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Rapid report

Cationic liposome and plasmid DNA complexes formed in serum-free medium under optimum transfection condition are negatively charged

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Abstract

In medium where in vitro transfection is routinely performed, DC-chol liposomes alone were nearly neutral, whereas the DC-chol liposome/DNA complexes were largely negatively charged which changed only slightly at all [liposome]/[DNA] ratios ($\zeta = -27.1$ to -21.8 mV). Three other commercial transfection reagents, Lipofectin®, LipofectAMINE™ 2000, and SuperFect™, were also largely negatively charged when complexed with DNA. The aggregation of liposomes in medium was prevented by the addition of DNA. Incubation of the complexes in medium did not change their size, charge or lipofection activity for 30 min. These results suggest that, in medium, the liposome/DNA complexes were formed at the time of mixing with negative charges.

Keywords: 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 [1]. Cationic liposomes are made of both cationic lipid and neutral phospholipid, conferring positively charged submicron particles, while plasmid DNA is a hydrophilic molecule with a highly negative surface charge due to the presence of phosphate groups on each nucleotide. Thus, cationic liposome spontaneously form complexes with the negatively charged DNA via charged interactions when they are simply mixed. A number of studies have been shown that condensation of DNA into cationic liposome and/or the overall positive charges of the cationic liposome/DNA complex enhance gene delivery [2]. Their mechanism of action, however, remains unknown.

All of the standard in vitro transfection has been routinely performed in serum-free medium that contains a variety of ions and charged molecules. However, no information has been reported on the size and surface charge density (zeta potential) of liposomes or the liposome/DNA complexes formed in medium and their relation to gene transfer efficiency. Here, using a recently developed laser light scattering software ZetaPals having the dual function of submicron particle analyzer and laser electrophoretic mobility analyzer (Brookhaven, NY), we determined the hydrodynamic diameter (d_H) and zeta potential (ζ) of the liposome/DNA complex formed under the optimum transfection condition in serum-free medium [3]. The parameters used to measure d_H were: temperature, 25°C; viscosity, 0.0890 cP; reflex index,

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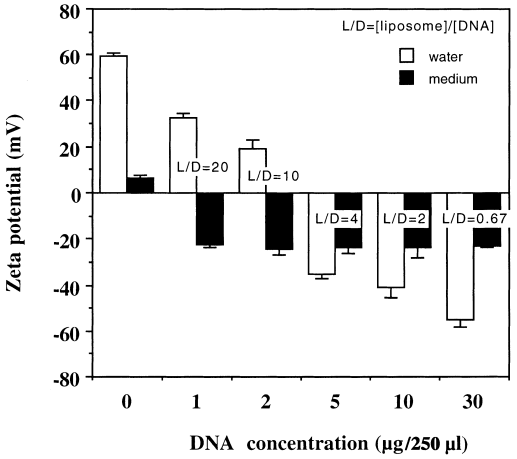

Fig. 1. The ζ of DC-chol liposome/CAT plasmid DNA complexes formed in serum-free medium or water under the optimum condition at constant liposome concentration. An appropriate amount of DNA (1–30 μg) was diluted in 250 μl of medium or water, to which 20 nmol of DC-chol liposome was added for ζ measurement. The diameter of the liposomes was 167–172 nm. All measurements were made in triplicate and expressed as the mean ± S.D.

1.330; angle, 90°; wavelength, 676 nm. The parameters used to measure ζ were: zeta potential model, Smoluchowski; field frequency, 2.00 Hz; voltage, 3.00 V. To form DC-chol liposomes, 3β[*N*-(*N*′*N*′-dimethylaminoethane)carbamoyl] cholesterol (DC-chol) and dioleoyl phosphatidylethanolamine (DOPE) (3:2 mol ratio) were combined in chloroform, dried over nitrogen gas, vacuum dried for 1 h, and hydrated with 1 ml water for 48 h at 4°C [4]. The dispersion was vortexed for 1 min and sonicated for 2–3 min using an ultrasonic water bath. We used deionized water (pH 7.50 ± 0.25) or Dulbecco's mod-

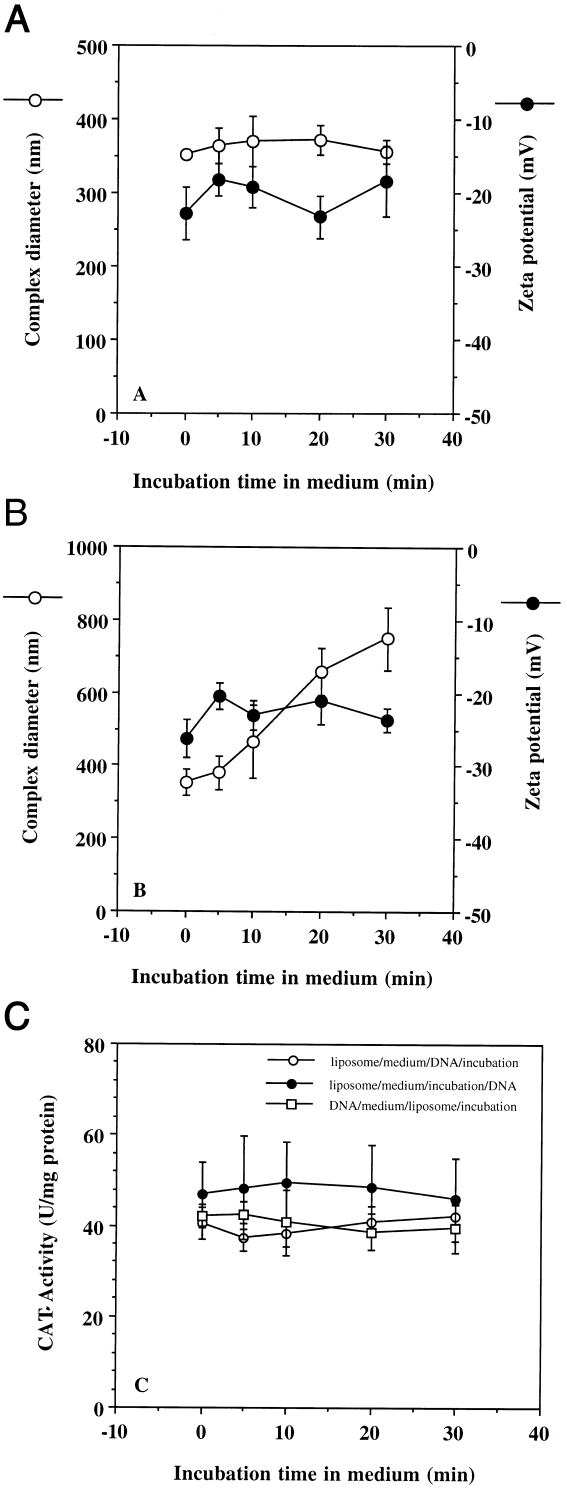

Fig. 2. The d_H, ζ, and transfection efficiency of DC-chol liposome/DNA complexes. (A) Time-course of liposome/DNA complex formation. One microgram of pUCCMVCAT plasmid DNA was diluted in 250 μl of medium and mixed with 10 nmol of liposome. The mixture was incubated at various times and measured for the d_H and ζ. (B) Time course of liposome–medium interaction prior to the complex formation. Ten nanomoles of liposomes were diluted in 250 μl of serum-free medium, incubated for 5, 10, 20 and 30 min before DNA was added, and measured for the d_H and ζ. (C) The lipofection activity of liposome/DNA complex formed in A and B. One microgram of DNA was diluted with 250 μl medium and added to 10 nmol of liposomes (□), 10 nmol of liposomes were diluted with 250 μl medium, without (○) or with (●) incubation and added to 1 μg of DNA. The complexes were incubated at the various time points and added to the cells and transfected as described [6]. All measurements were made in triplicate and expressed as the mean ± S.D.

ification of Eagle's medium (DMEM) (pH 7.27 ± 0.02, the ionic strength calculated from the formulation = 0.17) which was filtered through 0.2 μm membrane. DC-chol liposomes in water ($d_H = 172 \pm 3.1$ nm) were highly positively charged ($\zeta = +60.1 \pm 2.3$ mV); however, those in medium ($d_H = 167 \pm 5.3$ nm) approached to neutral ($\zeta = 6.25 \pm 2.72$ mV) (Fig. 1). In contrast, all the complexes formed between DC-chol liposomes (20 nmol) and the plasmid pUCCMVCAT DNA (1–30 μg) in 250 μl of serum-free medium were similarly negatively charged ($\zeta = -22.7 \pm 3.6$ mV). It was of interest that, in water, as the DNA concentration increased, the charge of the complexes dramatically decreased to negative ($\zeta = -59.3 \pm 3.5$ mV), whose formulation has been used for in vivo transfection [5].

To examine whether the complexes formed under the optimum condition depend on incubation time, 1 μg of DNA was diluted in 250 μl of serum-free medium and mixed with 10 nmol of liposomes. Then, the mixture was incubated at different time points ($t = 0$–30 min) and measured for its diameter and zeta potential. The mixture had $d_H = 353 \pm 5.4$ nm and $\zeta = -22.7 \pm 3.6$ mV at $t = 0$, which did not significantly change for at least 30 min (Fig. 2A). The complexes formed as soon as DNA was mixed with liposomes and remained a fairly consistent size for at least 30 min. We did not observe any changes in the size of liposomes incubated in water; however, liposomes incubated in medium at various time points resulted in remarkable aggregation with no significant change in their surface charges (Fig. 2B). It seemed that counter-ions in medium bound cationic liposomes and neutralized their surface charges, which increased liposome–liposome collisions and tended to stick together by van der Waals attraction. This liposome aggregation was prevented when plasmid DNA was added. This implies the formation of a unique stable structure of complexes. To test if the aggregation may lower the transfection activity, we performed in vitro transfection with the complexes formed in A and B using Chinese hamster ovary (CHO) cells grown in DMEM medium [5]. Plasmid pUCCMVCAT which contains a chloramphenicol-transferase (CAT) gene was purified by Qiagen Plasmid Giga Kit (Valencia, CA) and assayed for its expression [6,7]. Largely aggregated complexes in Fig. 2B ($d_H = 749 \pm 86$ nm) were as efficient as the unaggregated ones in Fig. 2A and an order of mixing of two components did not affect the activity.

Table 1
The d_H (hydrodynamic diameter) and ζ (zeta potential) of four different commercial transfection reagents at the optimum transfection condition in serum-free medium

Transfectant	[Transfectant]	DNA (μg)	d_H (nm)	ζ (mV)
DC-chol	10 nmol/250 μl	0	167 ± 5.3	+6.25 ± 2.72
	20 nmol/250 μl	0	174 ± 6.1	+4.98 ± 3.56
	10 nmol/250 μl	1	353 ± 5.4	−22.7 ± 3.60
	20 nmol/250 μl	1	334 ± 7.2	−22.4 ± 1.10
Lipofectin	2 μg/203 μl	0	308 ± 4.37	−2.57 ± 0.88
	20 μg/221 μl	0	312 ± 6.32	−2.34 ± 1.05
	2 μg/203 μl	1	431 ± 37.9	−20.7 ± 1.02
	20 μg/221 μl	1	1037 ± 258	−25.9 ± 1.44
LipofectAMINE (2000)	2.5 μg/250 μl	0	319 ± 21.4	−3.68 ± 0.73
	7.5 μg/250 μl	0	377 ± 31.3	−3.47 ± 2.56
	2.5 μg/250 μl	2.5	488 ± 54.5	−24.4 ± 2.67
	7.5 μg/250 μl	2.5	639 ± 12.4	−26.1 ± 1.23
SuperFect	60 μl/360 μl[b]	0	ND	ND
	12 μl/312 μl	5	419.0 ± 43.6	−12.3 ± 2.19
Effectene	60 μl/360 μl[b]	0	817.1 ± 4.03	+8.39 ± 4.06
	12 μl (8 μl[a])/312 μl	1	449.0 ± 15.8	+2.71 ± 0.83

Each transfection agent was prepared according to the manufacturer's instructions and then subjected to size and zeta potential measurement. All measurements were made in triplicate and expressed as the mean ± S.D. ND, not determined.

[a] Enhancer.

[b] Control suggested by the manufacturer.

To find if the optimum transfection condition depended on these two parameters, we have measured the diameter and zeta potential of the complexes at different [liposome]/[DNA] ratios (0.5–20) (Fig. 3). The complexes formed at any ratios we tested had $d_H = 322$ to 355 nm and $\zeta = -27.1$ to -21.8 mV. Whether the liposome concentration increased at fixed DNA concentration or DNA concentration increased at fixed liposome concentration, the diameter (d_H) as well as the negative surface charge of the complexes did not change significantly. This implies that the transfection complexes formed in medium at any ratios were negatively charged and thus the optimum transfection could occur at any ratios, depending on the type of cells to be transfected. Therefore, the transfection efficiency of the complexes may be still unpredictable and vary by up to a factor of 100 in different cell lines as reported [8].

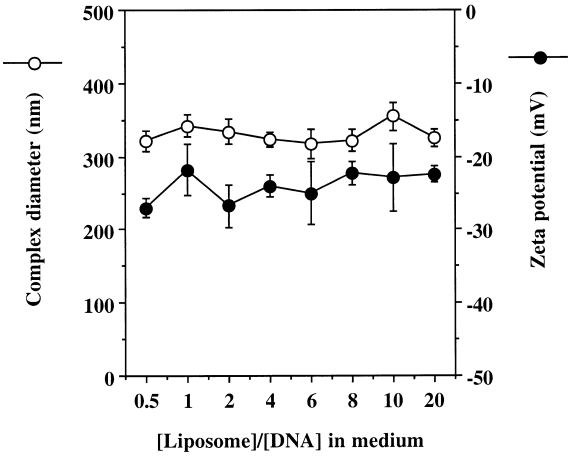

Fig. 3. The d_H and ζ of the DC-chol liposome/DNA complexes at different [liposome]/[DNA] ratios. One microgram of DNA was diluted in 250 μl of medium and mixed with an appropriate amount of liposomes. The complexes were subjected to the d_H and ζ determination. All measurements were made in triplicate and expressed as the mean ± S.D.

We have expanded our observation to four other commercially available transfection reagents at the optimum transfection condition as the manufacturer suggested (Table 1). Lipofectin® (Gibco/BRL, Rockville, MD) alone had $d_H = 308.1 \pm 4.37$ nm and $\zeta = -2.57 \pm 0.88$ mV. In the presence of DNA, its diameter (d_H) increased to 430.8 ± 37.9 nm, while the zeta potential decreased to -20.7 ± 1.02 mV. Likewise, LipofectAMINE™ 2000 (Gibco/BRL, Rockville, MD) had a very similar size and zeta potential to Lipofectin both with and without DNA present. Both Lipofectin and LipofecAMINE 2000 liposomes were approximately 2-fold larger in size than DC-chol liposome in medium (no DNA present). Surprisingly enough, in medium, these liposomes were also nearly neutral, although in water all were largely positively charged. At the optimum transfection conditions, both Lipofectin/DNA and LipofecAMINE 2000/DNA complexes were also negatively charged as DC-chol/DNA complex. All three complexes were 1.5–3-fold larger in size than liposomes alone, however, we have not observed any significant change in zeta potential. As Lipofectin or LipofectAMINE 2000 concentration increased the complex diameter increased to 1037 ± 258 and 639 ± 12.4 nm, respectively, with little change in zeta potential. It suggests that DNA condensation may not be essential for efficient gene transfer.

SuperFect™ (Qiagen, Valencia, CA), activated dendrimers, did not form particles in medium large enough to be detected by our laser light scattering (the detection limit of the lowest size being 2 nm). However, in the presence of DNA it formed the complexes with $d_H = 419 \pm 43.6$ nm and $\zeta = -12.3 \pm 2.19$ mV, which were comparable with those of DC-chol liposome/DNA complex. In contrast, Effectene™ (Qiagen, Valencia, CA), composed of enhancer molecules and micelles of a cationic non-liposomal lipid, had $d_H = 817 \pm 4.03$ nm with $\zeta = +8.39 \pm 4.06$ mV. When DNA present, Effectene formed the complexes with a smaller size ($d_H = 449 \pm 15.8$) and almost neutral zeta potential ($\zeta = +2.71 \pm 0.83$ mV). Effectene/DNA complex was about 2-fold smaller in size than Effectene alone and had a slightly positive charge close to nearly neutral. This may increase the interaction between the complexes and anionic cell membranes for enhanced transfection.

The structure and function of the liposome/DNA ionic complexes have been the subject of many recent studies. The main structural features of the complexes formed between cationic liposomes and plasmid DNA were very similar in several systems of different lipid composition. At low lipid/DNA ratios, the positively charged multilamellar liposomes adhered as an intact bead on the negatively charged DNA ('bead on string'), as observed by electron microscopy (EM) [9,10]. At high ratios, the DNA strands intercalated between the lipid bilayers or

the DNA strands coated by the cationic liposomes was a general structure in all studied systems, as observed by cryo-transmission EM, freeze-fracture EM, or synchrotron X-ray diffraction [11–13]. Templeton et al. demonstrated high level expression of the packaged gene in vivo with a unique structure in which DNA is condensed in the interior of invaginated liposomes between two-lipid bilayers [10]. However, these findings were not based on the studies in serum-free medium. For the first time, our studies of current lipofection formulations demonstrated that the complexes formed in medium, under the optimum transfection conditions, between cationic liposomes and plasmid DNA, were all largely negatively charged. Based on these observations, our working model for the liposome/DNA complexes is, that the negatively charged DNA wraps around liposomes which condense the DNA in a similar manner to that of histones in eukaryotic nuclei, rendering the complexes a net negative charge.

In conclusion, the present formulations for in vitro lipofection were negatively charged, which, nonetheless, efficiently delivered plasma DNA into the cells regardless of the complex size. To find if the positively charged complexes in culture medium indeed deliver plasmid DNA more efficiently, we are actively investigating the factors influencing the zeta potential of the complexes to develop a lipid or non-lipid formulation that may render positive charges to the complexes in culture medium.

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