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Ultrastructural characterization of cationic liposome-DNA complexes showing enhanced stability in serum and high transfection activity in vivo

Brigitte Sternberg [a,*], Keelung Hong [a,b], Weiwen Zheng [a,b], Demetrios Papahadjopoulos [a,b]

[a] *California Pacific Medical Center, Research Institute, 2340 Clay Street, San Francisco, CA 94115, USA*
[b] *Liposome Research Laboratory, 2200 Webster Street, San Francisco, CA 94115, USA*

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Abstract

We have investigated the morphology and transfection activity of cationic liposome-DNA complexes (CLDC) under conditions relevant to both in vivo and in vitro studies. Moreover we have attempted to establish structure-function relationships relevant for high transfection activities under both conditions. CLDC were composed of dimethyldioctadecylammonium bromide with either 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) or cholesterol (Chol) interacting either with pre-condensed DNA or with uncondensed plasmid DNA. Furthermore for steric stabilization 1% poly(ethylene glycol)-phospholipid conjugate was added to CLDC containing Chol and plasmid DNA. The in vivo studies were carried out in mice following i.v. injection, and the in vitro studies were performed on SK-BR-3 human breast cancer cells in the presence of media with serum. The morphology of the CLDC, monitored by freeze-fracture electron microscopy, was investigated after mixing with mouse serum or the medium where the cells were kept. The substitution of DOPE with Chol, and the addition of *N*-[ω-methoxypoly(oxyethylene)-α-oxycarbonyl-DSPE are producing CLDC which are stabilized with respect to time and serum, and are relatively small (100–300 nm). These stabilized complexes show high expression of a marker gene in mouse lungs reaching expression values up to 10 ng luciferase per mg tissue protein, but relatively low expression in SK-BR-3 cells in vitro. Additionally, some of the complexes containing pre-condensed DNA look like 'map-pin' structures showing heads of the size of liposomes and short, stiff and tapering tails. The in vivo transfection activity of these preparations is highest. Similar complexes containing DOPE rather than Chol as helper lipid precipitate in the presence of serum and especially of cell medium and convert into hexagonal lipid (H_{II}) phase. Such complexes, despite their high transfection activity in vitro, show very little transfection activity in vivo. These comparisons may help us to understand the fundamental difference between in vitro and in vivo activity of CLDC: high in vitro transfection activity seems to be associated with hexagonal lipid precipitates whereas high in vivo activity seems to be related with small, stabilized complexes, which in our case also exhibit some protrusions (map-pin structures).

Keywords: Cationic liposome; DNA delivery; In vivo and in vitro gene expression; Freeze-fracture electron microscopy

Abbreviations: Chol, cholesterol; CLDC, cationic liposome-DNA complexes; DC-Chol, 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)carbamoyl] cholesterol; DDAB, dimethyldioctadecylammonium bromide; dLS, dynamic light scattering; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOTAP, 1,2-dioleoyl-3-trimethylammonium propane; FCS, fetal calf serum; FFEM, freeze-fracture electron microscopy; H_{II}, inverse hexagonal lipid phase; MES, 2-*N*-(morpholino)ethanesulfonic acid; PEG-PE, *N*-[ω-methoxypoly(oxyethylene)-α-oxycarbonyl-DSPE; RLU, relative light units

* Corresponding author. Fax: (415) 8312813; E-mail: brigitt@itsa.ucsf.edu

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1. Introduction

Many diseases have become a target for somatic gene therapy, including acquired multifactorial diseases such as cancer, arthritis and AIDS as well as genetic disorders such as cystic fibrosis and Duchenne muscular dystrophy [1–4]. Gene therapy methods have been designed to introduce genetic information into patient's cells to enable these cells to correct or modulate a disease [5]. Over the past few years, rapid progress has been made in controlling the in vivo delivery and expression of therapeutic genes [6]. Presently, viral-based carriers of DNA are still the most common method of gene delivery, although there is a strong research effort for developing synthetic non-viral vectors, in particular, cationic liposome-DNA complexes (CLDC) [7–9]. When compared to viral vectors, liposomal gene delivery systems offer several advantages, including the lack of viral gene elements, low immunogenic and inflammatory responses, potential for transfer of expression units of essentially unlimited size, and possibility for cell-specific targeting [10–12].

During the last 10 years, in vitro studies have demonstrated convincingly that CLDC can mediate gene delivery by showing relatively high expression of reporter genes in cultured cells [13–15]. Such preparations appeared initially to be ineffective for many in vivo applications and showed instability in serum. More recently, however, CLDC have also been used for in vivo transfection in animals [16–21] as well as in humans [22] by direct intra-tumoral injection [16], by repeated intravenous injection [17], by aerosol inhalation [18] or administration to the nasal epithelium [22]. Since initially CLDC have not been as efficient as viral vectors for achieving successful gene transfer in vivo, much effort has been devoted in optimizing the complexes. This included synthesizing more efficient cationic lipids [5,19,20], alternative helper lipids [21,23], as well as plasmid expression vectors [24], but also stabilization of the whole CLDC by polyamines and poly(ethylene glycol)-phospholipid conjugates [21]. With about 5.5 mg of protein expressed per gram of packed cells in culture [25] and more than 1 μg protein expressed per gram lung tissue [21,23,26] trans-gene expression levels have recently approached those achievable with adenovirus. Furthermore, there is growing awareness of a discrepancy between in vivo and in vitro with respect to transfection activity, and that screening and optimization of CLDC must be done in vivo because in vitro assays are usually not predictive [25].

Since 1993, there has been an increasing number of publications describing the morphology of CLDC [26–35]. These studies presented evidence for DNA-induced fusion of the cationic lipid vesicles, but the morphological result of this fusion process was visualized differently, using such structural analogies as 'beads-on-string' [27], oligolamellar particles [26,29,32–34], bundled and folded loops of DNA strands [35], and 'spaghetti'-like fibrils depicting DNA coated by a lipid bilayer [28,31,36]. Hexagonally packed DNA, coated by lipid in the inverted H_{II} phase, was also proposed [30] and observed under certain conditions [31]. Even particles adopting the H_I hexagonal tubular phase and containing the DNA condensed in this tubular network, were observed when starting with lipopolyamines instead of cationic liposomes [37]. In most of these publications it is claimed that a lipid coating is able to protect the DNA from restriction enzymes or other degradative processes [26–33], irrespective as to whether this coat is made of a lipid bilayer sheet [27,29], tubule [28,31,36], stacks [32,33], invaginated liposomes [26], or non-bilayer lipid arrangements [30,31,37]. It is also proposed that the lipid coat is able to enhance the uptake of CLDC by recipient cells possibly via endocytosis [36–40] and/or local fusion [36], and possibly help the escape of DNA material from the endosomes into the cytoplasm [37–40].

Despite the increasing number of publications describing the morphology of CLDC, very little is known about the transfection-active structure(s) responsible for in vitro and/or in vivo transfection activity. Thus, in the present study, we have investigated the morphology and the transfection activity (in vitro and in vivo) of stabilized CLDC made of plasmid DNA, dimethyldioctadecylammonium bromide (DDAB), and cholesterol (Chol). Moreover, we have compared both parameters (morphology and transfection activity) with those obtained from similar but non-stabilized complexes containing 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) instead of Chol. Morphological stability was studied in buffer, culture media, and serum, with respect to time. DDAB was chosen as 'model' compound out

of 18 different cationic amphiphiles because DDAB/Chol was one of the most efficient vehicles for in vivo gene delivery which were screened recently in mice [21]. Furthermore, in a preliminary study with 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) instead of DDAB we have obtained similar results with respect to the effect of Chol versus DOPE and the relative differences between in vitro and in vivo activity.

2. Material and methods

2.1. Lipids and other reagents

DOPE and *N*-[ω-methoxypoly(oxyethylene)-α-oxycarbonyl-DSPE (PEG-PE) were purchased from Avanti (Alabaster, AL). Purified Chol was obtained from Calbiochem (San Diego, CA). Spermidine and DDAB were purchased from Sigma (St. Louis, MO). DDAB was recrystallized once from acetone-methanol solution. D-Luciferin was obtained from Boehringer Mannheim. Chloroform solution of each lipid was stored under argon in sealed ampules at −40°C. Other reagents of the highest possible grade were purchased and used without further purification.

2.2. Preparation of liposomes

DDAB was mixed with DOPE or cholesterol in 1:1 molar ratio, and the solvent removed slowly under reduced pressure at 50°C on a rotary evaporator. The dry lipid film was hydrated with 5% dextrose solution prewarmed to 50°C and the container was sealed under argon. The hydrated lipid suspension was sonicated in a bath sonicator (Lab Supplies, Hicksville, NY) for 5–10 min at 50°C. The final concentration of liposomes was 5 mM cationic lipid and the size of liposomes was measured by dynamic light scattering to be 195 ± 65 nm. Sonicated liposomes were stored under argon at 4°C until use.

2.3. Luciferase reporter system

Plasmid containing luciferase as a reporter gene (pCMV/*luc*$^+$) was constructed with CMV promoter [21]. Plasmids were purified using alkaline lysis procedures adopted and devised by Qiagen (Chatsworth, CA). Plasmid purity was measured by the ratio of absorbance at 260 nm vs. 280 nm, and purified plasmids were stored in buffer containing 10 mM Tris-Cl and 1 mM EDTA at pH 8.0 at concentrations of 1–2 mg/ml.

2.4. Preparation of transfection complexes

Prior to the transfection experiments, the optimal DNA/liposome ratio for forming complexes which were not large aggregates was determined by mixing a fixed amount of plasmid with various amounts of liposomes as described in [21]. In general, the transfection complexes were formed by pipetting the plasmid solution into a liposome suspension of equal volume and mixing rapidly. The final DNA concentration of the complexes was 200 μg/ml for in vivo studies and 10 μg/ml for in vitro studies. Routinely, liposomes containing 8–12 nmol of DDAB could complex with 1 μg plasmid without forming visible large aggregates. Such complexes have excess positive charge, but still tend to aggregate with time during storage at 4°C and lose transfection activity in 4 days. For in vitro experiments, which called for more diluted complexes, CLDC at 5–12 nmol DDAB per μg DNA have been formed.

In order to prevent CLDC from forming large aggregates and loosing their in vivo transfection activity with time the following two stabilization methods were used, as described before [21]. Small amounts of PEG-PE (from 0.5 to 2 mole percent of cationic lipid) were added to the DNA-liposome complexes within a few minutes after their preparation. Alternatively, the plasmid was partially condensed with spermidine (e.g. 0.5 nmole of spermidine per μg DNA) prior to mixing with the liposomes. The optimal amount of spermidine was determined by titrating the polyamine to DNA to a point before forming large aggregates. Both PEG-PE and spermidine were added as solutions in distilled water. The size of the complexes was estimated by dynamic light scattering (dLS) and freeze-fracture electron microscopy (FFEM). The unmodified DDAB/Chol complexes had average diameters of 388 ± 130 nm and 180 ± 120 nm respectively. The size of both types of stabilized DDAB/Chol complexes was somewhat smaller. The complexes stabilized by partial condensation of DNA by spermidine showed the smallest

size (293 ± 98 nm as measured by dLS and 140 ± 80 nm as measured by FFEM). Stabilization of the complexes by PEG-PE as well as by spermidine was performed only for the Chol containing complexes. Further studies are required to establish the effect of such stabilization on DOPE-containing complexes.

2.5. Assay of reporter gene expression

Purified luciferase was purchased from Boehringer Mannheim as a standard for calibrating the luminometer and constructing a standard curve for the relative specific activity of luciferase. Reporter gene expression in a tissue extract was presented in nanogram quantities of luciferase protein by converting relative light units measured from a luminometer into weight units according to a standard curve [21]. Luciferase expressed in cells or tissues was extracted with cell lysis solution. Effective lysis buffer consisted of 0.1 M potassium phosphate buffer at pH 7.8, 1% Triton X-100, 1 mM DTT and 2 mM EDTA.

2.6. In vivo delivery and gene expression

Female CD1 mice (4–6 weeks old, weighing approx. 25 g) were obtained from Charles River Laboratory. In two sets of independent investigations with three mice for every composition, each mouse received 60 μg CLDC at a concentration of 8 nmole DDAB per μg DNA by tail vein injection. Twenty-four hours later they were euthanized and the anesthetized animals perfused with cold phosphate-buffered saline (PBS) via heart puncture. Each tissue was dissected and washed in PBS, and then homogenized in 6 ml round-bottomed culture tubes containing 500 μl of lysis buffer. The samples were kept at room temperature for 20 min with occasional mixing. The homogenized samples were centrifuged for 10 min at 3000 rpm in an Eppendorf centrifuge. Luciferase activity of each tissue was measured by mixing 100 μl of the reconstituted luciferase substrate (Promega, Madison, WI) with 20 μl of the supernatant of tissue homogenate in the injection system of a luminometer. Peak light emission was measured for 10 s at 20°C. Relative light units of each sample were converted to the amount of luciferase in the tissue extract were comparing with a standard curve which was established for each set of experiments. The protein content of the extract was determined using protein assay kits (BioRad, Richmond, CA). Background was the count of corresponding lysis buffer in tissues of mice without treatment. We have obtained consistent negative controls of tissue samples from mice that were treated with liposomes only or with complexes with β-galactosidase DNA. The luciferase activity of these tissues measured was at the same level as lysis buffer analysis, i.e. 200–300 relative light units (RLU). After background subtraction, a standard curve was created to convert the RLU into weight units which was linear from 2×10^2 to 9×10^6 RLU, which corresponded to 3×10^{-5} ng up to 3 ng luciferase. According to this standard curve the reporter gene expression in a tissue extract was presented in nanogram quantities.

2.7. In vitro delivery and gene expression

SK-BR-3 cells were cultured in McCoy's 5A medium supplemented with 10% heat-inactivated bovine calf serum and in 5% CO_2 [21]. SK-BR-3 cells in monolayer culture were plated at 50 000 cells per well in 12-well plates and incubated overnight. In more than three sets of independent investigations with three wells per composition, each well received 1 μg of pCMV/*luc*$^+$ complexed with 8 nmole DDAB within 20 min of complex formation. The complexes were left in contact with the cells for 4 h at 37°C. Then they were removed and replaced by fresh medium containing 10% serum. After 24 h of transfection the cells were lysed with 200 μl per well of luciferase lysis buffer. Luciferase activity after transfection was determined as described above.

2.8. Freeze-fracture electron microscopy

For FFEM all CLDC were prepared under the same conditions and using the same DNA (200 μg/ml) and lipid concentrations (8.0 nmole cationic lipid/1 μg DNA) as described in Section 2.4 for the transfection experiments. The CLDC were stored at 4°C in dextrose buffer of low ionic strength. For final incubation for 10 min CLDC suspensions were diluted 1:1 v/v either with 2-*N*-(morpholino)ethanesulfonic acid (MES) buffer (1.0 mM, pH 5.5), or mouse serum (simulating in vivo conditions), or cell medium

as well (RPMI-1640 with 10% fetal calf serum (FCS); simulating in vitro conditions). Electron microscopical snapshots were taken after a storage time of 3 days when all suspensions of the Chol-containing CLDC were still entirely clear and after 6 days when the unmodified DDAB/Chol/DNA complexes started to precipitate. For FFEM the samples were quenched rapidly using the sandwich technique and liquid propane (cooling rate $>10^4$ K/s). The cryofixed specimens were fractured and shadowed with Pt/C in a Balzers BAF 400D freeze-fracture device at -120°C and 2×10^{-6} torr. The cleaned replicas were examined in a transmission electron microscope (Zeiss CEM 902 A) [41].

3. Results

Preparations of CLDC are metastable with time and since structural instability may be connected with the loss of transfection activity, it is highly desirable for in vivo applications to develop well-defined formulations which are stable in buffer and in serum over a reasonably long period of time. Stabilization of cationic-plasmid DNA complexes was achieved using Chol instead of DOPE, by addition of PEG-PE, and also by pre-condensation of the DNA by polyamines [21]. In order to study the influence of these stabilizing factors on the morphology of CLDC, we investigated by FFEM the structure of traditional DNA/DDAB/DOPE complexes and compared their morphology with those of the newly developed CLDC stabilized by Chol, and additionally either 1% PEG-PE, or spermidine-condensed plasmid DNA. The morphology of all CLDC, whether non-stabilized or stabilized, was investigated in buffer at low ionic strength, cell medium, and mouse serum as well.

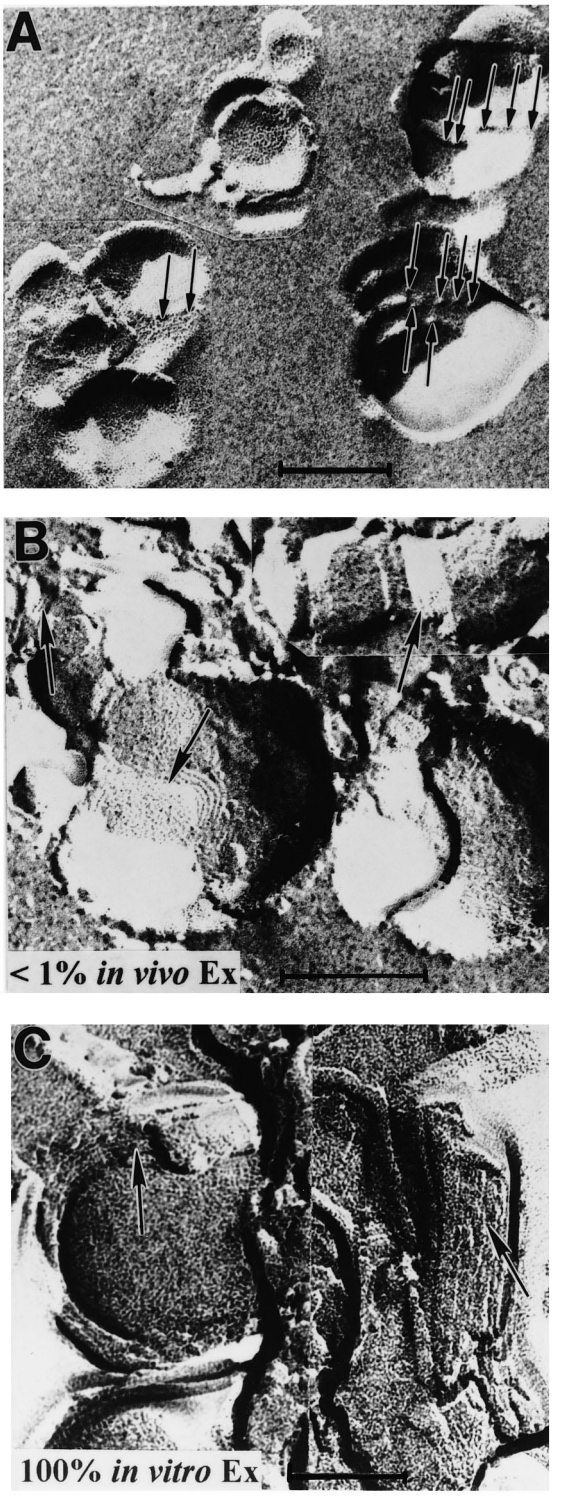

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Fig. 1. Morphology of CLDC (DDAB/DOPE/DNA). Effect of mouse serum and culture medium. (A) Sample prepared and stored in dextrose buffer, and diluted 50% v/v for final incubation at MES buffer (pH 5.5). (B) As in A, but incubated for 10 min in mouse serum, or (C) in cell medium. Some of the lipidic particles at the fusion area of the complexes are marked by arrows in A. In B hints for the formation of H_{II} lipid phase and in C areas with well-developed H_{II} tubules are marked by arrows. Bars on all freeze-fracture electron micrographs represent 100 nm and the shadow direction is running from bottom to top.

3.1. Morphology and transfection activity of DOPE-containing CLDC

DOPE is most commonly used as a helper lipid in cationic liposome-mediated gene transfer [42]. Although an increased transfection potency of DOPE-containing CLDC is described in most of the in vitro studies, their in vivo transfection activity is very low compared to Chol-containing CLDC [21,23,26]. Because of these contrary reports we undertook morphological analysis of CLDC containing DOPE and compared to similar complexes but containing Chol.

The morphology of DNA/DDAB/DOPE complexes is quite different compared to similar complexes but containing Chol (shown below). This is true in buffer (Fig. 1A), but especially in mouse serum (Fig. 1B), as well as cell medium (Fig. 1C). Even in buffer of low ionic strength (1.0 mM MES; pH 5.5) the liposomes fuse into larger aggregates after interaction with the DNA, and have a tendency to form non-bilayer structures such as lipidic particles. Some of the lipidic particles at the fusion areas of the liposomes are marked by arrows in Fig. 1A. This tendency of DOPE-containing CLDC to form non-bilayer structures such as the hexagonal (H_{II}) lipid phase is especially pronounced in mouse serum (Fig. 1B) and in cell medium (RPMI-1640, with 10% FCS) at high ionic strength (Fig. 1C), where CLDC are observed by FFEM as tightly packed, fused to bigger units, and forming hexagonal (H_{II}) lipid structures (marked with arrows in Fig. 1B,C). No fibrillar structures of any type are observed. Interestingly, these complexes show only 1% of the in vivo transfection activity compared to the DDAB complexes containing Chol. Contrary to the in vivo studies, the in vitro transfection activity of the DOPE containing complexes is very high (100%, Table 1).

3.2. Morphology of stabilized CLDC in low ionic strength buffer

In contrast to non-stabilized DNA/DDAB complexes containing DOPE, freeze-fracture electron micrographs of similar complexes stabilized by Chol (control) display small spherical particles (average diameter in the range of 100–250 nm) with few protrusions (marked by an arrow in Fig. 2A). This is true for the clear suspension in buffer of low ionic strength (1.0 mM MES; pH 5.5) and is still true after 3 days of storage time at 4°C (Fig. 2A). Within a storage time of 6 days, however, the suspension is not clear any longer and the CLDC start to precipitate. The spherical particles obviously adhere, fuse, and form larger complexes with diameters between 300 and 600 nm (Fig. 2B). In contrast, the morphology of similar complexes but additionally stabilized by PEG-PE is very similar to the 3 day old controls even when the electron micrographs were taken from 6 day old samples, displaying still small spherical

Table 1
Luciferase expression in vitro (SK-BR-3 cells) and in vivo (mouse lung) by CLDC composed of DDAB/DOPE or DDAB/Chol and DNA with or without stabilizing agents

Sample (age)	Luciferase activity calculated as ng luciferase protein per mg tissue protein ± S.E.[a]			
	DDAB plus DOPE	DDAB plus Chol	DDAB/Chol plus either PEG-PE or spermidine	
			PEG-PE (1 mole% of DDAB)	Spermidine (0.5 nmole/μg DNA)
In vitro (freshly prepared)	79.17 ± 33.0	15.93 ± 3.6	–	–
	(100 ± 41.2)	(20.1 ± 4.5)	–	–
In vivo (freshly prepared)	0.02	0.95 ± 0.29	0.52 ± 0.18	2.14 ± 1.75
	(< 1)	(100 ± 30.4)	(55.1 ± 19.3)	(224.6 ± 180.4)
In vivo (1 month old)	–	0.0014 ± 0.0005	1.15 ± 0.66	4.14 ± 1.22
	–	(< 1)	(120.7 ± 70)	(380 ± 128.0)

The in vitro data were obtained from more than three sets of independent experiments with three wells per composition within each set, using SK-BR-3 cells in culture. The in vivo data are based on two sets of independent experiments with three mice per composition in each set, using i.v. injection and measuring luciferase expression in lungs. Procedures for both in vitro and in vivo measurements as described in Section 2.

[a]Numbers in parentheses represent relative luciferase activity based on respective control values from [21] taken as 100% (DDAB/DOPE for in vitro studies and DDAB/Chol for in vivo studies).

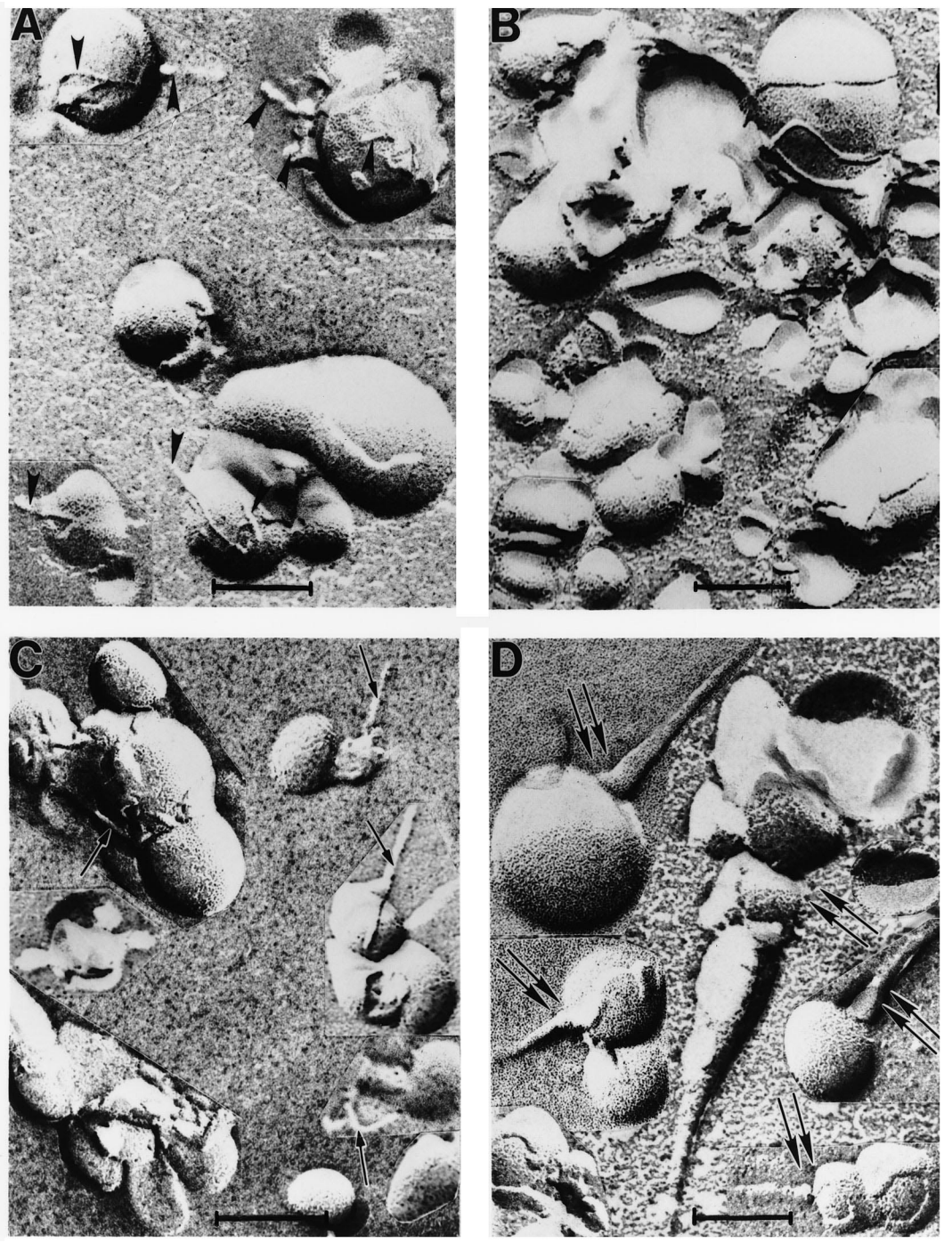

Fig. 2. Morphology of CLDC (DDAB/Chol/DNA) in buffer. Additional stabilization by PEG-PE or spermidine. (A) Chol-stabilized CLDC, 3 day old sample. Small complexes with some protrusions (marked by arrowheads) are observed. (B) Chol-stabilized CLDC, 6 day old sample. Much bigger aggregated/fused complexes without protrusions are observed. (C) Stabilized CLDC made with 1% PEG-PE added. (D) Stabilized CLDC made with spermidine-condensed plasmid DNA. Both samples, shown in C and D, are 6 days old, the additionally stabilized CLDC are quite small and show some short but stiffer protrusions (marked by small arrows in C). Some well-developed 'map-pin' structures, found at complexes where the plasmid DNA was pre-condensed by spermidine, are marked by two arrows in D. All samples were prepared and kept in low ionic strength dextrose buffer and diluted 50% v/v before final incubation with 1.0 mM MES buffer (pH 5.5). Bars on all freeze-fracture electron micrographs represent 100 nm and the shadow direction is running from bottom to top.

particles up to 300 nm in diameter and also showing few protrusions (marked by an arrow in Fig. 2C). Although 6 days old, DDAB/Chol complexes additionally stabilized by spermidine-condensed DNA are even smaller, and show additionally few but well developed, stiff and tapering protrusions ('map-pin' structures; Fig. 2D). The map-pin structures of the complexes, as seen and marked by two arrows in Fig. 2D, are characterized by spheroidal heads showing diameters of one or two liposomes (100–200 nm) and mainly short (about 200 nm) but sometimes up to 600 nm long and tapering 'pins'. Both types of preparations additionally stabilized by PEG-PE or by spermidine-condensed DNA are clear and do not show any precipitation after storage for months.

3.3. *Morphology in serum or culture medium and transfection activity of stabilized CLDC*

We investigated the transfection activity both in vivo and in vitro of plasmid DNA/DDAB/Chol complexes as a control, and of similar but additionally stabilized DDAB/Chol complexes either containing 1% PEG-PE and interacting with supercoiled plasmid DNA or without PEG-PE but interacting with spermidine-condensed plasmid DNA. In parallel, we have studied their morphology in serum as well as in cell medium by FFEM. The in vivo studies were carried out in mice following i.v. injection and therefore the morphology of the CLDC was investigated in mouse serum. The in vitro transfection activity of the CLDC was measured on SK-BR-3 cells and therefore we studied their morphology in the same medium where these cells were grown.

When examined in 50% mouse serum (10 min incubation time), Chol-stabilized, 3 day old CLDC are as small as they are in buffer at low ionic strength (100–250 nm) but show very few protrusions (Fig. 3A). Such complexes show high transfection activity in mouse lungs after i.v. injection (up to 3 ng expression per mg tissue protein) [21] and we take this level of activity as 100% for further comparisons. After the same incubation time in mouse serum, these Chol-stabilized CLDC of the same composition but 6 days old, appear as densely packed aggregates of spherical particles where the number of attached particles in each aggregate is quite high (approx. ten to 14; Fig. 3B). Such formulations have lost all their in vivo transfection activity within 4 days [21]. Residual fibrillar protrusions are not observed.

DDAB/Chol complexes additionally stabilized by PEG-PE and incubated with mouse serum, are quite small (approx. four to six attached particles) even when 6 days old (Fig. 3C). Interestingly, these stabilized complexes, freshly prepared, show about half of the in vivo lung transfection activity (about 60%, Table 1) observed with similar, freshly prepared complexes but Chol-stabilized only (100%, standard value, Table 1). Freshly prepared DDAB/Chol complexes where the DNA was pre-condensed by spermidine showed even approx. 200% compared to the Chol-stabilized, freshly prepared complexes (Table 1). Interestingly, neither of these additionally stabilized preparations loses activity within a storage period of 6 days. In this case, the resulting complexes are also quite small (approx. four to six attached particles) even after 6 days storage time at 4°C and 10 min incubation in serum, and additionally reveal map-pin structures, marked by two arrows in Fig. 3D. It is remarkable that among the stabilized, 1 month old samples there were much higher expression rates (about 130% for PEG-PE stabilized complexes and about 380% for complexes containing spermidine-condensed DNA, Table 1) than for complexes stabilized by Chol only, which, after the same storage time, showed less than 20% of the standard value (Table 1).

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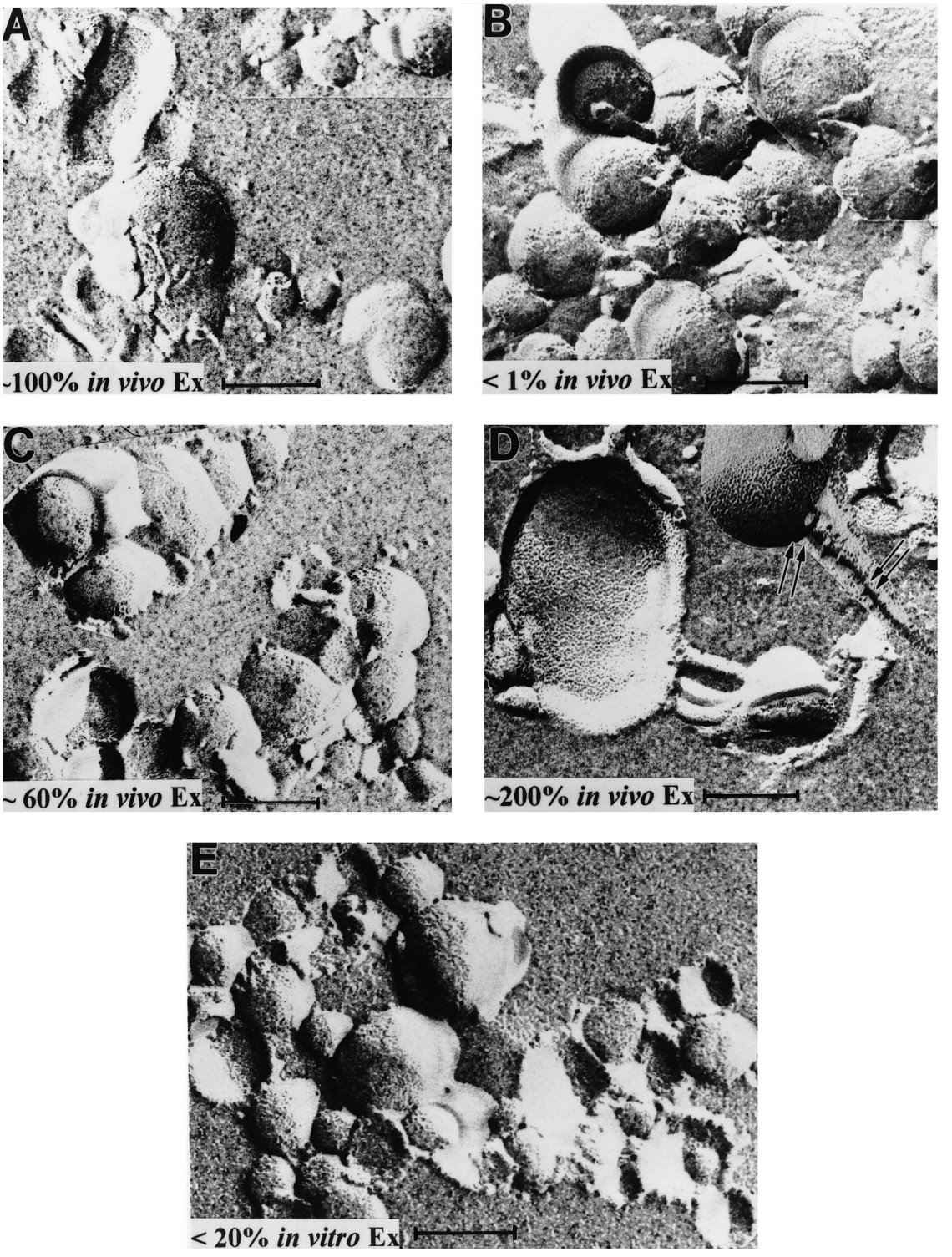
Fig. 3. Morphology of CLDC (DDAB/Chol/DNA) in mouse serum or culture medium. Additional stabilization by PEG-PE or spermidine. (A) Chol-stabilized CLDC, 3 day old sample, incubated in mouse serum for 10 min. (B) Chol-stabilized CLDC, sample 6 days old, incubated in mouse serum for 10 min. (C) Stabilized CLDC, incubated in mouse serum, made with 1% PEG-PE added. (D) Stabilized CLDC made with spermidine-condensed plasmid DNA. 'Map-pin' structures, found at complexes where the plasmid DNA was pre-condensed by spermidine and well preserved in mouse serum, are marked by two arrows in D. (E) Chol-stabilized CLDC, 6 day old sample, incubated in cell medium (RPMI-1640 with 10% FCS) for 10 min. All samples were prepared and stored as in Fig. 1, and diluted 50% v/v with either mouse serum or culture medium before final incubation. Bars on all freeze-fracture electron micrographs represent 100 nm and the shadow direction is running from bottom to top.

When investigated after 10 min incubation in cell medium (RPMI-1640, with 10% fetal calf serum (FCS)), Chol-stabilized, 6 day old CLDC are quite similar to those in mouse serum (aggregates of about 12 attached spherical particles), but packed looser and showing no fibrillar protrusions of any type



(Fig. 3E). This is also true for DDAB/Chol complexes additionally stabilized by PEG-PE as well as by spermidine-condensed DNA (not shown here). In contrast to their high in vivo transfection activity (60–200%), their in vitro transfection activity is relatively low, and shows less than 20% of luciferase expression in SK-BR-3 cells compared to similar but DOPE containing complexes, even when kept in the same cell medium (Table 1).

4. Discussion

Complexes formed during interaction of cationic liposomes with plasmid DNA display a variety of polymorphic and metastable structures. These include small or large aggregates, of the two oppositely charged macromolecular assemblies which maybe loosely or tightly packed or fused [26–36,38,39], fibrillar structures [28,31,36], and, last but not least, inverted hexagonal H_{II}-lipid arrangements [30,31]. They are metastable both with time [21] and in media at high ionic strength, such as cell media and serum [21,31,34,35]. Since structural instability seems to be connected with loss of transfection activity, it is highly desirable to develop well-defined formulations stable in buffer and in serum over a longer period of time, a condition critical for in vivo applications [21].

Our structural investigations, using freeze-fracture electron microscopy, show that stabilization of the morphology of CLDC can be achieved by substituting Chol for DOPE as the helper lipid, by adding poly(ethylene glycol)-phospholipid conjugates such as PEG-PE shortly after complex formation, and also by pre-condensation of the plasmid DNA by polyamines such as spermidine. Whereas non-stabilized complexes grow much larger during 6 days of storage time even in buffer at low ionic strength (as seen from Fig. 2B compared with Fig. 2A), the complexes containing Chol and additionally stabilized by PEG-PE as well as by spermidine maintain the same morphology, displaying in both cases small-size complexes (not bigger than 300 nm). In the case of stabilization by PEG-PE, the protrusions connected with the CLDC are relatively small (marked by an arrow in Fig. 2C), while with spermidine stabilization they are well developed and relatively big (pin of the mappin structures; marked by two arrows in Fig. 2D).

Non-stabilized CLDC made of DNA/DDAB and containing DOPE show quite a different morphology in buffer, serum as well as cell medium compared to those containing Chol as helper lipid. After interaction with DNA, the DDAB/DOPE liposomes fuse into larger aggregates and show a clear tendency to form non-bilayer structures [43,44] at a 1:1 molar ratio of cationic amphiphile to helper lipid and even in buffer of low ionic strength. FFEM is an excellent technique to recognize and to distinguish between non-bilayer structures such as hexagonal H_{II} phase [41], lipidic particles [28], or cubic phase. Some of the lipidic particles formed during the fusion process (marked by arrows in Fig. 1A) are decorating the fusion areas of the liposomes [28,31]. This tendency to fuse into large extended aggregates, thereby forming non-bilayer structures such as H_{II} lipid phases, is especially strong at high content of DOPE [31,45] but also in media of high ionic strength such as serum and cell medium (H_{II} lipid areas are marked by arrows in Fig. 1B,C) [45]. This is also true for complexes containing cationic amphiphiles other than DDAB as shown for 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)carbamoyl] cholesterol (DC-Chol) in buffer at low ionic strength but high DOPE:DC-Chol ratios (4:1 by mole) [31] or for DOTAP:DOPE at molar ratios of 1:1 and 1:2 but in DMEM known for high ionic strength [45].

Interestingly, these large lipid precipitates, found in mouse serum and extending here to areas of several micrometers, show a much lower in vivo transfection activity, only 1% of that shown by small (100–300 nm), serum-stable DDAB complexes containing Chol. However, these large extended lipid precipitates, formed by DOPE-containing complexes in cell medium, show very high transfection activity in vitro (100%) compared to quite low in vitro activity ($<20\%$) of similar complexes containing Chol instead of DOPE. Recent reports about high in vitro transfection activity of complexes containing DOPE in relation to their ability to form quite large complexes in buffer of low ionic strength (400–1400 nm) [38] or even non-bilayer structures such as cubic phase [46] support our observations with DOPE-containing complexes.

This discrepancy between high in vitro but low in vivo transfection activity of similarly composed preparations reveals a fundamental difference between in

vitro and in vivo gene delivery by means of CLDC. Obviously, for in vitro transfection activity, large extended hexagonal lipid arrays, precipitating the DNA onto the cells in culture similarly to $Ca_3(PO_4)_2$, seem to be associated with high transfection rates. Contrary to that, the in vivo transfection activity seems to be highest with small complexes, stabilized with respect to serum and time. It is possible that this stabilization allows the complexed DNA to travel within the blood stream avoiding immediate clearance by the RES, and eventually to be adsorbed on endothelial cells in lung capillaries where transfection can occur.

Additional stabilization of Chol-containing CLDC by addition of 1% of PEG does not change much the morphology of freshly prepared complexes kept in buffer at low ionic strength as seen by comparison of Fig. 2A with Fig. 2C. However, pre-condensation of the DNA has quite a remarkable effect and leads to the appearance of a new structure (the map pin), characterized by spheroidal heads showing diameters of one or two liposomes (100–200 nm) and mainly short (about 200 nm) but sometimes up to 600 nm long and tapering pins (Fig. 2D). These structures are different from the spaghetti-like fibrillar structures which are observed with CLDC made of some other monovalent cationic amphiphiles and DOPE at certain molar ratios, which represent a single bilayer tube coating the supercoiled plasmid DNA [28,31,36]. Interestingly, these pins do not show normal fracture behavior when freeze-fracture technique is applied [41]. They have a much thicker diameter at their base (near the head) of about 30 nm. It should be noted that condensation methods with a variety of polycations such as spermidine have been found to condense DNA molecules into toroids or rods, whereby the rods show rather similar diameters of 30 nm and lengths of 200–300 nm [9,47]. Therefore it is quite possible that the tapering pins represent exposed, partly condensed DNA rods interacting at one end with the CLDC. These map-pin structures were also observed in mouse serum although to a lesser extent (Fig. 3D), but not in cell medium (not shown here). Therefore they seem to be stable in serum but not in cell media.

Adding PEG-PE shortly after the complex formation, and also pre-condensation of the plasmid DNA by spermidine, are effective methods to stabilize additionally both the morphology and the in vivo transfection activity of CLDC made of DDAB/Chol/DNA. Whereas DDAB/DNA complexes stabilized by Chol alone, at the age of 1 day and incubated with 50% mouse serum for 10 min, are still small (100–200 nm; Fig. 3A) and show 100% in vivo transfection activity (standard value, Table 1) they form much bigger and tighter packed aggregates with time (tight aggregates of ten to 14 spherical particles after 6 days, Fig. 3B). Significantly, they lose totally their in vivo transfection activity within 4 days [21]. Similar complexes when stabilized by the methods described above are still quite small (aggregates of four to six particles) even when 6 days old and when incubated with 50% serum for 10 min (Fig. 3C,D). These preparations were functionally stable for a month at 4°C and gave reproducibly high transfection activity in vivo after i.v. injection in mice [21]. Interestingly, preparations additionally stabilized by pre-condensing the DNA, which show some serum-stable map-pin structures (Fig. 3D), exhibit even higher gene expression in mice lungs (approx. 200%) than PEG-PE stabilized complexes (approx. 60%) which are not showing any protrusions in serum (Fig. 3C).

When incubated with cell medium (RPMI-1640 containing 10% FCS), aggregates of about 12 spherical particles (fairly tightly packed) are observed with 6 day old preparations whether Chol-stabilized (Fig. 3E) or additionally stabilized by either method described above (not shown). The transfection activity of all stabilized preparations in vitro with SK-BR-3 cells when kept in the same medium is quite low and less than 20% of the activity of the same complexes containing DOPE instead of Chol. This discrepancy between high transfection activity in vivo but low in vitro for similarly composed preparations is indicative again of the different parameters involved in the transfection process of cells under in vivo or in vitro conditions. Parameters such as clearance rate of the CLDC, their removal by the RES system, and their reactivity with cells of the endothelial barrier [48] are certainly essential for in vivo applications but are irrelevant for in vitro transfection [37–40]. Our results also show clearly that screening for in vivo optimization of stabilized CLDC must be done with in vivo assays, because in vitro assays can introduce irrelevant and ineffective parameters [25].

It appears from our studies that small, stabilized, spherical particles (100–300 nm), forming only small aggregates of a few particles in serum, even after storage in buffer at 4°C for 6 days, correlate with the high luciferase expression observed in mouse lungs. Although much smaller, these stabilized particles might have some similarity to recently described heterogeneous particles in the shape of flat, concentric, bent or amorphous stacks of bilayers in the size range of 200–500 nm [32] or birefringent liquid-crystalline condensed globules with sizes in the order of 1000 nm [33]. Freshly prepared and investigated in buffer at low ionic strength, these structures appear as particles/globules with a short-range lamellar order in which 2D layers of oriented DNA are sandwiched between lipid bilayers being apart from each other with an interlayer spacing of 6.5 nm [32,33]. However, in both publications no activity was reported for the heterogeneous, mainly quite big, oligolamellar particles. In contrast, a very recent publication has reported the enhanced expression of chloramphenicol acetyltransferase gene in most tissues examined using a homogeneous population of DNA-liposome complexes between 200 and 450 nm in size, whereby the liposomes contributing to the complexes showed an invaginated morphology [26]. Whether or not invaginated liposomes or a regular fine structure of 2D DNA layers sandwiched between lipid bilayers are also structural components of the much smaller, stabilized, spherical particles observed by us in serum, and whether these DNA-lipid arrangements have some advantage for in vivo gene expression are still open questions. Hexagonal or cubic [46] non-bilayer DNA/lipid structures, formed in samples containing DOPE instead of Chol as helper lipid, can transfect very well in vitro. Therefore, their lack of in vivo transfection activity is due either to serum instability or a high clearance rate.

Our investigations on DDAB/Chol/DNA complexes show clearly that their in vivo transfection activity is primarily related to small complexes, stabilized with respect to serum and time. Similar results have also been obtained with DOTAP/Chol/DNA complexes (unpublished results). Moreover, we consider that the small protrusions as observed in freshly prepared non-stabilized CLDC, and the well-developed protrusions observed in stabilized complexes including the map-pin structures revealed by pre-condensing DNA, may contribute to their high in vivo transfection activity. Based on these observations we believe that the stabilization of formulation and morphology of the CLDC in biological fluids is at least equally important for high transfection activity in vivo or in vitro as compared to the specific chemical nature of cationic amphiphiles.

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References

[1] W.F. Anderson, Science 256 (1992) 808–813.
[2] W.F. Anderson, Sci. Am. 273 (1995) 124–128.
[3] F.D. Ledley, Expert Opin. Invest. Drugs 3 (1994) 913–921.
[4] F.D. Ledley, Hum. Gene Ther. 6 (1995) 1129–1144.
[5] R.G. Crystal, Science 270 (1995) 404–410.
[6] M. Blaese, T. Blankenstein, M. Brenner, O. Cohen-Haguenauer, B. Gansbacher, S. Russel, B. Sorrentino, T. Velu, Cancer Gene Ther. 2 (1995) 291–297.
[7] X. Gao, L. Huang, Gene Ther. 2 (1995) 710–722.
[8] P.L. Felgner, Y.J. Tsai, L. Sukhu, M. Manthrope, J. Marshall et al., Ann. NY Acad. Sci. 772 (1995) 126–139.
[9] D.D. Lasic, N.S. Templeton, Adv. Drug Deliv. Rev. 20 (1996) 221–266.
[10] Y. Yang, F.A. Nunes, K. Berencsi, E.E. Furth, E. Gonczol, J.M. Wilson, Proc. Natl. Acad. Sci. USA 91 (1994) 4407–4411.
[11] M.R. Knowles, K.W. Hohneker, Z. Zhou, J.C. Olsen, T.L. Noah, P.C. Hu, M.W. Leigh, J.F. Engelhardt, L.J. Edwards, K.R. Jones, R. Boucher, New Engl. J. Med. 333 (1995) 823–831.
[12] M.J. Stewart, G.E. Plautz, L. Del Buono, Z.Y. Yang, L. Xu, X. Gao, L. Huang, E.G. Nabel, G.J. Nabel, Hum. Gene Ther. 3 (1992) 267–275.
[13] J.-P. Loeffler, J.-P. Behr, Methods Enzymol. 217 (1993) 599–618.
[14] J.H. Felgner, R. Kumar, C.N. Sridar, C.J. Wheeler, Y.J. Tsai, R. Border, P. Ramsay, M. Martin, P.L. Felgner, J. Biol. Chem. 269 (1994) 2550–2561.
[15] J.P. Schofield, C.T. Caskey, Br. Med. Bull. 51 (1995) 56–71.
[16] G.E. Plautz, Z.Y. Yang, B. Wu, X. Gao, L. Huang, G.J. Nabel, Proc. Natl. Acad. Sci. USA 90 (1993) 4645–4649.

[17] Y. Liu, D. Liggitt, W. Zhong, G. Tu, K. Gaensler, R.J. Debs, J. Biol. Chem. 270 (1995) 24864–24870.
[18] R. Stribling, E.B. Brunette, D.L. Liggitt, K. Gaensler, R.J. Debs, Proc. Natl. Acad. Sci. USA 89 (1992) 11277–11281.
[19] I. Solodin, C.S. Brown, M.S. Bruno, C.-Y. Chow, E.-H. Jang, R.J. Debs, T.D. Heath, Biochemistry 34 (1995) 13537–13544.
[20] D.J. Stephan, Z.-Y. Yang, R.D. Simari, H. San, C.J. Wheeler, P.L. Felgner, D. Gordon, G.J. Nabel, E.G. Nabel, Hum. Gene Ther. 7 (1996) 1803–1813.
[21] K. Hong, W. Zheng, A. Baker, D. Papahadjopoulos, FEBS Lett. 400 (1997) 233–237.
[22] N.J. Caplen, E.W.F.W. Alton, P.G. Middleton, J.R. Dorin, B.J. Stevenson, X. Gao, S.R. Durham, P.K. Jeffery, M.E. Hodson, C. Coutelle, L. Huang, D.J. Porteous, R. Willjamson, D.M. Geddes, Nat. Med. 1 (1995) 39–46.
[23] Y. Liu, L.C. Mounkes, H.D. Liggitt, C.S. Brown, I. Solodin, T.D. Heath, R.J. Debs, Nat. Biotechnol. 15 (1997) 167–173.
[24] J. Hartikka, M. Sawdey, F. Cornefert-Jensen, M. Margalith, K. Barnhart, M. Nolasco, J. Norman, M. Manthorpe, Hum. Gene Ther. 7 (1996) 1205–1217.
[25] P.L. Felgner, Hum. Gene Ther. 7 (1996) 1791–1793.
[26] N.S. Templeton, D.D. Lasic, P.M. Frederik, H. Strey, D.D. Roberts, G.N. Pavlakis, Nat. Biotechnol. 15 (1997) 647–652.
[27] H. Gershon, R. Ghirlando, S.B. Guttman, A. Minsky, Biochemistry 32 (1993) 7143–7151.
[28] B. Sternberg, F.L. Sorgi, L. Huang, FEBS Lett. 356 (1994) 361–366.
[29] J. Gustafsson, G. Arvidson, G. Karlsson, M. Almgreen, Biochim. Biophys. Acta 1235 (1995) 305–312.
[30] P.L. Felgner, Y.J. Tsai, J.H. Felgner, in: D.D. Lasic, Y. Barenholz (Eds.), Handbook of Nonmedical Applications of Liposomes. Vol. IV. From Gene Delivery and Diagnostics to Ecology, CRC Press, Boca Raton, FL, 1996, pp. 43–56.
[31] B. Sternberg, J. Liposome Res. 6 (1996) 515–533.
[32] D.D. Lasic, H. Strey, M.C.A. Stuart, R. Podgornik, P.M. Frederik, J. Am. Chem. Soc. 119 (1997) 832–833.
[33] J.O. Rädler, I. Koltover, T. Salditt, C.R. Safinya, Science 275 (1997) 810–814.
[34] T. Boukhnikachvili, O. Aguerre-Chariol, M. Airiau, S. Lesieur, M. Ollivon, J. Vacus, FEBS Lett. 409 (1997) 188–194.
[35] D.D. Dunlap, A. Maggi, M.R. Soria, L. Monaco, Nucleic Acids Res. 25 (1997) 3095–3101.
[36] B. Sternberg, in: D.D. Lasic, Y. Barenholz (Eds.), Handbook of Nonmedical Applications of Liposomes. Vol. IV. From Gene Delivery and Diagnostics to Ecology, CRC Press, Boca Raton, FL, 1996, pp. 271–297.
[37] F. Labat-Moleur, A.-M. Steffan, C. Brisson, H. Perron, O. Feugeas, P. Furstenberger, F. Oberling, E. Brambilla, J.-P. Behr, Gene Ther. 3 (1996) 1010–1017.
[38] C. Kawaura, A. Noguchi, T. Furuno, M. Nakanishi, FEBS Lett. 421 (1998) 69–72.
[39] J. Zabner, A.J. Fasbender, T. Moninger, K.A. Poellinger, M.J. Welsh, J. Biol. Chem. 270 (1995) 18997–19007.
[40] F.C. Szoka Jr., Y. Xu, O. Zelphati, J. Liposome Res. 6 (1996) 567–589.
[41] B. Sternberg, in: G. Gregoriadis (Ed.), Liposome Technology, 2nd edn., CRC Press, Boca Raton, FL, 1992, pp. 363–383.
[42] S.W. Hui, M. Langner, Y.-L. Zhao, P. Ross, E. Hurley, K. Chan, Biophys. J. 71 (1996) 590–599.
[43] J.M. Seddon, Biochim. Biophys. Acta 1031 (1990) 1–69.
[44] T.M. Allen, K. Hong, D. Papahadjopoulos, Biochemistry 29 (1990) 2976–2985.
[45] I. Jääskeläinen, B. Sternberg, J. Mökkönen, A. Urtti, Int. J. Pharm. (1998) in press.
[46] K.W.C. Mok, P.R. Cullis, Biophys. J. 73 (1997) 2534–2545.
[47] R. Podgornik, D.D. Lasic, D. Papahadjopoulos, in: A.V. Kabanow, L.W. Seymour, P. Felgner (Eds.), Self-assembling Complexes for Gene Delivery: from Chemistry to Clinical Trial, J. Wiley and Sons, New York, 1997, in press.
[48] J.W. McLean, E.A. Fox, P. Baluk, P.B. Bolton, A. Haskell, R. Pearlman, G. Thurston, E.Y. Umemoto, D.M. McDonald, Am. J. Physiol. 273 (1997) H387–H404.