ELSEVIER

Single-molecule manipulation of nucleic acids

Ulrich Bockelmann

During the past decade, local force measurement techniques, such as atomic force microscopy and optical tweezers, were used to study the elastic properties and mechanically induced structural transitions of nucleic acids at the single-molecule level. Single-molecule manipulation has also increasingly been used to investigate DNA-dependent enzymatic processes, with implications for unfolding and modifying DNA, protein–DNA interactions, replication and transcription. Compared to classical techniques of molecular biology, single-molecule measurements avoid the need to average over a large number of events, and can thus potentially provide detailed and complementary information.

Addresses
Ecole Normale Supérieure, Laboratoire Pierre Aigrain, 24 rue Lhomond, 75005 Paris, France
e-mail: ulrich.bockelmann@lpa.ens.fr

Current Opinion in Structural Biology 2004, **14**:368–373

This review comes from a themed issue on
Nucleic acids
Edited by Carlos Bustamante and Juli Feigon

Available online 17th April 2004

0959-440X/$ – see front matter

DOI 10.1016/j.sbi.2004.03.016

Abbreviations

DIG	digoxygenin
dsDNA	double-stranded DNA
ssDNA	single-stranded DNA

Introduction

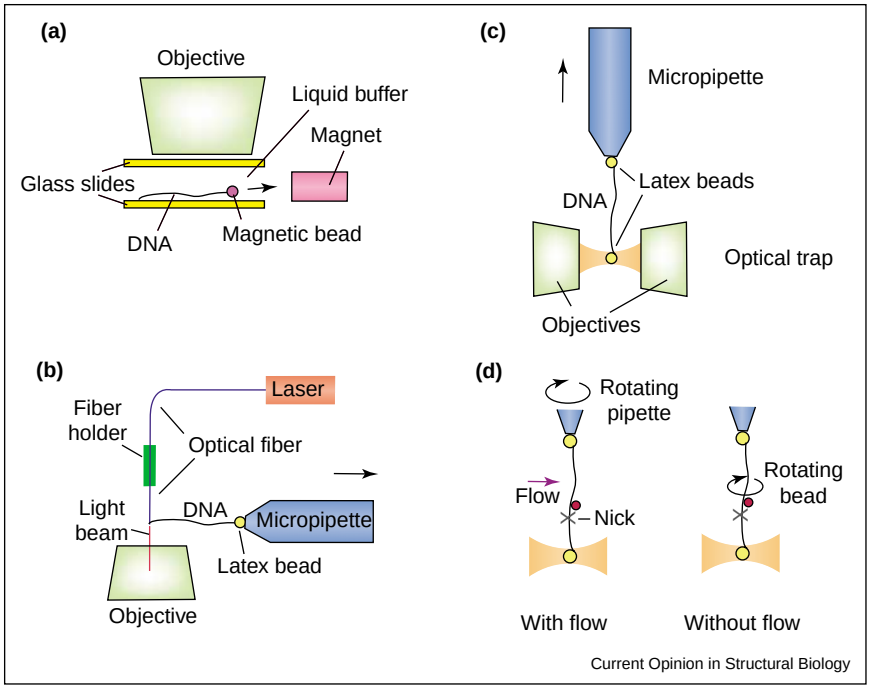
The nucleic acids DNA and RNA play a central role in biology, and our understanding of molecular processes involving nucleic acids is steadily growing. X-ray scattering and NMR techniques are very important to this field, as they provide high-resolution structures of RNA and protein–nucleic acid complexes. This structural information has to be complemented by dynamic studies to reveal the conformations, energy landscapes, length and timescales of the molecular rearrangements involved in the function of the macromolecule. Single-molecule manipulation of nucleic acids, the subject of this review, allows one to apply an external mechanical constraint (force and/or torque) in a well-defined way and to measure the time-dependent response in length, force, angle or torque. These measurements are usually done in solution, and can provide information on the folding dynamics and elastic properties of nucleic acids. Forces that are generated in the course of biochemical reactions can be measured and the reaction can be investigated as a function of an external mechanical load. Earlier reviews of single-molecule mechanics of nucleic acids are available [1–3]. Single-molecule fluorescence measurements have been reviewed [4,5] and are not considered here.

Stretching and twisting DNA

The field of single-molecule manipulation of nucleic acids started in 1992 with the first measurement of the elasticity of single DNA molecules by Smith, Finzi and Bustamante [6]. A dsDNA molecule was attached at one end to a glass surface and at its other end to a magnetic bead (see Figure 1a). Assembly of the system was done in solution using biotin–streptavidin and digoxygenin (DIG)–anti-DIG interactions to specifically bind the molecule to the two different solid surfaces; this choice remained the reference immobilization strategy of the field until today. Equilibrium positions of the beads were observed with a microscope while the beads were acted on by magnetic force. This way, extension versus force curves were measured for individual multimers of λ-DNA (48 502 base pairs) in aqueous buffers of different salt concentration and with forces of up to 10 pN. In this regime of low force, the elasticity of dsDNA is governed by competition between the entropy, which tends to coil the molecule, and the external force, which tends to stretch it. Important deviations from the force curve predicted by the freely jointed chain model of polymer elasticity were observed, suggesting that DNA has significant local curvature in solution. The worm-like chain model, which describes the DNA molecule as a thermally fluctuating stretched elastic tube, was found to provide a good theoretical description of dsDNA elasticity at low force.

A couple of years later, when forces in excess of 60 pN were applied to DNA, unexpected overstretching of the dsDNA molecule by about 70% was observed [7,8]. This property was attributed to a structural transition to a new DNA phase, called S-DNA. In spite of a significant body of experimental and theoretical investigation, the structure of S-DNA remains the subject of debate [3]. In the setup of Cluzel *et al.* [7], an optical fiber was used as the force sensor, and the DNA molecule was attached at one end to the fiber and at the other end to a microbead (see Figure 1b). The bead was caught by suction and held at the tip of a rigid micropipette. This pipette was displaced by a piezo-translation stage and the fiber deflection was recorded by detecting a transmitted laser beam with a position-sensitive photodiode after magnification with a microscope objective. In the setup of Smith *et al.* [8], the DNA molecule was stretched with a force-

Figure 1



Stretching and twisting single DNA molecules. Experimental configurations **(a–c)** were used in early measurements of DNA elasticity ([6–8], respectively). **(d)** The three-bead configuration allows the application and measurement of force and torque [11].

measuring optical tweezer (see Figure 1c). Two beads were attached to opposite ends of the DNA; one bead was held by the micropipette, the other one was captured in the optical trap. The optical trap was formed by bringing two counter-propagating beams to a common focus using two objective lenses and also functioned as a force transducer. The extension of the DNA was determined from the distance between the beads and the force was derived from the displacement of the laser beams on position-sensitive photodiodes. The elastic response of both ssDNA and dsDNA was measured using this experimental configuration.

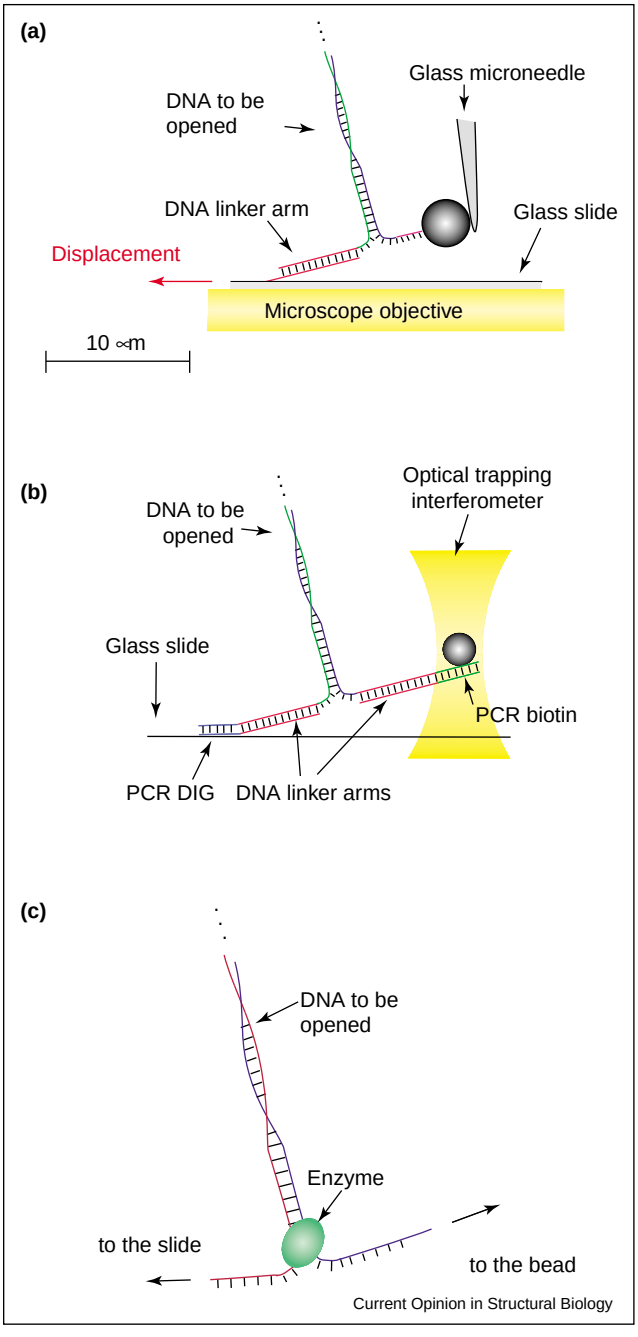
The elasticity of dsDNA is sensitive to coiling of the molecule. Using molecular constructs that prevent rotation at the attachment points (introduction of multiple biotin and DIG groups), the double helix can be twisted, and the coupling between linear elasticity and torsional strain investigated in detail. In these measurements, either magnetic beads in conjunction with rotating magnets [9] or a setup similar to that shown in Figure 1b with additional rotation of the pipette [10] were used. Bryant *et al.* [11] recently introduced an experimental configuration that allows the direct measurement of torque (see Figure 1d). The corresponding molecular construct contains three different attachment sites (using biotin, DIG and fluorescein modifications) and a single strand break, acting as a swivel. The molecule is stretched between two beads with a micropipette and an optical trap. A rotor bead is attached to the biotinylated central part of the molecule. This bead is held in place by fluid flow, while torsional strain is built up in the upper segment by rotating the pipette. Upon releasing the flow, the rotor bead turns to relieve torsional strain. The angular velocity of the rotor bead is proportional to the torque. Using this dynamic torque measurement, the torsional modulus of dsDNA was determined. The approach also confirmed an earlier experimental result regarding the structure of P-DNA [10], namely that the overstretching of dsDNA induces partial untwisting; when the molecular construct was linearly extended with a force above 65 pN, torque was generated that spun the rotor. An interesting perspective of this experimental technique is the measurement of torque generated by enzyme activity on DNA.

Unzipping and refolding nucleic acids

It is also possible to separately grasp the two strands at one end of the DNA double helix and pull the molecule open by mechanical force (see Figure 2). In this case, a sequence-dependent force signal has been measured, first with glass microneedles [12,13] and later with optical tweezers [14•,15], atomic force microscopy [16] and magnetic beads [17]. Sequence-dependent signals have also been observed in recent unfolding experiments on RNA [18,19,20•,21].

For unzipping DNA, the use of an optical trapping interferometer and an optimized molecular construct

Figure 2



Mechanical opening of the DNA double helix and force measurement by **(a)** soft microneedles or **(b)** a gradient optical trap. **(c)** Detection of protein–DNA interactions by unzipping DNA.

resulted in a two order of magnitude increase in stiffness compared to the earlier setup based on soft microneedles (Figure 2a,b). As a consequence, a significant increase in base pair sensitivity was obtained; sequence features at a scale of 10 base pairs became detectable in the force versus displacement curves. In addition, it was found that the unzipping force exhibits characteristic flips between different values at positions that are determined by the base sequence. These flips directly reflected transitions between different states involved in the time averaging of the molecular system close to equilibrium [14•]. Related flip events were observed in RNA folding/unfolding measurements by Liphardt *et al.* In the experimental configuration shown in Figure 1c, a constant force was applied to short RNA molecules and the extension of the single molecule could be measured as a function of time. The end-to-end distance was found to jump between two values, signaling repeated folding and unfolding of a short hairpin structure. By varying the applied force, it was possible to shift the equilibrium between the folded and the unfolded state [18]. These unzipping and unfolding measurements nicely illustrate how single-molecule manipulation could help to decipher the complex energy landscapes and structural dynamics of nucleic acids.

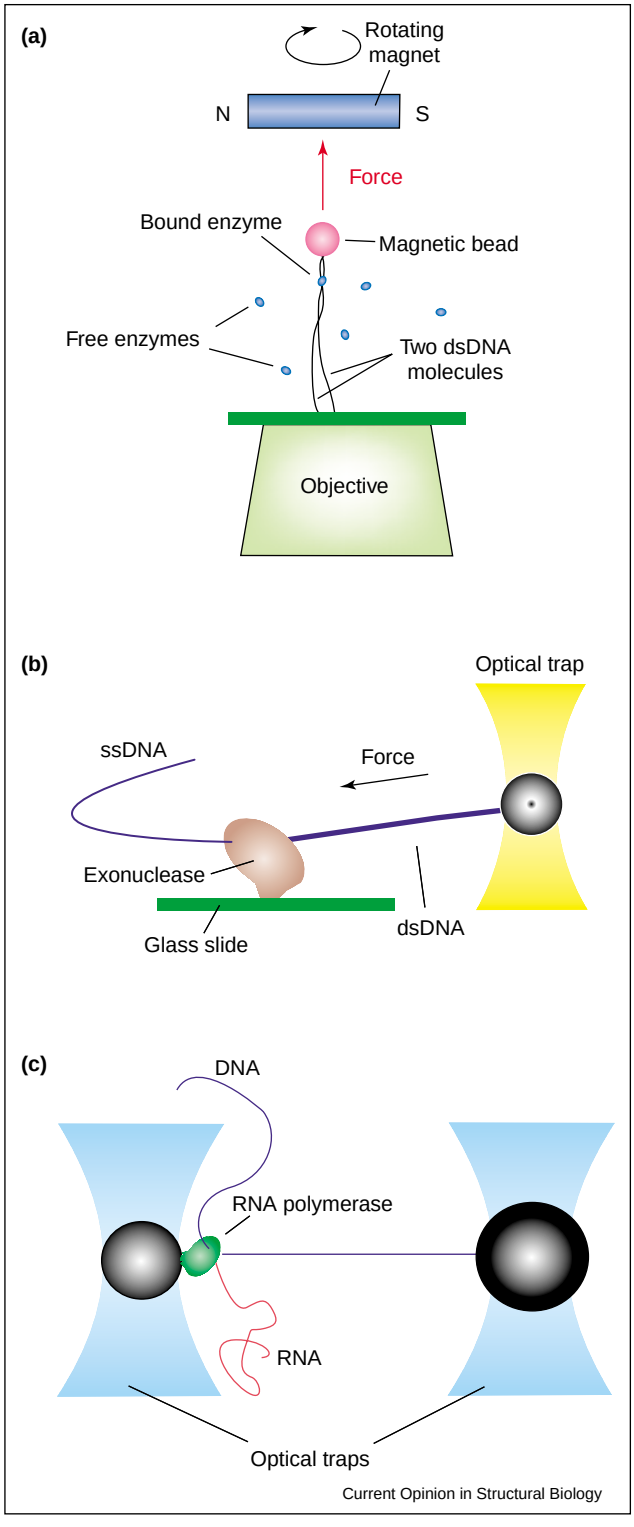
Recently, longer single RNA molecules, exhibiting more complex secondary and tertiary structures, were mechanically unfolded. Onoa *et al.* [20•] mechanically unfolded the *Tetrahymena thermophila* ribozyme, a 390-nucleotide catalytic RNA, using the setup shown in Figure 1c. Harlepp *et al.* [21] recently performed force measurements with a gradient optical trap (similar to Figure 2b) on an even longer RNA (i.e. 1540-base *Escherichia coli* 16S rRNA). The approach looks both promising and challenging; non-trivial single RNA molecular constructs have to be prepared and manipulated, and theoretical work is needed to correlate the measured force signal to the dynamics of RNA structural features.

DNA unzipping has recently also been studied under constant force, using magnetic beads (setup similar to Figure 1a). These measurements showed that the unzipped length as a function of time is characterized by jumps and pauses, in agreement with earlier theoretical predictions. The positions and durations of the pauses were found to be sequence dependent [17].

Single-molecule enzymology

Enzymatic processes related to DNA and protein–DNA interactions have increasingly been studied at the single-molecule level. For example, DNA–protein interactions can be detected using the unzipping apparatus, because the binding of an enzyme to dsDNA transiently blocks the opening fork (see Figure 2c). Once the opening fork reaches a bound protein, the measured force increases until sufficient elastic energy is accumulated to eject the protein. The beginning of the protein-induced peak in the sequence-dependent force signal gives the position of the enzyme along the DNA and the peak height provides information on the stability of the protein–DNA complex. In this way, we studied the interaction between DNA and the *Eco*RV restriction endonuclease [22]. Koch *et al.* [23,24] measured the protein-induced peaks as a function of velocity and compared the stability of different

Figure 3



Three different setups for single-molecule studies of the activity of DNA-dependent enzymes: **(a)** DNA unlinking by topoisomerases; **(b)** exonuclease digestion of one strand of the double helix; and **(c)** transcription. (a) In the topoisomerase experiment, two DNA molecules are attached between bead and surface. They are braided by rotating a magnet, which in turn causes rotation of the magnetic bead. Afterwards, topoisomerases are introduced. Their activity (i.e. the enzymatic unlinking of the two DNA molecules) induces length increase of the molecular system at constant applied magnetic force. This length increase is detected by video microscopy as a change in the Brownian motion of the bead [27]. (b) In the exonuclease experiment, a histidine-tagged λ-exonuclease was pre-incubated with 7100 base pair substrate DNA and anchored to a glass surface coated with a histidine-specific antibody. The position of the bead in the trap was measured by interferometry. A feedback loop was established, whereby the glass slide was displaced by a piezo-translation stage to keep constant the bead position in the trap. The imposed displacement thus measures the time-dependent advancement of the enzyme against a predetermined external force [28]. (c) In the double-trap transcription experiment, a stalled complex, consisting of a biotin-tagged RNA polymerase and a DIG-terminated DNA template, is attached between two polystyrene beads of different size. Each of the two beads is held in a separate optical trap. The tension in the molecular construct is kept nearly constant by feedback control of the position of the optical trap holding the larger bead. Transcription is recorded by monitoring the position of the smaller bead [32••].

protein–DNA complexes (interaction of DNA with *Bso*BI, *Xho*I and *Eco*RI endonucleases).

Another class of experiment uses the difference between the elasticities of ssDNA and dsDNA, or the dependence of dsDNA elasticity on twist. By pulling a single DNA molecule from its opposite ends, enzymatic activity that transforms ssDNA to dsDNA or that modifies the rotational state of dsDNA can be observed without enzyme immobilization. For example, the activity of DNA polymerases and topoisomerases was addressed this way [25,26]. Recently, the decatenation of two mechanically braided dsDNA molecules by *Drosophila melanogaster* topoisomerase II and *E. coli* topoisomerase IV was followed in time, using single-molecule micromanipulation [27] (see Figure 3a). It was found that the former enzyme relaxes left-handed and right-handed braids at a similar rate, whereas the latter shows a pronounced preference for left-handed braids. Topoisomerase IV, however, could also efficiently unlink right-handed braids when they form left-handed plectonemes. This work contributes to our understanding of the decatenation of the daughter strands during replication.

The single-molecule enzymology studies described above did not require immobilization of enzymes. We now consider experimental configurations whereby enzyme and DNA are immobilized on separate solid surfaces, allowing direct measurement of the interaction forces.

The digestion of single DNA molecules by λ-exonuclease was recently examined by Perkins *et al.* [28]. The apparatus consisted of a surface-attached enzyme bound to a DNA molecule, with the opposite end of the DNA molecule attached to a polystyrene bead (Figure 3b). The tethered bead was captured in a gradient optical trap and maintained at constant force. Digestion occurred at nearly constant rate (12 nucleotides/s) and pauses of variable

duration were observed. Long pauses were strand specific and sequence specific.

Very similar experimental configurations were used to investigate transcription of DNA at the single-molecule level [16,29–31]. One essential aspect of these studies is the measurement of the force/velocity relationship, because this relationship provides valuable insight into the function of polymerases as DNA-dependent motors. It is interesting to compare the corresponding results with curves obtained for other motor enzymes, such as myosin and kinesin, and also with theoretical models. The effects of hindering forces and, recently, also of assisting forces on the translocation of *E. coli* transcription elongation complexes have been studied. Recent measurements indicated that the translocation step is not rate limiting (even for sizeable hindering load), and that the vast majority of transcriptional pauses are short (a few seconds) and independent of polymerase backtracking [31]. The statistics and sequence dependence of pausing, and the polymerase 'proofreading' mechanism (including backtracking by as few as five base pairs) were investigated [32••] for *E. coli* RNA polymerase using an optical double trap (Figure 3c). Pausing and arrest during elongation by the *E. coli* enzyme have also been addressed in a two-bead single-molecule assay, in which one bead is held on a micropipette and a force is exerted on the second bead by fluid flow [33]. Recently, single-molecule force measurements of transcription have been applied to T7 RNA polymerase [34], a single-subunit enzyme that is widely used for *in vitro* synthesis of RNA.

Perspectives

Begun about a decade ago, experiments based on single-molecule manipulation of nucleic acids are increasingly providing information on biological function that would be difficult (or even impossible) to obtain by bulk measurements. Future work will probably bring further improvements in the manipulation techniques, in particular with respect to throughput, precision and the degree of control of the molecular system, including the method of surface attachment. Whether unconventional optical trapping schemes, with non-Gaussian laser light or interferometric patterns [35,36], will be used in this field remains to be seen. Combinations of mechanical micromanipulation with optical and/or microfluidics techniques are rapidly developing. In a recent single-molecule study of DNA unwinding by RecBCD helicases, for example, a microfluidic flow cell, optical trapping and fluorescence imaging were used together [37,38••]. Single-molecule manipulation of nucleic acids can also bring unexpected and original contributions to non-biological science. For example, using optical-tweezer-based micromanipulation, researchers recently tied a strand of DNA into knots of various complexity [39]. These knots are found to diffuse thermally along the DNA strand. They thus represent a very small model system for polymer reptation (the sliding of one strand on the other is here facilitated by electrostatic repulsion) and are close to the idealized knots considered in mathematics.

Acknowledgements

I thank D Côte, M Dreyfus and M Springer for helpful comments on the manuscript. LPA is associated with the CNRS (UMR 8551), and the universities Paris VI and Paris VII.

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