Interaction of Lipoplexes with Anionic Lipids Resulting in DNA Release is a Two-Stage Process

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We propose a mechanism for DNA release from lipoplexes in cells that accounts for various observations of lipoplex−anionic lipid interactions. We examined the structural evolution of lipoplexes upon interaction with cellular lipids by synchrotron small-angle X-ray diffraction (SAXD), and the extent of DNA release from lipoplexes was determined by gel electrophoresis. We find that the interaction of lipoplexes with anionic cellular lipids is a two-stage process. In the first step, anionic lipids laterally diffuse into the complex and neutralize the cationic lipids. As a result, the membrane charge density of lipoplexes decreases and interactions between cationic lipids and DNA become weaker, but DNA is extremely poorly released. Only after the cationic charge of lipoplex membranes is completely neutralized by anionic lipids does DNA starts to be released significantly.

Introduction

Complexes of DNA with cationic liposomes have attracted considerable attention recently, essentially because of their use as nonviral DNA delivery systems in gene therapy.[1] A critical obstacle for the clinical application of cationic liposome−DNA complexes (lipoplexes) is their insufficiently low transfection efficiency.

The transfection of cells by lipoplexes is a very complex process with distinct stages. The basic steps of lipid-mediated transfection (lipofection) include adsorption and endocytosis of lipoplexes inside the cell.[2,3] The second crucial process is the release of DNA from the lipoplex into the cytoplasm. About 10 years ago, it was proposed that electrostatic interactions between the cationic lipids of the lipoplex with the anionic phospholipids in the endosomal membrane would facilitate simultaneous dissociation of the DNA from the lipoplex and disruption of the endosomal membrane.[4] The experiments raise a question of fundamental importance to lipofection science: Which is the mechanism of DNA release from lipoplexes by anionic lipids? The current opinion is that lipoplexes interact with a number of cellular membranes, during which DNA may be released gradually only after the lipoplex has acquired enough anionic lipids to neutralize the cationic charge of lipoplexes.[4] Nevertheless, at present, little if anything is known about the exact physical mechanism by which DNA is released.

Here, we report an analytical model describing DNA release from lipoplexes by anionic lipids. We find that the interaction between cationic lipoplexes and anionic lipids is well described by a two-step mechanism. In the first step, anionic lipids neutralize cationic lipids, thereby modifying the membrane charge density of lipoplexes. In this stage, DNA is very poorly released. In the second step, the onset of major DNA unbinding results from complete charge neutralization of cationic membranes.

Materials and Methods

Cationic Liposomes. Cationic lipid 3-β-[*N*-(*N*, *N*-dimethylaminoethane)-carbamoyl]-cholesterol (DC-Chol) and neutral helper lipid dioleoylphosphatidylethanolamine (DOPE) were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. DC-Chol-DOPE cationic liposomes were prepared following standard protocols[5] at a molar ratio of neutral lipid in the bilayer of $\Phi = L_0/(L_C + L_0) = 0.5$, where L_0 and L_C are moles of neutral and cationic lipids, respectively. The final concentration of lipid solutions was 25 mg/mL. Anionic liposomes (AL) were prepared from 1,2-dioleoyl-*sn*-glycero-3-phosphate (DOPA), an anionic lipid that is common in the plasma membrane of mammalian cells.

Lipoplexes. Lipoplexes were prepared by mixing 100 μL of sonicated calf thymus Na-DNA (Sigma, St. Louis, MO) (DNA fragments between 500 and 1000 base pairs) at 5.3 mg/mL with suitable volumes of liposome solutions. DC-Chol-DOPE/DNA lipoplexes were prepared with the following cationic lipid/DNA molar ratio, $\rho = L_C/D =$ (moles of cationic lipid)/(moles of DNA bases) = 3.2 (positively charged lipoplexes).

Lipoplexes/Anionic Liposomes Mixed Systems. Lipoplexes/DOPA mixed dispersions were prepared by mixing solutions of DOPA and preformed lipoplexes at different charge ratios $R = A/L_C$ (moles of anionic lipid/moles of cationic lipid) between $R = 0$ and 5 in increments of 0.05. At present, it is not clear if lipid-mediated transfection involves single or multiple interactions with anionic membranes. If lipid-mediated transfection involved multiple interactions with anionic membranes, the dependence of structural changes of lipoplexes and DNA release on R would be a critical point to be deeply investigated.[6] After storage for 3 h (time scale of transfection experiments) at 4 °C, allowing the samples to reach equilibrium,[5] they were transferred to 1.5-mm-diameter quartz X-ray capillaries (Hilgenberg, Germany). The capillaries were centrifuged at room temperature for 5 min at 6000 rpm to consolidate the sample.

Synchrotron Small-Angle X-ray Diffraction. Small-angle X-ray diffraction (SAXD) was used to characterize the structure of lipoplexes and to study the structural changes of lipoplexes when interacting with anionic liposomes, which are intended to be model systems of biological membranes.[4] All SAXD measurements were performed at the Austrian SAXS station of synchrotron light source ELETTRA (Trieste, Italy).[6] SAXD patterns were recorded with a gas detector based on the delay line principle covering a q range ($q = 4\pi \sin(\theta)/\lambda$) of 0.05−0.6 Å^{-1}. The angular calibration of the detector was performed with silver behenate powder (d spacing = 58.38 Å). The exposure time for each sample at each temperature was 100 s. No evidence of sample degradation due to radiation damage was observed in any of the samples at this exposure. The

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data have been corrected for fluctuation of the primary beam intensity with detector efficiency, and the background has been subtracted.

Electrophoresis Experiments. Electrophoresis studies were conducted on 1% agarose gels containing Tris-borate-EDTA (TBE) buffer. Detailed experimental conditions can be found elsewhere.[6] After electrophoresis, ethidium bromide (Et−Br) was added and then observed. Lipoplexes were prepared by mixing 2 μL of lipid dispersions (20 mg/mL, Tris-HCl buffer) with 4 μg of pGL3 control plasmid. Plasmid preparation was checked by gel electrophoresis. It was found to contain two components: a high-mobility band attributed to the most compact (supercoiled) form and a less-intense one that was considered to be the non-supercoiled content in plasmid preparation. These complexes were allowed to equilibrate for 3 days at 4 °C before adding negatively charged liposomes. After 3 h, naked plasmid DNA, lipoplexes, and lipoplexes/AL systems with different R values were subjected to agarose gel electrophoresis for 1 h. (Unless otherwise specified, all gels were 1% agarose and were run at 5 V/cm at 20 °C). The electrophoresis gel was observed and digitally photographed using a Kodak Image Station (model 2000 R, Kodak, Rochester, NY). Digital photographs were elucidated using dedicated software (Kodak MI, Kodak) that allows us to calculated the molar fraction of released DNA, X_{DNA}.

Results and Discussion

Figure 1 shows the structural evolution of DC-Chol-DOPE/DNA lipoplexes upon interaction with anionic lipids in the $0 < R < 0.5$ range of the anionic/cationic charge ratio. At $R = 0$ (no anionic lipid added), the sharp peaks labeled at q_{00l} arose from the lamellar periodicity along the normal to the lipid bilayer, d, which is the sum of the membrane thickness (d_B) and the thickness of the water/DNA layer (d_W): $d = d_B + d_W = 2\pi/q_{001} = 69.1$ Å (Figure 1, top). The diffuse broader peak (marked by an arrow) resulted from 1D in-plane packing of the intercalated DNA. The DNA strands form a 1D ordered array where the interaxial spacing, $d_{DNA} = 2\pi/q_{DNA}$, decreases with the membrane charge density of lipoplexes, $\sigma_M = e(1 - \Phi)/a$ where e is the electronic charge and a is the average interfacial lipid headgroup area.[7,8] Membrane charge density has recently been identified as a key universal parameter regulating the transfection efficiency of lipoplexes.[9,10]

Although the (00l) peaks move only slightly with R toward smaller q,[6] the much broader and weaker peak arising from the DNA−DNA correlations (vertical arrows) shifts over a wide range corresponding to a change in d_{DNA} from closed packed at 35.1 Å ($R = 0$) to significantly dilute at 53 Å ($R = 0.5$). This finding indicated that the 1D in-plane rod DNA lattice was diluted by anionic lipids (i.e., d_{DNA}, reported in Table 1, increasing with growing R).[11] For $R > 0.5$, the DNA peak was not detected from the X-ray pattern. In principle, this finding may indicate that DNA was so easily released from lipoplexes that DNA−DNA in-plane interactions became too weak to be detected. Alternatively, the increasing amount of anionic lipid may disturb the DNA packing, eventually leading to the complete loss of short-range order in the DNA−DNA correlations.[6]

As a result, we asked whether the observed enlargement in DNA spacing (Table 1) was really due to DNA release from lipoplexes. To address this question, we performed electrophoresis experiments on agarose gels. Electrophoresis experiments can provide direct evidence for DNA release from lipoplexes after interaction with anionic liposomes[6,12] and quantify the molar fraction of DNA released, X_{DNA}. Figure 2 shows the changes in X_{DNA} as a function of R. Electrophoresis seemed to suggest that DNA release may be a two-step process. Indeed, DNA release was extremely low up to $R \approx 0.5$. Conversely, for $R > 0.5$, X_{DNA} started to increase significantly.

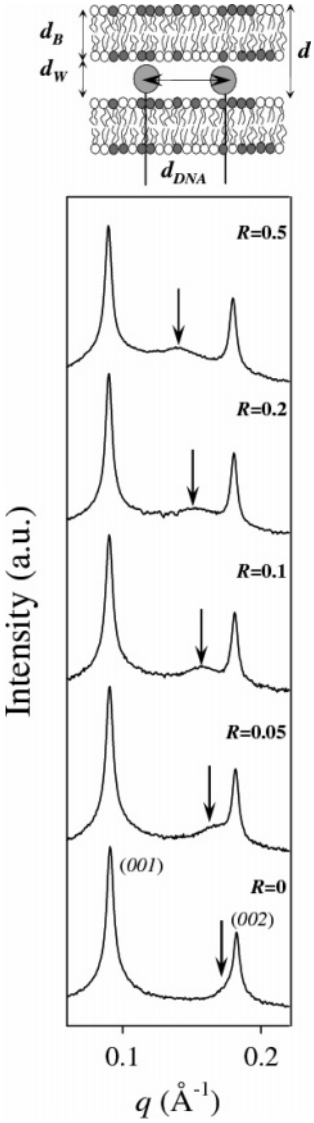

Figure 1. (Top) schematics of the structure of lamellar CL-DNA complexes. The nanostructure is composed of alternating lipid bilayers and DNA monolayers, and the repeat spacing is given by $d = d_W + d_B$. DNA monolayers are ordered in a 1D lattice with a well-defined spacing d_{DNA} between the DNA chains. (Bottom) representative SAXD patterns of mixed DC-Chol-DOPE/DNA/DOPA systems as a function of increasing R. Curves are arbitrarily shifted for clarity. As the mobile DNA peak (marked by an arrow) clearly shows, the 1D DNA in-plane rod lattice is diluted by anionic lipids. For $R > 0.5$, the DNA peak was not seen in the X-ray pattern.

When comparing SAXD and electrophoretic results, we did not find a clear relationship between changes in the 1D DNA packing density and DNA unbinding. We noted that for $0.05 < R < 0.5$ the 1D packing of DNA changed significantly (Table 1) but DNA was almost not released from lipoplexes (Figure 2). Here we address a basic question: why does the 1D DNA packing density change with increasing anionic lipid content? Answering this question means clarifying how and why the unbinding of DNA occurs.

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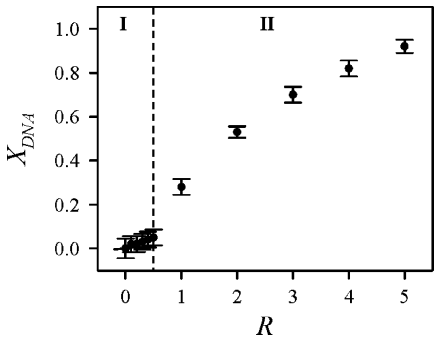

Figure 2. Molar fraction of released DNA, X_{DNA}, measured by electrophoresis as a function of increasing R. DNA unbinding appeared to be a two-stage process. The dashed line indicates two regimes of DNA release labeled I and II. In the first step ($0 < R < 0.5$), DNA is tightly bound to cationic liposomes and is poorly released. In the second step ($R > 0.5$), DNA starts to be released considerably.

Table 1. DNA Interstrand Distance, d_{DNA}, Molar Fraction of Anionic Lipid Diffusing into the Complex, X_{AL}, and Molar Fraction of Neutral Lipid Molecules, Φ', as a Function of the Anionic Lipid/Cationic Lipid Molar Ratio, R

R	d_{DNA} (Å)	X_{AL}	Φ'
0.05	37.2 ± 0.6	0.95 ± 0.10	0.581 ± 0.009
0.10	39.2 ± 0.5	0.91 ± 0.05	0.649 ± 0.008
0.15	41.3 ± 0.4	0.93 ± 0.04	0.722 ± 0.008
0.20	43.4 ± 0.4	0.91 ± 0.03	0.784 ± 0.007
0.25	44.9 ± 0.4	0.87 ± 0.03	0.829 ± 0.007
0.30	47.0 ± 0.4	0.87 ± 0.02	0.879 ± 0.007
0.35	48.8 ± 0.4	0.85 ± 0.02	0.932 ± 0.007
0.40	49.9 ± 0.4	0.81 ± 0.02	0.960 ± 0.007
0.45	51.5 ± 0.3	0.79 ± 0.02	0.997 ± 0.007
0.50	53.0 ± 0.3	0.77 ± 0.01	1.003 ± 0.006

It was found in SAXD experiments[7] that the interaxial distance d_{DNA} is related to charge ratio ρ by

$$d_{DNA} = \frac{a}{2l_B(1-\Phi)}\rho \qquad (1)$$

where l_B is the Bjerrum length. In principle, eq 1 is valid only for isoelectric complexes ($\rho = 1$) because it relies on the basic assumption of complete release of confined Manning counterions from cationic lipids and DNA.[7,8] Nevertheless, it can be successfully used in the so-called isoelectric regime both above ($\rho > 1$) and below ($\rho < 1$) the isoelectric point.[8] In particular, for $\rho > 1$, complexes continue to absorb excess cationic lipid and remain one-phase, and d_{DNA} increases linearly with ρ.[8] Finally, interbilayer repulsions are thought to set an upper limit on the amount of excess lipid that a complex can accommodate.[7,8,13] Starting from this "plateau point", the lipoplex starts to coexist with excess liposomes.[7,8,13] As a result, the actual composition of lipopexes becomes more and more different from the nominal one (in the following, all quantities referring to actual values are denoted by *, and nominal values will be indicated without *).[7,8,11,13]

For $\rho > 1$, all DNA molecules are incorporated within the complex whereas only part of the cationic lipids, $L_C^* \le L_C$, are so incorporated. (Analogously, we shall indicate by L_0^* the moles of neutral lipid incorporated within the lipoplexes.) As a consequence, the plateau charge ratio is given by $\rho_P = L_C^*/D$. The moles of cationic lipids in the complex, L_C^*, are therefore related to L_C by $L_C = L_C^*\rho/\rho_P = \gamma L_C^*$, with γ indicating the deviation of the nominal charge ratio, ρ, from the plateau value, ρ_P. High-resolution synchrotron SAXD measurements carried out on DC-Chol-DOPE/DNA lipoplexes as a function of ρ (Figure 3) allowed us to estimate the plateau charge ratio ($\rho_P \approx 2.4$) and, in turn, the deviation of ρ from ρ_P ($\gamma = \rho/\rho_P = 3.2/2.4 = 1.4$). It is also opportune to specify that the molar ratio of neutral lipid in the bilayer of lipoplexes $\Phi^* = L_0^*/(L_0^* + L_C^*)$ is assumed to be equal to that in the bilayer of cationic liposomes $\Phi = L_0/(L_C + L_0)$ ($\Phi^* = \Phi$).

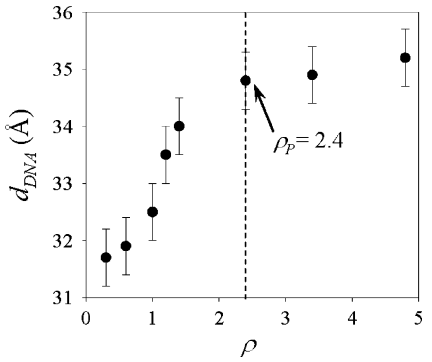

Figure 3. Variation of d_{DNA} in the lamellar DC-Chol-DOPE/DNA complexes as a function of ρ. The dashed line indicates the plateau charge ratio, ρ_P. Starting from ρ_P, the system is biphasic, and lipoplexes begin to coexist with excess lipid.

When adding A moles of anionic lipids ($A = RL_C$) with valence Z_A to preformed lipoplexes, two main effects have to be considered: (i) anionic lipids laterally diffuse into the complex[4,11] and locally neutralize cationic lipids[4] and (ii) DNA is released from lipoplexes. The first process is thought to modulate the membrane charge density of lipoplexes, σ_M, by changing the actual fraction of neutral lipids in the lipid bilayer, Φ^*.[11,14] We indicate by X_{AL} the molar fraction of anionic lipids added to preformed lipoplexes that really enter the complex. We also suppose that anionic lipids that laterally diffuse into the complex ($X_{AL}A$, $0 \le X_{AL} \le 1$) locally neutralize cationic lipids.[4] In particular, each anionic molecule can neutralize Z_A cationic lipids. As a result, the number of molecules that behave as neutral lipids is given by the sum of the number of neutral lipids in the complex, L_0^*, plus the number of neutralized molecules ($X_{AL}A + Z_A X_{AL}A$).

As a consequence, we can calculate the actual fraction of neutral lipid within the complex, $\Phi'(R)$,

$$\Phi'(R) = \frac{L_0^* + X_{AL}A + Z_A X_{AL}A}{L_0^* + L_C^* + X_{AL}A} = \frac{\Phi + X_{AL}R\gamma(1-\Phi)(1+Z_A)}{1 + X_{AL}R\gamma(1-\Phi)} \qquad (2)$$

We also suppose that electrostatic interactions between the cationic lipids of the lipoplex (L_C^*) and the anionic phospholipids that diffuse within it ($X_{AL}A$) result in simultaneous charge neutralization of cationic lipids and weakening of the cationic lipid/DNA interaction. The latter process is likely to induce the release of a fraction of the delivered DNA, D_R ($0 \le D_R \le D$). Both diffusion of anionic lipid and potential DNA release can modify the charge ratio of lipoplexes. As a result, we can immediately calculate the modified charge ratio, $\rho'(R)$, by the following relation

$$\rho'(R) = \frac{L_C^* - Z_A X_{AL}A}{D - D_R} = \rho_P \frac{(1 - Z_A X_{AL} R\gamma)}{(1 - X_{DNA})} \qquad (3)$$

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By combining eqs 1 and 3, obtaining the ratio between $d_{DNA}(R = 0)$ and $d_{DNA}(R)$ and substituting all of the relevant parameters, one can finally calculate the molar fraction of anionic lipids that diffuse within the lipoplex

$$X_{AL}(R) = \frac{\frac{d_{DNA}(R)}{d_{DNA}(R=0)}(1 - X_{DNA}) - 1}{R\gamma(1 - \Phi)} \quad (4)$$

By measuring interaxial DNA−DNA distances $d_{DNA}(R)$ and γ by SAXD and X_{DNA} by electrophoresis, one can therefore calculate the molar fraction of anionic lipid added to preformed lipoplexes that actually enters the complex, X_{AL}. Table 1 shows that X_{AL} decreases with R. This finding indicates that the affinity of anionic DOPA for cationic lipoplexes decreases with R (i.e., with an increasing number of interactions between lipoplexes and anionic membranes).[4,14] In fact, initial molecular exchange between the cationic lipid aggregates and the negatively charged membranes is expected to create cationic/anionic lipid mixtures that lower the electrostatic attraction between cationic lipoplexes and anionic membranes.[4,6,14]

By inserting X_{AL} values into eq 2, we can finally calculate the dependence of both Φ' and $\sigma_M' = e(1 - \Phi')/a$ on the anionic/cationic charge ratio, R. As eqs 2 and 3 clearly show, Φ' and, in turn, σ_M' depend on Z_A. Recent publications[15,16] have shown that the lipid headgroup charge of PA depends on lipid composition and may be increased by the inclusion of zwitterionic lipid species such as DOPE and cholesterol.[15] In particular, the headgroup of PE facilitates the deprotonation of PA in a mixed bilayer. Indeed, the headgroup of PE lies parallel to the membrane surface, and the positively charged amine is uniquely located to interact electrostatically with the phosphate group of the PA, bringing both molecules sufficiently close in space to allow hydrogen bond formation.[15] This intermolecular hydrogen bond can destabilize the intramolecular hydrogen bond within the phosphomonoester headgroup and thus lower the pK_{a2}. Similarly, the presence of a positive charge, as found in cationic lipid DC-Chol, increases the local pH and decreases the apparent pK of the same molecule.[16] Thus, the most realistic assumption is that in mixed DC-Chol-DOPE bilayers[15,16] DOPA releases both protons and acquires two negative charges at the membrane interface ($Z_A = 2$).

Table 1 shows the dependence of Φ' on the anionic/cationic charge ratio, R. To calculate $\sigma_M'(R)$ we used a mean interfacial area of $a = 48.2$ Å^2 according to ref 6 (and references therein). Figure 4 (top panel) shows the change in the charge density of DC-Chol-DOPE membranes, $\sigma_M'(R)$, with R. We noted that the cationic charge of DC-Chol-DOPE membranes monotonously decreased with R and was completely neutralized at $R \approx 0.5$ ($\sigma_M' \approx 0$). This finding means that the amount of DOPA that was needed to neutralize DC-Chol fully was half the amount of cationic lipid. As discussed above, this apparent contradiction disappears when it is recognized that DOPA is a diprotic acid whose physiologically relevant pK_{a2} is close to 7.[15] It is well established that in fluid mixed bilayers individual lipid components are free to move within the plane of the membrane. Thus, at the molecular level, we can assume that charge-neutral triplets made of one DOPA molecule plus two DC-Chol molecules form locally.

By combining the results of SAXD and electrophoresis experiments, the proposed model allowed the physical mechanism of DNA release from lipoplexes by anionic lipids to be clarified. As discussed in the literature,[4,12] it has been recently suggested that DNA may be released only after the lipoplex has acquired enough anionic lipids to counterbalance the cationic charge of lipoplexes. Our model quantifies such "enough anionic charge". Indeed, we showed that DNA starts to escape significantly from lipoplexes (Figure 2) only when the membrane charge density of lipoplexes has been completely neutralized by anionic lipids ($\sigma_M' \approx 0$, Figure 4, top panel). Thus, we show that the complete charge neutralization of cationic lipoplex membranes is the fundamental prerequisite leading to significant DNA release.

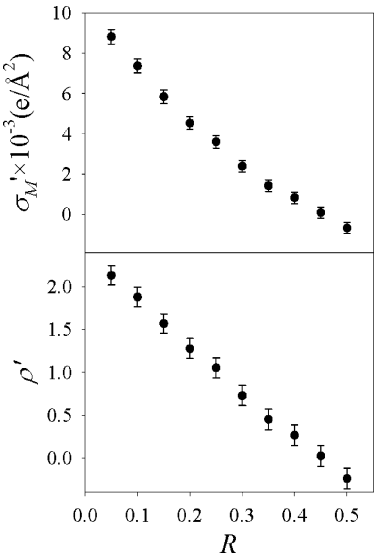

Figure 4. (Top) change in membrane charge density of lipoplexes, $\sigma_M'(R)$, induced by anionic lipids. The cationic charge of DC-Chol-DOPE membranes is completely neutralized at $R \approx 0.5$. (Bottom) change in the effective charge ratio of lipoplexes, ρ', with R.

We have provided evidence that the DNA release from lipoplexes by anionic lipids is a two-stage process. In the first step ($0 < R < 0.5$), anionic lipids penetrate the cationic membranes of lipoplexes and progressively neutralize the positive charge carried by cationic lipids. As a result, the membrane charge density, σ_M', decreases (Figure 4, top panel), but DNA is poorly released (Figure 2). The decrease in 1D DNA packing density (Table 1), experimentally observed by SAXD (Figure 1), was only slightly due to the unbinding of DNA. Conversely, the 1D DNA lattice was essentially diluted because the diffusion of anionic lipids increased the total membrane area.[11] In the second step ($R > 0.5$), when the membrane charge density of lipoplexes was completely neutralized by anionic lipids (Figure 4, top panel), DNA started to escape from lipoplexes appreciably (Figure 2).

The neutralization of the cationic charge of lipoplexes by anionic lipids also resulted in a monotonous decrease in the actual charge ratio $\rho'(R)$ (Figure 4, bottom panel). It has been proposed in the literature[10,12] that the unbinding of DNA from lipoplexes is a critical step along the transfection route. According to these suggestions, our findings suggest that the decrease in the positive charge of lipoplexes (Figure 4, bottom panel) upon interaction with anionic membranes could make further lipoplex−cellular membrane interactions more difficult.

Conclusions

We have clarified the physical mechanism by which DNA is released from lipoplexes by anionic lipids. We have supported the general expectation[4] that anionic lipids are the factors responsible for DNA release. What we have unambiguously shown and what was poorly appreciated before is that the complete

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electrostatic neutralization of cationic membranes by anionic lipids is the essential requirement for the release of DNA.

Given the consistent agreement between our results and the various experimental observations on lipoplex/anionic lipid interactions reported herein, we suggest that the presented model is an appropriate and promising tool for investigating the interactions of other anionic lipids and/or cell-mimicking anionic liposomes with lipoplexes for biological and biomedical applications.

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