Solubility and Charge Inversion of Complexes of DNA and Basic Proteins

Eric Raspaud,[1,*] J. Pelta,[2] M. de Frutos,[1] and F. Livolant[1]

[1]*Laboratoire de Physique des Solides, CNRS UMR 8502, Université Paris Sud, 91405 Orsay Cedex, France*

[2]*Groupe Microenvironnement et Comportements Cellulaires, Université de Cergy-Pontoise, 2 Avenue Adolphe Chauvin, 95302 Cergy-Pontoise Cedex, France*

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The basic proteins, protamines and histones H1, are known to condense DNA *in vivo*. We examine here their ability to condense and solubilize *in vitro* linear DNA [and a synthetic polyanion, Poly(Styrene-Sulfonate) or PSS] at low ionic concentrations by varying the charge concentration ratio. Phase separation is observed in a very narrow range of ratios for short DNA and PSS; on both sides of this range, polydisperse and charged complexes are formed. A charge inversion is detected. For long DNA chains however, a different behavior is observed: the complexes are not soluble in excess of proteins.

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From viruses to eukaryotic cells, DNA is tightly packaged in order to store, transport and preserve the genetic material. In eukaryotic cells, proteins such as histones and protamines condense DNA. They are basic (positively charged) and interact with the negative DNA charges. Their association originates primarily from electrostatic interactions. Understanding how these interactions control the formation of proteins-DNA condensate is therefore of fundamental interest in biology, with, moreover, potential therapeutic applications in gene delivery systems. It is also a fascinating challenge in physics because nontrivial electrostatic effects come out beyond a simple charge neutralization effect: attraction between like-charged chains and overcharging effect. According to recent theoretical approaches [1], one of the major forces driving the complexes formation derives from a strong correlation effect between condensed charges. According to other approaches, that were initially devoted to this specific question three decades ago [2], the force derives from the entropic gain due to the release of counterions initially condensed onto the polyelectrolytes.

How proteins and DNA attract each other avoiding or inducing soluble collapsed chains, an aggregation, or a macroscopic phase separation remains an open question that we tackle here. The typical charge number of the condensing proteins being about 20–50, we may wonder whether the formation of proteins and DNA condensates is comparable to the phase separation observed when small polycations condense DNA [3] or to the complexation of DNA by cationic vesicles and colloids [4,5]. In particular, are proteins able to overcharge DNA? Two proteins, histones H1 and protamines, were used here. Histones H1 achieve the chromatin compaction in the nucleus of eukaryotic cells while protamines replace histones during spermatogenesis and ensure the compaction of chromosomes inside the sperm heads. Our histones H1 (extracted from calf thymus [6]) and salmon protamines chloride (Sigma Chemical Co., St. Louis, MO) are composed of 220 (63 basic and 13 acidic) and 32 (21 basic) amino acids respectively [7]. This leads to net positive structural charges $q_{H1} = +50e$ and $q_{\text{protamine}} = +21e$, e denoting the elementary charge. Short DNA fragments about 150 base pairs long corresponding to one persistence length, and long semiflexible chains of lambda DNA (48500 bp) (Invitrogen, Carlsbad, CA) were used; the two structural charges are equal to $Q_{\text{DNA}} = -300e$ and $-9.7 \times 10^4 e$, respectively. In addition to these systems and in order to generalize this study, linear DNA was replaced by a synthetic polymer, highly charged and more flexible than DNA, the sodium Poly(Styrene-Sulfonate) Na-PSS of structural charge $Q_{\text{PSS}} = -1620e$ (Fluka, Buchs, Switzerland). The solubility, charge, and structure of the condensates were investigated.

The condensates solubility was studied by the centrifugation method previously used to build up the phase diagram of polyamine-DNA solutions [3]. While the final DNA or PSS concentration is fixed (mostly 10 mg/l), different amounts of proteins are added to different samples. After incubation and centrifugation, the percentage of PSS or DNA remaining in the supernatant is determined by UV absorption [8] and plotted versus the charge ratio [cations]/[anions] (cf. conversion factors in Ref. [9]). Typical results are illustrated in Figs. 1(a) for protamines and 1(b) for histones H1. The curves shape is similar for the short DNA fragments and for the PSS chains whatever the protein is. Precipitation occurs within a very narrow range centered around a ratio value that will be noted X_c in the following. This value may differ from one; it depends on the polyanion and protein species. In all cases, a lower X_c ratio is required to precipitate the PSS chains compared to the DNA fragments. Similarly lower concentrations of protamines than H1 induce the phase separation. These differences may come from different origins: the flexibility and the backbone hydrophobicity, the number of initially condensed counterions, and the translation entropy of the different components. For instance, a preferential binding of the H1 *C*-terminal domain to DNA has been already suggested [10].

This behavior may be compared to the case where DNA or PSS are condensed by small polycations like polyamines

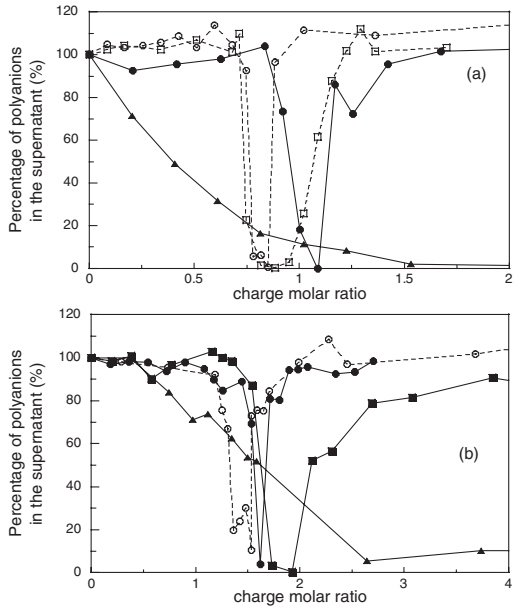

FIG. 1. Percentage of polyanions remaining in the supernatant and measured from UV absorbance. Protamines (a) or histones H1 (b) have been added to the solution of PSS (dashed line) or of DNA (solid line) at different molar ratios X. The experimental conditions are the following: PSS was diluted at 10 mg/l in water (○) or in 10 mM NaCl (□) and centrifuged at 11 000 g; DNA fragments were diluted at 10 mg/l in 2 mM (●) or 10 mM (■) monovalent salt and centrifuged at 2750 × g. Lambda-DNA (▲) was diluted at 5 mg/l in 2 mM monovalent salt without centrifugation but with an incubation time of two weeks (a). In (b), lambda-DNA was diluted at 10 mg/l in 10 mM monovalent salt and centrifuged at 11 000 g.

(3–4 positive charges). In contrast to the present case, the range of small polycation concentrations for which precipitation takes place may extend over about 4 orders of magnitude [3]. Therefore, increasing the charge and size of polycations from 3–4 to 21–50 strongly affects the conditions required to obtain a phase separation. Actually, in dilute solution and in the presence of monovalent salt, the onset of precipitation always occurs when polyamines are in excess, indicating that part of them remain free in solution (see for instance Ref. [11]). Here the narrow range located at a molar charge ratio close to 1 indicates that most of the proteins are bound. This difference could be understood in terms of translation entropy which cannot be neglected for the small polycations. In both cases, the macroscopic phase separation is due to some short-range attractive interactions inside the complexes. According to a certain approach [1,12], a short-range correlation energy is gained due to the regular arrangement of the condensed charges which repeal each others. These correlations can lead to an attraction between like-charged chains but also to overcharging. This short-range attraction competes with the long-range Coulomb repulsion between charged complexes, which may prevent their aggregation. For large polycations of negligible translational entropy, the phase separation domain is found very narrow and the polycations concentration of its boundaries may read as $S_{c,d}/S_0 = 1 \mp \sqrt{|E_{\rm coh}/E_{\rm elect}|}$ where S_0 corresponds to the polycations concentration required to get neutral complexes, $E_{\rm coh}$ to the cohesive energy in the condensate (per polycation) and $E_{\rm elect} = 1/2qV$ to the energy associated with the electrostatic interaction polycation-polyanion electrostatic interaction (V being the electrostatic potential created by the free polyanion and q the polycations charge). For small polycations, however, their translational entropy should be added to the other energetic terms; that leads to an exponential broadening of the phase separation domain $S_{c,d}/S_0 = \exp[\mp(1/k_BT)\sqrt{|4E_{\rm coh}E_{\rm elect}|}]$ with k_BT the thermal energy. Such an approach is qualitatively consistent with the difference observed experimentally when the polycation charge (and size) grows from the polyamines case to the present proteins case.

To go further, we investigated the salt effect as illustrated in Fig. 1 for the two protamines-PSS and H1–short-DNA complexes, respectively. Addition of monovalent salt (from 0 or 2 mM to 10 mM) leads to a broadening of the precipitation range, but only on the right side of the curves, where proteins are in excess: a larger amount of proteins is required to solubilize the complexes at higher salt concentration. According to the theoretical approach mentioned above, salt addition is expected to first screen interactions and to reduce the electrostatic energy $E_{\rm elect}$. Therefore the salt is expected to affect differently the boundaries $S_{c,d}$ for small and large polycations. For small polycations (entropy included), the difference $S_{c,d}/S_0 - 1$ goes to zero as long as salt is added, shrinking the phase separation domain as observed in the DNA-polyamide studies [3,13]. For large polycations (no entropy), the difference $S_{c,d}/S_0 - 1$ increases with the salt concentration, enlarging the precipitation domain. Both boundaries S_c and S_d are predicted to be affected in a symmetric way. For the two present couples, we observe an enlargement but the salt effect is not symmetric. Only the upper boundary S_d increases with the salt concentration in our conditions. Therefore the salt effect predicted in this approach cannot explain the present results. The asymmetric behavior rather suggests that the added salt acts on the complexes solubility (on their charge) mostly when proteins are in excess. In this range, the complexes charge is supposed to be inverted, of positive sign.

To determine the charge (or the electrophoretic mobility) as a function of the charge ratio, two types of zetameter were used (a Coulter Delsa 440SX and a Malvern Zetasizer Nano ZS) at relatively high concentrations (50–100 mg/l of PSS and short DNA) because of the low sensitivity detection. As suspected, mobilities turn from negative to positive values at a charge ratio close to X_c (indicated by arrows in Fig. 2). It can be noted, however, that the positive value measured for the Hl-DNA complexes was actually

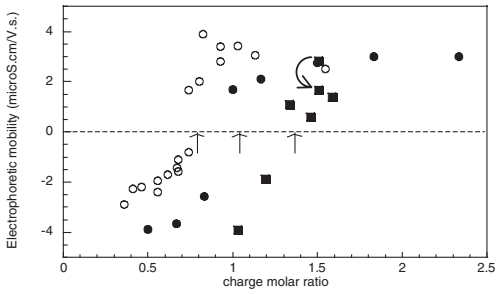

FIG. 2. Electrophoretic mobility of the different complexes in 10^{-8} S m/V s units as a function of the molar ratio. Symbols: ○ represents the couple protamine-PSS complex diluted at 0.1 g/l PSS in water, ● shows the couple protamine-DNA complexes fragment diluted at 100 mg/l DNA in 10 mM monovalent salt, and ■ is for the couple H1-DNA fragment diluted in 2 mM monovalent salt and at a DNA concentration ranging from 50 to 100 mg/l. The straight arrows point out the X_c locations which were estimated from the Fig. 1 for the three couples. The curved arrow indicates the variation of mobility observed during a measurement for the last couple.

not stable during the measurements. We suspect a kinetic growth of the complexes formed at this high DNA concentration.

The size of a few complexes was characterized by quasielastic light scattering for 10 mg/l of PSS diluted in water in the presence of different amount of protamines [14]. In Fig. 3 we report their typical diffusion coefficient $D = 1/\tau k^2$ versus the transfer vector k, the characteristic time τ being extracted from the relaxation function. As expected, two distinct behaviors are clearly distinguishable: (i) For [cations]/[anions] $= X_c$ (condition for macroscopic phase separation), D values are roughly independent of k and are very low compared to the others. On average D is equal to 3.5×10^{-13} m^2/s which leads to a micron size R_H according to the Stokes-Einstein relation $D = k_B T/(6\pi\eta_s R_H)$ (η_s being the solvent viscosity). (ii) For the other samples of charge ratios equal to $X_c/2$, 2 X_c and 3.8 X_c (soluble complexes), all the data superimpose and the diffusion coefficient D strongly depends on

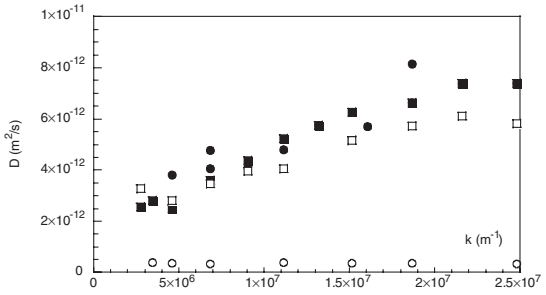

FIG. 3. Diffusion coefficient D (in m^2/s) as a function of the wave vector transfer k (in m^{-1}) for a few protamine-PSS complexes diluted in water at 10 mg/l PSS at ratios equal to X_c (○), $1/2$ X_c (●), $2X_c$ (■), and $3.8X_c$ (□) with $X_c = 0.79$.

k. A characteristic size of 90 nm is estimated from the intercept value. The high k dependence of D indicates that the complexes are highly polydisperse and are composed of several chains. It may be noted that for [cations]/[anions] $= 3.8X_c$, we observed a polydispersity and large dispersion of size from one sample to another that persists even after one month of incubation. This "nonreproducibility" suggests that the final structures are extremely dependent on the formation kinetics; ''kinetically trapped'' structures were already reported for the protamine–long-DNA complex [15].

Finally, the DNA length effect was also investigated (see in Fig. 1). Surprisingly the shape of the curves recorded for lambda-DNA and the two proteins clearly differs from the others. The percentage of DNA remaining in the supernatant first decreases progressively as the proteins amount increases and then stays at zero in excess of proteins. Therefore, in our conditions, an excess of proteins is not able to solubilize the protein–long-DNA complexes. This difference of solubility between long and short DNA is quite puzzling. The screening length of the Coulomb repulsion between complexes being lower than the DNA sizes, the repulsion should not depend on the DNA length. This is also true for the short-range attraction between complexed charges or for the entropic gain due to the counterions' release.

Because the concentration we used in Fig. 1 ($C_{\mathrm{DNA}} = 5$ or 10 mg/l) is not so far from the overlap concentration of the long lambda chain, we may wonder if large micron-sized trapped structures could be favored to the detriment of small isolated complexes, thus explaining the solubility difference between short and long DNA complexes. Using the light scattering technique on very diluted long DNA ($C_{\mathrm{DNA}} = 1$ mg/l) in excess of proteins, we found the instantaneous formation of quite small and isolated complexes. When using protamines, a diffusion coefficient $D = 5.3 \times 10^{-12}$ m^2/s is measured, corresponding to the typical size of long DNA toroidal globules (45 nm). In excess of H1, a lower value is observed $D = 2.0 \times 10^{-12}$ m^2/s leading to a typical size of 120 nm. Experiments have been repeated on the same samples one day later: we found lower D values indicating an increasing size. Therefore, even if the initial complexes formed with long DNA chains are isolated and small, they became micron-sized in agreement with the centrifugation data.

To sum up, (I) when proteins associate with flexible PSS chains and with DNA fragments (rods), they behave like cationic vesicles leading to self-assembled complexes of similar characteristic in terms of stability, size, and charge inversion [4]. These aggregates could be thermodynamically controlled or could be the result of kinetic barriers as predicted by different authors [16]. We plan to study their stability over long period. (II) We observe two different kinetics: the instantaneous phase separation and a slow aggregation process (for long DNA chains). A similar

behavior was observed when mixing colloids with oppositely charged polymers [17]. (III) Unlike the short polycations-DNA systems, addition of low amounts of salt enlarges the concentrations range where an instantaneous phase separation occurs. Only the upper boundary is affected, suggesting that the added salt screens the repulsion but also acts on the overcharging process which controls the aggregates stability. Such an effect could be related to the salt effect predicted when counterions (initially condensed) are released; the energy gained from the release depends logarithmically on the Debye screening length and diminishes when adding salt [2]. (IV) the two proteins behave in a similar manner. The size of long DNA collapsed chains differs, however. The size reduction when protamines are used instead of histones could be one of the benefit for the cells to exchange them during the spermatogenesis. (V) In sperm cells, protamines could compensate the DNA charges [18] and the condensed chains are intricate. In somatic cells, histones electro-neutralize about half of the DNA charges and the condensed chains are territorially separated and individualized. One may wonder if these two situations could correspond to our two dynamic states: the instantaneous phase separation in the first case and the isolated collapsed chains which slowly aggregate in the second case, their secondary aggregation being possibly prevented *in vivo* by the binding of other proteins like condensins which are known to stabilize chromosomes.

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*To whom correspondence should be addressed.
Email address: raspaud@lps.u-psud.fr

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