Phase diagram of 3β-[N-(*N*,*N*-dimethylaminoethane)-carbamoyl]-cholesterol–dioleoylphosphatidylethanolamine/DNA complexes suggests strategies for efficient lipoplex transfection

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Synchrotron small angle x-ray scattering and electrophoresis on agarose gels have been applied to construct the phase diagram of the ternary complex made up of the cationic lipid 3β-[N-(*N*,*N*-dimethylaminoethane)-carbamoyl]-cholesterol, the neutral lipid dioleoylphosphatidylethanolamine and DNA. We show that nominally charge-neutral complexes coexist with free DNA, while excess cationic charge is necessary to protect all the genetic cargo. Such an extra-charge requirement diminishes as the molar fraction of neutral lipid in the bilayer increases. Furthermore, complexes with very different membrane composition and charge ratio exhibit the very same DNA protection ability. The relevance of results for transfection studies is discussed. © *2010 American Institute of Physics*. [doi:10.1063/1.3427394]

Cationic liposomes (CLs) are self-assembled lipid vesicles currently investigated as possible nonviral carriers of nucleic acids in gene delivery.[1–4] Contrary to the virus-based technologies, CLs offer biocompatibility, biodegradability, and nonimmunogenity in carrying DNA and have emerged worldwide as the most prevalent synthetic gene delivery systems. Nowadays, it is accepted that complete DNA protection is a major step toward rational design of efficient CL/DNA complexes (lipoplexes). Previous investigations[5–7] aimed at elucidating the interactions determining structure, charge, and thermodynamic stability of lipoplexes, and pointed out the existence of three regions in the phase diagram as a function of the cationic lipid/anionic DNA charge ratio, ρ: complexes are monophasic at the isoelectric point ($\rho=1$), while they separate into complex plus excess liposomes for $\rho>1$ and complex plus excess DNA for $\rho<1$. As a result, mixing lipid and DNA at the isoelectric point has been long considered sufficient for assuming complete DNA protection within a cationic lipid envelope. On the opposite, a number of recent investigations unexpectedly showed that (i) nominally isoelectric lipoplexes coexist with a large part of unbound DNA (Refs. 8–11) and that (ii) a huge excess of cationic charge is required to guarantee 100% of DNA protection.[12] As evident, many aspects of the phase diagram of cationic lipid/DNA still remain unclear and need to be further investigated. The aim of this work was to study thoroughly the phase diagram of lipoplexes. We selected the lipoplex formulation made of binary 3β-[N-(*N*,*N*-dimethylaminoethane) - carbamoyl] - cholesterol–dioleoylphosphatidylethanolamine (DC-Chol–DOPE) CLs and plasmid DNA for two reasons as follows: it has been extensively studied from a biological point of view, and it has recently emerged as a potential candidate for the delivery of the chloride transporter gene to the lungs on the treatment of cystic fibrosis disease.[13]

Coexistence of complexes with unbound plasmid DNA was proofed by electrophoresis on agarose gels that provides very precise quantification of free DNA that is not protected by lipids. The nanostructure of complexes was characterized by means of high-resolution synchrotron small angle x-ray scattering (SAXS) that allows to determine the one-dimensional (1D) DNA packing density within complexes as well as to reveal the coexistence of complexes with pure liposomes.[6,14–17]

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 at molar ratios of neutral lipid to total lipid concentrations of Φ (mol/mol)=0, 0.3, 0.5, 0.7, and 0.75. Liposomes were sonicated to obtain small unilamellar vesicles (SUVs) (mean diameter less than 100 nm). DC-Chol–DOPE/DNA lipoplexes were prepared by mixing suitable volumes of SUVs (1 mg/ml, Tris-HCl 10^{-2} M pH 7.4 buffer solution) with pGL3 plasmid DNA at 12 cationic lipid/DNA charge ratios ρ (mol/base)=0.25, 0.33, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, and 5. Electrophoresis studies were conducted on 1% agarose gels containing ethidium bromide in tris-borate ethylenediamine tetraacetic acid buffer as elsewhere described.[17] Lipoplexes were electrophoresed on an agarose gel at 80 V for 1 h. The electrophoresis gels were visualized and digitally photographed using a Kodak Image Station, model 2000 R (Kodak, Rochester, NY). For SAXS experiments, lipoplexes were equilibrated for two days and lastly filled into glass capillaries. All SAXS measurements were performed at the Austrian SAXS station of the synchrotron light source ELETTRA (Trieste, Italy).[18]

Figure 1 shows the digital photograph of DC-Chol–DOPE/DNA ($\Phi=0.5$) lipoplexes as a function of ρ. Lipoplexes, due to size exclusion, remain at the site of appli-

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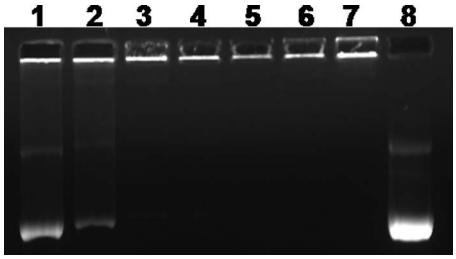

FIG. 1. Digital photograph of positively charged DC-Chol–DOPE/DNA lipoplexes ($\Phi=0.5$) with increasing cationic/anionic charge ratio: $\rho=1$ (lane 1), $\rho=1.5$ (lane 2), $\rho=2$ (lane 3), $\rho=2.5$ (lane 4), $\rho=3$ (lane 5), $\rho=3.5$ (lane 6), $\rho=4$ (lane 7), and control DNA (lane 8). Experiments revealed two major bands for the naked DNA (lane 8). The high-mobility band was attributed to the most compact (supercoiled) form, and the less-intense one was considered to be the nonsupercoil content in the plasmid preparation.

cation, while free DNA migrates toward the cathode. Experiments revealed two major bands for the naked DNA. The high-mobility band was attributed to the most compact (supercoiled) form, while the less-intense one was considered to be the nonsupercoil content in the plasmid preparation. As Fig. 1 clearly shows, isoelectric DC-Chol–DOPE/DNA complexes ($\rho=1$) did not protected all the plasmid DNA (Fig. 1, lane 1). Complexes coexisted with free DNA up to $\rho\sim2$ (Fig. 1, lanes 1–3). As evident, excess cationic charge to protect all the genetic cargo is necessary. As a general rule, such an extra-charge requirement was found to decrease as the molar fraction of neutral lipid in the bilayer increases (i.e., as Φ increases; data not reported for space consideration). Furthermore, the minimum charge ratio from which all plasmid DNA is protected by CLs and complexes start to be monophasic was precisely determined.

Figure 2 shows representative SAXS patterns of DC-Chol–DOPE/DNA ($\Phi=0.5$) lipoplexes as a function of ρ. The sharp periodically spaced peaks at q_{00l} are caused by alternating lipid bilayer-DNA-monolayer structure with periodicity $d=2\pi/q_{001}$. SAXS experiments revealed that DC-Chol–DOPE/DNA lipoplexes are lamellar even at high DOPE content. This was quite surprising since DOPE is the most frequently explored example of a membrane lipid prone to form nonbilayer phases. The much broader peak marked by arrow is the so-called "DNA peak" arising from the 1D in plane DNA lattice with repeat distance $d_{DNA}=2\pi/q_{DNA}$. According to literature, the DNA peak is mobile and shifts to lower q with increasing ρ (Fig. 2, from bottom to top). This can be explained considering that increasing ρ results in a growing amount of lipids that enter the complex and separate the DNA strands. At $\rho\sim4$ (Fig. 2, panel c) the DNA peak is fixed indicating that the 1D DNA lattice cannot be diluted by adding further lipid. Furthermore, the additional peaks at $q\sim1.25$ and 2.5 nm^{-1} (Fig. 2, panel C, dashed arrows) are consistent with a membrane stack of periodicity $d=5.03$ nm, equal to the periodicity of pure DC-Chol–DOPE bilayers in excess water.[14] This means that starting from $\rho\sim4$ complexes become biphasic and coexist with excess CLs. SAXS experiments performed on lipoplexes with varying Φ and ρ (data not reported) allowed us to determine the precise charge ratio from which each lipoplex formulation starts to coexist with a pure lipid lamellar phase. Interestingly, a general trend was found: the higher Φ, the lower ρ. This finding is in excellent agreement with electrophoretic

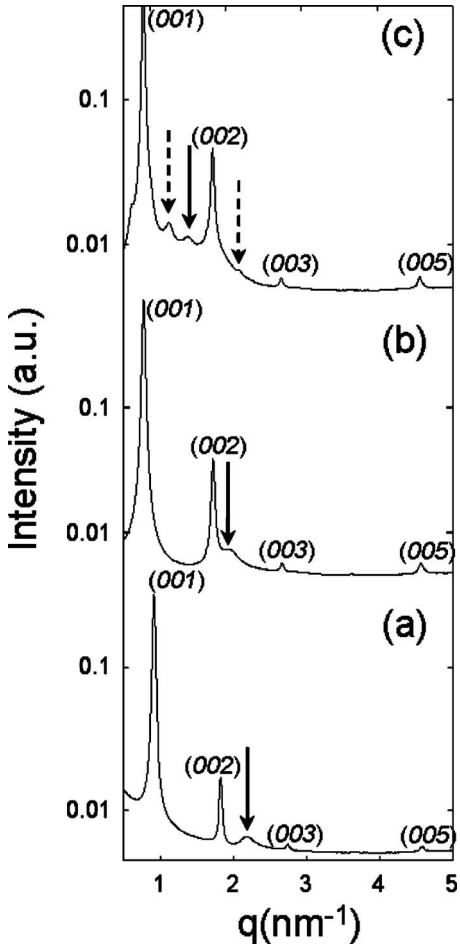

FIG. 2. Representative synchrotron SAXS patterns of lipoplexes DC-Chol–DOPE/DNA ($\Phi=0.5$) as a function of ρ: $\rho=0.5$ (panel a); $\rho=1$ (panel b); and $\rho=4$ (panel c). Arrows with dashed lines indicate Bragg peaks of the excess lipid phases, whereas arrows with solid lines indicate the Bragg peak that arises due to 1D DNA alignment.

data showing that extra-charge requirement decreases with increasing Φ.

Combining electrophoresis and SAXS data the phase diagram of DC-Chol–DOPE/DNA lipoplexes was constructed (Fig. 3). Since there are only three relevant chemical components in the system, namely, DNA and the two lipid species, the phase diagram is presented specifying the overall chemical composition of the ternary mixture. Corners of the triangle correspond to 100% molar fraction of DC-Chol, DOPE, and DNA, while black solid line indicates the nominally isoelectric DC-Chol/DNA ratio ($\rho=1$). Three different phase regions were identified: lamellar complexes coexisting with free plasmid DNA (black points, region A); one-phase complexes (white points, region B); complexes coexisting with excess CLs (gray points, region C).

Most remarkable features of the phase diagram reported in Fig. 3 are the following: (i) the extension of region A where the amount of lipid in solution does not suffice to complex all the DNA is much larger than those of regions B and C; (ii) complete association of the lipid and DNA molecules is limited to a very narrow portion of the phase diagram (region B); (iii) region B is not centered around the isoelectric line ($\rho=1$) as assumed so far. The latter observation is noteworthy since previous studies claimed that stoichiometrically charge-neutral complexes ($\rho=1$) are

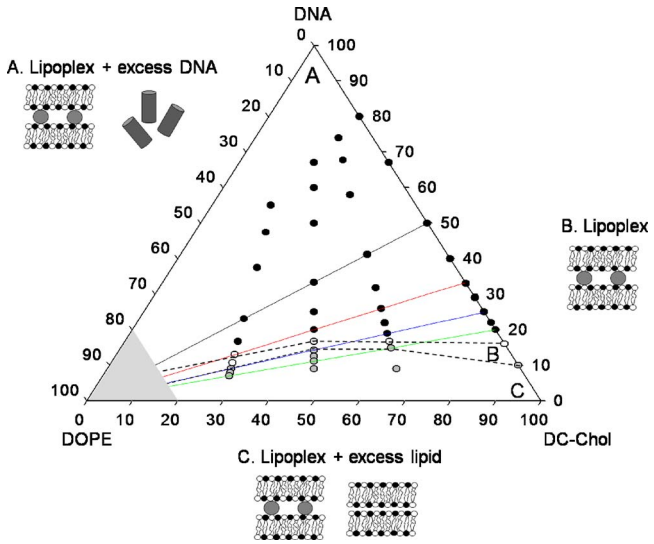

FIG. 3. (Color online) Phase diagram of DC-Chol–DOPE/DNA lipoplexes. Complexes are one phase in the very narrow region B only (white points), while they coexist with excess DNA in region A (black points) and with excess CLs in region C (gray points). Dashed lines give tentative phase boundaries. Lines of equal charge ratio are indicated as: $\rho=1$ (black solid line); $\rho=2$ (red solid line, second line from the top); $\rho=3$ (blue solid line, third line from the top); and $\rho=4$ (green solid line, fourth line from the top).

monophasic systems with all the DNA and the lipids associated within the complex. Our results are in very good agreement with the recent findings of Henriques *et al.*,[12] who showed that only a minor part of DNA was protected by CLs when lipoplexes were prepared at charge ratio $\rho=1$.

We also observed that phase coexistence of DC-Chol–DOPE/DNA lipoplexes with unbound DNA spanned over a large range of charge ratio whose extension decreased with increasing Φ. Solid lines in the phase diagram of Fig. 3 indicate complexes prepared at the same charge ratio, ρ, with different percentages of neutral lipids in the cationic membranes of lipoplexes. As evident, enriching the cationic membranes with neutral lipid at fixed cationic lipid/anionic DNA charge ratio (moving from the "DC-Chol corner" toward the "DOPE corner") results in protection of larger amounts of DNA. For instance, at $\rho=2$ (red solid line), lipoplexes with a low percentage of neutral DOPE in the bilayer (close to the DC-Chol corner) cannot complex all the DNA that remains free in solution (black points), while, when the percentage of DOPE in the mixed DC-Chol–DOPE increases (i.e., moving toward the DOPE corner), complete complexation of DNA occurs (white points). At $\rho=4$ (green solid line), complexes coexist with unbound DNA at extremely low Φ values, are monophasic in an extremely narrow region, while they become rapidly biphasic coexisting with excess lipid. Remarkably, we also observe (data not reported) that complexes with very different membrane composition and charge ratio (i.e., variation in Φ and ρ), but with the same total lipid/DNA molar ratio, exhibit similar DNA-protection ability and 1D DNA packing density. All these observations are likely to suggest that DNA-binding ability of CLs is mainly regulated by the total lipid membrane area available to DNA condensation. Since lipid species used for transfection studies are very different in chemical-physical properties, this would also explain why distinct lipoplex formulations exhibit very different DNA-protection ability.

Relevant implications arise for transfection studies where complete protection of DNA by lipid envelope is essential. CLs protect DNA from enzymatic degradation and ensure their transfer through the cellular membrane. As a consequence, an increase in DNA protection results in higher levels of transfection efficiency due to the lower extent of DNA degradation by DNases. On the other side, high doses of cationic lipids can lead to undesirable cytotoxicity. Transfection efficiency of lipoplex-mediated gene delivery is definitely a multifactorial process, but the correlation between the factors is not fully understood. However, each particular cell type is supposed to require lipoplex membranes having optimal lipid composition. This means that knowledge of the phase diagram of lipoplexes allows to accurately determine the minimum charge ratio from which all DNA molecules are protected by a specific lipoplex formulation.

In conclusion, we have reported the complete phase diagram of the widely used lipoplex formulation made of the highly fusogenic DOPE and the cholesterol-derivative DC-Chol. We have shown that isoelectric complexes coexist with free DNA, while an excess of cationic charge is required to protect all the genetic cargo. Interestingly, we have demonstrated that, at a fixed cationic lipid/DNA charge ratio, the DNA-binding ability of DC-Chol–DOPE membranes correlates with the neutral/total lipid ratio, Φ. This analysis should be useful for the interpretation of future experimental studies and may be relevant for the design of efficient lipoplex formulations.

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