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The Influence of Ionic Environment and Histone Tails on Ordered Columnar Phases of Nucleosome Core Particles

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Abbreviations: $CoHex^{3+}$, cobalt(III)hexammine^{3+}, $Co(NH_3)_6^{3+}$; NCP, nucleosome core particle; SAXS, small angle X-ray scattering; Spd^{3+}, spermidine^{3+}; Spm^{4+}, spermine^{4+}; Tris, 2-Amino-2-hydroxymethyl-propane-1,3-diol, $(HOCH_2)_3CNH_2$

ABSTRACT The nucleosome core particle (NCP) is the basic building block of chromatin. Nucleosome-nucleosome interactions are instrumental for chromatin compaction and understanding of NCP self-assembly is important for understanding chromatin structure and dynamics. Recombinant NCPs aggregated by multivalent cations form various ordered phases that can be studied by X-ray diffraction (small angle X-ray scattering; SAXS). In this work, the effects on the supramolecular structure of aggregated NCPs due to lysine histone H4 tail acetylations, histone H2A mutations (neutralizing the “acidic patch” of the histone octamer) and the removal of histone tails were investigated. The formation of ordered mainly hexagonal columnar NCP phases is in agreement with earlier studies; however, the highly homogeneous recombinant NCP systems used in this work display a more compact packing. The long range order of NCP columnar phase was found to be abolished or reduced by acetylation of the H4 tails, acidic patch neutralization and removal of the H3 and H2B tails. Loss of nucleosome stacking upon removal of the H3 tails in combination with other tails was observed. In the absence of the H2A tails, formation of an unknown highly ordered phase was observed.

INTRODUCTION

In most species of eukaryotes, genomic DNA is packed by nuclear proteins forming chromatin. Chromatin consists of regular linear arrays of DNA-histone complexes, the nucleosomes. A central part of the nucleosome is the nucleosome core particle (NCP) formed by wrapping of 145-147 bp DNA as a 1.70 - 1.75-turn superhelix around an octamer of four core histone proteins, two H2A/H2B dimers and an $(H3/H4)_2$ tetramer (1, 2). The NCPs are connected by linker DNA of variable (10-80 bp) length forming a beads-on-a-string chromatin fiber that is further folded into various dynamic secondary and tertiary structures that are still not fully understood. In higher organisms, an additional protein, the linker histone H1, is also regularly present and contributes to the chromatin folding but cells are remarkably tolerant to H1-depletion (3) and it has been shown that fibers with short nucleosome repeat length can fold to their "natural" degree of compaction even in the absence of the H1 histone (4).

NCPs show aggregation behaviour similar to chromatin fibers, and this phase separation is sensitive to the ionic conditions with presence of multivalent cations with charge $\geq +2$ provoking self-association and aggregation (5-14). This sensitivity is due to the polyelectrolyte nature of the NCP, which is a polycation-polyanion complex between the histone octamer (HO) and the DNA (15). The nucleosomal DNA carries a large negative charge (−290e - −294e), which is only partially neutralized by the histones (total net charge of the HO is +148e). The charge of the eight core histones is unevenly distributed between the octamer globular domain (+60e) and the eight flexible unstructured N-termini (the "histone tails") (1, 16) each carrying a charge of +9e - +14e due to a varying number of positively charged lysine and arginine residues. Consequently, the NCP, which has a significant net negative charge (about −148e) can be visualized as a central particle (−236e) to which the positive flexible tails are attached (15, 17). The tails can interact with the DNA of its own NCP, with the linker DNA and with the other NCPs and proteins present in chromatin; perform important roles in chromatin folding (4, 18) and are essential for primary nucleosome-nucleosome interactions (19). Folding and aggregation of chromatin is facilitated and regulated by tail bridging between adjacent NCPs (8, 11, 20).

Amino acids in the histone tails are subject to numerous post-translational modifications with charge quenching acetylations of the ε-amino groups of the lysines being the most frequent and dynamic. Acetylated chromatin is easier digested by nucleases (21, 22) and shows higher solubility in monovalent salt (NaCl, KCl) and in the presence of millimolar concentration of Mg^{2+} (22-24). The histone tails also play important roles in transcription, replication, repair and recombination, both as direct modulators of chromatin folding and as a recognition platform for regulatory proteins (25, 26). These functions of the tails are regulated through the histone modifications, and the presence of tail acetylations typically correlates with transcriptionally active chromatin (27-30).

The cation-induced aggregation of NCPs is similar to the behaviour exhibited by other polyelectrolyte systems such as DNA (15, 31), however DNA condensation can normally only be induced by cations with charge +3 or higher (32) whereas NCP and chromatin systems can be condensed by divalent cations such as Mg^{2+} and Ca^{2+} (15). NCP-NCP attraction is mediated by the histone tails and observed in solutions of monovalent salt (8, 11) as well as in the presence of Mg^{2+}, whereas under similar conditions, DNA as well as NCPs and nucleosome arrays lacking the tails do not show aggregation (4, 11) (and references cited in (4)). The close NCP-NCP contact between the flat surfaces of the HO core on both sides of the cylindrical wedge-shaped NCP, called nucleosome stacking, is a common structural feature of the condensed state of chromatin. This stacking has been observed in NCP crystals (1, 2, 33-35), in NCP liquid crystalline phases (9, 10, 13, 36, 37), in the crystal of the

tetranucleosome (38), in folded nucleosome arrays (39-41), and in cryo-electron microscopy images of frozen isolated native chromatin (42, 43).

Various aggregates of NCPs phase-separated by the presence of multivalent cations (or high concentration of monovalent salt and osmotic pressure) have been systematically characterized by Livolant and colleagues (13, 44) (and references cited therein). These phases include isotropic NCPs similar to solution state, NCP columnar phase with no long range order between columns, lamello-columnar and highly ordered columnar hexagonal phases (44) (schematically shown in Fig. 1). The phenomenon of supra-molecular ordering in NCP aggregates is determined by a complex combination of variables in the mixture and by intrinsic NCP properties. The former include NCP concentration, buffer conditions, cation type, concentration, and osmotic pressure. It was shown that cation-induced formation of condensed NCP phases follows a polyelectrolyte behaviour similar to DNA condensation (5). Aggregation of NCPs is dependent on the charge and nature of cations present in solution (5-7, 13) and the histone tails mediate NCP-NCP contacts in a salt-dependent manner (7, 8, 11, 12, 14). However, despite all these observations, knowledge about the molecular structure of the NCP-NCP contacts, its thermodynamics, the precise roles of the tails and tail modifications in the modulation of the NCP-NCP interactions and in the formation of the ordered phases is still limited. The contribution of intrinsic NCP features is less investigated and includes the cylindrical wedge shape, the inhomogeneous surface charge distribution, dyadic symmetry and chirality (45-47). Most of the earlier studies have used either cell-extracted NCPs or NCP reconstituted using histone octamers from chicken blood. These NCPs have intrinsic features with a certain degree of heterogeneity in DNA length and unknown degree and positions of histone post-translational modifications. The NCP tail and surface charge distribution can be varied in a controlled way using recombinant NCPs to address several aspects of nucleosome interactions, in particular, modification effects and roles of histone tails. The synchrotron X-ray diffraction method (using SAXS) is well suited to address the influence of NCP charge distribution through the detection and characterization of the order in the NCP phases (9, 10, 44-48). The effects of dyadic symmetry and NCP chirality on the order in NCP phases, however, can only be addressed conveniently by cryo-electron microscopy and optical microscopy methods.

Large quantities of homogeneous NCPs with well-defined DNA sequence can be obtained using recombinant histones (49). Such well-defined systems allow the determination of the contributions of individual tails and specific alterations within them, to the association behaviour of mono-nucleosomes. As noted above, much of the previously published data on NCP systems was obtained from cell extracted chromatin or in solutions of NCPs obtained by nuclease treatment of the chromatin (4, 6, 9, 10, 13, 50) (and references cited therein). A growing number of works performed with well-defined chromatin and NCPs prepared by recombinant methods is emerging (12, 14, 40, 51-58). However, there is limited amount of data available for systems with modifications of the individual histones, such as mutants lacking tails, containing only the globular part of the histone molecule (12) or with selected tail lysines replaced by glutamine (K→Q mutation), mimicking acetylation (40, 51-56, 59). Published results indicate that acetylation or the absence of tails stabilizes NCPs or arrays in the solution state over self-association and promote the unfolding of compact nucleosome arrays (12, 51-53, 58, 60). The H4 tail (12, 52, 61, 62) and specifically H4-K16 (51, 55, 58) has been found critical for intramolecular folding of individual nucleosome arrays. Noteworthy, one of the H4 K16-R23 regions (of the two H4 tails) is involved in NCP stacking since it is located between the two NCPs in practically all reported NCP crystal structures, interacting with the acidic patch located on the globular domain of H2A histone of the neighbouring NCP (1, 63).

In the present work characterization of X-ray diffraction spectra of various precipitated NCP samples and their Bragg peak patterns (using the synchrotron SAXS beamline) was used to investigate the supra-molecular structure in NCP aggregates complemented with SAXS dilute solution investigation for selected samples. We studied the effects caused by H4 tail acetylations or deletions of tails, on the supramolecular structure of NCPs aggregated by the Mg^{2+} and cobalt(III)hexammine ($CoHex^{3+}$) cations. Wild-type (WT) and mutant NCPs were reconstituted from recombinant *Xenopus laevis* histones with a 145 bp DNA fragment of the '601' nucleosome positioning sequence (64). Specifically, NCPs containing H4 histones with lysines acetylated at the K16 position (H4-K16Ac) and at the four positions K5, K8, K12, and K16 (H4-QuadAc) were investigated. To compare effects of acetylations with K→Q mutations (both quenching lysine charge), NCPs containing H4 tails mutated at the same K5, K8, K12, and K16 positions (H4-K16Q and H4-QuadQ) were studied. The effect of the H2A histone acidic patch neutralization was investigated. Additionally, the effects of deletion of individual tails and combination thereof were investigated. In our recent work using well-defined recombinant NCPs, the effect of H4 tail acetylation and deletion of tails on cation-induced NCP aggregation was investigated (20) and the present work complements this study.

Materials and Methods

Detailed description of materials and methods applied in this work is given in the Supporting Material (SM). Below a brief account of applied techniques is presented.

Preparation of nucleosome core particles (NCPs)

Recombinant wild-type, mutated and truncated *X. laevis* core histone proteins H2A, H2B, H3 and H4 were expressed in *E. coli* [BL21 (DE3) pLysS] strain and purified by gel-filtration and cation-exchange chromatography. The H4 histones containing acetylated lysine, H4-K16Ac and H4-QuadAc (H4 histone with lysine residues acetylated at positions 5, 8, 12 and 16) were synthesized by natural chemical ligation and purified as described earlier (58).

The histone octamers (HO) were refolded by dialysis from the mixture of wild-type or modified core histones H2A, H2B, H3 and H4 and purified by gel-filtration chromatography. The purity and completeness of the HO refolding was checked by 18% SDS-PAGE.

The 145 bp DNA of '601' Widom nucleosome-positioning sequence (64) was used in this work to reconstitute NCPs. In a few measurements, NCPs reconstituted with the 147 bp palindromic human α-satellite DNA fragment (1, 2) was also used. The 145 bp '601' DNA construct in the pUC19 plasmid was amplified using the *E. coli* HB101 strain, extracted by alkaline lysis method, released by EcoRV and separated by PEG 6000.

The NCPs were reconstituted from DNA and HO by a stepwise dialysis in a high- to low-salt buffer (49, 65, 66). The best yield was obtained for the wild type NCPs; reconstitution of the NCPs with mutated and deleted tails was less efficient but still was notably better than that quoted Bertin et al. for tailless NCP constructs (12, 14). The quality of the reconstitution was assayed by 5% PAGE (see SM Fig. S3C) yielding an estimated presence of less than 5% of free DNA. After NCP precipitation from solution by multivalent cations for the X-ray diffraction measurements, any free DNA will remain in the supernatant and this stage acts as a further purification.

Sample preparation, data collection and analysis

We investigated the structures of cation-induced NCP aggregates using SAXS, where ordered phases can be identified from the way they diffract X-rays. Table S1 (see SM**)** contains a

summary of more than 200 samples analysed. Along with WT-NCPs assembled with HO consisting of recombinant, fully charged histones, four variants of the NCP were reconstituted using various H4 histones with single- or tetra-acetylated or mutated lysine residues in the histone tail (H4-K16Ac, H4-QuadAc, H4-K16Q, H4-QuadQ). Additionally, one HO was prepared with three charge-neutralizing mutations in the acidic patch of the H2A histone (the mutations were D90S+E91T+E92T, abbreviated as H2A-STT). The other NCP samples contained various combinations of tailless histones. NCP aggregation was induced by addition of divalent Mg^{2+} or trivalent $CoHex^{3+}$ cations. Aggregation of a few samples of WT-NCP was also induced by the polyamines spermidine^{3+} (Spd^{3+}) and spermine^{4+} (Spm^{4+}). The final concentration of the NCP was 8 mg/mL. For comparison and as reference, solution SAXS spectra for WT-NCP solutions in a concentration range from 1.25 to 17 mg/ml NCP and at two concentrations of KCl (10 and 100 mM) were obtained. Furthermore, solution spectra of the H4K16Q, H4-QuadQ, gH2A and gH2BgH2B samples were collected for two concentration of KCl.

High energy synchrotron radiated X-rays were used in order to compensate for the weak scattering intensity of the biological samples. The samples were prepared as described in the SM. Measurements were made at the beamline 23A SWAXS end-station at the National Synchrotron Radiation Research Center (Hsinchu, Taiwan) (67). Some samples were repeatedly measured with several month intervals with no apparent changes in the spectral characteristics (data not shown).

The phases of the NCP aggregates were identified from the periodicity of the Bragg peaks as a function of scattering vector *q*. The NCP stacking and the hexagonally ordered columns generate sets of peaks with periodicity of 1:2:3:4 and 1: √3: √4: √7: √9, respectively (9, 44, 68). The NCP stacking distance *h* and the inter-columnar distance a_H in columnar hexagonal phases (Fig. 1C-D) were calculated from the position of the first peaks q_{1h} and q_1 using the equations $h = \frac{2\pi}{q_{1h}}$, and $a_H = \frac{2}{\sqrt{3}} \cdot \frac{2\pi}{q_1}$. Fig. 2 displays an example of an X-ray diffraction spectrum with annotated peaks.

Results and Discussion

The effects of lysine acetylations in the H4 histones tails, neutralization of the acidic patch on the H2A globular domain, and removal of histone tails in the recombinant NCP aggregated by Mg^{2+} and $CoHex^{3+}$ cations were studied by SAXS (SM Table S1). In addition, H4-K16Q, H2A-STT, gH2A and gH2AgH2B samples in solution at two concentrations of monovalent KCl were investigated.

Solution SAXS spectra

The diffraction spectrum intensity *I(q)* originates from X-rays scattered by the atoms of individual NCP (by the form factor, *P(q)*) and by the structures formed by interacting NCPs (by the structure factor, *S(q)*), hence $I(q) \sim P(q) \times S(q)$). Figure 2 shows spectra of the WT-NCP recorded in the presence of five cations, namely K^+, Mg^{2+}, $CoHex^{3+}$, polyamines Spd^{3+} and Spm^{4+}. It is well established that at low concentrations of monovalent cations (K^+ or Na^+), the interaction between the nucleosomes is weak and repulsive (7, 48). The SAXS spectrum obtained from the solution of homogenous and isotropic NCPs is therefore primarily defined by the form factor *P(q)* of the NCPs (top magenta curve in Fig. 2). For the WT-NCP, we recorded SAXS spectra of NCP solutions with 10 and 100 mM KCl in a range of NCP concentrations from 1.25 to 17 mg/mL (see SM, Fig. S1). Our results agree with the solution SAXS data reported by others (7, 14, 48). At low NCP concentrations (C_{NCP} = 1.25

and 2.5 mg/mL) in 10 mM KCl, the experimental SAXS profiles show almost perfect overlap with the simulated spectrum calculated from the atomic structure of the NCP obtained from molecular dynamics simulations (69), which compared to the NCP structure in the crystal (2) exhibits collapse of the histone tails on the globular part of the NCP. According to physicochemical principles (70) oligocationic tails should be bound to the polyanionic DNA under low salt conditions and this most realistic NCP structure gives the best agreement with the measured SAXS spectrum. NCP structures with the tails extended outside the globular part or with the tails removed showed worse agreement with experimental data. We used two programs simulating SAXS spectra from atomic coordinates, CRYSOL (71) and FoXS (72) (SM Fig. S2) both producing very similar results.

We used the experimental form factor of the WT-NCP obtained at C_{NCP} = 1.25 mg/mL in 10 mM KCl to estimate the structure factors of the WT-NCP in solution. The scattering profiles together with calculated structure factors (Fig. S1C,D) allow a better assessment of the NCP ordering, when broad maxima in scattering vector ranges of 0.09 - 0.10 and 0.16 - 0.17 $Å^{-1}$ of the *P(q)* profile contribute significantly and overlap with peaks from weak NCP-NCP interactions. Samples of WT-NCPs show little interaction both in 10 and 100 mM KCl. At 10 mM KCl, an increase in the NCP concentration results in a progressive drop of the intensity of the *S(q)* profile at low *q* values (SM Fig. S1,C) indicating repulsive NCP-NCP interactions. In contrast, at 100 mM KCl, weak maxima observed at 0.012-0.016 $Å^{-1}$ (SM Fig. S1,D) that are likely to indicate the weak attractive NCP-NCP interactions as reported by others for NCP solutions with increased concentrations of NaCl and NCP (7, 11, 48).

The scattering from solutions of selected mutated and tailless NCPs was recorded to assess the form factor that could indicate the change in shape of the particles caused by modifications. Selected SAXS profiles recorded for the WT-NCP, H4-K16Q, and H2A-STT NCPs are compared in Fig. S3A of the SM; Fig. S3B shows spectra in solutions of the WT-NCP, gH2A and gH2AgH2B NCPs; Figs. S4 and S5 present all solution SAXS spectra collected for the selected mutated and tailless NCPs. H4-K16Q, and H2A-STT samples produced scattering spectra with features almost identical to the WT-NCPs (Fig. S3A). The results in Fig. S3A demonstrate (together with the corresponding gels in Fig. S3C and other assays, data not shown) that modifications/mutations have only minor effect on the particle shape and size and that our systems are homogeneous, which is known from previous studies with these constructs. The tailless particles on the other hand, produced featureless spectra, distinct from the WT-NCP (Fig. S3B). These spectra are in agreement with the spectra reported by Livolant and colleagues (14). The absence of the minima as the most pronounced effect of tail deletion was suggested to originate from a change in DNA wrapping around the octamer core and may also indicate that DNA wrapping is more dynamic and flexible.

Cation-induced condensed phases of WT-NCP

The formation of ordered phases by WT-NCP (i.e. for histones without any modifications or tail deletions) in a range of concentrations of Mg^{2+}, $CoHex^{3+}$, Spd^{3+}, and Spm^{4+} was investigated (Figs. 2, 3 and SM Tables S1, S2 and Figs. S6, S7). Upon addition of multivalent cations, the highly homogeneous recombinant NCPs instantaneously aggregate, and precipitate upon gentle centrifugation (see SM Supporting Methods). In a certain cation concentration range, highly ordered phases were identified from SAXS spectra (Fig. 2). The spectral features are reproducible without significant changes for periods from immediate measurements within days of preparation up to several months (data not shown).

Columnar hexagonal phases are formed in solutions of WT-NCP in the presence of millimolar concentrations of Mg^{2+}, $CoHex^{3+}$, Spd^{3+} and Spm^{4+} as indicated by a clear appearance of the characteristic hexagonal order peaks, q_1, q_2, q_3, and q_4 at about 0.065,

0.113, 0.13 and 0.172 $Å^{-1}$, with the expected ratio of 1: √3: √4: √7 (Fig. 2). The set of peaks with a 1: 2 ratio at about 0.11 and 0.22 $Å^{-1}$, assigned respectively as q_{1h} and q_{2h} indicate columnar stacking of the NCPs. The most ordered WT-NCP aggregates were observed at 20 mM Mg^{2+}, 1.5 mM $CoHex^{3+}$, 3.88 mM Spd^{3+}, and 1.45 mM Spm^{4+} (Fig. 2 and SM Table S2). The presence of only $q_{1,2,3}$ and $q_{1h,2h}$ peaks, illustrated by spectra for WT-NCP with Spd^{3+} and Spm^{4+} indicates a two-dimensional columnar hexagonal phase. The presence of peaks in addition to the columnar hexagonal phase illustrated in spectra from the WT-NCP with Mg^{2+} and $CoHex^{3+}$, indicates the formation of a three-dimensional orthorhombic quasi-hexagonal phase. The additional peaks indicate correlation between columnar and hexagonal orders like in a crystal. This interpretation was developed by Livolant and colleagues, where they have used inter-columnar distances a_H and NCP stacking distance h to characterize the hexagonal phase, and a, b, c indices for the orthorhombic quasi-hexagonal phase (9, 44). The orthorhombic quasi-hexagonal phase has a slightly distorted hexagonal order with peak positions deviating by around 2% from the hexagonal periodicity. In our work while we distinguish between phases with two-dimensional columnar hexagonal and three-dimensional orthorhombic quasi-hexagonal order, we use the two-dimensional notation for both.

The NCP stacking distances h = 55 - 57 Å and the inter-columnar distances a_H = 110 - 112 Å calculated from the SAXS spectra of the WT-NCP are inversely related to the cation charge, being the shortest for the Spm^{4+} and largest for the Mg^{2+} ions, indicating the relative compactness of the NCP packing (SM Table S2). Previous computational studies have also shown more condensed inter-nucleosome packing induced by $CoHex^{3+}$, compared to Mg^{2+} (20, 57, 58). The characteristic distances observed for recombinant WT-NCPs that were precipitated by multivalent cations were generally comparable or less than the values reported for native cell-extracted NCPs condensed by osmotic shock in the presence of NaCl (h = 57.7 - 60 Å and a_H = 110 - 116 Å) (44).

The three-dimensional orthorhombic quasi-hexagonal symmetry in NCP aggregates is not unexpected given the orthorhombic packing obtained for single crystals of NCPs (1, 2). In Fig. 3 our spectra of ordered WT-NCP phases condensed by Mg^{2+} and $CoHex^{3+}$ are compared with the spectrum for highly ordered NCPs prepared from nuclease digested calf thymus chromatin obtained by Livolant and co-workers after 4 months of equilibration at high monovalent salt concentration, subjected to osmotic shock and 15-day orientation in magnetic field (9, 44). The comparison allows indexing some of the correlation peaks in our X-ray diffraction profiles. Not only our assignment is incomplete and should be considered tentative, but our samples were not oriented, thus more disordered. It is challenging to assign all diffraction peaks from the averaged one-dimensional X-ray diffraction spectrum from a polycrystalline sample. Two dimensional spectra with the phase information from single crystals are needed to unequivocally assign all peaks and determine the structure. It is, however, clear that the recombinant WT-NCP samples aggregated by Mg^{2+} or $CoHex^{3+}$ are indicative of a similar degree of ordering that was obtained under conditions that favour high order observed by Livolant and co-workers. This shows that the formation of highly ordered columnar hexagonal phases is a natural characteristic of mononucleosomes that likely corresponds to the equilibrium phase under physiological conditions.

The polyelectrolyte behaviour of NCP interactions in a wide concentration range of Mg^{2+} is noteworthy. A gradual loss of hexagonal order is observed at cation concentrations significantly away from 20 mM (SM Figure S6A). In the range of 8 - 14 and 24 - 50 mM Mg^{2+}, an NCP columnar phase with no long range order is observed. The broad peaks are arising from the average inter-columnar distance (q_1 ~ 0.065 $Å^{-1}$), the average NCP stacking distance within columns and the columnar form factor from the local arrangement (q_2 and q_{1h} ~ 0.11 $Å^{-1}$ and q_3 ~ 0.17 $Å^{-1}$) (44). At 3 and 80 mM Mg^{2+} an isotropic NCP phase is observed with spectra being similar to the form factor obtained from a solution of WT-NCP. At Mg^{2+}

concentrations lower than 3 or higher than 80 mM the NCPs remain soluble. In contrast, the stable columnar hexagonal phase is observed in a broad range of 1.5 – 30 mM $CoHex^{3+}$, with an isotropic NCP phase being observed at 1.2 mM $CoHex^{3+}$ (see SM Fig. S6).

We also obtained X-ray diffraction spectra for a few samples of NCPs reconstituted with a different DNA template, namely the 147 bp palindromic human α-satellite DNA fragment. Spectra are shown in SM Fig. S8 and the pattern shows a 2D hexagonal columnar phase, displaying sharp peaks, in addition to some weak additional peaks indicating distortion from the 2D hexagonal arrangement. The columnar hexagonal phase has consequently been obtained for mononucleosomes extracted from cells that likely contain an unknown amount of posttranslational modifications in the histone tails (previous work by Livolant and co-workers) as well as in reconstituted NCPs having different types of DNA template (44).

Supramolecular structures for NCPs with modified and tailless histones

Spectra representing the most ordered phases for NCPs with various histone modifications obtained in presence of Mg^{2+} or $CoHex^{3+}$ are summarized in Fig. 4. The concentration ranges of Mg^{2+} and $CoHex^{3+}$ investigated for each variant of NCP and the type of the most ordered phase are schematically presented in Fig. 5 (see also SM Table S2 and Figs. S9 - S22). In Fig. 4 the spectra are divided into three groups: i) Fig. 4A,B - NCPs with mutated and acetylated histones (H2A-STT, H4-K16Q, H4-K16Ac, H4-QuadQ, H4-QuadAc); ii). Fig. 4C,D - NCPs with one of the four histones tails truncated (gH2A, gH4, gH2B, gH3); iii). Fig. 4E,F - NCPs lacking two or more histones tails (gH2AgH2B, gH3gH4, gH2AgH3gH4, gH2AgH2BgH3, and gNCP). Within each graph the spectra are aligned in the order reflecting the change of the charge in the histone octamer relative to the charge of the WT-NCP (numbers are shown between the Mg^{2+} and $CoHex^{3+}$ panels). All modifications, excluding the H2A-STT, increase the net negative charge of NCPs. Changes in the NCP charge and charge distribution alone may alter the NCP structure and affect NCP-NCP interactions, which in turn may influence the degree of order within the NCP aggregates in a cation-dependent manner. In addition, acetylation or tail deletion may affect NCP structure and NCP-NCP interactions in a cation-independent manner, combining electrostatic and steric contributions.

All our results are subject to the assumption that we have been able to detect the most ordered state in the range of measured concentrations of counterions.

One noticeable feature of the SAXS spectra shown in Fig. 4 is that NCP samples with $CoHex^{3+}$ generally display columnar hexagonal order, except for the NCPs lacking two or more histone tails. Samples with the corresponding NCPs aggregated by Mg^{2+}, in contrast, display columnar hexagonal phases, columnar disordered phases and the isotropic NCP phase. The H4-K16Q, H4-QuadQ and H4-K16Ac NCPs show a degree of order and phase behaviour rather similar to the WT-NCP in the presence of $CoHex^{3+}$. The H2A-STT NCP in contrast, shows a columnar hexagonal order only at 2 mM $CoHex^{3+}$ and a disordered columnar phase for $CoHex^{3+}$ concentrations in the 1.2 – 4 mM $CoHex^{3+}$ range measured. The limited measurements with H4-QuadAc show a columnar hexagonal phase, and based on the similarity in effects for K16Q and K16Ac modifications, we assume that the H4-QuadAc modification effect is similar to the H4-QuadQ one. The H4-K16Q sample shows an isotropic NCP phase. The other samples show diffraction spectra with characteristics of an isotropic columnar organization. The loss of hexagonal order is cation-dependent because well-ordered columnar hexagonal phases were observed for the NCP variants with mutated or modified histones in the presence of $CoHex^{3+}$ (Fig. 4B). The characteristic NCP stacking and inter-columnar distances in the columnar hexagonal phases of NCPs with modified histones are in the range of h = 54.6 – 55.9 Å and a_H = 109.9 – 111.8 Å. Interestingly no apparent

dependence on the nature (acetylation versus K→Q mutation) and the number of modifications (single H4-K16 or tetra H4-K5, K8, K12, K16 change) was observed.

With the more biologically relevant Mg^{2+} the columnar hexagonal phase was not achieved for all five mutated NCPs. The NCP columnar phase was observed for all but H4-K16Q. The latter showed an isotropic NCP phase. The modifications in the H4 histone tails as well as STT mutation in the H2A histone lead to significantly reduced order compared to the WT-NCP in the presence of Mg^{2+} (Fig. 4A). The investigated modifications in the presence of Mg^{2+} seem to prevent the ordering of NCP columns. The two specific effects of mutations are the H2A acidic patch neutralization greatly reduces the $CoHex^{3+}$ range for the columnar hexagonal phase formation, and H4-K16Q prevents NCP stacking in the presence of Mg^{2+}. The neutralization of three negative charges on the NCP stacking interface has a destabilizing effect on columnar hexagonal phase, in comparison with neutralization of one or four positive charges on H4 tail. The observed difference may be due to the nature or the location of the neutralized charges and has analogy to a similar disruption of the NCP-NCP contacts in folding of nucleosome arrays reported for the H2A.Bbd, variant of the histone H2A with charge-quenching alteration of the acidic patch. (73).

The removal of histone tails from one core histone shows that not all tails are equal (Fig. 4C,D). The absence of H2B and H3 tails leads to formation of columnar NCP phase in the presence of Mg^{2+} and columnar hexagonal phases in the presence of $CoHex^{3+}$ in a cation-dependent manner. For both Mg^{2+} and $CoHex^{3+}$, removal of the H4 tails does not affect formation of columnar hexagonal phases, the behavior thus resembling the WT-NCP. Surprisingly, in the presence of either of the two cations (Mg^{2+} and $CoHex^{3+}$) the NCP with truncation of N-terminal as well as C-terminal tails in the H2A histones produced X-ray diffraction spectra with a large number of peaks not previously observed for NCPs here or in the literature. The gH3 samples display spectra with peak patterns similar to gH2A with 1.5 mM $CoHex^{3+}$. A possible interpretation of these spectra may be that there is a mixture of more than one phase, including one of hexagonal order. The removal of tails has no effect on phase structure in the case of the histone H4, is cation-dependent for the histones H3 and H2B, and leads to structural changes for the H2A histyone.

NCP systems lacking tails in two (gH3gH4), three (gH2AgH3gH4, gH2AgH2BgH3) or all four histones (gNCP) do not form columnar hexagonal phases with Mg^{2+} and $CoHex^{3+}$ (Fig. 4E,F). The gNCP samples display peaks characteristics of NCP stacking at 80 and 100 mM Mg^{2+}, and the isotropic NCP phase was observed in the 12-60 mM Mg^{2+} range. NCP samples gH3gH4, gH2AgH2BgH3 and gH2AgH3gH4 resemble gNCP in the 10 - 30 mM Mg^{2+} range forming an isotropic NCP phase. All NCP samples lacking more than one tail mentioned above form isotropic NCP phases in the range of 1.5 – 5 mM $CoHex^{3+}$ (gNCPs were measured in a broader range of 1.5 – 27 mM $CoHex^{3+}$).

In the presence of Mg^{2+} the gH2AgH2B samples display unusual spectra with sharp peaks indicating long range order like in a crystal (see the spectrum highlighted by red colour in Fig. 4E). These are similar to the spectra of the gH2A sample and the diffraction pattern reveals a crystal-like organization (Fig. 4G, bottom spectrum in red frame compared with the spectrum from the WT-NCP). It seems likely that these spectra are due to the formation of at least two phases, one of which could be the commonly observed columnar hexagonal structure (the expected hexagonal peaks can be tentatively assigned). It appears that the removal of the H2A and H2B tails resulted in the formation of highly ordered phases of yet unknown structure. Spectra of gH2AgH2B at 1.5 or 5 mM $CoHex^{3+}$ are very similar to gH2A spectra at 2 mM $CoHex^{3+}$. The same set of peaks of higher intensity for the former is shifted to higher q range indicating tighter packing. Thus, we suggest that the same phase of unknown structure and long-range order is formed by NCPs lacking H2A tails in the presence of Mg^{2+} and $CoHex^{3+}$ and by the NCP lacking H2A and H2B tails in the presence of 1.5 and

5 mM $CoHex^{3+}$. The formation of the columnar hexagonal phase by gH2AgH2B NCPs was also observed in the presence of $CoHex^{3+}$. At the moment, it is not possible to suggest what sort of structure is formed and additional experiments are needed in order to get an insight into this effect.

In general, it was observed that the ordering of the NCP aggregates decreases as the number of tailless histones increases (with the notable exceptions for the gH2A and gH2AgH2B systems). The order decreases in both the NCP stacking and the hexagonal columnar association.

Two broad diffraction peaks with a 1:2 positional ratio observed in spectra of gNCPs at 80 and 100 mM Mg^{2+} (Fig. 4E, SM Fig. S22) indicate a possible NCP stacking of short-range order with a mean distance h = 59.4 Å (SM Table S2). This distance is significantly larger than that of the other NCP variants, suggesting much loosely stacked NCP aggregates. It may be noted that the electrophoretic mobility of the gNCP is slower than that of the wild-type and the mutated/acetylated NCPs (20), suggesting that the gNCPs might have an extended size probably due to detachment of the entry/exit parts of the DNA from the histone core. A similar DNA detachment effect may apply to the gH2AgH3gH4 sample where a weak peak corresponding to h = 57.1 Å was observed at 3 mM $CoHex^{3+}$ (Fig. 4F, SM Table S2). The absence of columnar ordering in these spectra may suggest a smectic NCP organization with layers of NCP without proper stacking and laterally disordered due to extending DNA.

Studies of NCPs concentrated by variation of the osmotic pressure in the presence of monovalent salt have identified the formation of bilayers of NCPs stacked in columns also visible in EM images and showing a scattering peak q_{1L} at low q corresponding to a distance of around 350 Å (9, 10, 44). However, in our study as well as in the other studies of ordered phases in the presence of multivalent cations (13, 74) the bilayer phase was never observed; such loose bilayer arrangement was seen in NCP systems with monovalent salt and under osmotic pressure (44) (and reference therein). In general, multication-induced condensation of DNA (31, 75) and nucleosome arrays (76) proceeds as cooperative process, an all-or-nothing transition. It is reasonable to expect that NCP aggregation follows the same process. Therefore, under conditions applied in the present work one would expect the existence of either NCPs in solution or fully-condensed phases of NCPs without bilayer intermediates.

Estimation of the size of the columnar hexagonal NCP domains

A lower boundary of the linear domain size s of the ordered aggregates can be estimated from the fitted full width at half-maximum (*fwhm*) of the first diffraction peak, namely $s = 2\pi/fwhm$. In the columnar hexagonal NCP phase the length across the columnar hexagonal domain $s(a_H)$ and the length of NCP columns $s(h)$ can be estimated using the *fwhm* of q_1 and q_{1h} peaks, respectively. The domain sizes of columnar hexagonal phases are listed in Table S3 of SM. The number of columns and stacked NCPs in the column may be calculated by dividing the domain size $s(a_H)$ and $s(h)$ by a_H and h, respectively. A WT-NCP columnar hexagonal phases made of 20 - 30 columns, each consisting of 40 - 50 NCPs estimated in our work from the SAXS data agrees with earlier observations using cryo-EM (44). A reduction in domain size in comparison with the WT-NCP was observed for H4-QuadAc and gH2B with $CoHex^{3+}$, whereas the opposite effect was observed for H4-K16Q with $CoHex^{3+}$, and gH4 with Mg^{2+}. The general observation is to have a larger number of NCP stacked in the column, in comparison with the number of arranged columns.

CONCLUSIONS

In solutions with low (below 50 mM) monovalent salt, interactions between NCPs are repulsive. Addition of millimolar concentrations of Mg^{2+} or $CoHex^{3+}$ results in phase separation and formation of NCP aggregates with different arrangement of particles ranging from isotropic NCP phase, to NCP columnar and columnar hexagonal phases. The major contribution to free energy driving phase separation comes from the entropic gain of the release of monovalent counterions (15).

The X-ray diffraction method has enabled us to quantitatively assess the short- and long-range order in many NCP aggregates and to identify and distinguish a number of known phases with NCP stacking and columnar arrangement order. These advantages compensate for the limitations of polycrystalline samples of higher disorder, incomplete sampling that assumes the detection of the most ordered phases, and inability to solve the structure with regard to the order of dyad symmetry and NCP chirality in general, and for some of the tailless NCP in this work in particular.

Our results are in general agreement with the earlier X-ray diffraction studies where a variety of NCP phases has been observed. Compared with earlier studies performed on inhomogeneous nucleosomes extracted from native chromatin, more compact packing and faster equilibration of the highly homogeneous recombinant NCPs were observed in our work. A WT-NCP stacking distances in the range of h = 55 - 57 Å and inter-columnar distances of a_H = 110 -112 Å are shorter and more uniform than these parameters observed for cell-extracted NCPs.

In the 3 – 80 mM Mg^{2+} range the phase behavior of the WT-NCP aggregates is cation concentration dependent. With increasing Mg^{2+} concentration, the order changes from isotropic NCP, to columnar disordered and to columnar hexagonal. Further increase in Mg^{2+} concentration leads to the same set of phases in reversed order. A column formation by NCP stacking followed by the inter-columnar interactions is a suggested model of columnar hexagonal phase formation based on our observations. For the $CoHex^{3+}$ the isotropic NCP to three-dimensional to two-dimensional columnar hexagonal phase transition in the narrow range of 1.2 – 2.5 mM $CoHex^{3+}$ was observed. The columnar hexagonal phase persisted in the 2.5 – 30 mM $CoHex^{3+}$ range.

Single tail deletion effects result in diversity in spectra. The H4 tail deletion results in spectra similar to WT-NCP for both Mg^{2+} and $CoHex^{3+}$ with formation of columnar hexagonal phases. Deletion of gH2A tails in the presence of Mg^{2+} or $CoHex^{3+}$ produces spectra with multiple peaks indicating highly ordered structures with peak positions not in agreement with columnar hexagonal phase reported before. Deletion of H2B and H3 tails results in NCP columnar phase in the presence of Mg^{2+}. In the presence of $CoHex^{3+}$, multiple peaks at high $q > 0.15$ $Å^{-1}$ indicate ordering in the gH2B spectrum, but the low intensity of q_1 with the high intensity q_2 peak with a shoulder and the absence of regular NCP stacking peak makes the phase identification difficult. The gH3 spectrum in the presence of $CoHex^{3+}$ is similar to gH2A. The electrostatic contribution of the tail deletion effect may be because NCPs columns lacking H2B and H3 tails are more electronegative in comparison with columns lacking H2A and H4 tails that form columnar hexagonal phases. Recent coarse grained (CG) simulations from our laboratory with an advanced CG NCP model reproducing the detailed shape of the NCP core and geometry and flexibility of the tails, confirm that in phases of aggregated NCPs, H2A and H4 tails stabilize the NCP-NCP stacking contact while the H3 and H2B are responsible for screening and bridging interactions between lateral surfaces of the contacting NCPs observed (17). The gH2A and gH3 samples are special cases since an unknown structure is observed. The tail deletion also changes the shape of NCP with a different and probably more dynamic DNA wrapping around the HO, besides changing the charge and charge distribution, therefore allowing the formation of new contacts. One

particular effect of the tail removal is the unpeeling of the DNA ends that makes the NCP resemble the chromatosome, a complex of NCP with the linker histone H1 (77).

In the case of four samples with more than one tail removed, namely gH3gH4, gH2AgH3gH4, gH2AgH2BgH3, and gNCP the aggregates produced featureless spectra indicating the formation of isotropic NCP phase with no long range order in the presence of Mg^{2+} or $CoHex^{3+}$. We conclude that the removal of the H3 tail in combination with any other tail is sufficient to prevent the formation of NCP columns by possibly inhibiting the NCP stacking. The H3 tail is the largest among four tails and its essential role for long range NCP order is not surprising.

The effects of the absence of H2A and H2B tails resulting in novel highly ordered structures in the NCP aggregates observed in this work is intriguing. The effects may be biologically relevant because H2A/H2B dimers are more dynamic, in comparison with $(H3/H4)_2$ tetramers and H2A histone family has the largest number of variants. The highly ordered phases observed in this work deserve to be characterized using high-resolution methods like cryo-electron microscopy or crystallography. The role of individual tails may be addressed by NMR. In addition, studies on aggregates of NCP reconstituted with $(H3/H4)_2$ tetramers only, or H2A histone variants may contribute to the understanding of the role of histone tails.

Finally, it must be strongly emphasised that the reconstitution of tailless constructs results in NCPs with different particle shape, which is likely caused by a different more dynamic DNA wrapping around the HO with unwrapping at the DNA ends and resulting in a more dynamic heterogeneous system. For this reason, the effects observed for the tailless systems can be due both the change in NCP particle structure and to the absence of the tails, expected to result in decreased inter-NCP interactions and diminished order. The above conclusions and discussions on the tailless systems should therefore be considered tentative and further characterization of these systems is warranted.

SUPPORTING MATERIAL

Additional methods, figures and tables are available at ????????

AUTHOR CONTRIBUTIONS

NK and LN designed research; NVB, YL, C-JS and NK performed research; AA, C-FL and RY contributed analytical tools; NVB, YL and NK analyzed data; NVB, NK and LN wrote the manuscript.

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FIGURE LEGENDS

FIGURE 1 Schematic presentation of phases formed by nucleosome core particles: (**A**). Isotropic NCPs phase. (**B**). NCP columnar phase. (**C**). Two-dimensional columnar hexagonal phase. (**D**). Three-dimensional orthorhombic quasi-hexagonal phase. The distance between stacked NCPs is marked as *h*, the inter-columnar distance is a_H; star indicates direction of the dyad axis. (**E**). A single bilayer of the lamello-columnar NCP phase observed by Livolant and colleagues (9, 10, 44) in the systems where NCP condensation was caused by osmotic stress and addition of monovalent cations. Lines indicate NCP layers in the bilayer. Bilayers in turn formed higher order structures with bilayer-bilayer distance dependent on conditions.

FIGURE 2 SAXS spectra of solution and most ordered phases of WT-NCP with cations (nature and concentration of ions is indicated in the graph). The solution SAXS spectrum of WT-NCP at 1.25 mg/mL in 10 mM KCl (top spectrum) largely overlaps with the profile calculated from the atomic coordinates of the NCP crystal structure 1KX5 (2) (see SM Fig. S2) In the presence of multivalent cations (second to last spectra from top) NCPs precipitated from solution form ordered columnar hexagonal structures. On the spectrum in the presence of 20 mM Mg^{2+} the peaks characteristic of the 3D orthorhombic columnar order (q_1, q_2, q_3, and q_4) and due to NCP-NCP stacking within the columns (q_{1h} and q_{2h}) are indicated.

FIGURE 3 (**A,C**) Spectra of highly-ordered phases formed by wt-NCP in the presence of 20 mM Mg^{2+} (**A**) and 1.5 mM $CoHex^{3+}$ (**C**). The peaks characteristic of the columnar order (q_1, q_2, q_3, and q_4) and NCP-NCP stacking within the columns (q_{1h} and q_{2h}) are indicated. The inserts show corresponding SAXS diffraction patterns. (**B**). The spectrum for NCPs obtained from nuclease digested calf thymus chromatin in 160 mM NaCl under an osmotic pressure of 4.7×10^5 Pa after four month of equilibration and partially oriented in a 10 T magnetic field during 15 days reported by Mangenot et al (9) is shown for comparison. In **B**, peaks are marked according to periodicity of the orthorhombic lattice (a, b, c) with dimensions (a_H, $\sqrt{3}\cdot a_H$, ~h). Peaks indicated by letters from A to M are suggested to correspond to a superstructure along c-axis of a periodicity close to 6c: A (1, 1, 1/3), B (1, 1, 1/2), C (0, 2, 1 + 1/2), D (1, 3, 1/2), E (0, 0, 2 + 1/6), F (0, 0, 2 + 1/3), G (0, 4, 1/2) and (2, 2, 1/2), H (0, 0, 2 + 1/2), I (2, 1, 1 + 1/2), J (3, 1, 1/2), K (3, 3, 1 + 1/2), L (0, 0, 4 + 1/6), M (0, 0, 4 + 1/3). Panel **B** is reprinted with permission from the Biophysical Society.

FIGURE 4 (**A-F**) SAXS spectra for NCPs with various modifications of the histones and with added Mg^{2+} (**A,C,E**) or $CoHex^{3+}$ (**B,D,F**). (**A,B**) NCPs with octamers containing mutated or H4-acetylated histones; (**C,D**) NCPs with octamers lacking tails in one of the four histones (gH2A, gH2B, gH3, gH4); (**E,F**) NCPs with octamers lacking tails in two or more of the four histones. For each NCP modification, SAXS spectrum corresponding to the most ordered NCP phase from the range of investigated Mg^{2+} or $CoHex^{3+}$concentrations is shown. All spectra are given in the SM (Figs. S9 - S22). In each panel, the WT-NCP SAXS spectrum is given for comparison; the change of the net NCP charge relative to the WT-NCP is indicated next to the sample identifiers. (**G**) Two modified NCP variants lacking H2A tails (gH2A and gH2AgH2B) form highly ordered phases that differ from all other NCP variants. Panel **G** compares 2D SAXS patterns of WT-NCP (top frame) and gH2AgH2B (bottom frame) in the presence of Mg^{2+}; with the corresponding 1D spectra shown in panel **E**.

FIGURE 5 NCP samples with different modifications of core histones studied in a range of concentrations of Mg^{2+} (left) and $CoHex^{3+}$ (right) salts. Numbers show change of the net NCP charge relative to the WT-NCP. Circles show studied Mg^{2+} or $CoHex^{3+}$ concentrations with the unique symbol highlighting the concentration resulting in the most ordered NCP phase. The NCP phases corresponding to a given symbol are listed below the chart.