

## Self-association of polynucleosome chains by macromolecular crowding

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**Abstract** The crowding of macromolecules in the cell nucleus, where their concentration is in the range of 100 mg/ml, is predicted to result in strong entropic forces between them. Here the effects of crowding on polynucleosome chains *in vitro* were studied to evaluate if these forces could contribute to the packing of chromatin in the nucleus *in vivo*. Soluble polynucleosomes ~20 nucleosomes in length formed fast-sedimenting complexes in the presence of inert, volume-occupying agents poly(ethylene glycol) (PEG) or dextran. This self-association was reversible and consistent with the effect of macromolecular crowding. In the presence of these crowding agents, polynucleosomes formed large assemblies as seen by fluorescence microscopy after labelling DNA with the fluorescent stain DAPI, and formed rods and sheets at a higher concentration of crowding agent. Self-association caused by crowding does not require exogenous cations. Single, ~800 nucleosome-long chains prepared in 100  $\mu$ M Hepes buffer with no added cations, labelled with the fluorescent DNA stain YOYO-1, and spread on a polylysine-coated surface formed compact 3-D clusters in the presence of PEG or dextran. This reversible packing of polynucleosome chains by crowding may help to understand their compact conformations in the nucleus. These results, together with the known collapse of linear polymers in crowded milieu, suggest that entropic forces due to crowding, which have not been considered previously, may be an important factor in the packing of nucleosome chains in the nucleus.

**Keywords** Polynucleosome self-association · Macromolecular crowding · Entropic forces · Chromatin packing · Interphase chromosomes

### Introduction

Each chromosome of a eukaryotic cell is formed by a single chain of nucleosomes, which is compacted to occupy a distinct “territory” within the nucleus (Belmont and Bruce 1994; Albiez et al. 2006). The concentration of nucleosomes has been measured as 30–60 mg/ml in different regions of the nuclei of HeLa cells (Weidemann et al. 2003) and calculated as an average of ~70 mg/ml in human K562 cells (Hancock 2007), and reaches ~400 mg/ml in densely-packed regions (Bohrmann et al. 1993). The mechanism(s) which cause these high levels of compaction are not understood. Effects caused by cations have received considerable attention because *in vitro*, polynucleosome chains are compacted by low concentrations of  $Mg^{2+}$  ions or higher levels of monovalent cations (Thoma et al 1979; Giannasca et al. 1993; Hansen 2002; Cano et al. 2006). However, the resulting regular and symmetrical helical ~30 nm diameter fibre (Cano et al. 2006; Hansen 2002) does not reproduce the conformation of polynucleosome chains in the nucleus, which show no regular or helical folding as seen by electron microscopical methods which conserve the *in vivo* conditions as rigorously as possible (Giannasca et al. 1993; Horowitz et al. 1994), and some workers have questioned if cation-induced packing of chromatin can provide models which are relevant to conditions in the nucleus or may be instead like “chasing a mirage” (van Holde and Zlatanova 1995). This discrepancy suggests that chromatin packing *in vivo* may be influenced by other types of forces.

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Macromolecular crowding and entropic forces have recently received attention as additional factors which are predicted to influence the interactions of macromolecules in the nucleus (Hancock 2004, 2007; Marenduzzo et al. 2006; Richter et al 2007). At their measured global concentration in the nucleus, in the range of 65–220 mg/ml (Hancock 2007), macromolecules are subjected to strong entropic forces because close contact or a more compact conformation of larger macromolecules or particles is strongly favoured since this results in an increase of the system's entropy by increasing the volume available to smaller macromolecules or particles (Asakura and Oosawa 1954). The formation of at least some of the microscopically-visible compartments within the nucleus appears to be driven by these forces (Hancock 2004), and they cause nucleosome core particles to pack closely in ordered liquid crystalline conformations in vitro (Leforestier and Livolant 1997). Here, the first in vitro studies which show that polynucleosome chains self-associate in crowded conditions are reported.

## Experimental

### Polynucleosomes

Polynucleosomes were prepared from K562 (human erythroleukaemia) cells by two methods. In method A, cells grown for 15 h with (methyl-<sup>3</sup>H)thymidine were encapsulated in agarose beads, permeabilised, and chromatin fragments were released by cleaving DNA with HaeIII and electroeluted, all in a “physiological” buffer (130 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1 mM MgCl<sub>2</sub>, 1 mM Na<sub>2</sub>ATP, pH 7.4); (Jackson et al. 1988); 1 mM glutathione (GSH) was included to suppress the possible formation of protein S–S crosslinks (Garrard et al. 1977). The buffer was changed by two cycles of dilution 1/100 in 100 μM Hepes, pH 7.4 followed by concentration in a Centricon-30 device (Millipore) to ~20 μg DNA/μl. The average length of DNA in these polynucleosomes was ~4 kb. In method B, polynucleosomes were prepared using a cation-free medium for all steps (Hancock 2007). Cells were washed in 100 μM Hepes, 10 μM GSH, containing 12.5% 8 kDa poly(ethylene glycol) (PEG) (Sigma-Aldrich) (all concentrations are w/v), pH 7.4, and homogenised in this buffer supplemented with 0.5% (v/v) Triton X-100. Nuclei were pelleted at 300×g for 10 min, incubated in the same buffer with RNase (20 μg/ml, 30 min), centrifuged, resuspended in 100 μM Hepes, 10 μM GSH, pH 7.4, sonicated gently, and centrifuged at 600×g for 5 min to remove nuclear envelope fragments. The supernatant contained polynucleosomes of average DNA length ~150 kb as measured by pulsed-field gel electrophoresis, and handled using cut

micropipette tips to minimise shearing. Both polynucleosome preparations showed a stoichiometric content of the five major histones as seen by SDS-polyacrylamide gel electrophoresis and a canonical pattern of digestion by micrococcal nuclease (not shown).

### Sedimentation assays of polynucleosome self-association

Stock solutions of 8 kDa PEG, 35 kDa PEG, or 10.5 kDa dextran (Sigma-Aldrich) were prepared in 100 μM Hepes, pH 7.4 and diluted in the same buffer to the desired polymer concentration for all experiments. Polynucleosomes prepared by method A (2 μl) were mixed well with 18 μl of polymer solution and incubated for 10 min on ice (~100 ng DNA/μl in all samples). One-quarter volume of 10% formaldehyde in the same buffer with the same concentration of PEG or dextran as the sample was added with gentle mixing. After 10 min on ice, the samples were diluted ten-fold in 100 μM Hepes, pH 7.4 to minimise differences of density and viscosity, mixed, centrifuged at 10,000×g for 10 min, and polynucleosomes remaining in the supernatant were quantitated by liquid scintillation counting of <sup>3</sup>H.

### Visualisation of polynucleosome self-association

Polynucleosomes prepared by method A (2 μl, ~10 ng DNA) were placed on a glass microscope slide, mixed well with 2 μl of 100 μM Hepes, pH 7.4 or with 2 μl of 8 kDa PEG or 10.5 kDa dextran solution to give a final polymer concentration of 12.5%, and the fluorescent DNA label DAPI was added to 200 ng/ml (final nucleosome concentration ~2 ng DNA/μl). Alternatively, 10 μl (~200 μg DNA) were spread slowly on the surface of 50 μl of PEG or dextran (25%) on a slide and the fluorescent DNA label YOYO-1 (Molecular Probes) (5 μM in H<sub>2</sub>O) was added to 200 nM. Samples were mounted under a cover glass and imaged in solution. To visualise polynucleosomes prepared by method B, 5 μl (~5 μg DNA) was incubated for 30 min with YOYO-1 (200 nM) then 1 μl was mixed with 1 μl of 100 μM Hepes, pH 7.4 or with 1 μl of 8 kDa PEG (25%) or 10.5 kDa dextran (25%) (final nucleosome concentration: ~0.5 μg DNA/μl), incubated for 10 min on ice, and then placed on a microscope slide which had been previously immersed for 1 h in poly-L-lysine (0.5 mg/ml) (Sigma-Aldrich), drained, and dried in air. A coverslip held horizontally was lowered to spread the sample radially. Images were captured with a CoolSNAP camera (Photometrics) on a Nikon E800 microscope, or on a MRC1024 confocal microscope (Bio-Rad) for 3-D reconstruction of image stacks using Volocity software (Improvision, Waltham, USA).

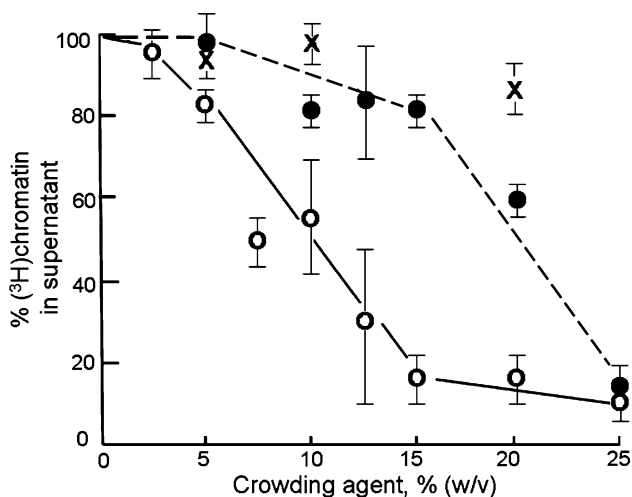
## Results

### Sedimentation assays of polynucleosome self-association by crowding

Polynucleosomes  $\sim 20$ -nucleosomes in length containing ( $^3\text{H}$ )thymidine-labelled DNA, prepared by method A in a buffer in which permeabilised nuclei show high transcriptional activity (Jackson et al. 1988), were mixed with the inert, uncharged, volume-occupying polymers PEG or dextran which are widely used as macromolecular crowding agents for in vitro studies (Zimmerman and Trach 1988; Zimmerman and Minton 1993; Murphy and Zimmerman 1995). A progressively larger fraction of the polynucleosomes was sedimentable as the concentration of crowding agent increased (Fig. 1); 8 kDa PEG and 10.5 kDa dextran showed similar effects, and 35 kDa PEG was less efficient. The parallel responses to PEG and dextran of a similar size, the inverse dependence of the responses on the size of the crowding agent in the case of PEG, and the reversibility upon dilution of the crowding agent (Fig. 1), satisfy the criteria for a macromolecular crowding effect (Zimmerman and Minton 1993).

### Visualisation of crowding-induced self-association

The self-association of polynucleosomes in solution caused by crowding could also be visualised by fluorescence



**Fig. 1** Self-association of polynucleosomes by incubation with a macromolecular crowding agent, examined by a sedimentation assay. Polynucleosomes containing ( $^3\text{H}$ )thymidine-labelled DNA prepared by method A were incubated with 8 kDa PEG or 10.5 kDa dextran (open circle) (values were essentially identical) or with 35 kDa PEG (filled circle). Samples ( $\times$ ) were incubated with 8 kDa PEG, then diluted ten-fold and incubated for a further 10 min on ice before fixation to assess the reversibility of the effect of crowding. After centrifugation, ( $^3\text{H}$ )-labelled polynucleosomes remaining in the supernatant were quantitated. Means and ranges from five experiments are shown

microscopy after staining DNA with a fluorescent label (Fig. 2a). When polynucleosomes in a more concentrated solution were spread on the surface of a solution of crowding agent so that they mixed slowly with the viscous polymer solution and encountered a higher concentration, larger structures, mostly rods (Fig. 2b, left) and sheets (Fig. 2b, right) up to several tens of  $\mu\text{m}$  in size were formed, whose internal organisation remains to be studied. In similar conditions, F-actin in solution spreads on the surface of a dense PEG solution self-associated to form large protein complexes (Hosek and Tang 2004).

### Self-association of polynucleosomes by crowding does not require exogenous cations

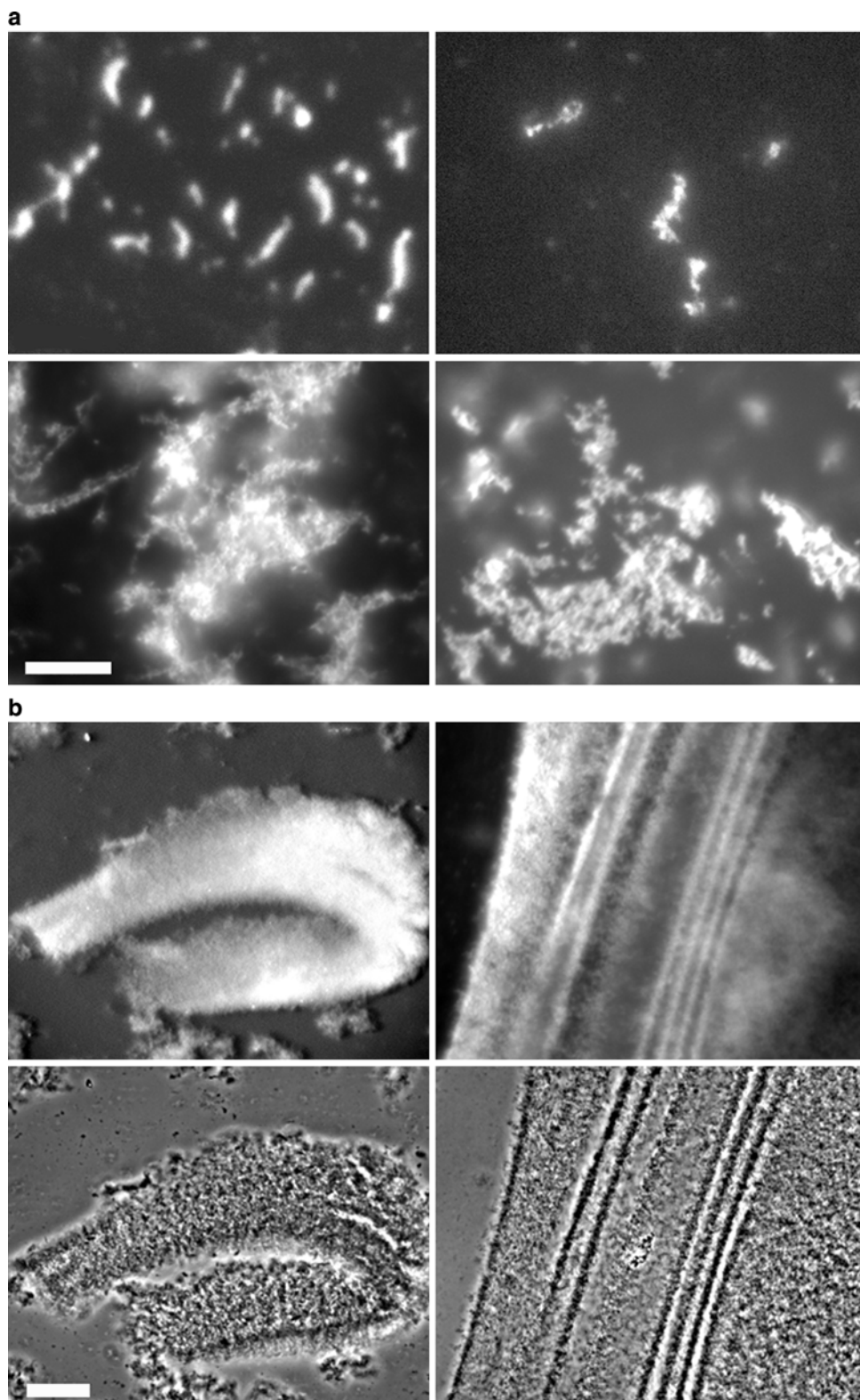
Exogenous cations in incubation media become bound to nuclei (Naora et al. 1961) and could influence the responses of polynucleosomes to crowding in view of their effects on self-association (Hansen 2002). To eliminate this possibility, a procedure (method B) was devised to prepare nuclei and polynucleosomes in a cation-free buffer; the dimensions, internal compartments, and ultrastructure of nuclei are preserved in these conditions (R. Hancock, unpublished results).

Polynucleosomes from these nuclei were labelled with the DNA stain YOYO-1, spread on a surface, and visualised by fluorescence microscopy, an approach which has not been described previously. Individual polynucleosomes could not be spread successfully on a silanised surface of the type used for DNA molecules (Lebofsky and Bensimon 2003), but they could be visualised after spreading on slides pretreated with polylysine (Fig. 3). Their average length was  $8.3 \pm 4.1 \mu\text{m}$  ( $n = 25$ ), close to that expected from the average length of their DNA ( $\sim 150 \text{ kb}$ ) reduced by a factor of 1/6 due to wrapping on nucleosomes. Their profiles were somewhat irregular (Fig. 3a), consistent with other reports describing the conformation of chromatin fibres in media of low ionic strength (Thoma et al. 1979; Leuba et al. 1994; Cano et al. 2006), although here it cannot be excluded that their conformation is influenced by interactions with positive charges on the surface. Compact 3-D clusters of polynucleosomes were formed after incubation with 12.5% PEG (Fig. 3b) or dextran (not shown), as seen by this procedure.

## Discussion and conclusions

Polynucleosome chains self-associate reversibly in conditions of macromolecular crowding, as seen using different experimental approaches. This process is distinct from that induced by cations (for example Cano et al. 2006; Hansen 2002) because it occurs in their absence. The folding of

**Fig. 2** Self-association of polynucleosomes prepared by method A visualised by microscopy. **a** In solution. *Upper panels* polynucleosomes alone labelled with DAPI; *lower panels* incubated with 8 kDa PEG (*left*) or 10.5 kDa dextran (*right*) (final polymer concentration 12.5%). **b** Polynucleosomes layered on a surface of 8 kDa PEG (25%) and labelled with YOYO-1. *Upper panels* fluorescence images; *lower panels* the corresponding phase contrast images. Bars 1  $\mu\text{m}$

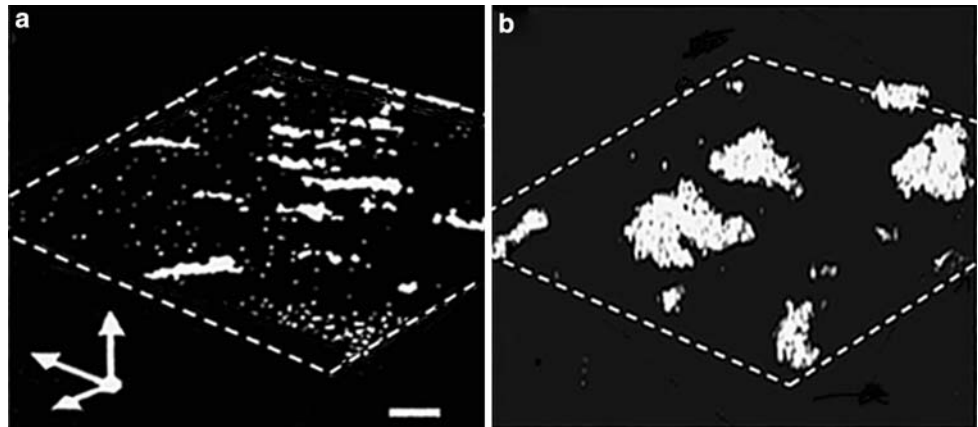


individual polynucleosome chain is also likely to be influenced by crowding, but other approaches such as real-time imaging will be required to study this aspect.

Considering that polynucleosome chains in the nucleus are in an environment where strong crowding forces are

predicted to occur (Hancock 2007), these results support the idea that crowding and entropic forces must contribute significantly to the compact lateral association of polynucleosome chains seen in vivo (Giannasca et al. 1993). Entropic forces in a crowded environment cause linear

**Fig. 3** Self-association of single polynucleosomes prepared by method B and labelled with YOYO-1, visualised by surface spreading. Polynucleosomes were incubated for 10 min in **a** 100  $\mu$ M Hepes, pH 7.4; **b** the same buffer with 8 kDa PEG (12.5%). Images are 3-D reconstructions from confocal image stacks. Bar 5  $\mu$ m



polymers to collapse to globular conformations (Van der Schoot 1998; Sear 1998; Cooke and Williams 2004; Dobrynin et al. 2005) as observed experimentally for DNA (Kojima et al. 2006), and they may demix to form a separate phase (Buitenhuis et al. 1995; Adams and Fraden 1998; Tuinier et al. 2003). These effects are believed to drive the compaction of bacterial chromosomes and their separation from the cytoplasm (Cunha et al. 2001) and are likely to contribute to the compaction of polynucleosome chains into discrete chromosome territories in the nucleus.

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