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Visualization of reconstituted solenoid chromatin structure by tapping mode atomic force microscopy†

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In this paper, tapping mode atomic force microscopy was applied to visualize the higher order chromatin structure reconstituted from calf thymus DNA and calf thymus whole histone *in vitro*. All the revealed reconstituted chromatin structures consisted of similar well-defined subunits. In some of the chromatin structures, four to six subunits per turn were wrapped right-handed to form a flattened solenoid with a height distribution of 12 ± 2 nm; and the other Y- and V-shaped chromatin structures were formed by aggregation of these solenoids, which presented similar heights as the solenoids. Typical atomic force microscopy (AFM) images revealed relatively regular solenoid structure (containing six nucleosomes per turn) with a ratio of diameter to pitch of 3 : 1, which is in good agreement with that of 30 : 11 in the chromatin solenoid model proposed by Finch and Klug. Bio-gel elution was used to separate reconstituted chromatin from uncombined histone particles, which proved to be helpful for the AFM imaging of the higher order chromatin structure with nearly no disturbance of histone particles and salt in the background. The AFM observations showed that the higher order chromatin structure was preserved well enough during the bio-gel elution process. The cause for the stability of higher order chromatin structure during the bio-gel elution was analysed. Electron microscopy analysis and circular dichroism spectroscopy investigation of the DNA–histone complexes indicated the formation of ordered aggregates. Copyright © 2001 John Wiley & Sons, Ltd.

KEYWORDS: AFM; solenoid chromatin structure; DNA; histone protein

INTRODUCTION

The structural organization of chromatin is fundamental to replication, transmission and expression of genetic information in eukaryotic cells. Although the location of core and linker histones relative to the path of DNA in the nucleosome has been established,[1–4] the mechanisms by which nucleosomes become compacted into higher order structures have not yet been determined. Several alternative structures such as the solenoid model[5–7] and the helical ribbon model[8,9] have been proposed for the 30 nm chromatin fibre thought to represent the primary level of chromatin folding. However, none of the several specific models that envisage the condensed fibre have any substantial experimental support, and the higher order structure of chromatin, even at the 30 nm fibre level, is poorly understood.[10,11]

To address this controversy, a study was performed using the three-dimensional imaging capability of tapping mode atomic force microscopy (AFM), which makes it possible to image chromatin fibres under less damaging conditions.[12,13] The samples are not vacuum dried and are scanned in air at ~50% relative humidity. Under these conditions a film of liquid water resides on the support surface.[14] Previous studies have shown that AFM can be used to image native and reconstituted chromatin fibres in 'beads-on-a-string'[15,16] and 'zig-zag' conformation.[17] Here we report the development of visualization of reconstituted solenoid and solenoid-aggregated chromatin structures by AFM with nearly no perturbance of histone protein particles and salt crystal in the background. To the best of our knowledge, it is the first time that the solenoid chromatin structure has been imaged by AFM.

MATERIALS AND METHODS

Reconstitution of calf thymus chromatin

The reconstitution was carried out under high NaCl salt conditions similar to those described elsewhere.[18] Calf thymus unfractionated whole histone (histone type IIA lyophilized powder from Sigma) was made 2.0 M with NaCl in 10 mM TE (TRIS and Na_2EDTA) buffer (pH 8.3) and mixed with double-strand calf thymus DNA obtained from Sigma. Before the reconstitution experiment, histone protein analysis carried out by electrophoresis in sodium

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dodecyl sulphate (SDS) polyacrylamide gels[19] guaranteed the composition of whole histone and nearly no other non-histone protein impurity, which showed that the whole histone was suitable for chromatin reconstitution. The final DNA concentration was always 40–50 μg ml^{-1} and the histone concentration was 1.5 μg μg^{-1} of DNA repeat. Sample were then dialysed at 4 °C against 0.5 l of 10 mM TE–NaCl (pH 8.3) as follows: 2.0 M NaCl, 4 h; 1.5 M NaCl, 4 h; 1.0 M NaCl, 4 h; 0.6 M NaCl, 6 h; 0.4 M NaCl, 4 h; 0.15 M NaCl, 6 h. The final dialysis was always overnight (>10 h) into 80 mM NaCl of 10 mM TE buffer. After reconstitution, the sample was stored at 4 °C prior to use.

Electron microscopy

The procedure is described elsewhere.[6] Reconstituted chromatin samples were fixed at 4 °C for at least 16 h in 80 mM NaCl of 10 mM TE buffer by adding glutaraldehyde to 0.1% of the solution. The fixed samples were diluted at room temperature to an absorbance (at 260 nm) of ~0.12, and benzyldimethylalkylammonium chloride was added to a final concentration of 2×10^{-4}% (w/v). After 30 min, 5 μl drops were adsorbed to freshly prepared carbon-coated grids (5 min), washed in redistilled water (10 min), dehydrated in ethanol (3 s), air-dried and rotary shadowed with platinum at an angle of 10°. Samples were examined in a JEM-100CX electron microscope.

Sample preparation by AFM

About 100 μl of the reconstituted chromatin sample in 80 mM NaCl–10 mM TE buffer was loaded onto a 1.5 × 25 cm bio-gel P-2 (from Bio-Rad Laboratories, Richmond, CA) column and eluted with redistilled water at a flow rate of 0.2 ml min^{-1}. Fractions of 0.6 ml were taken and the optical density (OD_{260}) of each was determined. Reconstituted chromatin was expected to elute first and histone particles were expected to elute later. These expectations were based on the nominal exclusion limit of the column material and were confirmed by our UV data of the column fractions. A column fraction of ~2 μl column fraction with a UV absorbance of 0.12 at 260 nm was distributed on freshly cleaved mica and immediately dried under an infrared lamp prior to the AFM observation.[20] Tapping mode images were obtained with a Nanoscope III in a multimode AFM using nanoprobe silicon tips. The volume of the eluted sample was minute compared with the volume of the redistilled water used during the elution process, and nearly all of the salts were depleted after the bio-gel elution process. Thus, the observed structures on mica (see Results and discussion) cannot have resulted from increasing salt concentration during the deposition process. One can be reasonably sure that the features observed on mica reflect the chromatin structures presented in solution.

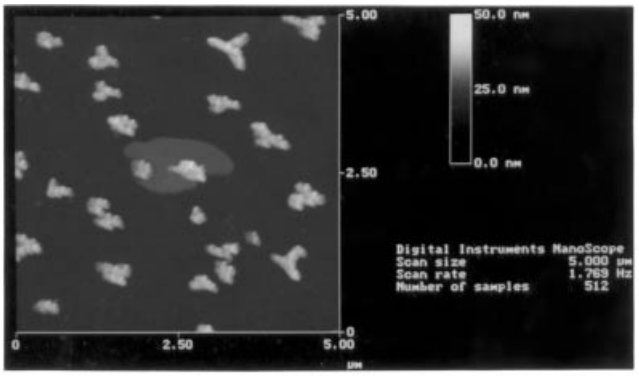
Circular dichroism spectroscopy

The circular dichroic spectra of the calf thymus DNA and the reconstituted DNA–histone complexes (both in 80 mM NaCl–10 mM TE buffer, pH 8.3) were recorded in a JACSO J-720 spectropolarimeter attached to a computer using a 1 mm path length cuvette and a slit width of 0.5 mm. Circular dichroism spectra measurements were accompanied by UV absorbance measurements (UV-1601 spectrometer) to determine the sample concentration. The spectra were recorded after the samples were kept at 4 °C overnight. The absorbances of histone–DNA complexes were checked routinely at 320 nm and were in the range 0.001–0.002, indicating that the spectra recorded were of soluble DNA–histone complexes.

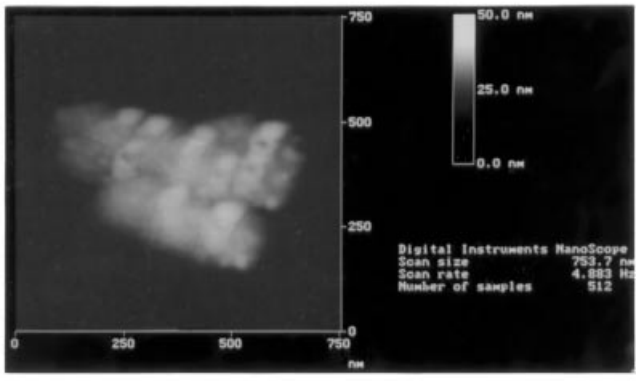
RESULTS AND DISCUSSION

Analysis of solenoid and solenoid-aggregated chromatin structure by AFM

Figure 1 shows the AFM images of reconstituted chromatin. Well-defined similar subunits in nearly all the reconstituted chromatin are observed, as shown in Fig. 1(a). A sheet of gauze-like material is also revealed, which is the result of the irregular aggregation of DNA under high NaCl salt conditions. Although a variety of chromatin conformations are presented, it seems that in some of the chromatin the



(a)



(b)

Figure 1. The AFM images of calf thymus DNA–calf thymus whole histone reconstituted complexes. The reconstituted sample was eluted by bio-gel P-2 column to separate DNA–histone complexes from uncombined histone particles, and most of the salts were depleted by the elution process. (a) Reconstituted solenoid and solenoid-aggregated V- and Y-shaped chromatin structures (scan size = 5.0 μm). (b) A typical solenoid chromatin (upper) aggregated with another condensed chromatin structure (below) (scan size = 750 nm).

subunits are assembled together to form a solenoid structure showing irregularity in diameter along the solenoid axis, and some V- and Y-shaped chromatin is formed by aggregation of these solenoid structures. The height at the highest point of the solenoid along the direction perpendicular to its axis is measured [Fig. 2(a)], and the statistical height distribution histogram is given (Fig. 3). All the observed solenoid and solenoid-aggregated structures show a height distribution of 12 ± 2 nm. One can discern from Fig. 1 that there are about four to six nucleosomes per turn in these solenoids and solenoid-aggregated chromatin. It is interesting to find that most of the solenoid chromatin structures have four nucleosomes in a turn at its end. The six nucleosomes per turn dominate in all the revealed chromatin structures, which indicates that this distribution of nucleosome in the solenoid represents a relatively more stable package configuration. One possible cause may concern the stress induced by coiling of nucleosomes to form solenoid. Six nucleosomes per turn induce less stress strength in the solenoid, which represents a relatively stable state and a denser package pattern as is common in higher order crystal structure. This package may be favourable for the condensation of chromatin in a more efficient way in order to accommodate long DNA molecules in a finite nucleus space. The histone H1 polymer may be smaller at its end and allow fewer nucleosomes to be packaged around it, which may account for the four nucleosomes in a turn at the solenoid end. The V- and Y-shaped chromatin consist of similar subunits and have a similar height to that of solenoid chromatin, which implies that the chromatin is an aggregation of solenoids. The tendency of solenoid chromatin to aggregate and its apparent flexibility are likely to be important properties for the further folding of chromatin *in vivo*,[21] which may account for the observed variability of chromatin conformations as shown in Fig. 1(a).

A typical image is shown in Fig. 1(b) and its corresponding dimension measurement is outlined in Fig. 2. One can discern from Fig. 1(b) that there are about three nucleosomes in every half-turn of the condensed chromatin. The image indicates that the observed structure is a flattened solenoid containing six nucleosomes per turn. Furthermore, from the height profile curve outlined in Fig. 2(a), one can clearly discern that there are six nucleosomes in a turn of the solenoid chromatin. The measured height information of the three nucleosomes at the right end of the solenoid, shown in Fig. 2(a) indicates that the structure is a right-handed solenoid, as is illustrated by the ideal solenoid shown elsewhere.[5,6] The right-handed solenoid is dominant in the revealed chromatin structure in Fig. 1, which may account for the circular dichroism spectra variation of DNA after its association with histone protein (see Fig. 5). The measured height value of an individual nucleosome (~6.7 nm, as indicated in Fig. 2(a)) is slightly higher than that of the nucleosome in the 'beads-in-a-string' chromatin conformation (5–6 nm), which may be (due to tilting of the disc-shaped nuclesomes (11 nm in diameter and 5.7 nm in thickness[16]) when combined with histone H1 and linker DNA to form solenoid chromatin structure. The height of the solenoid is measured to be 13.0 ± 1.0 nm. The measured

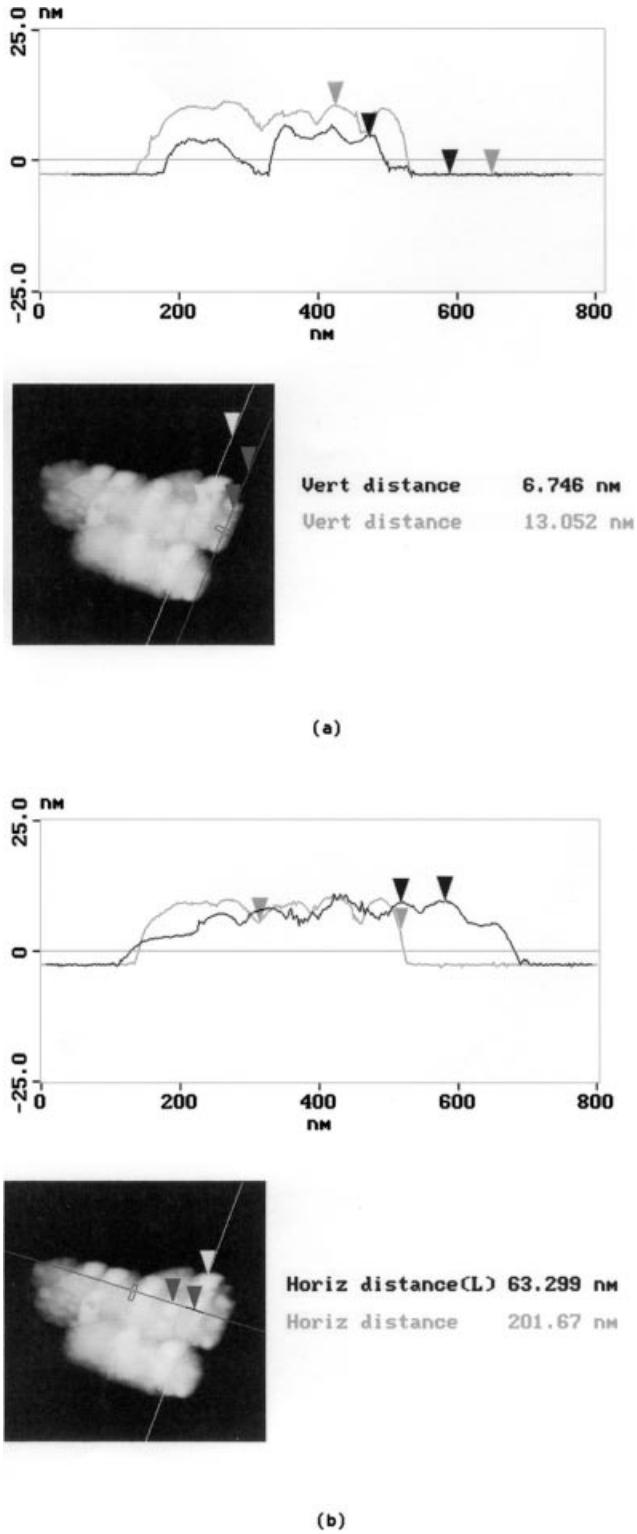


Figure 2. Dimension and height measurement profile curves of the typical solenoid chromatin shown in Fig. 1(b). (a) Height analysis profile curves of the solenoid and the three individual subunits in the half-turn at the right end of the solenoid. The measurements are done by drawing two parallel lines at the highest points of the subunits with their direction perpendicular to the axis of the solenoid. (b) Diameter and pitch measurements of the solenoid; these measurements are done by drawing two perpendicular lines at the highest points of corresponding subunits, with one line perpendicular and the other line parallel to the solenoid axis.

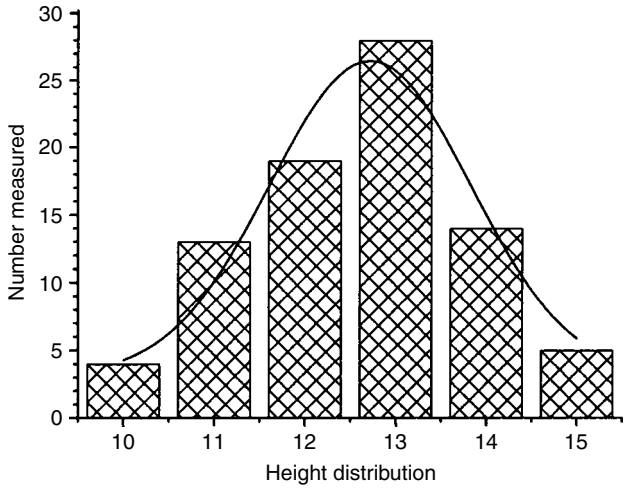


Figure 3. Histogram showing height distribution for the reconstituted higher order chromatin structure seen in Fig. 1.

value is smaller than anticipated for an ideal solenoid in which six nucleosomes per turn are packaged (the height of individual nucleosome is 5.7 nm), indicating that the sample undergoes a partial dehydration process and results in a flattened solenoid. The diameter of the solenoid is measured to be 201.7 nm and the pitch wideness is 63.3 nm as indicated in Fig. 2(b). These measured diameter and pitch values are sixtimes that of the proposed solenoid chromatin model with a diameter of 30 nm and a pitch of 11 nm.[5] It is interesting to note that in the revealed solenoid chromatin [Fig. 1(b)] the ratio of diameter to pitch is 3 : 1, in good agreement with that of the proposed solenoid model of 30 : 11.

The reconstituted higher order chromatin structures may present a relatively loose state after bio-gel elution treatment due to depletion of most of the salts in the solution, although the solenoid conformation is preserved. This loose of packing the solenoid and the solenoid-aggregated chromatin structures readily becomes flattened in the AFM sample preparation process. In addition, it is well known that a convolution effect of the tip existed in AFM measurements along a direction parallel to the substrate, and the convolution effect depends on the sharpness of the tip and may present different values for different tips. Allen *et al*. reported that the nucleosome core widths measured by AFM typically ranged between 30 and 40 nm in the 'beads-on-a-string' chromatin structure depleted of histone H1.[16] Thus, it is not difficult to understand that the dimensions and pitches of the imaged solenoid structures are much larger than that of the 30 nm chromatin fibre. In fact, the pitch per turn in the solenoid varies to a certain degree, as is revealed in Fig. 1(b). Thoma and co-workers[6] have measured the pitch of the solenoid obtained from rat liver chromatin. They estimated that each turn contains approximately six nucleosomes and observed a pitch of 10–15 nm. The preservation of solenoid conformation in the bio-gel elution and the AFM sample preparation and imaging process may attribute to histone H1 stabilizing the higher order chromatin structure, which may imply that H1 molecules form a polymer inside the solenoid. In addition, the flattened solenoid and solenoid-aggregated chromatin structures are anticipated to present similar height, in agreement with the measured height value, although the number of nucleosomes per turn may vary in different solenoids and/or in different turns of the same solenoid along its axis. Six nucleosomes per turn result in less stress strength and a larger hole inside the solenoid, which makes it easier to be flattened and compressed than for the solenoid with relatively fewer nucleosomes per turn in AFM sample preparation and imaging processes. The height difference of solenoids with different number of nucleosome per turn may be counteracted to a certain degree, as is shown in Fig. 3.

In the formation of solenoid chromatin structure, both NaCl and H1 are necessary.[22] The primary effect of NaCl is to screen DNA charge, thus increasing linker DNA flexibility and allowing a closer nucleosome–nucleosome approach. The presence of lysine-rich H1 either serves to constrain the exit angle of DNA from the nucleosome, as indicated in the zig-zag chromatin,[17,23] or promotes neighbouring nucleosomes to come closer together so that H1 might form a helical polymer inside the solenoid.[7] Perhaps the following five factors favour the formation, preservation and visualization of the higher order chromatin structure.

First, it is the mixture of calf thymus DNA with calf thymus unfractionated whole histone (but not that of other kind of histone or histone H1) in proper ratio under high salt condition that leads to the formation of the higher order structure chromatin. It is well known that core histones (H2A, H2B, H3 and H4) are highly conservative, but the kinds and relative amounts of the linker histone H1 vary with cell type[24] and each kind of H1 possesses a unique amino acid sequence.[25] It seems reasonable that the structural diversity exhibited by the H1 class serves as a basis for functional diversity. Analysis by circular dichroism, viscosity and nitrocellulose filter binding led to the conclusion that each kind of H1 differs in its ability to pack DNA fibres into highly ordered aggregates.[25] This suggests that the nature of the H1–DNA interaction involves different recognition sites on the DNA as well as on the H1 molecule.[25]

Second, it is possible that the relatively long length of DNA chromosomes does favour assembling of nucleosomes into solenoid arrays.

Third, the mild dialysis process helps to maintain the higher order chromatin structure.

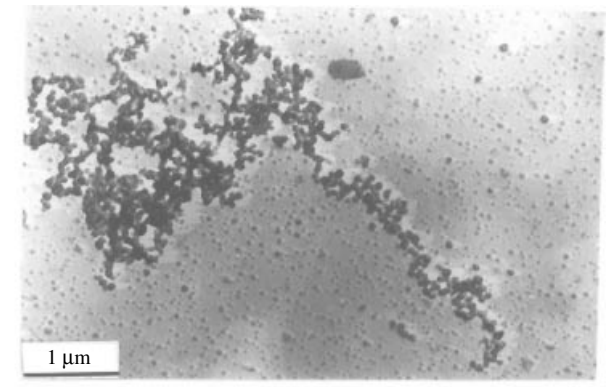
Fourth, the elution of chromatin by bio-gel P-2 column before AFM imaging contributes to visualize reconstituted solenoid-like chromatin with nearly no perturbance of histone particles and salt crystal in the background. The elution process of the chromatin sample by bio-gel P-2 makes the fraction containing chromatin separated from uncombined histone protein particles. Bio-gel P-2 is a porous polymer material with a pore dimension of 200–400 mesh. During the elution process, the uncombined histone particles are trapped among the porous polymer and flow through the interior of the porous spherical bio-gel material, whereas the reconstituted chromatin is excluded by the bio-gel polymer due to its relatively large size and flows through the interspace of the spherical bio-gel material. Thus, histone particles undergo a relatively longer distance and are separated from the reconstituted chromatin during the elution process. In addition, the low ionic strength

environment in the bio-gel column could contribute to suppress the aggregation of chromatin to a certain degree, which may favour visualization of the solenoid chromatin structure by AFM. It is interesting to learn from AFM observation that the higher order chromatin structure is not unravelled during the elution process. Early electron microscopy experiments showed that the higher order chromatin structure had been found at low ionic strength where the nucleosomes are still visible but join together to form 30 nm fibres.[25] Leuba *et al*.[26] demonstrated that chromatin fibre was three-dimensionally organized even at low ionic strength. Thoma and co-workers[6] showed that H1-containing chromatin did not unravel at low ion strength below 1 mM salt but only did s
removed. This points to the role of H1 in stabilizing the nucleosome and higher order structure of chromatin at very low ionic strength. We do not know the factor involved in the stabilization of the nucleosomal structure by H1. One contribution could simply be charge neutralization by lysine-rich positively charged histone H1. It is well known that H1 possesses the highest positive charge density among the histone protein family, which can condense double-strand DNA into loops, as does that of multications.[27] Clark and Kimura[28] have considered the role of electrostatic interaction in determining chromatin structure and single out repulsion between linker DNA as a probable major source of condensed fibre (depleted of H1) destabilization at low ionic strength. The binding of H1 to the linker DNA considerably reduces its electrostatic free energy by displacing bound cations and reducing the residual charge. Thus, H1-containing chromatin shows relatively higher stability at lower salt concentration than does H1-depleted chromatin. The H1 polymers formed inside the fibres under high salt condition were preserved in the chromatin structure during the elution process, whereas nearly all the salts were depleted in the mean time, though the H1 polymer may be presented in a relatively looser state after bio-gel elution treatment.

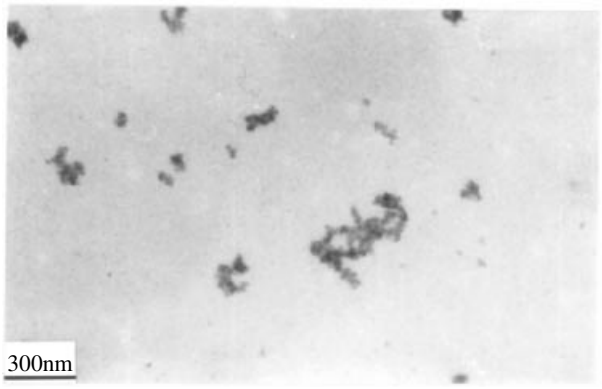
Fifth, the immediate infrared drying process of the sample on mica helps to preserve the higher order chromatin structure *in situ*. The conventional 'rinse and blow-dry' AFM sample preparation process on mica may distort the solenoid structure, and results in the 'zig-zag' chromatin pattern.[17] It is well known that an attraction force exists between chromatin and the mica surface; otherwise, the sample could not possibly be adsorbed on the surface of the mica. After deposition of the sample solution on mica and adsorption for a moment, the blow-dry operation with high-pressure nitrogen could exert a force on the adsorbed higher order chromatin structure at the liquid/mica interface and might distort its structure. The distortion induced by the blow-dry operation enables a relatively larger contact area of individual chromatin with the surface of the mica, which results in a relatively stable contact fashion of chromatin with mica and presents a 'zig-zag' chromatin pattern as reported by Martin *et al*.[17] This distortion effect cannot be neglected for the conventional 'rinse and blow-dry' AFM sample preparation process of the higher order chromatin structure, although this operation is preferable for the stretch of DNA for its base pair sequence analysis by AFM.[29] The infrared drying process can preserve the higher order structure on mica *in situ*, despite the structure possibly becoming flattened after undergoing a part-dehydration process. To the best of our knowledge, it is the first time a solenoid chromatin structure has been imaged by AFM.

Electron microscopy of reconstituted chromatin

The electron microscopy image shows that homogeneous subunits are closely packed together to form irregular fibres with uncombined histone particles dotted in the background [Fig. 4(a)]. The diameter of the subunits is ~70 nm, which is much larger than that is anticipated for the real dimension of nucleosomes (~11 nm in diameter). This dimension magnification effect may be attributed to shadow casting of the specimen at an angle of 10°. One can discern from its dimensions in the background that there are mainly two types of particles, the relatively larger particles correspond to histone octamer, and smaller particles relate to histone H1 particles (the latter being more prevalent). The relatively larger particles present a similar size to the subunits in the reconstituted chromatin,



(a)



(b)

Figure 4. Electron microscopy images of DNA–histone complexes. (a) Electron microscopy photograph of reconstituted DNA–histone chromatin. The sample with no bio-gel elution treatment was fixed with glutaraldehyde, washed in redistilled water, dehydrated in ethanol, air-dried and rotary shadowed with platinum. (b) Typical electron microscopy image of sample prepared from bio-gel-elution-treated reconstituted chromatin. Its corresponding sample preparation process is similar to that of (a) except that there was no glutaraldehyde fixation and no shadow casting of the specimen treatments.

which indicates that the core histone assembles to form histone octamer first and then reconstitutes with DNA strand to form a nucleosome. The revealed H1-containing chromatin fibre conformation shows a distinct difference from the appearance of the 'beads-on-a-string' of H1-depleted chromatin,[30] which is probably a partly ravelled product of the solenoid chromatin. However, solenoidal arrays of 30 nm chromatin fibre are not observed in Fig. 4(a).

It is well known that different sample preparation methods may result in variation of the chromatin structure. Chromatin structure may vary with the solution's ionic strength.[31] During electron microscopy sample preparation process, the deposition of chromatin on the carbon-coated grid, the wash and drying processing could distort the solenoid chromatin structure. Such conformation alterations have been observed also in other electron microscopy studies of chromatin structure.[32] Figure 4(b) is a typical electron microscopy image of a sample prepared from bio-gel-elution-treated reconstituted chromatin. Its corresponding electron microscopy sample preparation process is similar to that of Fig. 4(a) except that no glutaraldehyde fixation and shadow casting of the specimen treatments are adopted. Thus, the chromatin structures revealed in Fig. 4(b) show relatively lower contrast than that in Fig. 4(a). Figure 4(b) reveals a chromatin structure consisting of two and/or three spherical subunits every half-turn with nearly no uncombined histone particles in the background. The diameter of the subunits is ~30 nm. The imaged structure is flattened after adsorption to carbon-coated grids, which may account for the measured dimension discrepancy from the real value of the nucleosome.

The electron microscopy image shows structural similarity to the AFM image in Fig. 1 except that some of the chromatin fibres in Fig. 4(b) present a more aggregated state. This aggregation of fibres in Fig. 4(b) may be due to shrinkage of the fibres induced by the ethanol drying treatment and the dehydration process of the sample under high-vacuum electron microscopy conditions. The subunits in the chromatin fibre revealed in Fig. 4 are nucleosomes, despite the different sample preparation methods resulting in a measured dimension variation. From the electron microscopy and AFM data one can draw the conclusion that the subunits in the chromatin fibres revealed by electron microscopy observation (Fig. 4) are the same as the subunits in the solenoid and solenoid-aggregated chromatin structures shown in the AFM images (Fig. 1). Although a convolution effect exists in the latter method, the three-dimensional imaging capability and the operation in air of AFM provide useful information for analysis of the higher order chromatin structure.

Circular dichroism spectroscopy of DNA–histone complex

Condensation of DNA upon addition of histone protein has been studied extensively by employing the circular dichroism spectroscopy technique.[33–35] The basic parameter studied in this technique is the decrease in the positive ellipticity at 270 nm of the DNA spectrum upon interaction with histone. An increasing concentration of histone brings about a progressive decrease in the positive ellipticity,

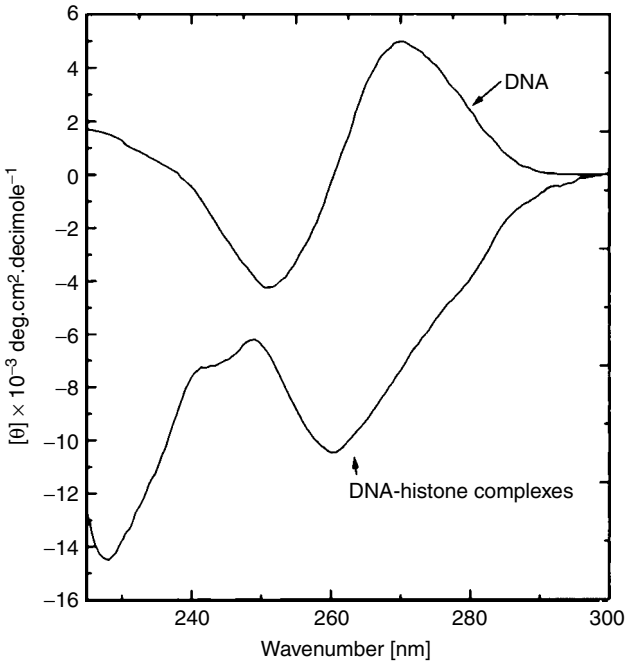


Figure 5. Circular dichroism spectroscopy of the DNA and the DNA–histone complexes.

which ultimately shows a steep negative $\theta_{270\,nm}$ value.[33] The spectrum generated is often referred to as a 'psi' type of spectrum and reflects the optical property of the soluble DNA–histone complexes formed. It is generally believed that the 'psi' type of spectrum is a result of base tilting in the DNA, a change of DNA hydration and the side-by-side aggregates of DNA duplexes.[35] The circular dichroism spectra of calf thymus DNA and complexes of calf thymus DNA with calf thymus histone are shown in Fig. 5. The uncomplexed DNA shows the conservative spectrum characteristic of the B form of DNA, with a positive ellipticity band at 270 nm and a negative band at 250 nm. Spectra of the calf thymus whole histone showed no ellipticity above 250 nm (results not shown), and thus do not interfere with the observations of changes in the DNA spectrum. The formation of DNA–histone complexes resulted in large distortions in the DNA spectrum; the positive ellipticity band at 270 nm completely disappeared and a very large negative band appeared at ~260 nm. This spectrum indicates the association of calf thymus DNA with calf thymus whole histone into higher order aggregates.

CONCLUSIONS

Atomic force microscopy in combination with the bio-gel P-2 elution method was applied to visualize the reconstituted higher order chromatin structure with nearly no disturbance of histone particles in the background. The AFM image showed that four to six nucleosomes per turn were wrapped right-handed to form flattened solenoid and solenoid-aggregated chromatin structures with a height distribution of 12 ± 2 nm. The AFM and electron microscopy observations showed that the reconstituted higher order chromatin structure is preserved well enough after bio-gel elution treatment. The cause for the stability of the chromatin during

the elution process is analysed. Circular dichroism spectra of the DNA–histone complexes indicated the formation of an ordered aggregation.

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