

On the correlation between phase evolution of lipoplexes/anionic lipid mixtures and DNA release

Giulio Caracciolo,^{a)} Daniela Pozzi, and Ruggero Caminiti

Department of Chemistry, University of Rome "La Sapienza," P.le A. Moro 5, 00185 Rome, Italy

Cristina Marchini, Maura Montani, and Augusto Amici

Genetic Immunization Laboratory, Department of Molecular Cellular and Animal Biology, University of Camerino, Via Camerini 5, 62032 Camerino (MC), Italy

Heinz Amenitsch

Institute of Biophysics and Nanosystems Research, Austrian Academy of Sciences, Schmiedelstrasse 6, A-8042 Graz, Austria

(Received 24 August 2007; accepted 16 September 2007; published online 4 October 2007)

We investigated the structural evolution of three lipoplex formulations when interacting with anionic lipids by synchrotron small angle x-ray diffraction, while the extent of DNA release from lipoplexes by anionic lipids was evaluated by gel electrophoresis. Lipoplexes formed lamellar phases when mixed with anionic dioleoylphosphatidylglycerol (DOPG), while promoting the formation of nonbilayer structures when mixed with anionic dioleoylphosphatidic acid (DOPA). However, lipoplexes exhibited a virtually identical extent of DNA release when mixed with DOPG or DOPA. Thus, the recently proposed correlation between the formation of nonlamellar phases in lipoplex/anionic lipid mixtures and the increase of DNA release does not seem to exist. © 2007 American Institute of Physics. [DOI: 10.1063/1.2794436]

Therapeutic procedures, such as gene transfection and gene silencing, require efficient delivery of genetic material to cells.¹ Nowadays, synthetic cationic lipids, which form stable complexes with polyanionic DNA (lipoplexes), are considered the most promising nonviral gene carriers.^{1,2} Formulations based on the exclusive use of zwitterionic lipids have also been investigated.³

For lipoplexes to become widely useful for gene therapeutic purposes, however, their efficiency needs to be improved.^{1,2} Progress in enhancing the lipid-mediated transfection is impeded because its mechanism is still largely unknown.^{3,4} Nevertheless, one of the major obstacles to efficient transfection is the low level of DNA release from lipoplexes. A current opinion is that the critical factor in DNA release is the structural evolution of lipoplexes upon interacting and mixing with cellular anionic lipids.⁵ In this context, recent studies⁵⁻⁷ put forward two main concepts: (i) anionic lipids adopting nonlamellar phases are more efficient in releasing DNA from lipoplexes than those forming lamellar phases; (ii) the ability of cationic lipids to promote nonbilayer structures in combination with anionic phospholipids facilitates DNA release and results in higher transfection efficiency.⁷

In this letter, we provide experimental evidence against that hypothesis. Here, we report on three lipoplex formulations that form lamellar phases when mixed with anionic dioleoylphosphatidylglycerol (DOPG), while promoting the formation of nonbilayer structures when mixed with anionic dioleoylphosphatidic acid (DOPA). However, despite dramatic differences in the phase evolution, the lipoplexes were found to exhibit a virtually identical extent of DNA release when mixed with DOPG or DOPA.

Calf thymus Na-DNA was purchased from Sigma (St. Louis, MO). DNA was dissolved in tris-HCl buffer and

was sonicated for 5 min, inducing DNA fragmentation with a length distribution between 500 and 1000 base pairs. Cationic lipids 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and 3 β -[N-(N,N-dimethylaminoethane) carbamoyl] cholesterol (DC-Chol), anionic lipids DOPG and DOPA, and neutral helper lipids dioleoylphosphatidylethanolamine (DOPE) and dioleoylphosphocholine (DOPC) were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. DOTAP-DOPC (A), DC-Chol-DOPE (B), and DOTAP-DOPC-DC-Chol-DOPE (AB) cationic liposomes were routinely prepared.⁷ Molar ratio of neutral lipid in the bilayer was Φ =(neutral lipid/total lipid)=(mol/mol)=0.5, while the final concentration of lipid dispersions was 10 mg/ml. DOTAP-DOPC/DNA(A/DNA), DC-Chol-DOPE/DNA (B/DNA), and DOTAP-DOPC-DC-Chol-DOPE/DNA (AB/DNA) lipoplexes were prepared by mixing 100 μ l of calf thymus DNA at 5.3 mg/ml with suitable volumes of A, B, and C liposome solutions. Lipoplexes were prepared at the cationic lipid/DNA ratio ρ =cationic lipid DNA (mol/base)=2 (positively charged lipoplexes).

Synchrotron small angle x-ray diffraction (SAXD) was applied to study the structural changes of lipoplexes upon interaction with anionic liposomes (ALs) prepared from DOPG and DOPA, two anionic lipids common in mammalian cells. Lipoplex/AL mixed dispersions were prepared by mixing solutions of AL and preformed lipoplexes at different charge ratios $R=A/L_c$ (moles of anionic lipid/moles of cationic lipid), between $R=0$ and $R=5$ in increments of 0.1, equilibrated for two 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).⁸

Figure 1 [panel (A)] shows some representative SAXD patterns of A/DNA/DOPG mixtures as a function of the anionic/cationic charge ratio R . At $R=0$ (no anionic lipid added), the sharp (00 l) peaks (marked with an asterisk) are caused by alternating lipid bilayer/DNA monolayer lamellar

^{a)} Author to whom correspondence should be addressed. Electronic mail: g.caracciolo@caspur.it

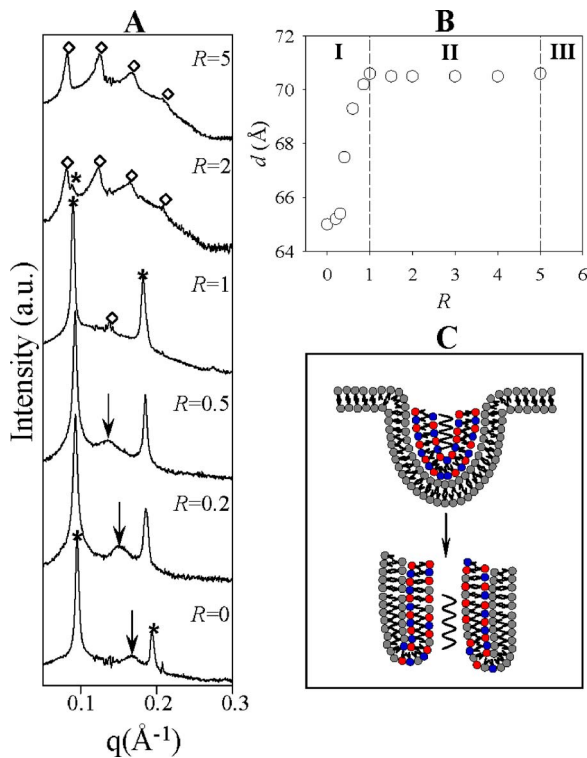


FIG. 1. (Color online) Panel (A): SAXD patterns of DOTAP-DOPC/DNA/DOPG (A/DNA/DOPG) mixtures as a function of the anionic/cationic charge ratio R . Bragg peaks arising from the lamellar structure of A/DNA lipoplexes are indicated with *, while the DNA peak is marked by an arrow. Diffraction maxima of pure DOPG are indicated with \diamond . Panel (B): evolution of the d spacings of A/DNA/DOPG mixture with differing DOPG content. Lamellar d spacings of lipoplexes are indicated with open circles (d spacing of pure DOPG with no DNA embedded is not reported). Dashed lines separate regions occupied by lamellar lipoplexes (I), lamellar lipoplexes plus excess DOPG bilayers (II), and pure DOPG swollen bilayers (III). Panel (C): mechanism of interaction between lamellar lipoplexes and DOPG liposomes (gray). DOPG molecules diffuse within A membranes made of cationic DOTAP (red) and neutral DOPC (blu) and form a charged neutralized ion pair with the cationic lipids. As a result, the lamellar structure of lipoplexes is progressively disintegrated by DOPG and DNA is released.

structure (L_{α}^C phase)⁹ with periodicity $d=2\pi/q_{001}=65$ Å. The diffuse broad peak, usually referred to as “DNA peak”^{9–11} (marked by an arrow), resulted from a one-dimensional ordered array of DNA molecules with an interstrand spacing, $d_{\text{DNA}}=2\pi/q_{\text{DNA}}=38.6$ Å.

As the DOPG concentration is increased in the mixture, the lamellar phase of A/DNA lipoplexes changed remarkably. Indeed, for $0 < R < 1$, the SAXD patterns were dominated by diffraction maxima of the L_{α}^C phase of A/DNA lipoplexes, while, for $R > 1$, Bragg peaks of the highly swollen lamellar phase of pure DOPG [$d=131.2$ Å (Ref. 12)] were observed (indicated by diamonds).

Panel (B) of Fig. 1 shows the evolution of the d spacings of the different phases of A/DNA/DOPG mixture with differing DOPG content (i.e., as a function of R). The approximate locations of the phase boundaries are indicated with dashed lines. In regime I, only lamellar lipoplexes were found to exist. In this regime, a characteristic structural feature of the A/DNA/DOPG mixture was the extensive swelling of the lamellar phase of A/DNA lipoplexes that accompanied its enrichment with DOPG molecules (lamellar d spacings are indicated with open circles).¹³ In regime II, lamellar lipoplexes were progressively disintegrated and started to coexist with excess DOPG. In regime III, only the

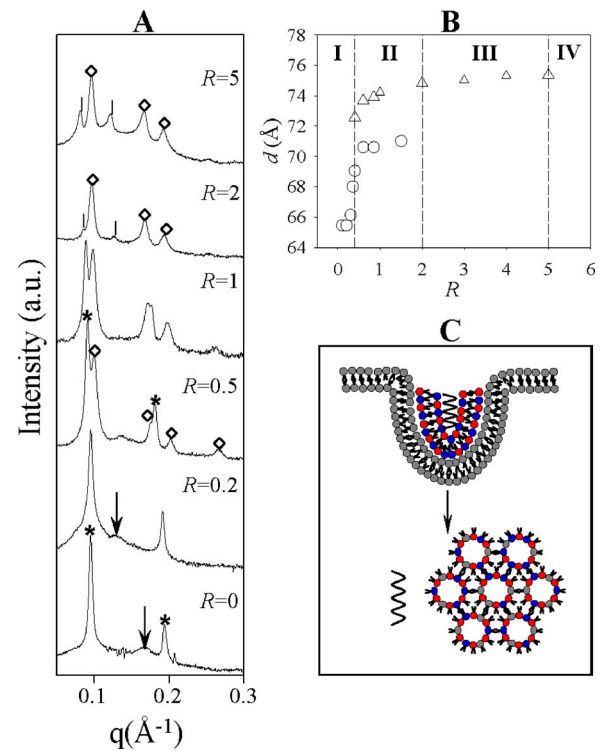


FIG. 2. (Color online) Panel (A): SAXD patterns of DOTAP-DOPC/DNA/DOPA (A/DNA/DOPA) mixtures as a function of the anionic/cationic charge ratio R . Bragg peaks arising from the lamellar structure of A/DNA lipoplexes are indicated with *, while the DNA peak is marked by an arrow. Diffraction maxima of hexagonal complexes are indicated with \diamond , while reflections of pure DOPA with |. Panel (B): phase diagram of A/DNA/DOPA mixture with differing DOPA content. Dashed lines separate regions occupied by lamellar lipoplexes (I), coexisting lamellar and hexagonal lipoplexes (II), and hexagonal lipoplexes coexisting with pure DOPA forming the cubic phase $Pn3m$ (III). Repeat spacings of lamellar and hexagonal lipoplexes (d spacing of pure DOPA with no DNA embedded is not reported) are indicated with open circles and triangles, respectively. Panel (C): mechanism of interaction between lamellar lipoplexes and DOPA liposomes (gray). DOPA molecules diffuse within A membranes promote a lamellar-to-hexagonal phase transition and DNA is released.

fluid DOPG bilayers were found. We conclude that, all over the compositional range investigated, A/DNA lipoplexes retained their lamellar arrangement up to complete disintegration by anionic DOPG [Fig. 1, panel (C)].

Figure 2 [panel (A)] shows SAXD patterns of A/DNA/DOPA mixtures as a function of R . For $0 < R < 0.2$, only lamellar lipoplexes were found to exist. In this stage, the DNA lattice was diluted by the anionic lipid.¹³ This can be seen in SAXD scans where d_{DNA} changes from 38.6 Å at $R=0$ up to about 41.9 Å at $R=0.2$. As the DOPA concentration is increased in the mixture, a lamellar-hexagonal phase transition occurred. At $R=0.5$, the diffraction pattern was still dominated by lamellar peaks, but we also observed three Bragg peaks that were indexed as the (10), (11), and (20) reflections of an inverted hexagonal phase with a 71.3 Å structural unit. The initial lamellar phase of A/DNA lipoplexes persisted up to $R \sim 2$. For $R > 2$, the SAXD patterns of the A/DNA/DOPA mixture were dominated by a hexagonal phase, with a unit cell of 75.5 Å. Additional five reflections, fitting the ratios $\sqrt{2}, \sqrt{3}, \sqrt{4}, \sqrt{6}, \sqrt{8}$, were also seen. Such reflections, characteristic of a cubic $Pn3m$ phase with a 150 Å unit cell size, arose from pure DOPA coexisting with hexagonal lipoplexes.⁵ Panel (B) of Fig. 2 shows the phase diagram of A/DNA/DOPA mixtures. In regime I, only lamel-

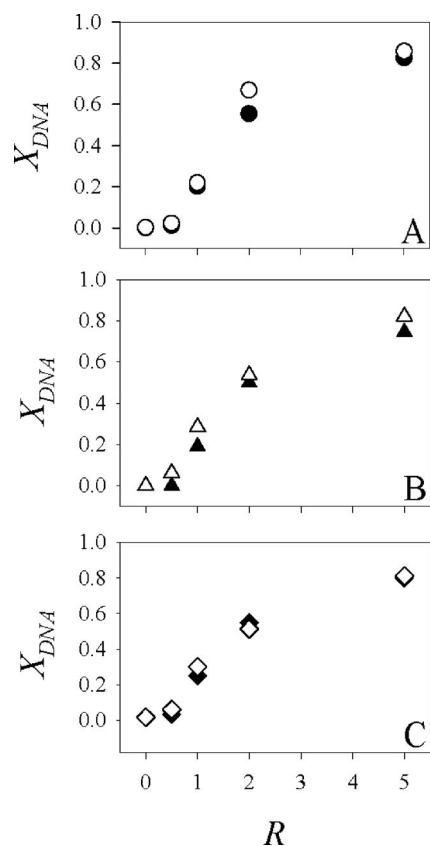


FIG. 3. (Color online) Fraction of DNA release from A/DNA [panel (A)], B/DNA [panel (B)], and AB/DNA [panel (C)] lipoplexes by DOPG (black symbols) and DOPA (white symbols).

lar lipoplexes were detected (lamellar d spacings are indicated with open circles). In regime II, lamellar and hexagonal lipoplexes coexisted. The increase of both the lamellar (open circles) and the hexagonal (open triangles) spacings is consistent with the inclusion of DOPA molecules within A membranes.⁵ This finding indicates that DOPA molecules penetrated within A membranes and promoted a lamellar-hexagonal phase transition [Fig. 2, panel (C)]. Our results are consistent with previous reports in the literature showing that DOPA holds the potential to promote the adoption of an inverted hexagonal structure of phospholipids.^{14,15} Lastly, in regime III, hexagonal lipoplexes were found to coexist with pure DOPA.

Very similar results (not reported because of space considerations) were obtained with B/DNA and AB/DNA lipoplexes.

As mentioned above, one factor that is likely to be important in the efficiency of transfection is the dissociation of DNA from cationic lipids and its release into the cytoplasm. A current opinion is that the structural evolution of lipoplexes upon interaction with cellular lipids may be a controlling factor in DNA release.⁵⁻⁷ We applied gel electrophoresis to examine the release of DNA from lipoplexes by anionic lipids. Details of the experiments are given elsewhere.¹³ Figure 3 shows the molar fraction of DNA released, X_{DNA} , from A/DNA, B/DNA, and AB/DNA lipoplexes [panels (A), (B), and (C), respectively] after the addition of negatively charged DOPG (black symbols) and DOPA (white symbols). For clarity, error bars are not shown, but each data point represents an average value of at least three experimental observations with standard deviation

being $<10\%$. Figure 3 unambiguously shows that the DNA-releasing activity of DOPG and DOPA was very similar for all lipoplex formulations. Recent studies showed that DOPA was a very good DNA releaser in the case of *o*-ethylidoleoylphosphatidylcholinium-containing lipoplexes, while DOPG was not.⁵ On the opposite, electrophoresis data shown in Fig. 3 did not reveal any significant difference in the DNA releasing capacity of DOPG and DOPA. A possible explanation is that the efficiency of anionic lipids in terms of DNA release could be meaningful only in the context of specific lipoplex formulations.

In the same studies,⁵⁻⁷ DOPA generally expressed a greater ability to destroy the initial lamellar structure of the lipoplexes than did DOPG. In contrast, our SAXD data showed that at $R=5$, A/DNA lipoplexes were completely destroyed by DOPG while they partly resisted the disintegration by DOPA. Some evidences have been reported that the anionic lipids that are more efficient in releasing DNA adopt nonlamellar phases with high interfacial curvature when mixed with preformed lipoplexes, while the anionic lipids that are poorly efficient DNA releasers form mostly lamellar phases.⁵⁻⁷ As evident, our combined synchrotron SAXD and electrophoresis results disagree with that opinion. Indeed, SAXD experiments showed that A/DNA lipoplexes retained their lamellar structure in mixtures with negatively charged DOPG membrane, while exhibiting a lamellar-hexagonal phase transition in mixtures with DOPA. However, electrophoresis experiments showed that such a very different phase behavior did not result in a diverse DNA release efficiency (Fig. 3).

In conclusion, we have shown that (i) the anionic lipids with a disposition to form lamellar phases (such as DOPG) may be DNA releasers as good as the anionic lipids with a propensity to form nonlamellar phases (such as DOPA); (ii) the extent of DNA release from lipoplexes by anionic lipids does not universally correlate with the propensity of the lipoplex/anionic lipid mixtures to adopt nonbilayer motifs. Thus, our findings suggest that the proposed general correlation between the formation of nonlamellar phases and the extent of DNA release⁵⁻⁷ does not exist.

¹M. C. Pedroso de Lima, S. Simões, P. Pires, H. Faneca, and N. Duzgunesü, *Adv. Drug Delivery Rev.* **47**, 277 (2001).

²P. Felgner, *Sci. Am.* **276**, 102 (1997).

³J. McManus, J. O. Rädler, and K. A. Dawson, *J. Am. Chem. Soc.* **126**, 15966 (2004).

⁴Y. Xu and F. J. Szoka, *Biochemistry* **35**, 5616 (1996).

⁵Y. S. Tarahovsky, R. Koynova, and R. C. MacDonald, *Biophys. J.* **87**, 1054 (2004).

⁶R. Koynova, L. Wang, and R. C. MacDonald, *Proc. Natl. Acad. Sci. U.S.A.* **103**, 14373 (2006).

⁷L. Wang, R. Koynova, H. Parikh, and R. C. MacDonald, *Biophys. J.* **91**, 3692 (2006).

⁸H. Amenitsch, M. Rappolt, M. Kriechbaum, H. Mio, P. Lagner, and S. Bernstorff, *J. Synchrotron Radiat.* **5**, 506 (1998).

⁹J. O. Rädler, I. Koltover, T. Salditt, and C. R. Safinya, *Science* **275**, 810 (1997).

¹⁰I. Koltover, T. Salditt, J. O. Rädler, and C. R. Safinya, *Science* **281**, 78 (1998).

¹¹G. Caracciolo, R. Caminiti, D. Pozzi, M. Friello, F. Boffi, and A. Congiu Castellano, *Chem. Phys. Lett.* **351**, 222 (2002).

¹²G. Caracciolo, C. Marchini, D. Pozzi, R. Caminiti, H. Amenitsch, M. Montani, and A. Amici, *Langmuir* **23**, 4498 (2007).

¹³G. Caracciolo, D. Pozzi, R. Caminiti, C. Marchini, M. Montani, A. Amici, and H. Amenitsch, *Appl. Phys. Lett.* **89**, 233903 (2006).

¹⁴Y. Shai, *Biochim. Biophys. Acta* **1462**, 55 (1999).

¹⁵J. Bondeson, J. Wijkander, and R. Sundler, *Biochim. Biophys. Acta* **777**, 21 (1984).