DNA release from cationic liposome/DNA complexes by anionic lipids

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(Received 8 September 2006; accepted 20 October 2006; published online 5 December 2006)

The authors found that recently developed multicomponent cationic liposome DNA complexes (lipoplexes) exhibit higher transfection efficiency with respect to usually employed binary lipoplexes in NIH 3T3 and A17 cell lines. Interaction of lipoplexes with anionic liposomes (model of cellular membranes) was investigated by synchrotron small angle x-ray diffraction. The authors used one-dimensional DNA packing density to estimate the molar fraction of DNA released from lipoplexes by anionic lipids. © *2006 American Institute of Physics*. [DOI: 10.1063/1.2399356]

Cationic liposome DNA complexes, named lipoplexes, currently account for the largest sector of nonviral gene delivery systems that are under clinical trials.[1] As a general rule, cationic liposomes (closed bilayer membrane shells of lipid molecules) are binary mixtures of cationic lipids and neutral "helper" lipids which are able to spontaneously form complexes with negatively charged DNA.[2] Formulations based on the exclusive use of zwitterionic lipids have also been investigated.[3]

From the initial basic view of gene delivery, the issue that has assumed importance is the possible transition from a simple cationic liposome to a complex engineered delivery system. Within this frame, we have recently prepared multicomponent (MC) cationic liposome DNA complexes (MC/DNA lipolexes).[4,5]

In this letter, we show that such multicomponent systems may represent efficient gene vectors. On the basis of our TE and structural results, we claim that structural stability upon interaction with anionic lipids may be identified as a likely reason for why lipoplexes transfect cells with varying efficiency.

Monovalent cationic lipids 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and 3-[*N*-(*N*,*N*-dimethylaminoethane)-carbamoyl]-cholesterol (DC-Chol) and neutral helper lipids dioleoylphosphatidylethanolamine (DOPE) and dioleoylphosphocholine (DOPC) were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and used without further purification.

In the present study, we chose the well characterized DOTAP–DOPC and DC-Chol–DOPE binary liposomes and the MC lipid system incorporating all the four lipid species simultaneously. Multilamellar DOTAP–DOPC and DC-Chol–DOPE binary cationic liposomes were prepared following standard protocols[6] at a molar ratio of neutral lipid in the bilayer ϕ=(neutral lipid/total lipid) (mol/mol)=0.5. Final concentration of lipid dispersions was 1 mg/ml.[6]

TE of the above lipid formulations was investigated by performing transient transfection assay of two cell lines, namely, NIH 3T3 and A17.[7,8]

Thus DOTAP–DOPC/DNA, DC-Chol–DOPE/DNA and MC/DNA, were prepared at the same charge ratio $\rho=L_C/D$ (mol/mol)=3.2, where L_C and D are cationic lipids and DNA bases, respectively. At charge neutrality the numbers of cationic (monovalent) lipids and DNA bases are equal and $\rho=1$. Figure 1 shows TE of the three lipid formulations with each bar representing an average value over at least three independent observations (standard deviation $\leqslant$10%).

In both the cell lines, the TE of the formulations was in the following order: DOTAP–DOPC<DC-Chol–DOPE <MC. These results suggest potential utility of MC/DNA lipoplexes as efficient vector of genes. But how to explain the much higher TE shown by MC/DNA lipoplexes? It has been recently shown that the structure of lipoplexes can be strongly modified, and even destroyed, by interaction with anionic lipids (such as those the cellular membrane is rich in) and that such structural changes may critically affect the TE.[9–11]

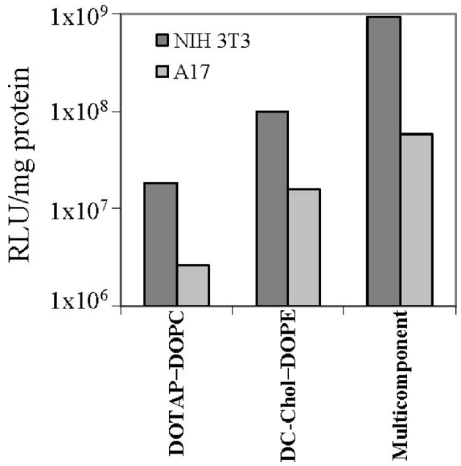

FIG. 1. Transfection efficiency of DOTAP–DOPC/DNA, DC-Chol–DOPE/DNA, and MC/DNA lipoplexes in mouse fibroblast (NIH 3T3, dark gray) and tumoral myofibroblastlike (A17, light grey) cell lines. MC/DNA lipoplexes exhibited higher transfection ability than those of both binary lipoplexes.

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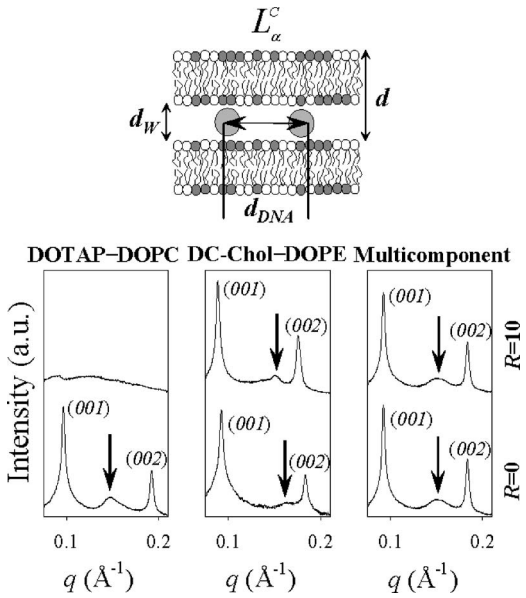

FIG. 2. Synchrotron SAXD patterns of DOTAP–DOPC/DNA, DC-Chol–DOPE/DNA, and MC/DNA lipoplexes as a function of the charge ratio, R =anionic charge/cationic charge. At $R=0$, all lipoplexes were assembled into a multilayer lamellar structure (L_α^C phase). DNA was completely released from DOTAP–DOPC/DNA lipoplexes by anionic lipids ($R=10$) while DC-Chol–DOPE/DNA lipoplexes released a minor part of their gene cargo as shown by the small shift of the DNA peak (marked by arrow). On the other side, MC/DNA lipoplexes definitely resisted solubilization and DNA packing density did not change.

We therefore applied synchrotron small angle x-ray diffraction (SAXD) to study the structural changes of lipoplexes upon interaction with anionic liposomes (models of cellular membranes) prepared from diphosphatidylglycerol (DOPG), an anionic lipid common in mammalian cells, and added to preformed lipoplexes. Lipoplexes/DOPG* mixed dispersions were prepared at different charge ratios R=(anionic charge/cationic charge)=0, 1, 5, and 10, equilibrated for 2 days and lastly filled into glass capillaries. All SAXD measurements were performed at the Austrian SAXS station of the synchrotron light source ELETTRA (Trieste, Italy).[12]

For SAXD experiments calf thymus Na-DNA (Sigma, St. Louis) was used (DNA fragments between 500 and 1000 base pairs) and lipoplexes were prepared by adding appropriate amounts of DNA solution to lipid dispersions, followed by mild vortexing and overnight equilibration. This procedure resulted in the spontaneous formation of visible large aggregates. Here we underline that such an aggregation may cause anionic/cationic charge ratio to be different than its nominal value.

In the absence of anionic lipids ($R=0$), the SAXD patterns of Fig. 2 show that DOTAP–DOPC/DNA, DC-Chol–DOPE/DNA, lipoplexes form highly organized lamellar phases (L_α^C phase[13,14]). The sharp periodically spaced peaks at q_{00n} are caused by alternating lipid-bilayer-DNA-monolayer structure with periodicity $d=2\pi/q_{001}$. The lamellar periodicity along the normal to lipid bilayer, d, is the sum of the lipid bilayer thickness (d_B) and the thickness of the DNA region (dW):$d=d_B+d_w=2\pi/q$. In Fig. 2, the much broader peak (marked by an arrow) is the 'DNA peak' arising from the one-dimensional (1D) in plane lattice with repeat distance $d_{\mathrm{DNA}}=2\pi/q_{\mathrm{DNA}}$.

In excess of anionic charge ($R=10$), the SAXD pattern of the less efficient DOTAP–DOPC/DNA lipoplexes changed remarkably and we could only detect a broad and weak first-order Bragg peak while there was no trace of higher order reflections and of the DNA peak. It means that DOTAP–DOPC/DNA lipoplexes were unstable against solubilization by anionic DOPG and that the most part of delivered DNA was released. DC-Chol–DOPE/DNA lipoplexes were more stable than DOTAP–DOPC/DNA lipoplexes, but a part of the gene freight was released as shown by the enlargement in the DNA distance (Table I). On the other side, the lamellar structure of the most efficient MC/DNA lipoplexes was absolutely not affected by interaction with anionic lipids.

How are these findings related to the mechanism of transfection? Our SAXD experiments suggested possible events occurring at the early stages of internalization of lipoplex inside the cytoplasm.[16,17]

In what follows we will show that DNA packing density can be used to estimate the molar fraction of released DNA as a function of R. In lamellar lipoplexes, the DNA-DNA distance is well described by the following equation.[18]

$$d_{\mathrm{DNA}}=\frac{A_D\rho_L}{d_B\rho_D}(M_{\mathrm{CL}}/M_{\mathrm{DNA}})\frac{1}{(1-\phi_W)}\rho, \tag{1}$$

where A_D is the cross section area of DNA molecule, ρ_D and ρ_L are densities of DNA and lipid, ϕ_W is the neutral lipid/total lipid weight ratio, and M_{CL} and M_{DNA} are the molecular weights of a cationic lipid molecule and of a DNA base. In principle, Eq. (1) should be only valid for charge-neutral complexes ($\rho=1$), because it relies on the basic assumption of complete release of confined Manning counterions.[18] Nevertheless, it can be used in the so-called isoelectric regime. In this scenario, electrostatic repulsions play a key role to adjust

TABLE I. DNA-DNA distances d_{DNA}, actual charge ratios ρ_{eff}, and molar fraction of released DNA X_{DNA} as a function of increasing anionic/cationic charge ratio R. Actual charge ratios were calculated by using Eq. (1). For the calculation the parameters used were $A_D=190$ Å^2; $\rho_D=1.7$ g/cm^3; $\rho_L=1.07$ g/cm^3; $M_{\mathrm{CL}}=698.5$ and 537.7 for DOTAP and DC-Chol, respectively; $M_{\mathrm{DNA}}=324.5$; and $\phi_W=0.52$ and 0.57 for DOTAP and DC-Chol, respectively (corresponding to the same neutral/total lipid molar ratio $\phi=0.5$).

	DOTAP−DOPC/DNA				DC-Chol–DOPE/DNA			
R	d_{DNA} (Å)	ρ_{eff}	X_{DNA}	R^*	d_{DNA} (Å)	ρ_{eff}	X_{DNA}	R^*
0	42.6	1.21	0	0	38.1	1.40	0	0
1	53.2	1.51	0.2	0.25	38.5	1.41	0.01	0.01
2	56.8	1.61	0.25	0.66	39.6	1.45	0.04	0.07
10	N.D.	N.D.	1	N.D.	42.3	1.55	0.1	0.1

DNA packing density in that they set upper and lower limits on the amount of excess material that a complex can accommodate.[18,19] Starting from these "plateau points," the actual composition (effective charge ratio ρ_{eff}) becomes more and more different from the nominal one (ρ).[20] By extrapolating ρ from Eq. (1), we used experimental DNA-DNA distances d_{DNA} to estimate the actual composition of lipoplexes ρ_{eff} as a function of increasing R (Table I),

$$\rho_{\mathrm{eff}}(R) = \frac{d_B \rho_D}{A_D \rho_L}(M_{\mathrm{DNA}}/M_{\mathrm{CL}})(1-\phi_W) d_{\mathrm{DNA}}(R). \tag{2}$$

Changes in DNA packing density (i.e., in DNA-DNA distances d_{DNA} as listed in Table I) may therefore be used to estimate the fraction of released X_{DNA}. It is easy to recognize that X_{DNA} depends on d_{DNA} by the following relationship:

$$X_{\mathrm{DNA}}(R) = 1 - \frac{\rho_{\mathrm{eff}}(R=0)}{\rho_{\mathrm{eff}}(R)} = 1 - \frac{d_{\mathrm{DNA}}(R=0)}{d_{\mathrm{DNA}}(R)}. \tag{3}$$

In the case of DOTAP–DOPC/DNA lipoplexes, DNA was completely released at $R=10$ ($X_{\mathrm{DNA}}=1$), DC-Chol–DOPE/DNA lipoplexes exhibited a much lower X_{DNA} ($X_{\mathrm{DNA}} \sim 0.1$), and MC/DNA lipoplexes did not release DNA ($X_{\mathrm{DNA}}=0$). Equation (3) allows for easy determination of X_{DNA} in the case that anionic lipids do not diffuse into the complex and the enlargement of the DNA-DNA distance can only be ascribed to the DNA release from lipoplexes. On the other side, if anionic lipids laterally diffuse within the lipoplex and form ionic charge pairs with cationic lipids, the amount of lipid into the complex would certainly increase, thus separating the DNA strands. If we suppose that DNA is not released from lipoplexes, the calculated actual composition of lipoplexes ρ_{eff} (R), reads

$$\rho_{\mathrm{eff}}(R) = \frac{L_C + L_A}{D} = \rho_{\mathrm{eff}}(R=0) + \rho_A, \tag{4}$$

where L_C, L_A, and D are the numbers of moles of cationic lipid, anionic lipid, and DNA inside the complex and ρ_A is an "apparent increase" in charge ratio actually due to the increase in lipid content.[17] As a result, we could give a good estimation of the molar fraction of anionic lipid X_{AL}, that diffused into the complex,

$$X_{\mathrm{AL}} = \frac{\rho_A}{\rho_{\mathrm{eff}}(R=0)} = \frac{\rho_{\mathrm{eff}}(R) - \rho_{\mathrm{eff}}(R=0)}{\rho_{\mathrm{eff}}(R=0)} = \left(\frac{d_{\mathrm{DNA}}(R)}{d_{\mathrm{DNA}}(R=0)} - 1 \right), \tag{5}$$

by which the effective anionic/cationic charge ratio $R^* = RX_{\mathrm{AL}}$ was calculated (Table I).

The most probable mechanism of DNA release, the so-called flip-flop mechanism first proposed by Xu and Szoka,[21] is an intermediate link between the two scenarios described here (some anionic lipids diffuse into the complex and a part of DNA is released).

In conclusion we have shown that MC/DNA lipoplexes are extremely promising vectors of gene and that their superiority with respect to usually employed binary lipoplexes is probably due to their structural stability against solubilization by anionic lipids. We have also presented a general formalism for calculating upper and lower limits of the molar fraction of DNA released from lamellar lipoplexes and of the molar fraction of anionic lipid the lipoplex accommodates by using 1D DNA packing density as calculated by synchrotron SAXD patterns. Even though we applied the model to the case of lamellar lipoplexes made of cationic liposomes and linear DNA, we emphasize that the conclusions of our model should not suffer from the kind of DNA (linear or circular). Indeed, one-dimensional DNA packing density we used to estimate the molar fraction of DNA released from lipoplexes by anionic lipids very slightly depends on the kind of DNA employed.[22]

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