Aggregation of Nucleosomes by Divalent Cations

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ABSTRACT Conditions of precipitation of nucleosome core particles (NCP) by divalent cations (Ca^{2+} and Mg^{2+}) have been explored over a large range of nucleosome and cation concentrations. Precipitation of NCP occurs for a threshold of divalent cation concentration, and redissolution is observed for further addition of salt. The phase diagram looks similar to those obtained with DNA and synthetic polyelectrolytes in the presence of multivalent cations, which supports the idea that NCP/NCP interactions are driven by cation condensation. In the phase separation domain the effective charge of the aggregates was determined by measurements of their electrophoretic mobility. Aggregates formed in the presence of divalent cations (Mg^{2+}) remain negatively charged over the whole concentration range. They turn positively charged when aggregation is induced by trivalent (spermidine) or tetravalent (spermine) cations. The higher the valency of the counterions, the more significant is the reversal of the effective charge of the aggregates. The sign of the effective charge has no influence on the aspect of the phase diagram. We discuss the possible reasons for this charge reversal in the light of actual theoretical approaches.

INTRODUCTION

Nucleosome core particles are the structural units of eukaryotic chromatin. They are formed by the association of a 146-bp DNA fragment coiled around a protein octamer composed of four different histones (H2a, H2b, H3, H4). The particle has the shape of a cylinder, 110 Å in diameter and 60 Å thick. Its structure has been determined with high resolution (Lüger et al., 1997; Harp et al., 2000) with the exception of parts of the histone tails, which are highly positively charged and protrude from the particle. These nucleosome core particles are linked together by DNA to form ordered nucleosomal arrays, which are themselves highly compacted into chromatin by association with H1 histones and other proteins. However, chromatin is not a homogeneous and frozen structure. Cells regulate chromatin folding both temporally and spatially, and histones are dynamic components involved in this regulation through post-translational modifications (including acetylation, phosphorylation, methylations, etc.) which may take place on the histone tails. Many proteins have been shown to be involved in this remodeling of chromatin, which is suspected to be of great importance in the regulation of transcription or induction of mitosis for instance (Strahl and Allis, 2000). The compaction of chromatin arrays has also been extensively studied in vitro. It has been demonstrated that the polyelectrolyte character of DNA, nucleosome, and chromatin was responsible for the compaction of the fiber (Widom, 1986; Clark and Kimura, 1990). It was shown also that the condensation of the fiber can be achieved by addition of cations in the absence of H1 histones, but the integrity of the histone tails is absolutely required (Fletcher and Hansen, 1996; Widom, 1998). The presence of divalent cations is also necessary to reach the ultimate states of condensation of the fiber. Nevertheless, numerous questions remain open due to the complexity of the system.

In the present work we investigate the polyelectrolyte properties of solutions of isolated nucleosome core particles over the range of ionic conditions maintaining the stability of the nucleoprotein complexes. NCP can be viewed as colloids whose charges are heterogeneously distributed at the surface of the particle: negative charges carried by the DNA phosphate groups and positive charges carried by the lysine and arginine residues on the histone tails. We analyzed the effects of divalent cations Mg^{2+} and Ca^{2+}, which are widely distributed in biological systems and play an important role in many enzymatic activities related to replication, transcription, and recombination. These divalent cations can induce the compaction of the chromatin fiber (Widom, 1986) but are inefficient in condensing pure DNA in aqueous solution (Bloomfield et al., 1994, 2000). We show that both cations may induce the aggregation of isolated NCP under defined ionic conditions and we question the reasons for this aggregation. Indeed, the stability of the solutions of negatively charged polyelectrolytes in the presence of added multivalent salts depends on the chemical nature of the functional groups carrying the polyion charges and on their interaction with the cations. Two different mechanisms have been proposed to explain the aggregation phenomenon observed at low ionic strength in solutions of polyelectrolytes (Oosawa, 1971; Record et al., 1978). They correspond to two extreme cases depending on the value of the chemical affinity constant between the charged groups and the cations. For a low affinity, the electrostatic interaction leads to the counterion condensation in the vicinity of the macroion. In this case, the aggregation phenomenon is due to the electrostatic interaction resulting from the counterion condensation. On the opposite, for a strong affinity, a

Received for publication 27 December 2000 and in final form 20 April 2001.

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0006-3495/01/08/1127/06 $2.00

specific interaction of the multivalent cation at a particular binding site of the polyelectrolyte leads to the formation of a complex. This chemical association is thought to produce a hydrophobic complex by dehydration of the cation and of the charged group (Sabbagh and Delsanti, 2000). For an intermediate value of the affinity constant, in a static approximation, site-specific binding and condensed states can coexist.

The conditions of precipitation of nucleosome core particles have been determined experimentally and compared to the previous results obtained with spermine (4^+) (Raspaud et al., 1999). The role of the electrostatic interactions in the aggregation phenomena is analyzed. Moreover, we have investigated by electrophoretic measurements the effects of the addition of multivalent cations on the effective charge of the aggregates, which let us get information on the repulsion between the nucleosome core particles, for the different added salt concentrations.

MATERIALS AND METHODS

The nucleosome core particles (NCP) were prepared following the procedure described in Leforestier and Livolant (1997). After selective digestion of H1-depleted calf thymus chromatin with micrococcal nuclease (Pharmacia, Uppsala, Sweden), NCP were purified by gel chromatography over a Sephacryl S300 HR column. NCP were dialyzed against 3.5 mM TE buffer (Tris HCl 3.5 mM, EDTA 0.35 mM, pH 7.6) and concentrated up to 250 mg/ml by ultrafiltration in a pressurized cell (Amicon 8010 with a YM100 membrane). The stability of the core particles was checked by polyacrylamide gel electrophoresis. The length of the DNA extracted from the NCP was measured by polyacrylamide gel electrophoresis as 160 ± 10 basepairs.

Two experimental procedures were used to determine the phase diagram. For concentrations below 7 mg/ml NCP, different quantities of divalent salts ($MgCl_2$ and $CaCl_2$) were added to the NCP solution. The samples were vortexed and incubated at room temperature for at least 15 min and centrifuged at 11,000 × *g* for 10 min. We checked that a longer incubation (up to 48 h) and a faster centrifugation (40,000 × *g*) do not change the results. The NCP supernatant concentration was determined by absorbance measurements at 260 nm with a U-1000 Hitachi spectrophotometer. We calculated an absorption coefficient $A_{260} = 9.87\ cm^2\ mg^{-1}$ for our particles containing a 160-bp DNA segment. The curve obtained as a function of the salt concentration, C_{multi}, allows us to determine the precipitation and redissolution thresholds C_{precip} and $C_{redissol}$ (Fig. 1). These values are defined as indicated in Fig. 1. For higher NCP concentrations, the aggregation produces a turbidity of the solution. In these conditions, C_{precip} and $C_{redissol}$ were determined by visual inspection of the solution. An overlap region between the measured C_{precip} and $C_{redissol}$ ensures the coherence of both methods.

In conditions of phase separation, we determined the charge of the NCP aggregates by electrophoretic measurements performed with a Delsa 440 SX instrument (Coulter). These experiments were performed in noncentrifuged solutions at concentrations $C_{NCP} \sim 0.1$ mg/ml. An electric field is applied to the solution, inducing the movement of the particles. The measured mobility is related to the effective charge at the shear surface of the aggregates (Overbeek, 1952). This method does not allow measurement of the mobility of isolated NCP.

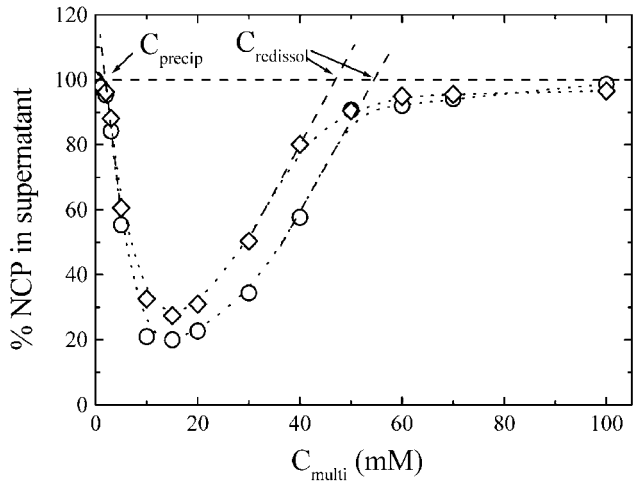

FIGURE 1 Percentage of NCP in the supernatant as a function of the Mg^{2+} (◇) and Ca^{2+} (○) concentration C_{multi}. The plotted data correspond to $C_{NCP} = 1$ mg/ml in a 3.5 mM TE buffer. The values of C_{precip} and $C_{redissol}$ are estimated as indicated by the arrows.

RESULTS AND DISCUSSION

Precipitation curves (Fig. 1) have been obtained for NCP concentrations ranging from 0.1 to 240 mg/ml in 3.5 mM TE and for divalent cation concentrations varying from 0 to 100 mM. Resulting phase diagrams are given in Fig. 2: the NCP concentration expressed in mM of accessible phosphates C_{ap} is plotted as a function of $z_{multi}C_{multi}$, where z_{multi} and C_{multi} are, respectively, the valence and concentration of

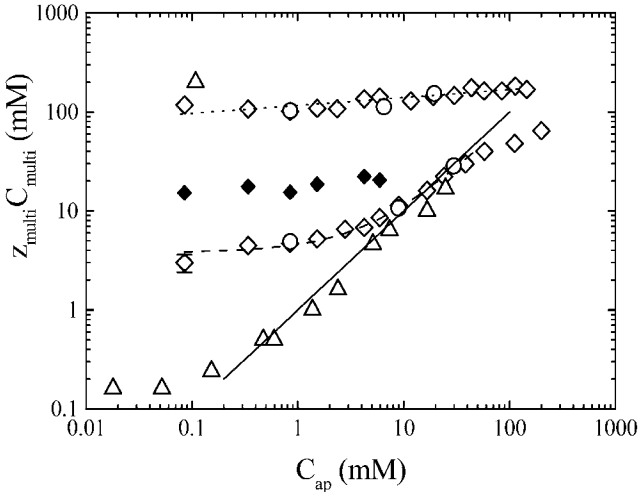

FIGURE 2 Phase diagram of NCP in TE 3.5 mM in the presence of different amounts of Mg^{2+} (◇), Ca^{2+} (○), and spermine^{4+} (△). The NCP concentration is expressed as the concentration of accessible phosphates C_{ap} (mM). The solid line corresponds to $z_{multi}C_{multi} = C_{ap}$. The dashed line corresponds to the fit of the precipitation concentrations $z_{multi}C_{precip} = 0.77 \times C_{ap} + 3.8$ and the dotted one to the redissolution values $z_{multi}C_{redissol} = 116.4 \times C_{ap}^{0.08}$. The magnesium concentration inducing the maximum precipitation of NCP is indicated by the symbol (◆).

the multivalent cations. We assume that the positively charged protein octamer neutralizes 130–134 of the 320 negative phosphate DNA charges (Khrapunov et al., 1997). As a consequence, the NCP structural charge Z_s is taken equal to 188 negative charges, and the relation between the NCP concentration C_{NCP} expressed in mg/ml and C_{ap} (accessible phosphates) in mM is given by $C_{ap}(mM) = 0.879 \times C_{NCP}(mg/ml)$.

The precipitation curves obtained by addition of $MgCl_2$ and $CaCl_2$ (Fig. 1) are similar, but a slightly higher efficiency is observed for $CaCl_2$. Despite this small difference, in a log-log plot the phase separation conditions for $MgCl_2$ and $CaCl_2$ correspond to the same concentration region: precipitation is observed for a low concentration of divalent salt and redissolution is obtained by further addition of salt. For polyelectrolytes carrying sulfonate and sulfate groups, Sabbagh and Delsanti (2000) have demonstrated that the existence of a redissolution limit is the signature of a system driven by the electrostatic interaction resulting from cation condensation. On the contrary, for polyacrylates, the absence of redissolution for the precipitation induced by divalent cations or La^{3+} indicates a complexation phenomena due to a site-specific interaction. In the present case, the reversibility of the phase separation by addition of multivalent cations points to an electrostatic origin of the mechanism. The similarity between phase diagrams obtained for Ca^{2+} and Mg^{2+} strengthens the idea of a nonspecific interaction.

Similar phase diagrams were obtained by Raspaud et al. (1998, 1999) for DNA, NCP, and chromatin fragments precipitated with polyamines (spermidine 3^+ and spermine 4^+). For comparison, the NCP/spermine data are plotted in Fig. 2. The precipitation region is strongly reduced when divalent cations are used instead of the tetravalent cations spermine. Raspaud et al. have proposed a division of the phase diagram into three regions corresponding to three regimes that have been interpreted within the framework of the "ion-bridging" model developed by Olvera de la Cruz et al. (1995). The present data reveal at least two of these regions.

For low NCP concentrations, the data can be fitted by the linear relation $z_{multi}C_{precip} = 0.77 \times C_{ap} + 3.8$ expressed in mM units (indicated in Fig. 2 by a *dashed line*). This regime extends to concentrations C_{ap} of the order of 30 mM. We can note that in this concentration range $z_{multi}C_{precip} > C_{ap}$, meaning that part of the multivalent cations are free in solution. This regime is reduced when divalent cations are replaced by spermine. Note that the NCP precipitation was determined in the presence of different concentrations of monovalent cations: for spermine experiments, NCP were diluted in 10 mM TE + 5 mM NaCl, and for divalent salts (Mg^{2+} and Ca^{2+}), in 3.5 mM TE (the latter value corresponds to the minimum monovalent salt concentration required to preserve the integrity of the core particles). At identical monovalent salt concentrations, the difference between spermine and magnesium would be enlarged. In agreement with our previous results (Raspaud et al., 1999), a reduction of the coexistence domain is observed by addition of monovalent cations. We checked that at $C_{ap} = 4$ mM the precipitation by Mg^{2+} is completely suppressed by addition of 70 mM NaCl. For higher NCP concentrations, the suppression of the precipitation is expected to occur at higher monovalent cation concentrations. This shrinking of the coexistence domain reveals a competition between the condensation of the different counterion species onto the surface of NCP. Consequently, a fraction of monovalent cations is probably condensed. As monovalent condensed cations do not produce any aggregation of the NCP, the attraction due to multivalent cations is reduced when the monovalent cation concentration increases.

For the intermediate range of DNA, NCP, and chromatin concentrations, a linear dependence of C_{precip} with the polyelectrolyte concentration was observed with spermine. This universal regime is independent of the monovalent salt concentration and appears to be a common feature for linear polyelectrolytes, and also for particles that can be considered as charged colloids, such as NCP (Raspaud et al., 1998, 1999). In this regime, precipitation occurs when the added cations nearly counterbalance the charges carried by the polyelectrolyte, that is, $z_{multi}C_{precip} \approx C_{ap}$ (indicated in Fig. 2 by a *continuous line*). As a consequence, the multivalent cations are mainly condensed while the monovalent ones are mainly free in the solution. Compared to the NCP/spermine system, this linear regime is only obtained here within a very small range of NCP concentration, at the junction of the two other regimes. This reduction is due to the important shrinking of the phase separation region. Such a situation was previously reported for DNA precipitated with spermine in the presence of 75 mM NaCl (Raspaud et al., 1998).

The redissolution process observed above a certain multivalent cation concentration was already described by Delsanti et al. (1994), Pelta et al. (1996), Saminathan et al. (1999), and Sabbagh and Delsantis (2000). Our results reveal a redissolution limit increasing with the NCP concentration. The data follow the power law $C_{redissol} = 58.2 \times C_{ap}^{0.08}$. Such an increase is also observed on poly(styrene sulfonate) chains (Delsanti et al., 1994) but was not observed experimentally for DNA (Raspaud et al., 1998). According to the model proposed by Olvera de la Cruz et al. (1995), the redissolution limit dependence on the polyelectrolyte concentration is given by the translational entropy of the chains. This term decreases as the chain length increases. As a result, the translational entropy term is negligible for long chains and the redissolution limit is governed by the interactions with the solvent molecules. This description is consistent with the constant value of the redissolution concentration observed for DNA and for long poly(styrene sulfonate) chains. For NCP and short poly(styrene sulfonate) chains, the variation of the redissolution limit reveals

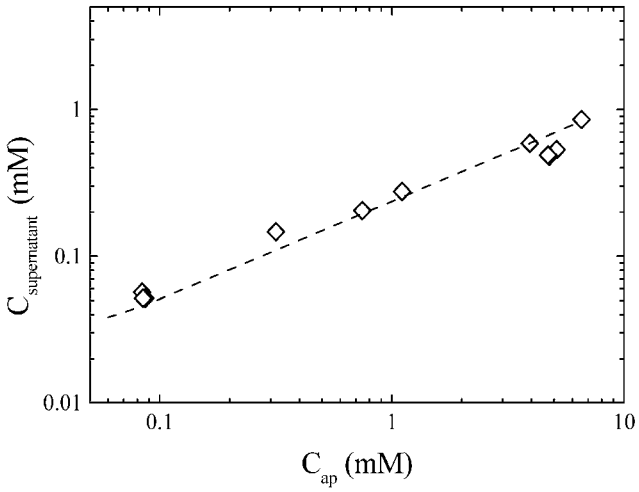

FIGURE 3 Minimum concentration of NCP in the supernatant as a function of the initial NCP concentration. NCP were diluted in a TE 3.5 mM buffer. The dashed line corresponds to a power law with an exponent 2/3.

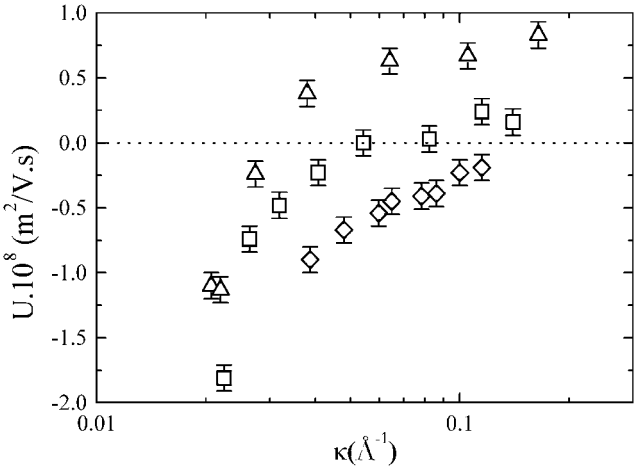

FIGURE 4 Electrophoretic mobility U of nucleosome aggregates obtained by addition of Mg^{2+} (◇), spermidine^{3+} (□), and spermine^{4+} (△) in 3.5 mM TE as a function of the inverse Debye screening length κ. The explored concentration ranges from 3.5 to 40 mM for Mg^{2+}, from 0.2 to 30 mM for spermidine^{3+}, and from 0.1 to 25 mM for spermine^{4+}. The aggregates are negatively charged for all the Mg^{2+} concentrations. A charge inversion is observed for spermine and in a smaller extent, for spermidine.

an increase of the entropy term. This dependence should also be observed experimentally for short enough DNA fragments. The influence of this term is also revealed by the strong dependence on the molecular species observed in the low concentration range (Raspaud et al., 1998).

As shown in Fig. 1, the precipitation rate presents a maximum for a certain multivalent cation concentration. This cation concentration is indicated in Fig. 2 for Mg^{2+} as a function of the initial NCP concentration. The corresponding NCP concentration in the supernatant $C_{\text{supernatant}}$ (Fig. 3) increases as a function of the initial NCP concentration. Such a behavior was also observed for DNA/polyamines with different monovalent salt concentrations (Raspaud et al., 1998). For NCP, the dependence is close to the ⅔ power law described for the DNA (indicated by the *dashed line* in Fig. 3). Because the precipitation rate is sensitive neither to the incubation time nor to the centrifugation conditions, we can hypothesize the presence of only two populations of clusters: large ones, which precipitate readily, and small ones (or single core particles), which remain in suspension for all tested conditions.

To study how the charge of the aggregates depends on the ionic conditions, the electrophoretic mobility of the NCP aggregates was measured over a large range of Mg^{2+} (3.5–40 mM), spermidine (0.2–30 mM), and spermine (0.05–25 mM) concentrations (Fig. 4). To allow a comparison whatever the valency of the aggregating agents, the mobility is plotted as a function of the inverse of the Debye screening length $\kappa = (2\pi l_B)^{1/2}[z_{\text{multi}}(z_{\text{multi}} + 1)\, C_{\text{multi}} + 2C_{\text{monoval}}]^{1/2}$, where l_B is the Bjerrum length (in water l_B = 7 Å) and C_{monoval} the monovalent cation concentration. A charge inversion is observed between 0.35 and 1 mM spermine. Compared to previous results (Raspaud et al., 1999), the neutrality is reached at a slightly lower spermine concentration. This difference can be related to the initial monovalent concentration (3.5 versus 10 mM TE). For spermidine, the charge inversion is observed to a smaller extent: the maximum mobility value remains close to zero. For Mg^{2+}, the aggregates remain negative over the whole explored concentration range.

These measurements characterize the charge on the shear surface of the NCP clusters. Shear occurs at a typical distance of the order of the Debye length κ^{-1} from the particle because, in our ionic concentration range, κ^{-1} is equal to or smaller than the NCP (radius ≈ 50 Å) and cluster size (Viovy, 2000). As a consequence, at this scale, the cluster charge variation should follow the charge variation of isolated NCP. The solvent layer moving with NCP contains ions, but also the basic protein tails of the core histones.

In a first-order approximation we neglect the effects due to the tail extension and we consider that they only contribute to the structural charge of the NCP. In this case, the value of the electrophoretic mobility gives information on the contribution to the total interaction of the electrostatic repulsion between the effective charge of isolated NCP. This effective charge is only regulated by the ion condensation phenomena. Several theories have been proposed to describe the precipitation and redissolution processes. In the mean-field approximations based on the Poisson-Boltzmann approach and on its linear Debye-Hückel form, the description of the condensation process neglects the ion-ion interactions. An equilibrium is reached between the thermal energy of the counterions and the electrostatic attraction between the ions and the polyelectrolyte. As a consequence,

the macroion is only partially neutralized, and whatever the particle geometry, the instability of the system occurs for a negative effective charge (Manning, 1978). The short-range attraction responsible for the aggregation phenomena is explained as due to the fluctuations of the condensed counterions, resulting in charge inhomogeneities (Oosawa, 1971; Rouzina and Bloomfield, 1996; Olvera de la Cruz et al., 1995). These charge fluctuations are related to the correlations between multivalent counterions. Recent models taking into account the strong counterion correlations (Shklovskii, 1999; Nguyen et al., 2000) show that this effect leads to a larger amount of condensed counterions, inducing a charge reversal of the macroions for the redissolution conditions. For a charged sphere, this approach predicts (Shklovskii, 1999) that the effective charge saturates at a positive value proportional to $z_{multi}^{1/2}$. Other authors (Solis and Olvera de la Cruz, 2000) have demonstrated within a two-state model that, depending on the cation size and valence, the redissolution can occur for a partial neutralization or for an inverted charge of the polyelectrolyte. This approach reveals the importance of the electrostatic contribution of ion fluctuations in the diluted phase. The comparison between our data and these results cannot be done in a simple way because in our experiments, the cation valence varies with its size.

In our mobility experiments, when the valence of the counterion increases, the effective charge of the aggregates is shifted to positive values. Such experimental variation is consistent with the given theoretical descriptions. The higher the valence of the added cation, the higher the deviation from the negative charge predicted for the aggregate within the mean-field approximations. This suggests that correlations between condensed cations responsible for the predicted charge inversion increase with the valence.

Another approach to explain the experimental results is to consider the influence of the basic histone tails, which may extend more or less out of the particle depending on the ionic conditions. In this case, NCP may be viewed as polyampholytes where, at high salt concentrations, the basic protein tails are more hydrodynamically exposed than DNA with its condensed counterions. As predicted by Long et al. (1998), the resulting electrophoretic mobility of linear polyampholytes in high salt solutions does not depend only on the structural charge, but also on the surface charge distribution. However, because the tail extension varies with the ionic environment, no simple law can be established. Moreover, in this situation, electrophoretic mobility only gives information on the apparent charge localized at the shear surface, and not on the effective charge contributing to the total interaction between isolated NCP.

The validity of these two descriptions depends on the importance of the influence of the protein tails. The first description is valid if the tails introduce negligible charge inhomogeneities at the NCP surface; that is, if the electromobility is mainly determined by DNA with its condensed counterions. For a strong influence of the proteic tails, it would be essential to take into account the exact conformation of the particle.

In conclusion, we have shown that the NCP precipitation due to divalent salts can be related to the short-range electrostatic attraction produced by the cation condensation. No evidence was found for a specific interaction between divalent cations and NCP leading to a complexation phenomenon. As observed for other polyelectrolytes, the NCP phase diagram can be divided into three regions depending on the value of $z_{multi}C_{multi}/C_{ap}$. The regime corresponding roughly to $z_{multi}C_{multi}/C_{ap} \approx 1$ is independent of the object specificities (molecular weight, charge density, structure, etc.). The similarity for polyelectrolytes of different shapes confirms that precipitation is driven by local interactions between polyions. For this regime and for $z_{multi}C_{multi}/C_{ap} < 1$, the aggregation is mainly governed by the electrostatic attraction involving counterion condensation. The third regime ($z_{multi}C_{multi}/C_{ap} > 1$) is characterized by a large excess of free cations relative to the polyelectrolyte low concentration. As a consequence, the electrostatic interactions are strongly screened and the phase diagram is highly sensitive to the fine structure and chemical composition of the particles. For NCP, the histones tails and their exact conformation are therefore expected to play an important role. In this respect, it would now be interesting to investigate the effect of histone tail removal or acetylation on this phase diagram. We can expect a strong influence of histone tails in the low concentration region, where precipitation is therefore likely to be suppressed. However, in the high concentration regions where electrostatic interactions dominate, this effect should be negligible and the precipitation could well be maintained. As a consequence on the phase diagram, histone tail removal or modification can be expected to result in shrinkage and closure on the low concentration side of the biphasic domain. In any case, this work highlights the need of considering the whole range of concentration for further understanding of these phenomena. High concentrations that correspond to physiological conditions should in particular not be disregarded.

The NCP aggregation observed in the presence of divalent cations has to be related to the folding and precipitation of native and H1-depleted chromatin. Contrary to NCP, no aggregation is observed for DNA in the presence of divalent cations. In H1-depleted chromatin it thus appears that condensation is promoted by the attraction between NCP and limited by the repulsion between linker DNA. By trypsinizing the tails (Schwarz et al., 1996), which carry about half of the positively charged amino acids of the NCP, this attractive interaction is lowered compared to DNA repulsion, and precipitation of nucleosomal arrays is suppressed. In the same way, lowering the density of NCP on a DNA fragment of a given length below a given value also prevents precipitation of nucleosomal arrays by divalent cations (Schwarz et al., 1996). The addition

of H1 reduces the repulsion between linker DNA. As a result, the native chromatin condenses at lower multivalent cation concentration than H1-depleted chromatin chromatin (Widom, 1986; Watanabe and Iso, 1984; Clark and Kimura, 1990).

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