Surface area of lipid membranes regulates the DNA-binding capacity of cationic liposomes

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We have applied electrophoresis on agarose gels to investigate the DNA-binding capacity of cationic liposomes made of cationic DC-cholesterol and neutral dioleoylphosphatidylethanolamine as a function of membrane charge density and cationic lipid/DNA charge ratio. While each cationic liposome formulation exhibits a distinctive DNA-protection ability, here we show that such a capacity is universally regulated by surface area of lipid membranes available for binding in an aspecific manner. The relevance of DNA protection for gene transfection is also discussed. © *2009 American Institute of Physics*. [DOI: 10.1063/1.3074444]

Over the past few years, cationic liposome (CL)-DNA complexes (lipoplexes) have attracted considerable attention as gene vectors due to their safety and other inherent advantages over viral delivery methods.[1,2] Currently, the most critical obstacle for clinical application of lipoplexes is their unsatisfactorily low transfection efficiency (TE) (a measure of the amount of exogenous DNA transferred into cells followed by gene expression).

A starting point to develop more efficient lipoplexes may be to acquire fundamental knowledge on lipoplex formation.[3,4] Through DNA-lipid condensation, the cationic lipid tends to completely neutralize the phosphate groups on the DNA in effect replacing and releasing the originally condensed counterions in solution.[5–7] Thus, the driving force for lipoplex formation is the entropy gain through the release of counterions, which were one-dimensionally bound to DNA and two-dimensionally bound to cationic membranes, into solution.[6]

The counterion release mechanism, as described above,[7] predicts the formation of only charge-neutral complexes because it implies one-to-one binding of opposite macroion charges. On the other hand, overcharging has been extensively investigated as a function of cationic lipid/DNA charge ratio ρ and overcharged lipoplexes have been observed experimentally. The currently accepted mechanism of lipoplex formation led to conception that the lipoplex is one-phase complex close to the isoelectric point (with one cationic lipid per negatively charged nucleotide base, i.e., $\rho \sim 1$), while separates into complex plus excess liposomes for $\rho > 1$ and complex plus excess DNA for $\rho < 1$.[7]

In this letter, we provide experimental evidence against this statement. Here we have applied electrophoresis on agarose gels to investigate the DNA binding capacity[8,9] of binary CLs made of cationic 3β-[*N*-(*N*,*N*-dimethylaminoethane)-carbamoyl]-cholesterol (DC-Chol) and neutral dioleoylphosphatidylethanolamine (DOPE) as a function of membrane charge density and cationic lipid/DNA charge ratio. This is a point of great general interest, even though only marginally addressed so far, in view of better understanding the mechanism of DNA interacting with CLs.

Lipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and used without further purification. Binary DC-Chol–DOPE CLs were prepared following standard protocols[10] at molar ratios of neutral lipid in the bilayer Φ=(neutral lipid/total lipid) (mol/mol) =0, 0.3, 0.5, 0.7. Liposomes were sonicated to obtain small unilamellar vesicles (SUVs) (mean diameter ~100 nm).[10] DC-Chol–DOPE/DNA lipoplexes were prepared by mixing suitable volumes of SUV liposomes dispersions (1 mg/ml, Tris-HCl buffer solution) with pGL3 plasmid DNA at seven cationic lipid/DNA charge ratios: ρ=cationic lipid/DNA (mol/base)=1, 1.5, 2, 2.5, 3, 3.5, 4. Electrophoresis studies were conducted on 1% agarose gels containing ethidium bromide in tris-borate-ethylenediaminetetraacetic acid (EDTA) buffer as elsewhere described.[9] Lipoplexes with different charge ratios were loaded on agarose gel. The electrophoresis gels were visualized and digitally photographed using a Kodak Image Station, model 2000 R (Kodak, Rochester, NY). Digital photographs (not reported) were elaborated using a dedicated software (KODAK MI, Kodak) that allows to calculate the molar fraction of free plasmid DNA, X_{DNA} (i.e., unprotected by lipids). Thus, the molar fraction of bound plasmid DNA is simply given by $(1-X_{DNA})$.

Formed lipoplexes, due to size exclusion,[11] remain at the site of application, while free DNA migrates toward the cathode (data not reported for space considerations). Figure 1 shows the molar fraction of plasmid DNA protected by lipids, $(1-X_{DNA})$, as a function of ρ. Unexpectedly, at $\rho=1$, $(1-X_{DNA})$ was found to be lower than 1 for all lipoplex formulations. This finding clearly indicates that lipoplexes containing excess cationic charge coexisted with unbound plasmid DNA. For $\rho > 1$, phase coexistence of cationic DC-Chol–DOPE/DNA lipoplexes with free DNA spanned over a relatively large range of ρ ratios with extension increasing with decreasing Φ. Since hypothesizing the coexistence of unprotected plasmid DNA with DNA-free SUV CLs is not convincing,[12] a compelling explanation is that there are not enough lipids to complex all the DNA.[11]

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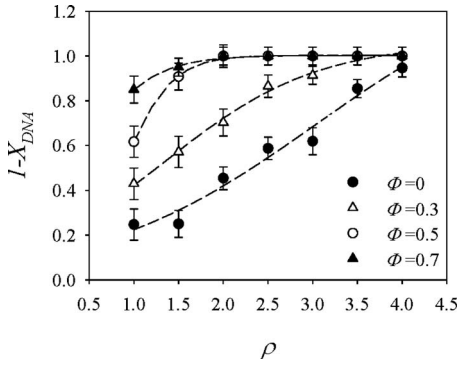

FIG. 1. Molar fraction of plasmid DNA protected by CLs, $(1-X_{\mathrm{DNA}})$, as a function of the cationic lipid/DNA charge ratio ρ. Dashed lines are the best fits to the data.

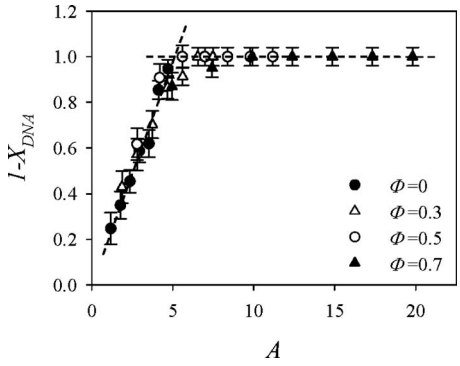

FIG. 2. Molar fraction of plasmid DNA protected by CLs, $(1-X_{\mathrm{DNA}})$, as a function of A, the dimensionless ratio between the area of cationic membranes A_L and that occupied by DNA molecules A_D. Dashed lines are guides to the eye.

These findings are noteworthy since previous x-ray structural studies[7,13] stated that stoichiometrically charge-neutral lipoplexes ($\rho=1$) are one phase systems with all the DNA and the lipids associated within the complex, while lipoplexes can be only found in coexistence with CLs when their preparation takes place above the isoelectric point ($\rho>1$).

To the best of our knowledge, our results are the first electrophoresis findings reported in the literature showing that positively charged lipoplexes can coexist not only with excess lipid, as assumed so far, but also with unbound DNA. Our findings are in good agreement with those previously reported by Pozharski and MacDonald[14] who showed that lipoplexes contain excess cationic charge at the end point of calorimetric titration of DNA into lipid solutions.

Our observations indicate that enriching the system with neutral lipid, by increasing Φ, enables complexation of larger amounts of DNA. Bearing in mind that, at fix ρ, complexes with higher Φ have larger lipid surface area, we put forth the concept that lipid surface available for binding DNA is a major factor in controlling the DNA-binding capacity of lipoplexes.

To test this suggestion, we introduce a dimensionless quantity expressing the ratio between the area of cationic membranes A_L and that occupied by DNA molecules A_D,

$$A = \frac{A_L}{A_D} = \rho \frac{a_C + a_0\left(\frac{\Phi}{1-\Phi}\right)}{2R_D l_D}, \tag{1}$$

where $a_C=40$ Å^2 and $a_0=55$ Å^2 denote the cross-sectional area per cationic (DC-Chol) and neutral (DOPE), R_D is the radius of DNA molecules plus a hydration shell ($R_D=12$ Å), and l_D is the mean distance between two adjacent negative charges projected on the DNA axis ($l_D=1.7$ Å).

Remarkably, given the diversity of lipoplex formulations, the data, spread out when plotted as a function of ρ (Fig. 1), coalesce into a "universal" curve as a function of A (Fig. 2), with the molar fraction of bound DNA monotonously increasing with A until a plateau is reached. Notably, the end points of DNA complexation occurring at different ρ ratios in Fig. 1 converge to a single end point ($A\sim6$) most likely representing full interaction of all DNA with all lipid. According to recent findings by some of us,[15] we suggest that, above the observed end point the system most likely remains one phase over a further range of A. Indeed, lipids are held in bilayers by hydrophobic interactions and a change in supramolecular organization, such as breakage of liposome and release of lipid molecules exceeding those needed to protect all the DNA, are markedly opposed by the hydrophobic effect. Finally, unbound liposomes are expected to be repelled from the cationic lipoplexes, and to coexist as a second phase with the condensed lipoplexes. This hypothesis is currently under analysis.

The most striking observation is that complexes with distinct membrane charge density (Φ) and charge ratio (ρ) but with similar lipid surface area (A) exhibit the same DNA binding capacity.

As a result, the marked differences in bound DNA between lipoplex formulations (Fig. 1) may be explained by a simple consideration that the plasmid-binding capacity of CLs does strictly correlate with lipid surface area available for binding DNA. The primary outcome of such an observation is that the association of CLs and DNA is essentially regulated by size effects (the cationic lipid surface area) more than by electrostatic interactions. This finding is in agreement with recent publications[16,17] and indicates that universal assumptions about the phase diagram of lipoplexes[7] need to be strongly revised.

The absolute requirement of a complete DNA protection may correlate in some way with higher gene expression levels.[8] As such, the consequences for the transfection efficiencies of different DNA binding capacities were also investigated. Figure 3 (panel a) shows the TE of all lipoplex formulations as a function of ρ. As evident, lipoplex formulations do not show any significant trend of TE with ρ. However, when plotted against A (Fig. 3, panel b), transfection data exhibit a clearer behavior. Indeed TE raises with A and

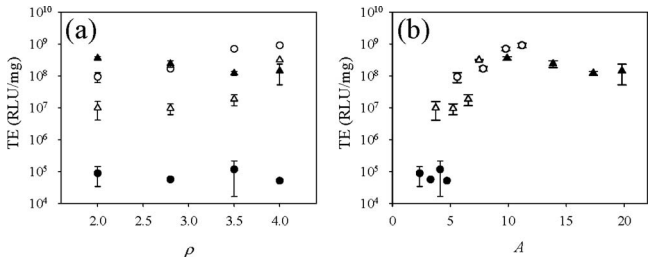

FIG. 3. TE of lipoplex formulations as a function of ρ (panel a) and A (panel b) ($\Phi=0$, black circles; $\Phi=0.3$, white triangles; $\Phi=0.5$, white circles; $\Phi=0.7$, black triangles).

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reaches a clear maximum at $A \sim 8$ ($TE \sim 10^9$ RLU/mg). These results do suggest that maximizing DNA binding ability of lipoplexes (by increasing A) may be an essential requirement for efficient gene transfection. We observe that the value of A required for achieving the highest efficiency ($A \sim 8$) is a little larger than that needed to complex all the DNA ($A \sim 6$, Fig. 2). However, in this narrow range ($6 < A < 8$), lipoplexes are expected to remain one phase,[15] and larger values of A should result in excess cationic charge that may favor lipoplex-cell interactions.[18]

As A further increases, TE decreases monotonically, reaching a likely asymptotic behavior ($TE \sim 10^8$ RLU/mg). Such a diminution in TE may realistically depend on the presence of DNA-free liposomes coexisting with cationic lipoplexes.[18] This coexistence is potentially unfavorable since it is widely known that the use of an excess amount of lipid is detrimental in terms of lipid toxicity to the cells.[18] Indeed, the larger the amount of lipid employed, the higher the metabolic effort for the elimination of the lipids from the cells.

In summary, the present study allowed us to draw some generalities that are as follows: (i) mixing lipid and DNA at an excess cationic charge ($\rho > 1$) does not assure complete DNA protection by lipids, (ii) at a given cationic lipid/DNA molar ratio ρ the DNA-binding capacity of CLs does correlate with the molar fraction of neutral lipid in the lipid bilayer of CLs: the highest Φ, the highest the molar fraction of plasmid DNA protected by lipids, and (iii) even though the mechanisms of lipid-mediated transfection largely remain to be explored, the DNA-binding capacity of CLs does play a key role in transfection. Our methodology can thus be used to predict which formulations will be most effective for transfection purposes. Those for which protection of DNA is full without needing a large excess of cationic lipid.

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