Lipid mixing upon deoxyribonucleic acid-induced liposomes fusion investigated by synchrotron small-angle x-ray scattering

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Multipart cationic lipid/deoxyribonucleic acid (DNA) complexes (lipoplexes) were prepared, for the first time, by adding linear fragments of DNA to mixed lipid dispersions containing two distinct populations of cationic liposomes exhibiting different lipid headgroups and a number of systematic variations in relevant physical-chemical parameters. Upon DNA-induced fusion of liposomes, large lipid mixing at the molecular level occurs. As a result, highly organized mixed lipoplexes form spontaneously. By varying the composition of lipid dispersion, the physical properties of the emerging lipid carrier, i.e., the surface charge density and membrane thickness, can be modulated and distinct DNA packing densities can also be achieved. © *2005 American Institute of Physics*. [DOI: 10.1063/1.2058202]

Cationic liposomes are closed bilayer membrane shells containing cationic lipids and zwitterionic "helper" lipids. They are extensively used as gene delivery systems[1] in therapeutic applications worldwide and represent the most reliable alternative to viruses.[1]

Upon mixing of cationic liposomes with deoxyribonucleic acid (DNA), supramolecular organizations of DNA and cationic lipid change remarkably, and a liquid-crystalline multilamellar structure is usually formed in which DNA monolayers are embedded between oppositely charged lipid bilayers.[2–7] The formation of such a highly organized lipid-DNA structure, namely lipoplex, implies that lipid vesicles break during complex formation.

A number of significant experimental and theoretical studies established the equilibrium structures of lipoplexes, and related the structures to the physical-chemical properties of its components and the experimental conditions of their formation.[2–7]

Despite the wealth of empirical data available, underlying interactions and physical mechanisms are not completely understood.[8,9] This is unfortunate since a more complete understanding of the process is needed to open novel possibilities also for therapeutic intervention.

In this letter, we give the first experimental evidence that, upon DNA-induced fusion of two distinct populations of cationic liposomes, a well-ordered four component lipoplex emerges and that, at the molecular level, a complete lipid mixing occurs.

Cationic lipids 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and (3_[*N*-(*N*_,*N*_-dimethylamino-ethane)-carbamoyl]cholesterol (DC-Chol), and neutral lipids dioleoylphosphocoline (DOPC) and dioleoylphosphatidylethanolamine (DOPE), were purchased by Avanti Polar Lipids in the lyophilized form and used without further purification.

Multilamellar DOTAP-DOPC (A) and DC-Chol-DOPE (B) liposomes were prepared following routine protocols.[2] Briefly, binary lipid mixtures, at a weight ratio of neutral lipid in the bilayer Φ=(weight of neutral lipid)/(weight of total lipid)=0.5, were dissolved in chloroform and the solvent was evaporated under a stream of nitrogen and then under a vacuum overnight. The obtained lipid film was hydrated with the appropriate amount of Tris-HCl buffer solution (10^{-2} M,*p*H 7.4) to achieve the desired final concentration of 100 mg/ml. The solution was incubated at 30 °C for 6 h to allow formation of liposomes. Calf thymus Na-DNA solution (10 mg/ml) was sonicated inducing a DNA fragmentation with length distribution between 500 and 1000 base pairs which was determined by gel electrophoresis. Isoelectric A-DNA and B-DNA lipoplexes were finally prepared by injecting DNA into lipid dispersions. In the isoelectric regime, the number of positive charges carried by the cationic lipid headgroups exactly matches the number of negative charges localized on the phosphate groups on the DNA backbone.[10–12]

A-DNA and B-DNA lipoplexes were characterized by means of high-resolution synchrotron small-angle x-ray scattering (SAXS). SAXS experiments were conducted at the Austrian SAXS beamline of Elettra (Trieste, Italy) and the details of these experiments are given elsewhere.[13] SAXS patterns of isoelectric A-DNA and B-DNA lipoplexes (T =20 °C) are shown in Figs. 1(a) and 1(d). The set of sharp lamellar peaks at q_{00n} is caused by the alternating lipid bilayer-DNA monolayer structure with periodicity d =$2\pi/q_{001}$=6.45 and 6.85 nm for the A-DNA and B-DNA lipoplexes, respectively. The Bragg peaks marked by arrows arise from diffraction from the one-dimensional lattice of DNA chains sandwiched between lipid bilayers.[2–7] Despite of similar lamellar periodicities d, very different DNA-DNA in plane repeat distances d_{DNA}=4.32 (A-DNA). and 3.25 nm (B-DNA) were detected. It means that, at the isoelectric point, A-DNA lipoplexes exhibit a lower surface charge density with respect to B-DNA lipoplexes resulting in a lower DNA packing density within the complex, i.e., in a higher d_{DNA} repeat distance.

Then, we mixed initially separated A and B vesicles at five relative molar ratios R=B/(A+B)=0.2, 0.4, 0.5, 0.6,

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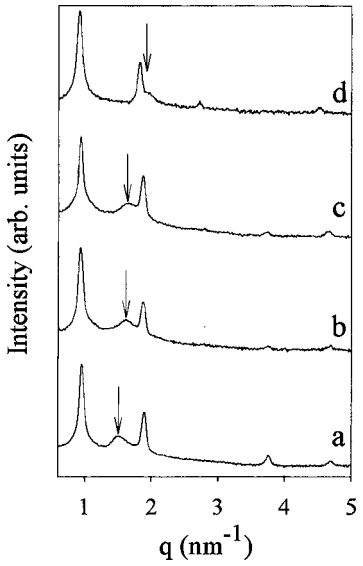

FIG. 1. Representative SAXS patterns of A-B-DNA complexes as a function of increasing molar percentage of B liposomes in the lipid dispersions $R = B/(A+B)$ from the bottom to the top: (a) $R=0$; (b) $R=0.4$; (c) $R=0.6$; and (d) $R=1$. Intermediate SAXS patterns [(b) and (c)] do not arise from the linear combination of (a) and (d) patterns. DNA-DNA peak is marked by an arrow.

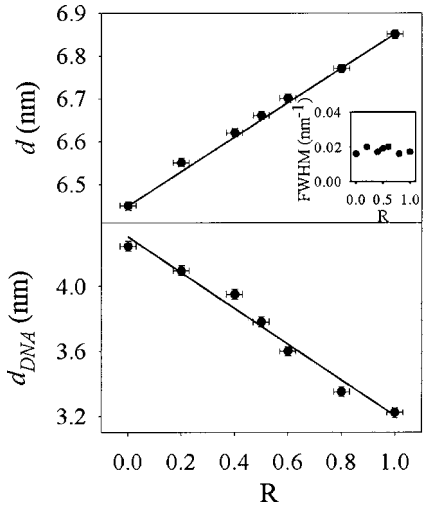

FIG. 2. Interlayer distance d (top panel) and DNA-DNA interaxial distance d_{DNA} (bottom panel) as a function of R. Solid lines are the best linear fits to the data. In the inset of the top panel, the FWHM of the first-order Bragg peak is shown. Higher-order reflections exhibited similar trends.

and 0.8. Mixed lipid dispersions were equilibrated at 30 °C for 24 h. As expected,[14] SAXS experiments revealed (data not reported) that, upon mixing A and B liposomes, interparticle electrostatic repulsions dominate over short-range attractive van der Waals forces and no fusion between A and B occurs.

Afterward, proper amounts of DNA solution were added to the mixed lipid dispersions paying attention to exactly neutralize the positive charge carried both by DOTAP and DC-Chol molecules. SAXS patterns of Figs. 1(b) and 1(c) show that, for each R value, only one kind of mixed A-B-DNA lipoplexes exist. Indeed, if A-DNA and B-DNA lipoplexes coexisted within the sample volume, the SAXS pattern would arise from the superposition of their own patterns.

Even if one could not exclude the presence of A-DNA- and B-DNA-rich domains, there are several reasons that a high degree of lipid mixing at the molecular level occurs.[15] First of all, both d and d_{DNA} vary monotonously as a function of R as shown in Fig. 2 (top and bottom panel). In addition, the full width at half maximum (FWHM) of all the Bragg peaks is approximately constant as a function of R (inset of Fig. 2, top panel). Furthermore, all the SAXS patterns exhibit a single DNA peak. It is well-established that the DNA-DNA distance d_{DNA} only depends on the physical properties of the lipid membrane.[2–7,10–13] So, if lipid mixing was not almost complete, distinct DNA peaks would be certainly detected.[2–7]

All of this evidence suggests that, upon liposomes fusion, a mixed lipid system emerges with physical membrane properties intermediate between those of A and B.

Undoubtedly, mixed A-B-DNA lipoplexes can exist only if A and B liposomes break during complex formation.[8,9] As liposomes fusion was not observed simply by mixing A and B, it is therefore the DNA which reduces the intermembrane repulsive barrier due to electrostatic, steric, and hydration forces.[9,15]

Our results show that, upon lipoplex formation, lipid mixing occurs (Fig. 3). Such a mixing, like any self-assembling process, is ruled by thermodynamics and requires a decrease of free energy to be spontaneous.[8–12] Lipid mixing occurring during DNA-induced fusion of one kind of phosphatidylcholine vesicles has been recently reported.[15] Nevertheless, to our knowledge, no experimental evidence of lipid mixing—upon DNA-induced fusion of very different liposomic formulations—has been provided so far.

To conclude, we have provided the first experimental evidence of the high lipid mixing occurring upon lipoplex formation. It is a central point since deciphering the physical mechanisms underlying lipoplex formation can be expected to yield a new therapeutic means for a variety of disorders.[16]

Our research has also highlighted, for the first time, the possibility of forming well-ordered self-assembled lipoplexes composed of four lipid components, merely by adding DNA to dispersions containing two distinct populations of binary cationic liposomes. By varying the composition of the mixed lipid dispersion, the physical properties of the emerging lipid carrier, such as the surface charge density and membrane thickness, can be modulated and distinct DNA packing densities may also be achieved.[2–7]

The development of such arrays is a very rich strand for future research in that it opens the gateway to synthesize multipart gene delivery vectors. Indeed, it is commonly accepted that there will not be a "universal vector," but each clinical indication may require a specific system.[17]

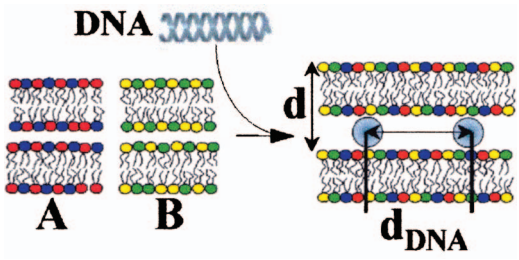

FIG. 3. Schematics of the process occurring, at the molecular level, upon DNA-induced fusion of two distinct populations of cationic liposomes. A and B represent a sketch of the lipid bilayer membranes of DOTAP-DOPC (A) and DC-Chol-DOPE (B) liposomes. For clarity, each color refers to a single lipid species.

To this purpose, engineering of multicomponent lipoplexes incorporating the specific physical-chemical properties of very different lipid species may represent a fascinating starting point to rationally design highly specific gene vectors.[18–22]

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