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Rapid report

Efficient in vivo gene delivery by the negatively charged complexes of cationic liposomes and plasmid DNA

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Abstract

We examined changes in zeta potential (the surface charge density, ζ) of the complexes of liposome (nmol)/DNA (μg) (L/D) formed in water at three different ratios (L/D = 1, 10 and 20) by changing the ionic strength or pH to find an optimum formulation for in vivo gene delivery. At high DNA concentrations, ζ of the complexes formed in water at L/D = 10 was significantly lowered by adding NaCl ($\zeta = +8.44 \pm 3.1$ to -27.6 ± 3.5 mV) or increasing pH from 5 ($\zeta = +15.3 \pm 1.0$) to 9 ($\zeta = -22.5 \pm 2.5$ mV). However, the positively charged complexes formed at L/D = 20 ($\zeta = +6.2 \pm 3.5$ mV) became negative as NaCl was added at alkaline pH as observed in medium ($\zeta = -19.7 \pm 9.9$ mV). Thus, the complexes formed in water under the optimum condition were stable and largely negatively charged at L/D = 1 ($\zeta = -58.1 \pm 3.9$ mV), unstable and slightly positively charged at L/D = 10 ($\zeta = +8.44 \pm 3.7$ mV), and unstable and largely positively charged at L/D = 20 ($\zeta = +24.3 \pm 3.6$ mV). The negatively charged complexes efficiently delivered DNA into both solid and ascitic tumor cells. However, the positively charged complexes were very poor in delivering DNA into solid tumors, yet were efficient in delivering DNA into ascitic tumors grown in the peritoneum regardless of complex size. This slightly lower gene transfer efficiency of the negatively charged complexes can be as efficient as the positively charged ones when an injection is repeated (at least two injections), which is the most common case for therapy regimes. The results indicate that optimum in vivo lipofection may depend on the site of tumor growth. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: In situ lipofection; Liposome/DNA complex; Surface charge; Particle size

Cationic liposome-mediated transfection (lipofection) has been used as an efficient method for direct gene transfer both in vitro and in vivo, an alternative to viral infection [1]. Cationic liposomes are made of both cationic lipids and neutral phospholipids, conferring positively charged submicrometer particles, while plasmid DNA is a hydrophilic molecule with a highly negative surface charge due to the presence of phosphate groups on each nucleotide. These two oppositely charged molecules spontaneously form complexes when simply mixed. The structure and function of the liposome/DNA ionic complexes have been extensively studied. At low lipid/DNA ratios, liposomes adhered to DNA as 'beads on a string', while at high ratios the DNA strands either intercalated between the lipid bilayers or are coated by the liposomes [2–4]. These models did not support the complex structure formed in serum-free medium,

Abbreviations: DC-chol, 3β[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl] cholesterol; MOT, murine ovarian teratocarcinoma; CAT, chloramphenicol acetyltransferase; L/D, liposome (nmol)/DNA (μg); DMEM, Dulbecco's modification of Eagle's medium

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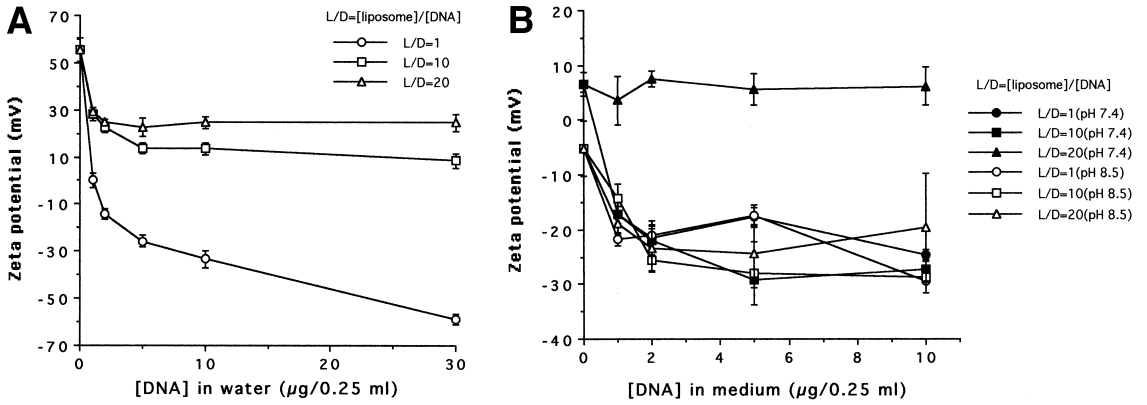

Fig. 1. ζ of liposome/DNA complexes formed in water (A) and in serum-free medium (B) at three different ratios. Plasmid CAT DNA (1–30 μg) was mixed with DC-chol liposome (10–600 nmol) in either water or medium to give L/D = 1, 10 and 20 and subject to ζ measurement. The pH of water was 7.50 ± 0.25, the pH of fresh medium was 7.43 ± 0.19, while the pH of old medium (stored in a refrigerator for at least 4–5 days) was 8.47 ± 0.17.

being all largely negatively charged ($\zeta = -22.7 \pm 3.6$ mV) at all liposome/DNA ratios as we previously reported [5]. This discrepancy may be due to the solvent system that was used to prepare the complexes. In this paper, we report the surface charge density (zeta potential, ζ) and size (the hydrodynamic diameter, d_H) of the liposome/DNA complexes formed in water (very low ionic strength) and their relation to in vivo gene transfer efficiency using laser light scattering (ZetaPal, Brookhaven, New York) to measure both ζ and d_H. The parameters used to measure ζ were: zeta potential model = Smoluchowski, field frequency = 2.00 Hz, voltage = 3.00 V. The other parameters, temperature = 25°C unless specified, viscosity = 0.0890 cP, reflex index = 1.330, angle = 90°, and wavelength = 676 nm, were used for measuring both ζ and d_H.

The liposome/DNA complexes for in vitro transfection were prepared with a small quantity of DNA (1–5 μg) in serum-free DMEM (calculated ionic strength = 0.17) [6]. On the other hand, the complexes for in vivo transfection were prepared with a large quantity of DNA (30–200 μg) in water (ionic strength < 0.002) which has been used to treat ovarian and breast tumors grown in mice [7,8]. Liposomes were composed of 3β[*N*-(*N*′,*N*′-dimethylaminoethane)carbamoyl] cholesterol (DC-chol) and dioleoylphosphatidyl ethanolamine (DOPE) (3:2 molar ratio) which were combined in chloroform, dried thoroughly, and hydrated with deionized water (pH 7.50 ± 0.25) as previously described [9,10]. To examine the dependence of ζ on DNA concentration, we determined ζ of the complexes formed in low and high ionic strength at one low liposome/DNA ratio (L/D = 1) and two high liposome/DNA ratios (L/D = 10 and 20) at different DNA concentrations (1–30 μg) but keeping the liposome/DNA ratio constant. In water, increasing the DNA concentration from 1 to 30 μg slightly changed ζ of the complexes, being still positive at L/D = 10 ($\zeta = +22.7 \pm 2.1$ to $+8.44 \pm 3.0$) and L/D = 20 ($\zeta = +29.4 \pm 1.7$ to $+24.7 \pm 3.6$ mV), however, significantly decreased at L/D = 1 from 0 ± 3.0 to -58.8 ± 2.1 mV (Fig. 1A). In medium, increasing the DNA concentration (1–10 μg) slightly changed ζ of the complexes at all ratios (Fig. 1B). We accidentally found that ζ of complexes formed at L/D = 20 decreased with the age of medium. The pH of fresh medium (red color) was $+7.43 \pm 0.19$, while the pH of old medium (pink color) was $+8.47 \pm 0.17$. The complexes formed in fresh medium were slightly positively charged ($\zeta = +3.58 \pm 4.5$ to $+7.56 \pm 1.4$ mV), while those in old medium were largely negatively charged ($\zeta = -24.3 \pm 4.8$ to -18.9 ± 1.9 mV) (Fig. 1B). However, the pH of the medium did not affect ζ of the complexes formed at L/D = 1 and 10, being all negative ($\zeta = -29.5 \pm 0.2$ to -14.2 ± 2.4 mV) (Fig. 1B). Thus, in vitro and in vivo transfection efficiency can be affected by the pH of the medium.

To determine an optimum formulation for in vivo gene delivery, we examined the effect of the ionic strength and pH on ζ of the DNA/liposome com-

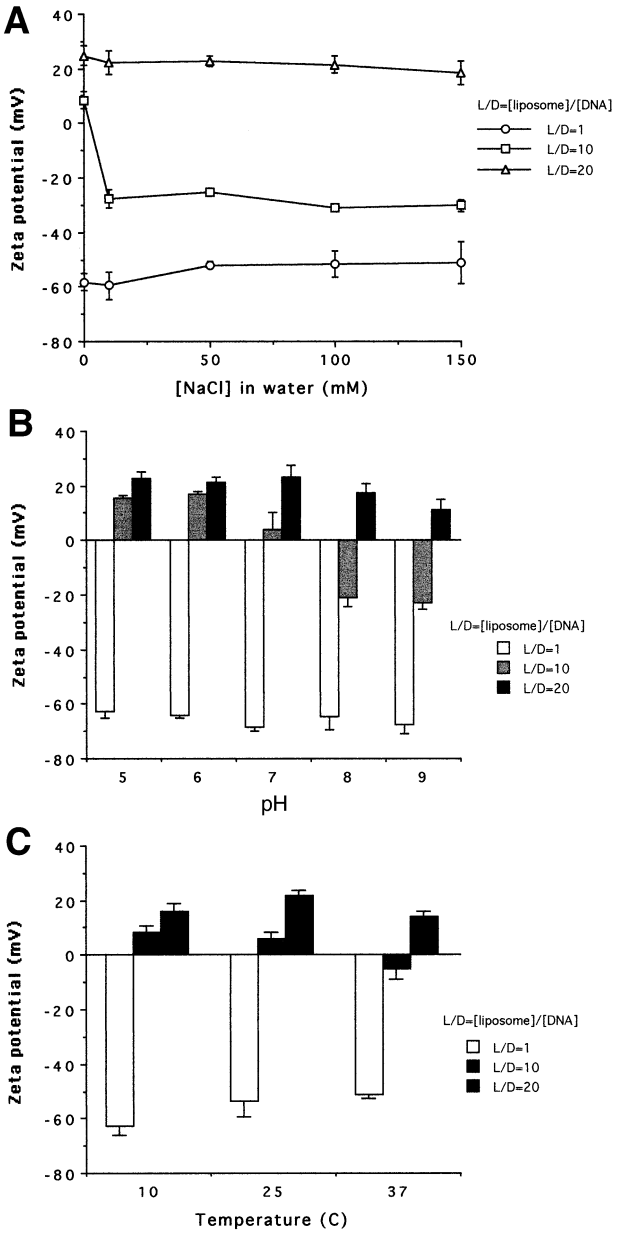

Fig. 2. The effect of ζ of liposome/DNA complexes on NaCl concentration, pH and temperature. 30 μg of CAT plasmid DNA was mixed with 30–600 nmol of liposomes in water to give L/D = 1, 10 and 20, at different NaCl concentrations (A), pHs (B), or temperatures (C) and subject to ζ measurement.

plexes formed in water. The addition of NaCl in a formulation did not significantly change the charge of the complexes formed at L/D = 1 ($\zeta = -58.2 \pm 3.0$ mV at [NaCl] = 0, $\zeta = -51.3 \pm 7.7$ mV at [NaCl] = 150 mM) or at L/D = 20 ($\zeta = +18.5 \pm 4.2$ mV at [NaCl] = 0, $\zeta = +24.7 \pm 3.6$ mV at [NaCl] = 150 mM) (Fig. 2A). However, the charge of the complexes formed at L/D = 10 dramatically decreased in the presence of NaCl ($\zeta = +8.44 \pm 3.1$ mV at [NaCl] = 0, $\zeta = -27.6 \pm 3.5$ mV at [NaCl] = 10 mM or higher) (Fig. 2A). Likewise, an increase in pH of a formulation slightly changed the charge of the complexes formed at L/D = 1 ($\zeta = -62.9 \pm 2.5$ mV at pH 5 to -68.7 ± 1.5 mV at pH 9) or at L/D = 20 ($\zeta = +22.5 \pm 2.7$ mV at pH 5 to 11.1 ± 3.9 mV at pH 9) (Fig. 2B). These positively charged complexes formed at L/D = 20 became negative as NaCl was added at alkaline pH, as observed in Fig. 1B ($\zeta = -19.7 \pm 9.9$ mV). A dramatic decrease in the charge of the complexes formed at L/D = 10 was also observed as the pH increased ($\zeta = +15.3 \pm 1.0$ mV at pH 5 to -22.9 ± 2.5 mV at pH 9) (Fig. 2B). We also examined the effect of temperature on ζ. This was because the formulation to be injected was prepared at room temperature but in vivo gene transfer occurred at body temperature. Unexpectedly, temperature did not seem to affect the charges of the complexes formed at any ratio, although the complexes at L/D = 10 were slightly negative at 37°C (Fig. 2C). Thus, one should be aware of a decrease in complex charges by increasing the ionic strength and/or pH of a formulation, when high L/D is chosen for injection.

To examine the relationship between two physical parameters and in vivo gene transfer efficiency, we performed in situ lipofection in two different types of tumors, solid tumors grown subcutaneously and ascitic tumors grown in the peritoneum of mice. The complexes formed under the optimum condition (no NaCl at neutral pH) with 30 μg DNA and 30 nmol of liposomes were stable and largely negatively charged at L/D = 1, unstable and slightly positively charged at L/D = 10, and unstable and largely positively charged at L/D = 20 (Fig. 1A and Table 1). The complexes formed at L/D = 10 and 20 were largely aggregated and precipitated with incubation due to flocculation (Table 1). The negatively charged complexes at L/D = 1 remained unchanged in size for at least 1 week stored in a refrigerator (data not shown). Surprisingly, their in situ lipofection activities were dependent on the type of tumor (or the site of tumor growth). For ascitic tumors, 6–7-week-old female C3HeFeB/J mice (Jax Lab., Bar Harbor, ME) were injected i.p. with 0.2 ml ascites (2×10^6 cells) of murine ovarian carcinoma (MOT) (Dr. F.M. Sirot-

Table 1
ζ, d_H and in situ lipofection activities of liposome/DNA complexes formed in water

	L/D = 1	L/D = 10	L/D = 20
Final concentration			
DNA (μg/μl)	0.167	0.167	0.167
Liposomes (nmol/μl)	0.167	1.67	3.33
ζ (mV)[a]	−58.1 ± 2.1	+8.44 ± 3.7	+24.3 ± 3.6
CAT activity (% conversion)[b]			
SKBR3 solid tumors (intratumor injection)	24.7 ± 4.4 (11.9 ± 5.1)[c]	2.6 ± 3.3 (3.1 ± 2.7)[c]	5.1 ± 6.2 (4.3 ± 5.5)[c]
MOT solid tumors (intratumor injection)	10.9 ± 3.1	0.5 ± 0.9	0.6 ± 0.4
MOT ascitic tumors (single i.p. injection)	44.2 ± 6.2	59.9 ± 7.3	55.1 ± 4.6
MOT ascitic tumors (multiple i.p. injection)	62.5 ± 3.7	63.3 ± 4.4	60.8 ± 5.3
d_H (nm)[a]	218 ± 4.7	408 ± 23	418 ± 19
Physical stability[d]	stable	aggregation[e] precipitation	aggregation[e] precipitation

30 μg of CAT DNA for solid tumors or 200 μg of CAT DNA for ascitic tumors was mixed with 30–4000 nmol of DC-chol liposomes to give L/D ratios of 1, 10 and 20 in water. The complexes were injected directly into the tumors implanted s.c. or i.p. and grown in mice for CAT gene expression following cisplatin injection or subjected to ζ and d_H measurements.
[a]No NaCl added at 25°C.
[b]Acetylated forms divided by acetylated plus non-acetylated forms after separation on TLC and quantification by Phosphorimager.
[c]CAT activity of SKBR3 solid tumors in the absence of cisplatin treatment.
[d]Changes in physical appearance of a formulation.
[e]The complexes were injected immediately after mixing to minimize aggregation or precipitation.

nak, Memorial Sloan Kettering, New York) using a 22-gauge needle as described [7]. After 4 days, cisplatin (7 mg/kg) was injected i.p. into mice. For solid tumors, MOT ascites or SKBR3 human breast carcinoma (ATCC) (8×10^6 cells/0.1 ml) grown in RPMI 1640 supplemented with 10% fetal bovine serum and penicillin and streptomycin were injected s.c. to 6–7-week-old female athymic nude mice (Harlan Sprague Dawley, Indianapolis, IN) using a 25-gauge needle as described [6]. When the tumor grew to 8–10 nm, mice were injected i.p. with cisplatin (5 mg/kg). One week after cisplatin injection, liposome/DNA complexes were directly injected into the tumors grown in mice. 200 μg of DNA for ascitic tumors or 30 μg of DNA for solid tumors was mixed with an appropriate amount of DC-chol/DOPE liposomes to give L/D ratios of 1, 10 and 20. After 2 days, the animals were sacrificed, the ascitic fluids or s.c. tumors were taken and processed for the chloramphenicol acetyltransferase (CAT) assay using [^{14}C]chloramphenicol as a substrate as described previously [11].

SKBR3 solid tumors were very poorly transfectable with the positively charged complexes formed at L/D = 10 and 20 but significantly transfected with the negatively charged ones at L/D = 1: the CAT activity of SKBR3 tumors without and with cisplatin pretreatment was 11.9 ± 5.1% and 24.7 ± 4.4%, respectively (Table 1). MOT tumors were very poorly transfectable with the complexes formed at any ratio without pre-injection of cisplatin [7]. Cisplatin-sensitized MOT s.c. tumors were significantly transfectable with the complexes formed at L/D = 1 only (CAT activity = 10.9 ± 3.1%) (Table 1). In contrast, cisplatin-sensitized MOT ascitic tumors were significantly transfectable with the complexes formed at all three ratios where higher gene transfer efficiency was observed with the positively charged ones (L/D = 10 and 20) (Table 1). This slightly lower gene transfer efficiency of the negatively charged complexes can be as efficient as the positively charged ones when the injection is repeated (at least two injections), which is the most common case for therapy regimes. These results indicate that the negatively charged complex also efficiently delivered DNA to both solid and ascitic tumor cells. This is a very important finding

because it is known that the positively charged complexes deliver DNA more efficiently inside the cells, which is not the case for solid tumors. Therefore, it seems reasonable for one to inject the complexes formed at any ratio between 1 and 20 to treat ascitic tumors, regardless of the complex charge, size or stability, taking into consideration, however, that at higher ratios the complexes are unstable and more toxic. Further, MOT solid tumors were approximately two-fold less transfectable than MOT ascitic tumors (Table 1). This is because (i) the solid tumor microenvironment is different from ascitic tumors [12,13] and (ii) cisplatin injected i.p. which was used to facilitate gene transfer is in direct contact with ascitic tumor cells [14]. Our studies suggest that the development of a new formulation or the synthesis of new lipids or non-lipids for improving gene transfer efficiency to solid tumors should be different from ascitic-like tumors.

In conclusion, the cationic liposomes and DNA complexes formed at a low liposome/DNA ratio (L/D = 1) in water were stable and negatively charged, which efficiently delivered DNA to both solid and ascitic tumors. In spite of a number of reports on the dependence of efficient in vitro and in vivo gene delivery on positive charges of transfection complexes, our data showed that the positively charged complexes were very poor in delivering DNA into solid s.c. tumors, although they were somewhat more efficient than the negatively charged ones to deliver DNA into ascitic tumors grown in the peritoneum regardless of complex size or stability.

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