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Single Chromatin Fibre Assembly Using Optical Tweezers

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Abstract

Here we observe the formation of a single chromatin fibre using optical tweezers. A single λ-DNA molecule was suspended between two micron-sized beads, one held by a micropipette and the other in an optical trap. The constrained DNA molecule was incubated with Xenopus laevis egg extract in order to reconstitute a single chromatin fibre. An eight-fold compaction of the DNA molecule was observed in real-time. The compaction kinetics were found to be strongly dependent upon the tension applied to the DNA molecule. We incorporated the analysis of Brownian motion to accurately determine the tension throughout the compaction process. At forces exceeding 10 pN complete inhibition of compaction was observed for the time scale of the experiment. We have previously shown that stretching of a reconstituted chromatin fibre results in discrete and quantized structural opening events that we can attribute to the unravelling of single nucleosomes. Assembly kinetics therefore provide insight into rates of nucleosome formation and we demonstrate the possibility of probing these kinetics under different experimental conditions.

Introduction

Eukaryotic DNA, when packaged into the cell nucleus, is in the form of a highly ordered nucleoprotein complex, chromatin. In the case of H. Sapiens, this DNA compaction is on the order of 10^5. The first structural level of this process is the formation of an array of nucleosomes. Eight histone proteins (two copies of H2A, H2B, H3 and H4) make a particle, about which the DNA is wrapped, forming a nucleosome. The nucleosomes are spaced approximately every 200 base pairs along the DNA [1], resulting in a "beads on a string" type of structure. Within the cell nucleus, biological processes such as transcription, replication and repair involve gaining access to a condensed DNA template that has undergone several more stages of compaction. It is proposed that interactions with the DNA sequence are gained through the disruption of the higher-order structure of chromatin, and that either unwrapping or loosening of the DNA from around the histone octamer is one essential component [2]. Chromatin is hence dynamic in terms of structure; regulation of these processes requiring not only disruption but also the formation of

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nucleosomes in maintaining or restoring a more compact structure.

With the advent of single molecule biophysical techniques, such as atomic force microscopy and optical tweezers, it is now possible to study the structural and mechanical properties of individual biological molecules under near-physiological ionic conditions. The molecule is attached between two surfaces, and the forces required to elongate the molecule are measured. For example, several groups have reported the force-induced opening of individual domains within a single protein molecule such as titin using atomic force microscopy [3, 4, 5] or optical tweezers [6, 7].

Our group has recently focused on using optical tweezers to investigate the structural and mechanical properties of a reconstituted chromatin fibre through force-induced disassembly [8]. Other studies include the use of optical tweezers to disrupt native chicken erythrocyte chromatin, extracted directly from the cell nucleus [9], and AFM force spectroscopy of chromatin reconstituted solely from core histones [10]. However, these single molecule techniques are not limited to the study of dissociation forces and mechanical properties, but can also provide a wealth of information about dynamic structural changes and interactions. For example, here we report on the use of optical tweezers for assessing the kinetics of the formation of nucleosomal structure.

Presently, a study of the literature reveals that an overwhelming number of experiments have been undertaken focusing on the study of mechanical and structural properties of biomolecules. This information is gained through force-induced stretching and rupture of structural domains or motifs. Furthermore, the pioneering work of Evans has shown that insight into the energetics of interactions between biomolecular pairs, held together by weak non-covalent bonds, is possible through the measurement of dissociation forces as a function of the rate at which the interaction is loaded [11, 12, 13]. However, it is noticeable that single molecule experiments have only just scratched the surface in terms of exploring the possibilities of measuring kinetics in terms of association, and the formation of structure rather than disruption [14, 15, 16].

Experimental

Instrument Design

The experimental set-up used for this study has been described in detail elsewhere [8]. The basic features include: a single-beam gradient optical trap, generated using 1 W of a continuous wave laser (1064 nm, 10 W, Millennia, Spectra Physics, Mountain View, CA, USA) and expanded to fill the back aperture of a high-NA water immersion lens (100 x, 1.2 NA, Leica, Wetzlar, Germany); a piezo-driven flow cell, incorporating a fixed micropipette; a quadrant detector monitors deflections of the transmitted light from the trapped bead; the entire experiment is observed by projection of the sample plane onto a ccd camera; a semi-automated flow system is used to control the exchange of different buffers and the rate of the flow within the purpose-designed flow cell.

Instrument Calibration and Force Measurement

Single molecule force spectroscopy requires the accurate determination of two parameters; tension (force) within the molecule as a function of molecular extension (distance). For our optical tweezers experiment the distance between our two beads is determined using video microscopy at a frame rate of 25 Hz. Conversion from pixels to absolute distances is made using a calibration slide. The stiffness of the trap is determined using hydrodynamic flow. This calibration involves recording the detector deflection signal and the bead position with respect to the centre of the trap as a function of drag flow on the bead. The flow rate at the trap position was determined by measuring the velocity of beads as they pass the field of view of a microscope. The drag force was then calculated using Stokes' law:

$$F_{drag} = 6\pi\eta r v \qquad (1)$$

where η is the viscosity of the medium, r is the radius of the bead, and v is the flow speed. A plot of drag force versus bead displacement gives a linear plot of which the gradient corresponds to the trap stiffness. Once these calibrations have been carried out, the tension within the molecule under investigation, as a function of its extension, is easily evaluated.

Materials

10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 0.05% BSA and 0.01% NaN_3 was used as the main flow buffer. Linearised bacteriophage λ-DNA (c1857ind1 Sam7, New England Biolabs, Beverly, MA) was end-biotinylated by incubation of a 50 μg ml^{-1} solution with 100 mM dGTP, 100 mM dCTP, 0.4 mM bio-14-dATP and 0.3 mM bio-11-dUTP and 10 units of Klenow DNA polymerase (50 mM phosphate buffer, 10 mM $MgCl_2$, pH 7.5) for three hours at 37°C. Following purification, using standard procedures, a 0.25 μg ml^{-1} λ-DNA solution in TE buffer was prepared for use in the experiment. Streptavidin-coated 2.6 μm polystyrene beads were prepared by incubating carboxylated beads at a concentration of 2% w/v, with a 2 mg ml^{-1} solution of streptavidin (Roche Molecular Biochemical, Almere, the Netherlands) for 15 minutes at room temperature. 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) (Sigma) was

added to activate the carboxyl groups in order to enable the streptavidin coupling. The pH was adjusted to 6.5 and the beads were incubated for 2 hours at 37°C. 100 mM glycine was used to block unreacted sites and the beads were then washed for a number of times with 50 mM phosphate buffered saline (PBS, pH 7.5). A solution of approximately 10^5 beads ml^{-1} in TE buffer was used in the experiment. *Xenopus laevis* cell extract was used as described previously [17].

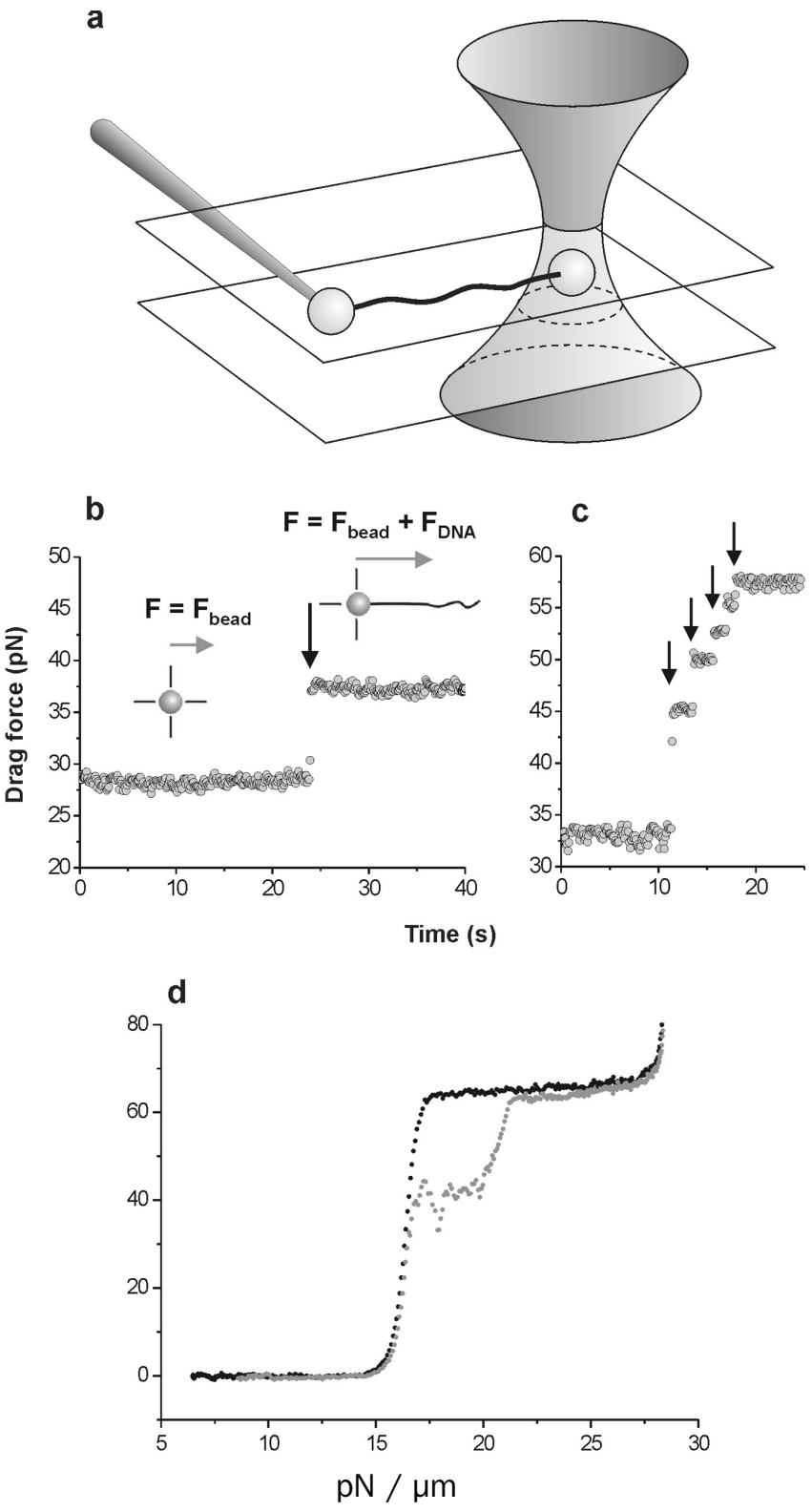

Fig. 1. *Attaching a single λ-DNA molecule between two polystyrene beads.* **(a)** *Schematic representation of a single DNA molecule suspended between two beads. After immobilisation of the first bead on a micropipette and the second in the optical tweezers, end-biotinylated DNA is introduced into the flow cell. The deflection of the transmitted laser light through the polystyrene bead is used to monitor the drag force on the bead under a constant flow rate (approx. 1000 mm* s^{-1}*).* **(b)** *The attachment of one single DNA molecules is detected as a sudden increase in drag force on the trapped bead. Using optimised conditions it is possible to have only a single molecule attached to the bead (~10 µl, 0.25 µg* ml^{1} *λ-DNA).* **(c)** *Here at a higher DNA concentration (2.5 µg* ml^{-1}*) we observe the drag force increasing in a stepwise manner as a result of multiple DNA molecules attaching to the trapped bead. The vertical arrows indicate the stepwise force increases detected per additional attached DNA molecule.* **(d)** *A typical force-extension curve from a single λ-DNA molecule.*

Results

Chromatin Assembly

Prior to each chromatin assembly experiment it was necessary to catch a single DNA molecule between two polystyrene beads (Figure 1(a)). These kinds of manipulations have been described in detail previously [8, 16, 18, 19, 20]. The concentration of the DNA solution and the amount introduced into the flow cell, in order to promote binding of only one DNA molecule, was optimised through monitoring changes in the drag force on the trapped bead (Figure 1(b - c)). Once the DNA is suspended between the two beads it can then be manipulated with piezo-controlled precision. Prior to chromatin assembly the force extension characteristics of the molecule are measured in order to ensure that it is indeed a single molecule (Figure 1(d)).

In order to assemble a single chromatin fibre, buffer containing nuclear extract from *Xenopus laevis* eggs is introduced into the system [17]. The extract (12 µl of high speed supernatant was diluted in 1 ml of assembly buffer (50 mM HEPES-KOH, pH 7.6, 50 mM KCl, 1 mM EDTA, 2 mM β-mercaptoethanol). The cell extract contains core histones H2A, H2B, H3 and H4 and other DNA-binding non-histone proteins, but lacks somatic linker histones. It has been shown that the extract has all of the necessary proteins required to assemble nucleosomal arrays on naked DNA molecules [21]. During the assembly process the optical trap is switched off and the instrument used in laminar-flow mode. This allows the collection of distance versus time data under very low forces, the lowest limit being set by the force required to prevent the beads from bumping into one another. The application of a tension within the DNA/chromatin is carefully controlled through the flow rate. Chromatin formation is evident as the distance between the two beads drastically decreases as cell extract enters the flow cell. Evidence that this compaction is due to histone binding comes from biochemical studies. These show that histone depleted extract is incapable of DNA compaction but that extract activity is restored by the addition of histones [15]. Structural evidence of a nucleosomal array is supported by both biochemical studies and our own force spectroscopy [15, 8].

Kinetics of Chromatin Assembly as a Function of DNA Tension

The assembly procedure was carried out for a number of different DNA molecules under a range of flow rates, and hence as a function of DNA tension. Figure 2 shows a schematic picture of the assembly procedure (a-c) along with typical length versus time data (d) from video microscopy analysis of the inter-bead distance. The tension applied to the DNA molecule during the assembly was determined using two independent methods. Firstly, the tension could be determined using Stokes' law as described earlier. However, this procedure was thought to be problematic since the micropipette with the bead on its tip could shield the flow in front of the freely suspended bead and hence result in a lower force than expected on the DNA molecule. In order to evaluate this shielding effect, an independent check of the DNA tension throughout the assembly was carried out by monitoring the Brownian motion of the freely suspended bead. Video analysis software was used to extract the positions of the two beads with a time resolution of 25 Hz (Figure 2(e)). The mean square displacement $\langle \Delta x^2 \rangle$ in the direction perpendicular to the flow, was determined by the force on the tethered bead [22]:

$$\frac{1}{2} k_B T = \frac{1}{2} \frac{F}{L} \langle \Delta x^2 \rangle$$
$$F = \frac{L k_B T}{\langle \Delta x^2 \rangle} \qquad (2)$$

where k_B is the Boltzmann constant, T is the absolute temperature and L the end-to-end distance of the connecting polymer. From this investigation it was found that the tension did indeed vary as a function of inter-bead distance. The tension reported in this study was taken from the point of inflection of the force versus time curve (see Figure 2 (e)). This value was found to be consistent with that measured using Stokes' law, however it is evident that precise control of force over the entire assembly will require detailed characterisation of its dependence on bead separation and control over the flow speed to correct for this effect.

Through careful control of the extract flow speed it was possible to study assembly kinetics for a range of DNA tensions. It was found that at tensions exceeding 10 pN in force, no apparent shortening was observed on the time scales that were studied, suggesting significant inhibition of nucleosome formation and therefore DNA compaction. At a tension of ~5 pN the rate of shortening was approximately 2 nm s^{-1}. A decrease in tension resulted in a dramatic increase in the compaction rate; at tensions of ~2 pN a rate of just over 10 nm s^{-1} was observed and for ~1 pN tension the compaction rate was almost 200 nm s^{-1}. The effects of tension on association kinetics are illustrated in Figure 3. We have previously suggested that our chromatin fibre consists of an array of nucleosomes [8], and due to the lack of somatic linker histones we expect that the reconstituted fibre has a "beads-on-a-string" type of structure. Hence, at the lowest tension studied we calculate that the assembly corresponds to the formation of 2 to 3 nucleosomes per second.

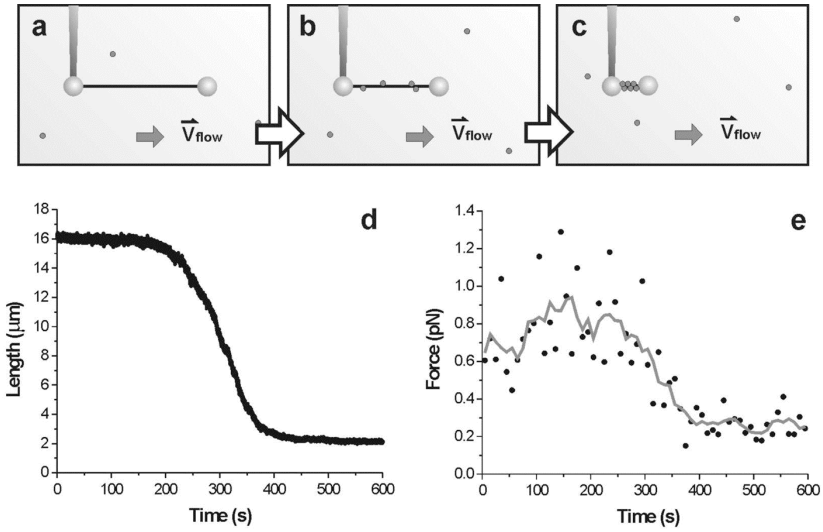

Fig. 2. *Assembly of chromatin with Xenopus laevis egg extract on a single λ-DNA molecule.* **(a-c)** *Schematic of chromatin assembly in the optical tweezers set-up.* **(d)** *Measurement of the length of DNA as a function of time. From these data it is evident that a saturation point is reached after approximately an 8-fold compaction of the DNA. The DNA changes in length from 16.4 μm to approximately 2 μm.* **(e)** *The tension within the DNA molecule, over the time course of the assembly procedure, is monitored by the Brownian motion of the trapped bead.*

Discussion

Here we have shown that through monitoring the drag force on an optically trapped bead, the number of DNA attachments can be monitored. This has proven to be essential for optimisation of the volume and concentration of our DNA solution in order to achieve just one attachment to the bead. Following on from our previous studies, in which we suggest that the reconstituted chromatin comprises of a nucleosomal structure, we now present data on assembly kinetics. Using this technique we have investigated chromatin formation at molecular tensions as low as 1 pN. Assembly kinetics were monitored through the collection of distance versus time data, and the tension during this process was determined using two independent methods; Stokes' law and Brownian motion of the freely suspended bead. In general the optical trap may be used to maintain a constant tension within the molecule using a force-feedback system. However, due to the presence of cell debris within the extract this was not possible. Instead forces were exerted using a constant laminar flow. Over the assembly period it was found that shielding of the flow by the micropipette and the bead attached to it, resulted in forces that were dependent on inter-bead distance (Figure 2(e)). In order to carry out a more detailed study on assembly kinetics at precisely set DNA tensions, it will be necessary to characterise force as a function of inter-bead distance for a wide range of flow rates. This will then allow us to automate adjustment of the flow in order to maintain a constant tension.

It can be estimated that a reduction in the length of our DNA from 16.4 to 2 mm during assembly corresponds to the formation of approximately 220 nucleosomes (a reduction of 65 nm in length per nucleosome formed [8]). At tensions above 10 pN no apparent shortening of the DNA is observed. From our force-induced disruption of nucleosomal structure we know that nucleosome core particles are removed at forces between 20 and 40 pN (loading rates of ~ 40 pN s^{-1}) [8]. Therefore we can predict that at higher tensions the kinetics of DNA-core histone interactions will have an equilibrium that is shifted further toward dissociation, hence assembly may be undetectable. At tensions below 10 pN we observe that the equilibrium between association and dissociation is drastically shifted as a function of force. At the lowest tension studied here (1 pN) we record a chromatin assembly rate of 2 to 3 nucleosomes per second.

We can compare our results to those of Ladoux *et al.* in which fluorescence videomicroscopy was used to follow single molecule chromatin assembly in real-time [15]. For this experiment one end of a λ-DNA molecule was attached to the surface whilst the other was free in solution. The DNA was fluorescently labelled with YOYO-1 dye and chromatin assembly was monitored under constant flow rates upon addition of *Xenopus laevis* extract. It is interesting to note that our experiments are comparable in terms of rates of compaction for similar estimated DNA tensions. From the sigmoidal form of the compaction curve (similar to our Figure 2(d)) they suggested that a minimum of three sequential events, of a similar time scale, occur during the wrapping of DNA around the histone octamer and that these events could possibly be binding of the H3-H4 tetramer, the first H2A-H2B dimer and then the second H2A-H2B dimer. However, we should note that in our study we typically use relatively slow flow rates hence it is difficult to identify the exact point at which the extract enters the flow cell and the time that it takes to completely exchange the original buffer within the flow cell. This could falsely lead to a sigmoidal curve of compaction and care must be taken in the interpretation of these data in its present form. One

solution to this problem could be the use of a combined optical tweezers with fluorescence, whereby the binding of fluorescently labelled histones may reveal a three step binding process; and this would ideally be in combination with a more efficient exchange of the buffer for cell extract at the start of the experiment.

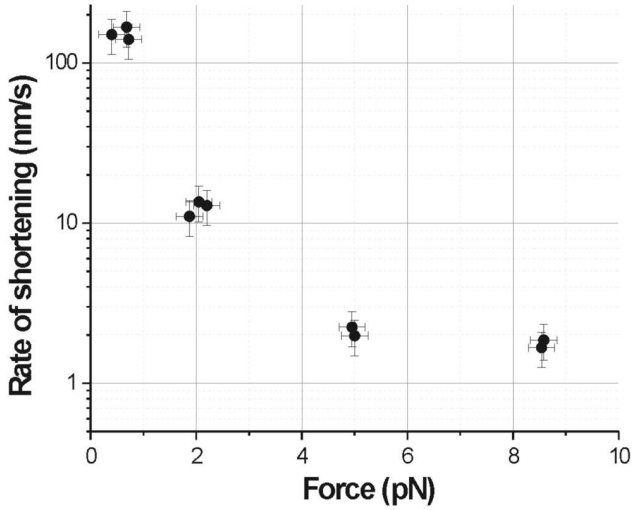

Fig. 3. *Rate of DNA compaction as a function of tension. Chromatin was assembled using cell extract for a number of different DNA molecules over flow rates corresponding to a range of 1 pN to > 10 pN tension within the DNA. At forces exceeding 10 pN no shortening was observed. A significant increase in rate of assembly was observed as the tension decreased below 10 pN. Tension within the polymer was calculated from the Brownian motion of the freely suspended bead perpendicular to the flow direction. These forces were found to be consistent with those calculated using Stokes' law.*

Our future experiments will focus on a more in depth study of both association and dissociation kinetics and will involve an extensive study of the effects due to DNA tension. In order to eliminate problems associated with flow shielding, an accurate force clamp technique will require careful flow rate control, based on the characterisation of tension as a function of inter-bead distance. This will be carried out in both low and high force regimes where we can examine the shift in equilibrium between association and dissociation respectively as a function of template tension. Furthermore, we would like to investigate the association/dissociation kinetics of a single nucleosome. However, we envisage a number of limitations using our current optical tweezers set up, particularly in terms of the viscous drag of the trapped bead, hence the detection response time is likely to be on a much longer time scale than the nucleosome interaction under investigation. This is a problem that will have to be addressed in terms of experimental design.

Conclusion

Throughout the lifetime of a cell, chromatin undergoes structural rearrangements, such that the accessibility of the DNA template is carefully regulated. This accessibility is crucial for processes such as gene expression, replication and repair. Therefore the study of dynamic interactions between histone proteins and DNA is biologically relevant. Investigations of the influence of force on biological interactions are pertinent since single molecule studies have recently revealed that enzymes such as RNA and DNA polymerases are capable of producing piconewton scale forces [23, 24, 25]. Indeed the generation of these forces may be central to the remodelling of chromatin structure, for example through movement, loosening, or complete dissociation of nucleosomes from the DNA template. Following disruption, nucleosomal structure must reform and be maintained, a process that may occur for a DNA template that is still under tension. Hence the dynamic structure of chromatin in terms of nucleosome disruption and formation is important in the regulation of all biological processes that require access to nuclear DNA.

We have previously shown that disruption of a single chromatin fibre provides information about physical properties such as mechanical strength and allows structural motifs such as the amount of DNA bound or associated with a single nucleosome, to be determined. Here we also report the unique ability of a single molecule technique to study association kinetics. It is evident that future optical tweezers studies and other force spectroscopy techniques such as atomic force microscopy can be used to study interaction kinetics and dynamic changes in structure under many different environments and could allow one to elucidate important biological mechanisms under the careful control of parameters such as force and torque [26, 27, 28].

Continuing from these pilot studies we are also focusing our research towards studying the exciting field of dynamics in chromatin structure in response to histone modification. It is known that different stages in the cell cycle require chromatin in different structural forms and that histone modification is an important parameter in this structural regulation. Modifications such as acetylation, phosphorylation and methylation have all been biochemically identified and are correlated to specific events in the cell cycle. Using recombinant histones, our aim is to investigate the structure and mechanics of a well-defined single chromatin molecule. Furthermore, enzymatic modification of the histones using single molecule optical tweezers studies will allow the study of chromatin remodelling in real-time.

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