Osmotically Induced Reversible Transitions in Lipid-DNA Mesophases

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ABSTRACT We follow the effect of osmotic pressure on isoelectric complexes that self-assemble from mixtures of DNA and mixed neutral and cationic lipids. Using small angle x-ray diffraction and freeze-fracture cryo-electron microscopy, we find that lamellar complexes known to form in aqueous solutions can reversibly transition to hexagonal mesophases under high enough osmotic stress exerted by adding a neutral polymer. Using molecular spacings derived from x-ray diffraction, we estimate the reversible osmotic pressure-volume (Π-V) work needed to induce this transition. We find that the transition free energy is comparable to the work required to elastically bend lipid layers around DNA. Consistent with this, the required work is significantly lowered by an addition of hexanol, which is known to soften lipid bilayers. Our findings not only help to resolve the free-energy contributions associated with lipid-DNA complex formation, but they also demonstrate the importance that osmotic stress can have to the macromolecular phase geometry in realistic biological environments.

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The complexes that spontaneously form when DNA is mixed with lipids have drawn great interest as potential vectors in gene therapy (1–6). Moreover, because these ''lipoplexes'' form regular ordered phases, they are also remarkably convenient tools for studying the link between molecular DNA-lipid interactions and the macroscopic ordered phases that they form. It has been shown that the structure of the ordered mesophases can be controlled through the delicate interplay of electrostatic, elastic, and entropic-mixing forces, as determined by the composition of lipids, DNA, and salt (1–3, 7–11). For example, it is possible to favor formation of hexagonal lipoplexes (''honeycomb'' shaped, H_{II}^C), or lamellar structures (''sandwich-like,'' L_α^C) by simply choosing lipid mixtures with appropriate elastic properties (2).

An important link has been established between lipoplex structure and composition, and transfection efficiency (1). In particular, it was recently shown that although the transfection efficiency of H_{II}^C complexes is generally high and independent of membrane charge density, for L_α^Ccomplexes this efficiency sensitively depends on the membrane charge density, but can match the transfection efficiency gained with the hexagonal.

We study the response of lipid-DNA complexes to osmotic stress exerted by adding polyethylenglycol (PEG), at various final concentrations, to lipoplex solutions (Fig. 1). This osmotic stress technique is an effective tool to investigate changes in hydration accompanying macromolecular assembly (12), and affords several advantages. First, it is widely appreciated that water plays an important role in determining macromolecular binding energies, but only a few methods are available for measuring the forces associated with changes in hydration that accompany phase transitions in aqueous solutions. Second, crowded with salts, sugars, amino acids, and other biopolymers including proteins, the physiological and intracellular milieu is far different from the dilute aqueous conditions typically used to study macromolecular assembly in vitro. Applying osmotic stress allows us to better simulate the response of lipoplexes to possible cellular and physiological crowded environments. Specifically, en route to its target inside the cell nucleus, DNA must traverse multiple barriers by first enduring stressed conditions in the bloodstream, then passing through the cell outer membrane, escaping internal cellular organelles, and finally shedding its lipid sheath. These barriers can all be modulated by the effect of crowding.

We show that by using x-ray diffraction and freeze-fracture cryo-transmission electron microscopy (TEM), we are able to assess the extent of hydration at the molecular level under a given applied osmotic stress and to derive an equation of state for lipoplexes. We study isoelectric mixtures of DNA and lipid at ratios corresponding to equal amounts of negative (DNA) and positive (cationic lipid) charges. All samples contained premixed, extruded lipid mixtures of dioleoyl-phosphatidylcholine and dioleoylphosphatidyl trimethylammonium propane at a molar ratio of 3:2, and short DNA fragments of ~146 basepairs (see Supporting Material for experimental details). Osmotic pressures as high as $\Pi = 100$ atm were applied, corresponding to solutions of ~50% PEG of molecular weight 8000 (the equivalent of ~4 Osmolal).

Fig. 2 shows the x-ray diffraction patterns from lipoplexes at different applied osmotic stresses. We find that when the complexes are unstressed they form lamellar assemblies with DNA intercalated between membranes as shown in Fig. 1.

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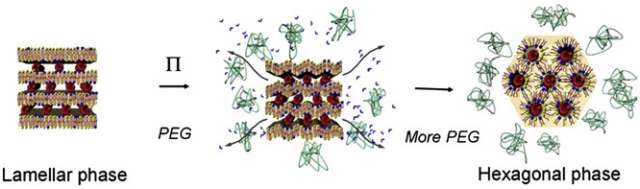

FIGURE 1 Schematic of the effect of osmotic stress on lipid-DNA complexes. Osmotic stress tends to draw water out of lipoplexes (*arrows*), so that at high enough osmotic stress lamellar lipoplexes transition to hexagonal phases.

Using freeze-fracture electron microscopy, we verify that the structure of these unstressed complexes is lamellar (Fig. 3 *A*).

As osmotic pressure increases from low to intermediate values, we find lamellar phases with smaller repeat spacings, both between adjacent membranes and DNA strands (Fig. 2, $\Pi = 45$ atm). Finally, when stressed with high osmotic pressure, the lipoplex mesophase changes and assumes hexagonal geometry (Figs. 2 and 3 *B*). This abrupt phase transition

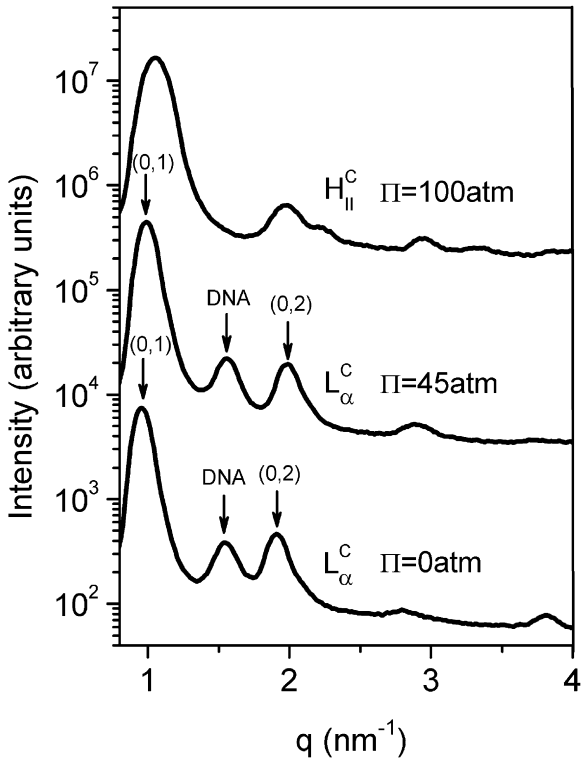

FIGURE 2 Small angle x-ray scans of lipid-DNA complexes at the isoelectric point, as a function of applied osmotic stress. At $\Pi = 0$ atm, the Bragg reflection indicated by the arrows showing first and second orders, correspond to periodic lamellar structures, whereas the arrow marked DNA corresponds to distances between the intercalated DNA strands of the L_α^C phase. As osmotic pressure grows to $\Pi = 45$ atm, we find that the Bragg reflections shift to larger *q* values corresponding to smaller molecular spacings (as indicated by the *arrows*). At even higher osmotic pressures of $\Pi = 100$ atm we find a series of reflections with ratios of $1:\sqrt{3}:2$ that correspond to the hexagonal symmetry and unit cell spacing of the H_{II}^C phase.

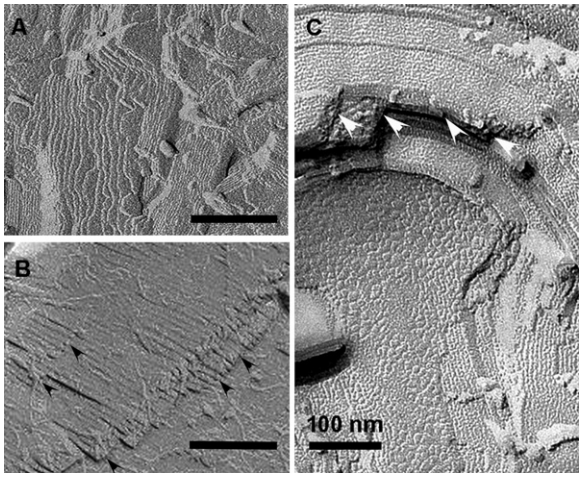

FIGURE 3 Freeze-fracture TEM images of the lipoplexes as a function of applied osmotic stress. (*A*) Lipoplexes with lamellar geometry that are created in the absence of PEG ($\Pi = 0$ atm). (*B*) Hexagonal DNA-lipid phase is formed after osmotic pressure of 100 atm is applied. The image clearly discloses multiple arrays of densely packed elongated rods composing the ordered mesophase. Note also the edges of the cylinders, some of which are marked by black arrowheads. (*C*) A dense hexagonal phase is created already at $\Pi = 45$ atm in the presence of hexanol. White arrowheads in *C* point to edges of rods that become visible upon fracturing.

can be expected to be of first order because the symmetry is different in the two phases. The transition to the hexagonal structure at high osmotic stress is also consistent with the fact that the hexagonal phase has smaller water content, because DNA and lipid are more closely bound. This structure should, therefore, be favored when the free energy cost of water in the bulk becomes higher, i.e., chemical potential (or water activity) becomes lower.

Using the information on the repeat spacings at different pressures and applying a simple geometrical ''box-model'' (see Supporting Material) we can estimate the volume occupied by water in a lipoplex unit cell. Using these estimates, we plot the pressure versus volume per unit cell, as shown in Fig. 4. The figure represents the equation of state for the system, assuming data points represent lipoplexes at equilibrium. In fact, we find that the lipoplexes that first form in the absence of osmotic stress, and then transform to the hexagonal phase with added polymer at large concentrations, return to the lamellar structure when osmotic pressure is lowered by PEG dilution (Fig. S1 in the Supporting Material). This indicates that the transitions between different lipoplex geometries are reversible under our experimental conditions.

Evaluating the equation of state, we can estimate the Gibbs free energy difference associated with transitioning from the L_α^C to the H_{II}^C phase by measuring the area under the dashed line in Fig. 4, from the unstressed lipoplexes to the transition point on the H_{II}^C side. This area represents the reversible work exerted in the transition.

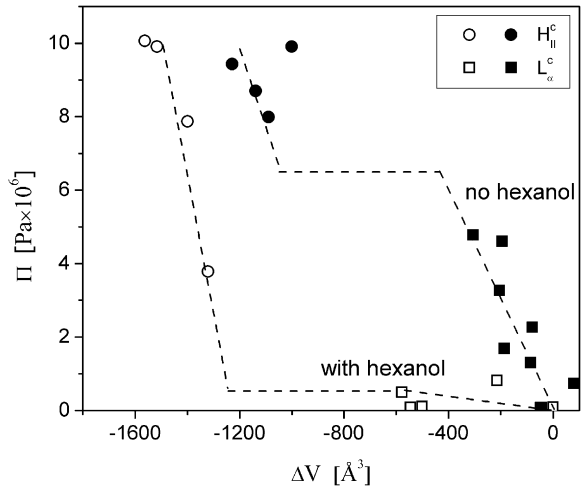

FIGURE 4 Changes in volume of hydration per lipoplex unit cell derived from small angle x-ray scattering molecular spacings. Symbols correspond to lipid mixtures in the absence of hexanol (*solid*) and with hexanol (*open*). With applied osmotic pressure, a transition in seen from L_{α}^{c} (*squares*) to H_{II}^{c} (*circles*) mesophases. Lines are guides to the eye.

We find that it takes $\approx 1.6\ kT$ of work (where kT is thermal energy) per DNA basepair to force the lamellar into hexagonal structure, with estimates varying up to 20% depending on the positioning of the plateau representing the coexistence.

We repeated the experiment using the same lipid mixtures, but adding the lipid-soluble cosurfactant hexanol, at 1:5 alcohol/lipid ratio. Isoelectric complexes show lamellar structure, but those complexes transition to hexagonal complexes at much lower applied osmotic pressure (Fig. 3 *C*), and the required work for transition is $\approx 0.2\ kT$, as shown in Fig. 4. Our findings are consistent with the known action of hexanol to reduce membrane rigidity, estimated to decrease the Helfrich bending rigidity from $\approx 20\ kT$ to under 10 kT for our experimental conditions, and hence we can expect easier bending of a membrane around DNA molecules (2,13).

We have previously used a Poisson-Boltzmann based mean-field theory to account for electrostatic, elastic, and entropic mixing terms related to both lipid and salt (9,10) Our calculations show that the amount of work required to bend a flat lipid membrane exactly to the extent that it is bent in the hexagonal phase is close to 1.4 kT per DNA base when the membrane rigidity is 20 kT, and drops to $\approx 0.3\ kT$ when the membrane rigidity is 1 kT. This implies that the free energy required to transition from lamellar to hexagonal lipoplexes is predominantly devoted to counteract lipid bending elasticity. Thus, we show that the changes in the lipoplex free energy can be measured directly by adjusting environmental conditions, without the requirement to vary lipid composition or lipid content as done previously.

Our work demonstrates that it is now possible to evaluate the free energy changes associated not only with lipolex response to modified properties of lipid and salts in solution, but also with changes in water activity. We have also been able to show that large changes in lipoplex structure and geometry are possible in stressed and crowded environments. Such response to confined environments should be important for the action of lipoplexes and their efficiency in vivo.

SUPPORTING MATERIAL

Experimental procedures and one figure are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(09)00213-6.

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REFERENCES and FOOTNOTES

1. Safinya, C. R., K. Ewert, A. Ahmad, H. M. Evans, U. Raviv, D. J. Needleman, A. J. Lin, N. L. Slack, C. George, and C. E. Samuel. 2006. Cationic liposome-DNA complexes: from liquid crystal science to gene delivery applications. *Phil. Trans. Royal Soc. a*. 364(1847):2573–2596.
2. Koltover, I., T. Salditt, J. O. Radler, and C. R. Safinya. 1998. An inverted hexagonal phase of cationic liposome-DNA complexes related to DNA release and delivery. *Science*. 281:78–81.
3. Koynova, R., L. Wang, and R. C. MacDonald. 2006. An intracellular lamellar-nonlamellar phase transition rationalizes the superior performance of some cationic lipid transfection agents. *Proc. Natl. Acad. Sci. USA*. 103:14373–14378.
4. Alfredsson, V. 2005. Cryo-TEM studies of DNA and DNA-lipid structures. *Curr. Opin. Coll. Inter. Sci.* 10:269–273.
5. Simberg, D., D. Danino, Y. Talmon, A. Minsky, M. E. Ferrari, et al.. 2001. Phase behavior, DNA ordering, and size instability of cationic lipoplexes - Relevance to optimal transfection activity. *J. Biol. Chem.* 276:47453–47459.
6. Sternberg, B., K. L. Hong, W. W. Zheng, and D. Papahadjopoulos. 1998. Ultrastructural characterization of cationic liposome-DNA complexes showing enhanced stability in serum and high transfection activity in vivo. *Biochim. Biophys. Acta.* 1375:23–35.
7. Dan, N. L. 1997. Multilamellar structures of DNA complexes with cationic liposomes. *Biophys. J.* 73:1842–1846.
8. Bruinsma, R. 1998. Electrostatics of DNA cationic lipid complexes: isoelectric instability. *Eur. Phys. J. B*. 4:75–88.
9. May, S., D. Harries, and A. Ben-Shaul. 2000. The phase behavior of cationic lipid-DNA complexes. *Biophys. J.* 78:1681–1697.
10. Harries, D., S. May, W. M. Gelbart, and A. Ben-Shaul. 1998. Structure, stability, and thermodynamics of lamellar DNA-lipid complexes. *Biophys. J.* 75:159–173.
11. Podgornik, R., D. Harries, J. DeRouchey, H. H. Strey, and V. A. Parsegian. 2008. Interactions in macromolecular complexes used as nonviral vectors for gene delivery. *In* Gene and Cell Therapy: Therapeutic Mechanisms and Strategies 3rd ed. N. Smyth Templeton, editor. CRC press, Boca Raton. 443–485.
12. Parsegian, V. P., R. P. Rand, and D. C. Rau. 1995. Macromolecules and water: probing with osmotic stress. *Methods Enzymol*. 259:43–94.
13. Safinya, C. R., E. B. Sirota, D. Roux, and G. S. Smith. 1989. Universality in interacting membranes - the effect of cosurfactants on the interfacial rigidity. *Phys. Rev. Lett*. 62:1134–1137.