99	1,01
1	0,08	0,77	0,95	1,05	1,09	1,04
0,5	0,35	0,30	0,98	1,13	1,05	1,08
0,25	0,38	0,77	0,89	1,07	1,12	1,00
0,125	0,30	0,58	0,93	0,85	1,04	0,93
0,063	0,46	0,66	0,79	0,73	0,98	0,94
0,031	0,39	0,74	0,86	0,83	0,92	0,94
0	0,23	0,54	0,67	0,72	0,85	0,86

DOCSPER 100

	10	5	2,5	1,25	0,63	0,31
2	0,94	1,31	1,12	0,83	0,96	0,87
1	0,88	1,03	0,91	1,07	0,89	0,97
0,5	1,19	1,47	0,92	1,04	0,95	0,94
0,25	0,92	1,45	1,05	0,98	0,83	0,76
0,125	0,62	1,45	1,05	0,92	0,93	0,86
0,063	0,81	1,59	1,15	0,77	0,80	0,87
0,031	0,66	1,46	1,01	1,07	0,68	0,76
0	0,83	1,46	0,94	0,69	0,77	0,69

DOCSPER 75

	10	5	2,5	1,25	0,63	0,31
2	0,98	0,78	0,74	0,85	0,85	0,84
1	0,44	0,66	0,86	0,84	0,87	0,91
0,5	1,15	0,77	0,97	0,88	1,00	0,97
0,25	1,12	0,89	0,73	0,95	0,99	0,89
0,125	1,27	0,86	0,82	0,69	0,80	0,83
0,063	1,08	0,79	0,75	0,64	0,83	0,82
0,031	0,88	0,82	0,80	0,75	0,75	0,82
0	0,76	0,74	0,63	0,60	0,62	0,71

DOCSPER 50

	10	5	2,5	1,25	0,63	0,31
2	0,87	1,00	1,00	0,88	0,88	0,91
1	0,71	0,83	0,92	0,89	0,90	0,85
0,5	0,96	0,92	0,91	0,90	0,81	0,86
0,25	1,03	0,99	0,97	0,79	0,80	0,81
0,125	0,93	0,97	0,85	0,85	0,85	0,88
0,063	0,92	0,93	0,80	0,86	0,82	0,78
0,031	1,09	0,90	0,85	0,75	0,76	0,74
0	0,78	1,06	0,77	0,92	0,67	0,78

DOCSPER 25

	10	5	2,5	1,25	0,63	0,31
2	0,82	0,86	0,79	0,76	0,78	0,85
1	0,66	0,86	1,10	0,94	0,98	0,91
0,5	0,55	0,71	0,86	0,89	0,89	0,90
0,25	0,61	0,81	0,79	0,86	0,83	0,80
0,125	0,70	0,80	0,87	0,76	0,79	0,85
0,063	0,71	0,70	0,78	0,91	0,76	0,80
0,031	0,60	0,75	0,74	0,75	0,67	0,72
0	0,69	0,62	0,66	0,56	0,59	0,76

F98 BG-Expression

DOTMA 50

	10	5	2,5	1,25	0,63	0,31
2	13	22	11	5	2	1
1	6	24	12	4	1	0
0,5	13	15	21	4	1	1
0,25	4	12	27	7	2	1
0,125	1	3	18	6	1	0
0,063	0	1	1	2	1	0
0,031	0	1	1	1	1	0
0	0	0	0	0	0	0

DOTMA 75

	10	5	2,5	1,25	0,63	0,31
2	2	7	3	1	0	0
1	2	16	4	1	1	0
0,5	5	10	14	2	1	1
0,25	2	6	17	4	1	0
0,125	1	1	3	8	1	0
0,063	0	0	0	0	1	0
0,031	0	0	0	0	1	0
0	0	0	0	0	0	0

DOCSPER 100

	10	5	2,5	1,25	0,63	0,31
2	0	0	0	0	0	0
1	4	1	0	0	0	0
0,5	3	2	1	0	0	0
0,25	0	0	7	0	0	0
0,125	0	0	0	2	0	0
0,063	0	0	0	0	0	0
0,031	0	0	0	0	0	0
0	0	0	0	0	0	0

DOCSPER 75

	10	5	2,5	1,25	0,63	0,31
2	4	0	2	1	1	0
1	20	3	1	2	0	0
0,5	21	19	1	0	0	0
0,25	1	1	6	1	0	0
0,125	1	0	1	0	0	0
0,063	1	0	0	0	0	0
0,031	0	0	0	0	0	0
0	0	0	0	0	0	0

DOCSPER 50

	10	5	2,5	1,25	0,63	0,31
2	5	1	0	0	0	0
1	20	2	0	0	0	0
0,5	12	33	0	0	0	0
0,25	1	3	4	0	0	0
0,125	0	0	0	1	0	0
0,063	0	0	0	0	0	0
0,031	0	0	0	0	0	0
0	0	0	0	0	0	0

DOCSPER 25

	10	5	2,5	1,25	0,63	0,31
2	18	6	2	1	0	0
1	36	6	2	1	0	0
0,5	26	21	4	1	0	0
0,25	11	27	6	1	0	0
0,125	3	2	3	2	0	0
0,063	1	1	1	1	0	0
0,031	0	0	1	0	0	0
0	0	0	0	0	0	0

Fig. 5. (*Continued*)

effective because of high toxicity with the exception of the F98 cell-line. However all four DOCSPER liposomal formulations were significiantly less toxic than DOTMA 50 and DOTMA 75 liposomes.

The maximal β-galactosidase expression achieved after transfection of MCF-7 cells differed only with a factor of two for all DOCSPER formulations, but the total β-galactosidase expression for all 48 wells was increased with higher DOPE amounts, because a broader range of lipid and DNA concentrations were active in transfection (compare Fig. 5A). In MaTu (Fig. 5B), F98 (Fig. 5C) and CC531 cells DOCSPER 100 was less effective, but in Jurkat cells DOCSPER 100 was the most effective formulation tested. Results for all five cell-lines tested are shown in Fig. 6. To sum up, DOCSPER formulated with DOPE was more effective in four of five cell-lines than DOCSPER without DOPE. Only in case of the cell-line Jurkat DOCSPER 100 being more aciitive than DOCSPER formulated with DOPE. However as described above prolonged incubation of lipid/DNA mixture can enhance transfection efficiency of DOCSPER 100/DNA complexes.

In addition the DOCSPER liposomes were compared with the DOSPER reagent obtained from Boehringer Mannheim. Results in toxicity and transfection efficiency were comparable to the DOCSPER 100 liposomal preparation (data not shown). The effect of higher serum concentrations on transfection efficiency were investigated. Transfections in the 96-well assay with DOCSPER liposomes were not inhibited by the presence of 10% fetal calf serum (data not shown).

4. Discussion

Here a new cationic lipid, DOCSPER is presented, which was synthesized for the delivery of DNA. In contrast to a comparible substance, DOSPER, it consists only of biological components, glycerol, oleic acid and spermine and it is easier to produce. Recently several other cationic amphiphiles, containing a spermine head group and a double chain anchor group have been investigated for their transfectability of DNA. These compounds spontaneously form liposomes in aqueous solutions. They are able to deliver DNA efficiently into primary and tumor cells (Loeffler and Behr, 1993; Hawley-Nelson et al., 1993). However use of these spermine derivatives is limited by either prices or quite complicated syntheses. In contrast cationic cholesterol derivatives DC-Chol (Gao and Huang, 1991) and DAC-Chol (Reszka et al., 1995) can be synthesized in a simple one step reaction, but they require a common neutral lipid for liposome formation and optimal gene delivery. The cationic lipid dioctadecylammonium bromide (Rose et al., 1991) has to be formulated with a neutral helper lipid as well.

In the present work the intention was to characterise one member of this lipospermine group, regarding the mechanism and requirements for optimal gene delivery.

In the past the size of lipid/DNA complexes was one aim of investigation. Recently Rädler et al. (1997) investigated lipid/DNA complexes by dynamic light scattering. They found a strong dependence of complex size on the L/D ratio. At a L/D ratio of about five, using liposomes formulated with dioleoyltrimethylammonium propane and DOPE, their complexes had a size of several micrometers. At lower and higher ratios, their complexes were only several hundred nanometers

Fig. 5. Comparison of toxicity and transfection efficiency of DOTMA and DOCSPER liposomes in the human mamma tumor cell-lines MCF-7 (A) and MaTu (B) and the rat glioblastoma cell-line F98 (C). The viability of cells in comparison to untreated control cells (set at 1) is shown on the left, the transfection efficiencies (micro units β-galactosidase per well) on the right for each lipid tested. In the left column the amounts of pUT 651 DNA (μg) used for transfection, in the upper row the amounts of lipid (μg) are shown. Cell viabilities of more than 50% and gene expressions with more than 50% maximal achieved in this cell-line (MCF-7 545 mU with DOCSPER 25; MaTu 124 mU with DOTMA 50; F98: 36 mU with DOCSPER 25), are highlighted by a grey underground. For example, transfecting MCF7 cells with 2.5 μg DOTMA 50 liposomes and 1 μg DNA pUT 651 resulted in cell viability of 4% in comparison to control cells and the β-galactosidase expression was 295 mU per well.

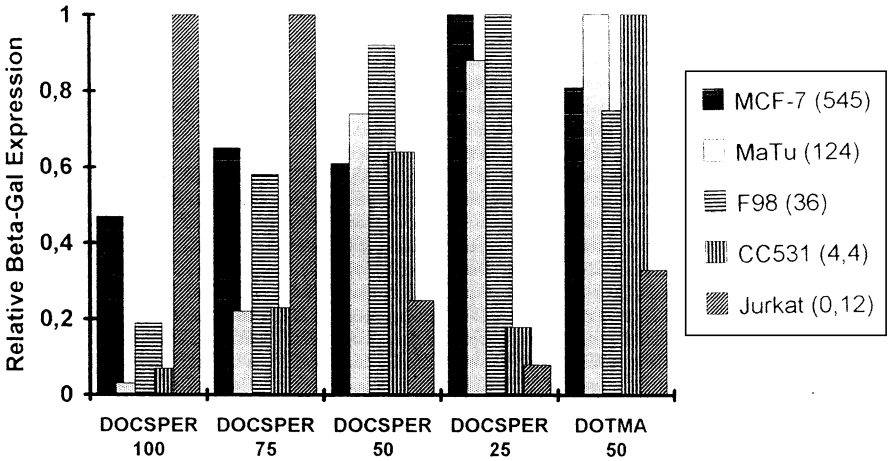

Fig. 6. Comparison of maximal transfection efficiencies of DOTMA and DOCSPER liposomes among different cell types. The maximal β-galactosidase expression from each 6×8 matrix was determined and expressed here in comparison to the highest value achieved in this cell-line. The highest amount for all liposomal formulations is written in parenthese as micro units per well.

in size. Also Emi et al. (1997) found that complexes prepared from polyarginine and DNA were only effective in mediating transfection if size of these complexes was about 10 μm. The size of the complexes depends on the DNA/poly-arginine ratio.

The electron microscopic data showed, that the size of formed liposomes depends on the DOPE amount. A higher net positive surface charge leads to a reduction in liposomal size. These observations are in agreement with previous reports, demonstrating that vesicle size decreases with increased surface charge density (Kraayenhof et al., 1993, 1996). For the characterisation of lipid/DNA complexes turbidity measurements were used, they were determined directly before the complexes were given to the cell culture. Turbidity of lipid/DNA complexes can give information about formation progress. During complex formation the size of the particles increases, leading to an increase in light scattering. The strong increase especially for DOCSPER liposomes/DNA complexes makes it possible to determine an optimal lipid/DNA ratio for transfection of cells by turbidity measurements. The results indicate, that the size of DOCSPER/DNA complexes depends on the L/D ratio and that maximal transfection efficiency is achieved at high turbidity. This is a great improvement in minimizing time and material consumption for in vitro experiments. Furthermore it was found that lipid/DNA complex formation can be completed within a few minutes if higher lipid/DNA concentrations are used. The detailed structure and size of the lipid/DNA complexes will be the aim of further investigations.

In spite of the possibility to transfect DNA with lipospermines alone, it was recently shown, that formulation of DOGS with DOPE can enhance transfection efficiencies (Thierry et al., 1997). Also 2,3 – dioleyloxy – *N* – [2(sperminecarboxyamido)ethyl]-*N*,*N*-dimethyl-1-propanaminium (DOSPA) is only available as a DOSPA/DOPE (3/1 w/w) formulation from Life Technologies, (Gaithersburg, MD) as well. Wheeler et al. (1996) have shown that converting the alcohol of an hydroxyethyl group on a quaternary nitrogen to an ethylamine lowers the co-lipid requirement and alters structures of lipid/DNA complexes. This amino compound termed β-aminoethyl-DMRIE (dimyristyl Rosenthal inhibitor ether) is strucuraly related to lipospermines. β-Aminoethyl DMRIE formulated with DOPE was more efficient than β-aminoethyl DMRIE alone only in three of the ten cell-lines tested.

The requirement of a neutral helper lipid for transfections with DOCSPER was tested. For the liposomal formulations DOPE was used, because of its endosomal disrupting properties (Zhou and Huang, 1994). Replacement of DOPE by another neutral phospholipid, such as dioleoylphosphatidycholine (DOPC), containing a quaternary amine instead of a primary, lowers transfectability of liposomes (Farhood et al., 1992, 1995).

As many other double chain amphiphiles, DOCSPER is able to form spontaneously stable liposomes in aqueous solutions, avoiding difficult formulation procedures, undesired oxidation products and unknown biological properties of DOPE. It was found that formulating DOCSPER with DOPE lowers the time, required for optimal complexations of DNA which can enhance transfection efficiency. In four of five cell-lines DOCSPER/DOPE formulations were more effective if an incubation time of 30 min was used for DNA complexing. DOCSPER 100 showed similar properties in complexing behaviour, toxicity and transfection effciency in comparison with the DOSPER reagent, which contains one positive charge more. For both formulations the correct lipid/DNA ratio and prolonged incubation times for complex formation are essential. The differences in transfection between DOCSPER 100 and 50 may be a result of the smaller size of the DOCSPER 100 liposomes. Plasmid DNA has a size of several hundred nanometers, DOCSPER 100 liposomes have a size of approximately 20–100 nm as documented by electron microscopy, while DOCSPER 50 liposomes have a size similar to DNA, thereby making complex formation easier and faster. Formulating DOCSPER with DOPE diminishes the DNA concentration dependent complex formation and promise an easier transfection procedure in vitro. However, in vivo, a defined complexing of plasmid DNA could be advantageous for stabilisation. To solve this problem further investigations are neccessary.

Liposomes prepared from DOCSPER were compared with liposomes prepared from DOTMA/DOPE in the transfection of four adherent and one suspension cell-line. It was found that lipid reagent is superior to DOTMA because of the much lower toxicity. The observation, that DOCSPER was less toxic than DOTMA was somewhat surprising, because the main cause of toxicity in cell culture was thought to be the positive charge of the cationic lipid. DOCSPER, with one additional positive charge, contains biodegradable carbamoyl bonds in contrast to DOTMA, which contains as many other cationic lipids (DMRIE, DOSPA) ether bounds, which are nonbiodegradable. This could be a possible explanation for the high toxicity of DOTMA. Furthermore it was found that also other spermine derivatives were less active in transfection of CC531 cells (unpublished observations), a possible change of the cationic head group of glycerol should increase the transfection rates on CC531 cells as well. It is possible, that the mechanism of uptake is different among various cell-lines. This will be the objective of further investigation. Thus the glycerol backbone should be a promising component for preparation of a nontoxic, ideal gene delivery system.

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