Direct Evidence for Counterion Release upon Cationic Lipid−DNA Condensation

K. Wagner,[†] D. Harries,[‡] S. May,[‡] V. Kahl,[†] J. O. Rädler,[†] and A. Ben-Shaul*[,‡]

Lehrstuhl für Biophysik, E22, Technische Universität München, James-Franck-Strasse 1, D-85748 Garching, Germany, and Department of Physical Chemistry and the Fritz Haber Research Center, The Hebrew University of Jerusalem, Jerusalem 91904, Israel

Received September 27, 1999. In Final Form: November 19, 1999

The cooperative condensation of DNA and cationic liposomes to form ordered aggregates in aqueous solution is associated with the release of partially bound counterions. We directly determine the extent of counterion release by separating the supernatant from the precipitated condensates, measuring the conductivity of the solution before and after the phase transition. The extent of counterion release is calculated for a range of lipid/DNA concentration ratios based on the nonlinear Poisson−Boltzmann theory. Both experiment and theory show maximal, essentially complete, release of counterions at the isoelectric point, where the positive (lipid)/negative (DNA) charge ratio is 1:1. Furthermore, at this point the entropic contribution to the condensation free energy is maximal and dominant.

I. Introduction

Oppositely charged macroions in solution attract each other, tending to form a bound complex. When separated, each macroion is surrounded by a diffuse layer of spatially confined counterions. Upon approach the fixed macroion charges partially (sometimes fully) neutralize each other, allowing the release of mobile counterions into the bulk solution, thereby increasing their translational entropy. This scenario suggests that macroion association in solution is to a large extent an "entropically driven" process.[1] Yet, the actual contribution of the counterion entropy to the association free energy depends on the detailed geometries and charge distributions of the separated and bound macroions, as well as on the salt concentration in solution.[1−3]

Counterion release has been studied extensively in the context of "bimacromolmolecular" association processes (e.g., the binding of a protein to DNA), which can be described in terms of chemical equilibrium theory.[1,2] Treating the "condensed" counterions as bound ligands, the association process can be expresses as $A \oplus n_A + B \oplus n_B \rightleftharpoons AB \oplus n_{AB} + \Delta n$, where $A \oplus n_A$ denotes a macroion A with n_A bound counterions etc., and $\Delta n = n_A + n_B - n_{AB}$ is, by definition, the number of counterions released upon pair formation; Δn can be inferred from the salt dependence of the (standard) reaction free energy. The theoretical interpretation of these results relies on structural and thermodynamic-electrostatic models for calculating the n_i values.[1−3]

In this Letter we study the release of counterions in a thermodynamic *phase transition* rather than in a bimolecular association process. The specific transition considered here is the mutual condensation of DNA and oppositely charged (cationic) lipids into an *ordered composite phase* of a well-defined, experimentally resolved, structure.[4−9] Furthermore, the macroscopic aggregates formed in this process can be separated from the embedding solution, allowing direct experimental determination of the amount of counterions released by conductivity measurements of the supernatant solution.

The collapse of a polyelectrolyte gel upon the addition of oppositely charged surfactant molecules is another interesting phase transition involving the release of counterions.[10] The surfactants micellize within the polyelectrolyte network, releasing the counterions into the external solution. Concomitantantly, the osmotic pressure inside the gel decreases and the network collapses. Unlike in the condensation process studied in the present work, the surfactant-induced collapse of a polyelectrolyte network involves important elastic (polymer) and self-assembly (surfactant) free energy contributions, which complicate the evaluation of the purely electrostatic effects. Clearly, however, in both types of transitions counterion release is a major mechanism.

In the particular system investigated here we have used linear DNA and mixed vesicles, composed of the cationic lipid (CL) dioleoyltrimethylammonium-propane (DOTAP) and the neutral ("helper") lipid dioleoylphosphatidylcholine (DOPC). The CL−DNA condensates are of great interest as DNA delivery vectors in gene therapy, in which context they are often called "lipoplexes".[4] Here, however, we focus on their unique physicochemical characteristics. In particular, their ability to optimize the charge distribution of the cationic lipids so as to achieve maximal stability.

The aggregates formed in the above lipid−DNA mixtures are smectic-like arrays of stacked lipid bilayers with DNA monolayers sandwiched between them; hereafter the L_α^C phase.[5,6] Their detailed structure has been determined by X-ray diffraction experiments, revealing that the DNA strands within each monolayer are parallel and

[†] Technische Universität München.

[‡] The Hebrew University of Jerusalem.

(1) Record, M. T., Jr.; Anderson, C. F.; Lohman, T. M. *Q. Rev. Biophys.* II **1978**, *2*, 103.

(2) Sharp, K. A.; Friedman, R. A.; Misra, V.; Hecht, J.; Honig, B. *Biopolymers* **1995**, *36*, 245.

(3) Palkar, S. A.; Lenhoff, A. M. *J. Colloid Interface Sci.* **1994**, *165*, 177.

(4) Felgner, P. L.; Gadek, T. R.; Holm, M.; et al. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 7413. Felgner, P. L. *Sci. Am.* **1997**, 27, 86.

(5) Rädler, J. O.; Koltover, I.; Salditt, T.; Safinya, C. R. *Science* **1997**, *275*, 810. See also: Salditt, T.; Koltover, I.; Rädler, J. O.; Safinya C. *Phys. Rev. Lett.* **1997**, *79*, 2582.

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

(7) Harries, D.; May, S.; Gelbart, W. M.; Ben-Shaul, A. *Biophys. J.* **1998**, *75*, 159.

(8) May, S.; Ben-Shaul, A. *Biophys. J.* **1997**, *73*, 2427.

(9) Bruinsma, R. *Eur. Phys. J. B* **1998**, *4*, 75.

(10) Khokhlov, A.; Starodubtzev, S.; Vasilevskaya, V. V. *Adv. Polym. Sci.* **1993**, *109*, 123.

10.1021/la991268a CCC: $19.00
Published on Web 12/28/1999

equidistant. The repeat distance, d, depends on the mole fraction of cationic lipid (CL) in the mixture, ϕ, and the ratio between the total negative (DNA) and positive (CL) charges, ρ.

Knowing the detailed geometry of the L_α^C phase we can directly compare *theory and experiment* for the very same system. To this end we have solved, numerically, the nonlinear Poisson−Boltmann (PB) equation for the L_α^C phase and its separate (DNA and lipid bilayer) constituents, as a function of ϕ and ρ. Our PB calculations account explicitly for the fluidlike nature of the lipid layers and their ability to modulate their 2D charge distributions. The details of the electrostatic calculations as well as of the thermodynamic equations governing the phase behavior of the system are given elsewhere.[7] Here we focus on the extent of counterion release in CL−DNA condensation and calculate the entropic and energetic changes in this process.

The L_α^C phase is stable over a range of ρ values: from negative ($\rho < 1$, i.e., excess DNA over CL) to positive "overcharging" ($\rho > 1$).[5,7,9] Of special interest is the *isoelectric point* ($\rho = 1$), in which vicinity the L_α^C phase is most stable.[7] In this work we show that the isoelectric point also marks maximal, nearly complete, release of counterions, as well as maximal and dominant entropic contribution to the condensation free energy.

II. System and Model

Consider an aqueous salt solution of volume V at temperature T, containing double-stranded DNA of total length D and $N = N_+ + N_0$ lipid molecules per unit volume, N_+ cationic and N_0 neutral lipids. The concentration of fixed negative (DNA) charges in the system is $M_- = D/bV$, where $b \approx 1.7$ Å is the mean distance between negative charges along the DNA axis. Thus, $\phi = N_+/N$ and $\rho = N_+/M_-$.

At and around the isoelectric point the system is monophasic; that is, all lipid and DNA molecules are incorporated in L_α^C aggregates.[5,7] This implies $d = (a/2\phi)\rho$ where a is the average area per lipid molecule. For the lipid mixture considered here $a \approx 70$ Å^2 is nearly constant for all ϕ. Upon increasing ρ beyond $\rho^* = 1$, the added lipids are first accommodated by the complexes, thereby d increases and the condensates become "positively overcharged". Beyond a certain $\rho = \rho_2 > 1$, the L_α^C phase coexists with an excess bilayer phase (typically $\rho_2 < 2$). Similarly, when ρ falls below a certain $\rho = \rho_1 < 1$ the system is again biphasic, containing complexes and excess ("naked") DNA. Both ρ_1 and ρ_2 depend on ϕ and salt concentration, n^0.[5,7]

The L_α^C condensates are periodic structures in the plane (x,y) perpendicular to the DNA axis, translationally invariant along the z direction (at least over a range comparable to the DNA persistence length, $l_P \approx 500$ Å). We define the L_α^C unit cell as consisting of two (halves of) DNA segments of length b, intercalated between two lipid monolayers, each of area $b \times d$. Note that, by definition, the unit cell contains one DNA charge.

Let m_+^C and m_-^C denote the excess numbers of counterions within a unit cell relative to the bulk solution. Thus

$$m_\pm^C = b\int\int \mathrm{d}x\,\mathrm{d}y\,[n_\pm^C(x,y) - n^0] \qquad (1)$$

where $n_\pm^C(x,y)$ denotes the local concentrations of counterions within the complex. The integration extends over the cross-sectional area, $d \times h$, of the unit cell; $h \approx 26$ Å denoting the distance between apposed bilayer surfaces. We calculate the local ion concentrations using $n_\pm^C = n^0 \exp(\mp e\psi/k_BT)$, where $\psi = \psi(x,y)$ is the electrical potential, k_B is Boltzmann's constant, and e is the elementary charge. For given ρ, ϕ, and n^0 we obtain ψ by solving, numerically, the nonlinear PB equation for the unit cell parameters. The DNA molecules are modeled as rigid rods, 20 Å in diameter, with the negative phosphate charges uniformly distributed over their cylindrical surface. This implies the familiar (constant surface charge density) boundary condition at the rod surface. For a given cationic/nonionic lipid composition the average surface charge on the lipid surfaces is fixed. However, because the lipid monolayers are 2D fluid mixtures, the cationic and neutral lipids may "demix" locally, thus lowering the electrostatic interaction energy. This, in turn, requires the addition of a lipid demixing entropy term to the "usual" PB free energy, resulting in a self-consistent (constant electrochemical potential) boundary condition on ψ at the lipid surface.[7]

For an isolated lipid bilayer, charge neutrality implies that the surface excess concentration of counterions is $m^B = 2db\int \mathrm{d}z[n_+^B(z) + n_-^B(z) - 2n^0] = 2db\phi/a$; for monolayer area equal to that in a unit cell. The excess number of counterions around a DNA segment of length b is $m^D = 1$. The number of counterions released into solution upon complex formation is $\Delta m = (m^D - m_+^C) + (m^B - m_-^C) = (1 + 2db\phi/a) - (m_+^C + m_-^C) = 2(1 - m_+^C)$, per unit cell. (The last equality reflects charge neutralization, $m^D + m_-^C = m^B + m_+^C$.) The corresponding (small) change in free ion concentration is $2\Delta n^0 = K\Delta m$, where K is the number of unit cells, per unit volume. As an operational definition of the extent of counterion release we use the quantity

$$\xi = \frac{2\Delta n^0}{N_+ + M_-} = 1 - \frac{K(m_+^C + m_-^C)}{N_+ + M_-} \qquad (2)$$

The first expression for ξ is measurable while the second can be calculated using PB theory (and the thermodynamic coexistence equations), enabling comparison of theory with experiment.

On the basis of PB theory we have calculated ξ as a function of ρ for a low salt solution (4 mM NaCl, corresponding to a Debye screening length of $l_D = 50$ Å) containing DNA and an equimolar ($\phi = 0.5$) cationic/neutral lipid mixture. The phase boundaries, ρ_1 and ρ_2, were determined by solving the thermodynamic coexistence equations.[7] It can be shown theoretically that ξ exhibits different functional dependencies on ρ in the three regimes of the phase diagram. When the system is monophasic ($\rho_1 \le \rho \le \rho_2$), $K = M^-$ and hence $\xi = 1 - (m_+^C + m_-^C)/(1 + \rho)$. This expression is also valid for $\rho > \rho_2$, i.e., when complexes coexist with an excess bilayer phase, implying a linear decrease of ξ with $1/\rho$ in the limit $\rho \to \infty$. When $\rho < \rho_1$, i.e., when complexes coexist with naked DNA, $K = N^+a/2d_1b\phi = N^+/\rho_1$ and hence $\xi = 1 - (m_+^{1,C} + m_-^{1,C})/(\rho_1(1 + 1/\rho))$, implying $\xi \sim \rho$ as $\rho \to 0$.

III. Experimental Section

The experiment is schematically illustrated in Figure 1. CL−DNA complexes were formed spontaneously upon mixing cationic vesicles and DNA. At high lipid−DNA concentrations the complexes are well above micrometer size and could easily be separated from the aqueous phase by centrifugation and filtration. Subsequently the number of released counterions was determined by the increase in conductivity of the supernatant. The concentration of free DNA in the supernatant was also measured by optical absorption at 260 nm. The experiments were carried out for 4 mM NaCl solutions, enabling direct comparison with theory.

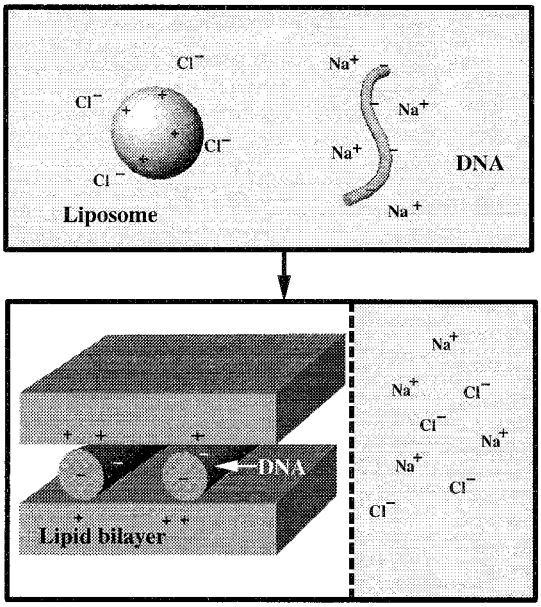

Figure 1. Schematic illustration of cationic liposomes and DNA condensing into a lamellar lipid−DNA complex. Counterions are released into the excess water phase.

To prepare the solutions, calf thymus DNA (Sigma) was exhaustively dialyzed against 4 mM NaCl salt solution. Mixed DOPC/DOTAP (Avanti Polar Lipids) vesicles were prepared in chloroform, dried in a vacuum, and subsequently resuspended and sonicated in salt or Millipore solution. Typically 200 μL of the vesicle stock solution (5 mg/ml) was added to a known volume of DNA solution (1 mg/mL) in an Eppendorf cup. The samples were well mixed, allowed to come to equilibrium overnight, and then centrifuged at 12000*g*, and the supernatant filtered through 0.2 μm filters. The optical density at 260 nm was measured in a Perkin-Elmer UV−vis spectrometer.

The conductivity of the supernatant (40 μL) was measured in a home-built setup designed for small sample volumes. The sample was filled into a glass capillary 1.2 mm in diameter and 5 cm in length (Brand, Wertheim, Germany). Two Ag/AgCl electrodes (EP08, WPI, Berlin, Germany) were inserted at the ends of the capillary. A Lab View routine automatically applied a voltage ramp of ±5 V, determining the conductivity from the slope of the voltage−current characteristic. A calibration curve of standard NaCl solutions was obtained, which showed good agreement with the classical Kohlrausch dependence of the molar conductivity on concentration, $\Lambda = \Lambda_\infty - ac^{1/2}$. The molar conductivity of dilute solutions of NaCl, free DNA, and sonicated lipid vesicles yielded $\Lambda_\infty^{NaCl} = 12 \times 10^{-3}$ S m^2/mol,[11] $\Lambda_\infty^{DNA} = 1.22 \times 10^{-3}$ S m^2/mol(bp), and $\Lambda_\infty^{lipid} = 0.15 \times 10^{-3}$ S m^2/mol, respectively. To determine the number of released counterions, the DNA conductivity was subtracted from the total conductivity of the supernatant. This correction applies to the $\rho < \rho_1$ regime, where free DNA coexists with complexes. In the $\rho > \rho_2$ regime some free lipid vesicles might be present in the supernatant despite filtration but do not significantly affect the total conductivity, as the molar conductivity of liposomes is a hundred times smaller than that of NaCl.

IV. Results and Discussion

The experimental and theoretical results for ξ are shown in Figure 2a. Theoretically, we find that $\xi \simeq 1$, i.e., complete counterion release, at the isoelectric point, $\rho = 1$. The experiments show maximal release at a slightly larger value of ρ; the difference may be due to the finite size of the condensate and other experimental uncertainties. The fractional release of counterions decreases monotonically on both sides of the isoelectric point. Within the one-phase region ($\rho_1 \le \rho \le \rho_2$) the decrease is linear, reflecting the negative ($\rho < 1$) and positive ($\rho > 1$) "overcharging" of the complex by fixed charges. For the system considered, the calculations yield $\rho_1 \simeq 0.77$ and $\rho_2 \simeq 1.3$. At both ρ_1 and ρ_2 there is a change in the slope of the ξ vs ρ curve, corresponding to passages between the one- and two-phase regions. At ρ_1 the change in slope is hardly noticeable, both theoretically and experimentally. However, the complete complexation of DNA at ρ_1 is clearly reflected in Figure 2b, which shows α, the fraction of free DNA in solution. The second phase boundary ($\rho = \rho_2$) is indicated, both experimentally and theoretically, by the change in slope of the ξ versus ρ curve.

In Figure 3 we show how $\Delta F = \Delta E - T\Delta S$, the formation free energy of the L_α^C phase (per unit cell), varies with ρ, for $\phi = 0.5$, and $n^0 = 4$ mM. $-T\Delta S$ is the contribution to ΔF from the entropy gain associated with the release of

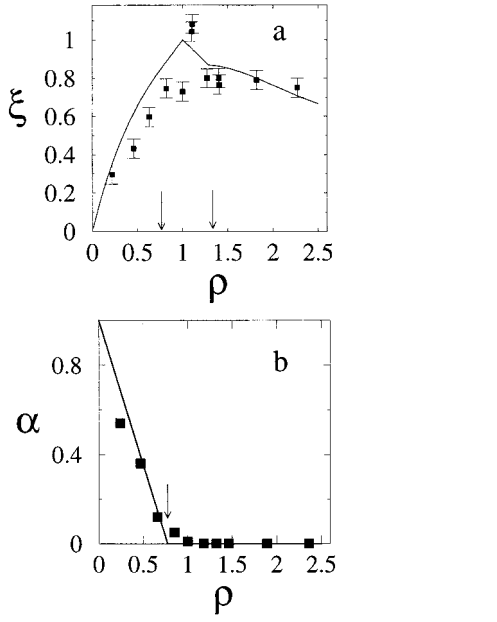

Figure 2. (a) The fraction of counterions released upon complex formation as a function of the CL/DNA charge ratio, for an equimolar lipid mixture, $\phi = 0.5$. The results from conductivity measurements are shown as solid squares. The solid curve is the theoretical prediction for the 4 mM NaCl solution. The arrows mark the theoretical phase boundaries, ρ_1 and ρ_2. (b) The fraction, α, of free DNA in solution, as determined by optical absorption at 260 nm (squares) and the theoretical calculations (solid curve). The arrow marks ρ_1.

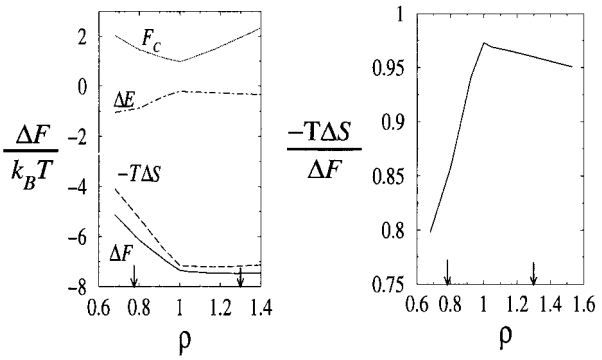

Figure 3. (left) ΔF the formation free energy of the L_α^C phase, per unit cell, as a function of the lipid/DNA charge ratio, ρ. $-T\Delta S$ and ΔE are the counterion entropy and electrostatic energy contributions, respectively. F_c is the changing free energy of the complex. (right) The relative contribution of the counterion entropy to the formation free energy. The arrows mark the phase boundaries.

(11) *Handbook of Chemistry and Physics*, 78th ed.; Chemical Rubber Publishing Co.: Boca Ratan, FL, 1997; p 5.

counterions, whereas ΔE is the change in the electrostatic energy (in which we include the very small contribution resulting from the lipid demixing entropy). ΔF is the difference between the charging energies of the condensate and its separate components. The most striking result of these calculations is the large contribution of the counterion release entropy, reaching its absolute and relative maximum ($-T\Delta S/\Delta F \approx 0.97$, $\Delta F \approx -7.5k_BT$) at the isoelectric point, $\rho = 1$. Note that ΔF is minimal at ρ slightly larger than 1; the moderate increase of ΔF at larger ρ arises from the weak repulsion between the apposed cationic monolayers.[7]

The maximum of ΔS at isoelectricity is not surprising, because at this point electrical neutrality can be achieved by the fixed macroion charges. When ρ is less than 1, positive counterions must be present within the condensate. Since d, the distance between DNA strands, decreases with ρ, a larger number of highly confined counterions enter the complex as ρ decreases, explaining the steep rise in $-T\Delta S$ on this side of the isoelectric point. Additional, negative, counterions also enter the complex when ρ increases beyond 1. Here, however, d increases while the spacing between the confining bilayer surfaces, h, is constant. Since h is comparable to the screening length of the isolated lipid layers the increase in $-T\Delta S$ in this regime is quite moderate.

Somewhat less expected is the small value of $|\Delta E|$, reaching its minimum at $\rho = 1$. This is because the electrostatic attraction between the DNA and lipid surfaces is largely compensated by the electrostatic repulsion between the apposed DNA and cationic lipid surfaces.

A qualitative estimate of the entropy gain associated with counterion release can be obtained as follows. Let $\bar{n}_+$ and $\bar{n}_-$ denote, respectively, the "effective" concentrations of counterions in the vicinity of the lipid bilayer and DNA surfaces prior to complexation. Now suppose, based on our calculations, that all counterions which are not needed for charge neutrality in the bound state are indeed released. For $\rho = 1$, this implies $\Delta S \approx 2k_B \ln(\bar{n}/n^0)$, where $\bar{n} = (n_+n_-)^{1/2}$ is the mean "effective" concentration of counterions in the diffuse layers. Rather than estimating ΔS using $\bar{n}$, let us estimate $\bar{n}$ using the calculated value $T\Delta S = 7.5k_BT$. For $n^0 = 4$ mM this implies $\bar{n} \approx 0.17$ M. Noting that the areas per fixed charge on the membrane and DNA surfaces are similar, $\approx$120 Å, we obtain $l_{eff} \approx 80$ Å for the "effective thickness" of the diffuse counterion layers. This value is comparable to the screening length of the, separated, charged surfaces, which should be on the order of the Debye length $l_D = 50$ Å.

V. Conclusion

We have shown that the formation of the condensed lamellar CL−DNA phase is driven by the release of counterions. Our calculations reveal that the entropic contribution to the association free energy is dominant, essentially complete at the isoelectric point. More generally, the entropic and energetic components of the complex formation free energy should depend sensitively on its geometry. It is expected, for instance, that the energetic contribution to the formation free energy of the hexagonal (H_{II}^C) CL−DNA phase is larger than that of the L_α phase, owing to the more favorable (concentric) configuration of the oppositely charged surfaces in this geometry.[6,8] Also, while our experiments and calculations apply for CL−DNA complexes, the phenomenon of counterion release is relevant to any system of oppositely charged macroions, for instance, complexes involving high molecular weight polyelectrolytes.[10,12]

Acknowledgment. We thank Erich Sackmann, Bill Gelbart, Barry Honig, and Adrian Parsegian for helpful discussions. We also thank the BMBF-03SA5TU10 and the SFB 266-C14 (K.W., V.K., and J.O.R.). A.B.-S. thanks the US−Israel Binational Science Foundation (Grant 97-205) and the Israel Science Foundation (Excellence Center Grant 8003/97) for financial support. D.H. thanks the Clore Foundation for a doctoral Fellowship and S.M. the Minerva foundation for a postdoctoral Fellowship. The Fritz Haber Center is supported by the Minerva Foundation, Munich.

LA991268A

(12) Dautzbenberg, H. *Macromolecules* **1997**, *30*, 7810.