

## Single-molecule experiments in biological physics: methods and applications

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## TOPICAL REVIEW

# Single-molecule experiments in biological physics: methods and applications

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**Abstract**

I review single-molecule experiments (SMEs) in biological physics. Recent technological developments have provided the tools to design and build scientific instruments of high enough sensitivity and precision to manipulate and visualize individual molecules and measure microscopic forces. Using SMEs it is possible to manipulate molecules one at a time and measure distributions describing molecular properties, characterize the kinetics of biomolecular reactions and detect molecular intermediates. SMEs provide additional information about thermodynamics and kinetics of biomolecular processes. This complements information obtained in traditional bulk assays. In SMEs it is also possible to measure small energies and detect large Brownian deviations in biomolecular reactions, thereby offering new methods and systems to scrutinize the basic foundations of statistical mechanics. This review is written at a very introductory level, emphasizing the importance of SMEs to scientists interested in knowing the common playground of ideas and the interdisciplinary topics accessible by these techniques.

The review discusses SMEs from an experimental perspective, first exposing the most common experimental methodologies and later presenting various molecular systems where such techniques have been applied. I briefly discuss experimental techniques such as atomic-force microscopy (AFM), laser optical tweezers (LOTs), magnetic tweezers (MTs), biomembrane force probes (BFPs) and single-molecule fluorescence (SMF). I then present several applications of SME to the study of nucleic acids (DNA, RNA and DNA condensation) and proteins (protein–protein interactions, protein folding and molecular motors). Finally, I discuss applications of SMEs to the study of the nonequilibrium thermodynamics of small systems and the experimental verification of fluctuation theorems. I conclude with a discussion of open questions and future perspectives.

(Some figures in this article are in colour only in the electronic version)

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## 1. Introduction

The study of single molecules has become a major theme of research in modern biophysics. Specialized journals are devoted to this emerging field and every year more researchers become attracted to it. The history of single-molecule experiments is related to that of single-molecule imaging and has its roots in the invention of optical tweezers and the scanning tunnelling microscope. The possibility of manipulating individual entities has always attracted the scientist. Since the experimental discovery of the nucleus of the atom by Rutherford it has become a major goal in physics to search for the ultimate constituents of matter. A similar trend is followed in modern biology. There the main goal has been to characterize and understand the function of all constituent parts of living organisms and, ultimately, the chemistry of life. Despite the fact that biology and physics are very different sciences (most students in biology rarely feel attracted by physics and vice versa), the true fact is that these two sciences are to become much closer than ever in the new starting century. Although there have been many notable contributions to molecular biology by physicists (like Max Delbruck, Francis Crick and the Braggs just to cite a few names) the two sciences have diverged over the past 30 years. Molecular biology has developed very specific experimental methods, most of them borrowed from biochemistry. These methods are largely unknown to physicists (PCR amplification, gene cloning, ...). The current tendency is for this temporary gap to progressively narrow. More physicists will learn about the subtleties of biological matter and therefore become acquainted with some of the techniques and methods used by biologists. The marriage between physics and biology may still take years, chemistry becoming the privileged witness of the union.

What is the benefit of such a marriage? On the one hand, physicists are becoming steadily interested in the properties of biological matter. The kinetics of molecular motors, the folding of biomolecules, the viscoelastic and rheological properties of the cell, the transport of matter through pores or channels, the physical properties of membranes and the structure of biological networks are just a few examples of subjects akin to the expertise and interests of the physicist. On the other hand, biologists are interested in the physical techniques and methods available from physics. Physics is a quantitative science while biology has been traditionally mostly descriptive [1]. It is not surprising that the discovery of the double helix was made possible thanks to x-ray diffraction, an experimental technique discovered and used by physicists to

determine atomic structures in crystals. More important, the biologist is steadily aware of the great complexity of biological matter, the large variety of biological forms and the relevance of trying to unify such knowledge. Physical abstraction can be important to single out common themes and variations throughout this vast phenomenology. In addition, current experimental methods applied to biological systems are providing a huge number of data that must be quantitatively analysed by using sophisticated methods. The physicist can help a lot in this task.

It is fair to say that single-molecule experiments will contribute to bridge the gap between physics and biology. Single-molecule experiments (hereafter referred as SMEs) provide a new tool in physical biochemistry that allows us to explore biochemical processes at an unprecedented level. They offer a quantitative description of biological processes reminiscent of the physicist's approach. SMEs are made possible thanks in part to the advent of nanotechnologies. These, combined with microscale manufacturing techniques, provide the technology required to design and build scientific instruments of enough sensitivity and precision to manipulate individual molecules and measure microscopic forces, thereby allowing experimentalists to investigate various physical and biological processes. Nowadays, the most widespread and commercially available single-molecule technique in biophysics laboratories is the atomic force microscope (AFM). This technique allows one to take images of individual molecules adsorbed onto surfaces. At the same time with the AFM it is possible to grab molecules one at a time by attaching one end of the molecule to the AFM tip, the other being immobilized on the surface. By moving the tip relative to the substrate it is then possible to pull the molecule away from the surface and exert mechanical force. The value of the breakage force, the distance that the tip has to be retracted before the contact breaks and the dependence of these numbers on the speed of the moving tip are important information about the mechanical strength and location of the probed molecular bond. By pulling apart many molecules one at a time it is possible to quantitatively characterize the breakage process, thereby giving precious information about molecular interactions.

There are several excellent reviews on SMEs, applications and methodologies. Most of them fall into two categories. Either they are very introductory and cover generic topics on single-molecule manipulation or they are more specialized and specifically devoted to discussing particular topics. It could not be otherwise. The field of single molecules is developing very fast and at the same time diversifying into many different areas. Therefore, it is very difficult to cover all the subjects in a review. SMEs deal with aspects related to instrumentation, their application to study many different systems (belonging to physics, chemistry and biology), theoretical modelling and numerical simulations. In the area of optical tweezers alone, there is a complete resource letter available with useful references until the year 2003 (which includes nearly 400 references) [2]. This number of references is steadily growing every year.

The present review attempts to partially fill this gap by presenting an overview of various topics from an experimental perspective, first exposing the most important experimental methodologies and later reviewing various molecular systems where such techniques have been applied. I also include a brief section describing the applications of SMEs to investigate thermodynamics at the molecular level. This review covers SMEs applied to biomolecules; it therefore does not touch upon applications of single-molecule techniques to other interesting subjects such as living cells or non-biologically inspired problems. It briefly discusses theoretical modelling and numerical simulations. When appropriate a few references about theoretical models and simulations are listed for those readers who want to delve deeper into the subject. The selection of topics has been naturally biased by my own taste and expertise. Although I have tried to cover the most relevant existing literature it is unavoidable that

some important work and papers have been unduly omitted. I apologize in advance to these colleagues whose work may have been overlooked.

Very introductory reviews to SMEs can be found in [3–6]. There are also more focused reviews on the mechanical properties of biomolecules [7–11], the elastic properties of proteins [12–14], mechanochemistry [15], single-molecule fluorescence [16–18] and instrumentation [19, 20]. Whole journal issues devoted to reviewing SMEs, some of which have been published as books [21], can be found in [22], and proceedings of biophysics conferences often include a section on SMEs [23, 24]. We must also mention web pages with detailed information about specific single-molecule techniques (for example, laser tweezers [25]) or excellent review journals [26]. Finally, reviews about the usefulness of SMEs to investigate the thermodynamics of molecular systems can be found in [27–29].

## 2. Why single molecules?

SMEs are central to biological physics research [30]. These offer a complementary yet different perspective to understand molecular processes. What are the advantages of SMEs compared to traditional bulk assays? The main difference between single-molecule and traditional biochemistry methods lies in the kind of average done when measuring the properties of the system. SMEs allow experimentalists to access biomolecular processes by following individual molecules. Using SMEs it is possible to measure distributions describing certain molecular properties, characterize the kinetics of biomolecular reactions and observe possible intermediates. SMEs provide additional information about thermodynamics and kinetics that is sometimes difficult to obtain in bulk experiments. All this is complemented by powerful visualization methods (which allow us to capture images and produce movies) that greatly help the scientist in the interpretation and understanding of the experiments.

To better understand the advantages of SME let us consider the example of protein folding. A protein in water solution can exist in two possible conformations (folded and unfolded). In one conformation the protein is folded into its native state, forming a compact globular structure. Roughly speaking, the hydrophobic core is buried inside the globule and stabilized by specific amino acid–amino acid interactions, whereas the hydrophilic amino acids are exposed to the outside on the surface of the globule. In the other conformation the protein is denatured or unfolded, forming a random coil. At room temperature (e.g. 25 °C or 298 K) the protein is in the native state as this state has a free energy that is lower than that of the random coil. Upon heating (or increasing the concentration of denaturants such as urea), the protein can denature and change conformation from the native to the unfolded state. Most proteins typically denature at temperatures in the range 50–80 °C, each protein being characterized (under given solvent conditions of salinity and pH) by a melting temperature,  $T_m$ , where the protein denatures. The full characterization of this transition is possible by using calorimetry bulk measurements where the protein is purified inside a test tube. The enthalpy curve obtained in such measurements shows a jump in the enthalpy and a latent heat (similar to that observed in water at its boiling point). This is the characteristic signature of a first-order phase transition separating two conformations. The same conclusion is reached and the same melting temperature found by carrying out other bulk measurements (such as UV absorbance).

What additional information can be obtained with single-molecule techniques? It is a well known fact that during the folding process some proteins transiently visit an intermediate state, the *molten globule* state, characterized by a short lifetime. In such a state proteins form a globular structure where, roughly speaking, the hydrophobic and hydrophilic parts are separated between the core and the surface of the globule but specific contacts between amino acids are not yet fully formed. Due to its short lifetime, the fraction of molecules that are in the molten globule state inside the test tube can be small enough to go unobserved in calorimetry

measurements. The tiny signal they produce can be masked by that of the overwhelming number of correctly folded or totally unfolded molecules. In SMEs one can follow one protein at a time, therefore it is possible to separate molecules into three families: the native ( $N$ ), the intermediate molten globule ( $I$ ) and the unfolded ( $U$ ).

By using single-molecule fluorescence it is possible to attach fluorescent molecules to proteins, detect them by focusing light into a tiny spot, and watch proteins go through that spot for a short interval of time. Fluorescent proteins are chemically synthesized by attaching fluorescent dyes to specific residues of the amino acid chains of the protein (see figure 1). In single-molecule fluorescence resonance energy transfer (FRET) techniques, two different colour dyes (e.g. *green* and *red*) can be positioned at specific locations of the protein. These locations stay close to each other when the protein is in its native state, only slightly close in the intermediate state and far away in the unfolded state. Upon radiation of light with the appropriate frequency the *green* dye (the donor) absorbs the radiation. A fraction of this intensity of light is emitted to the observer ( $I_D$ ). The rest of the intensity is emitted by the *red* dye (the acceptor) through a non-radiative resonance energy transfer mechanism between donor and acceptor (the condition being that the emission spectrum of the donor overlaps with the absorption spectrum of the acceptor). The light emitted by the acceptor always has lower frequency than that emitted by the donor and part of the energy transferred to the acceptor is lost to the environment in the form of heat. The amount of non-radiative energy transfer between the *green* and the *red* dye depends on the distance between the dyes and defines the FRET efficiency,  $E = I_A/(I_A + \eta I_D)$ , where  $\eta$  is a correction factor that depends on the quantum yields of donor and acceptor. For  $E = 1$  all light absorbed by the donor is transferred to the acceptor, whereas for  $E = 0$  the light is emitted only by the donor. The intensity of light emitted by the donor and the acceptor that is detected by the observer changes as the distance between the two dyes changes. However, the total amount of light emitted by the donor and the acceptor is constant, until photobleaching occurs. Therefore, the intermittent exchange between the amount of light emitted at both wavelengths is an indirect measure of the distance between the dyes, i.e. of the different conformations of the protein (providing a spectroscopic ruler).

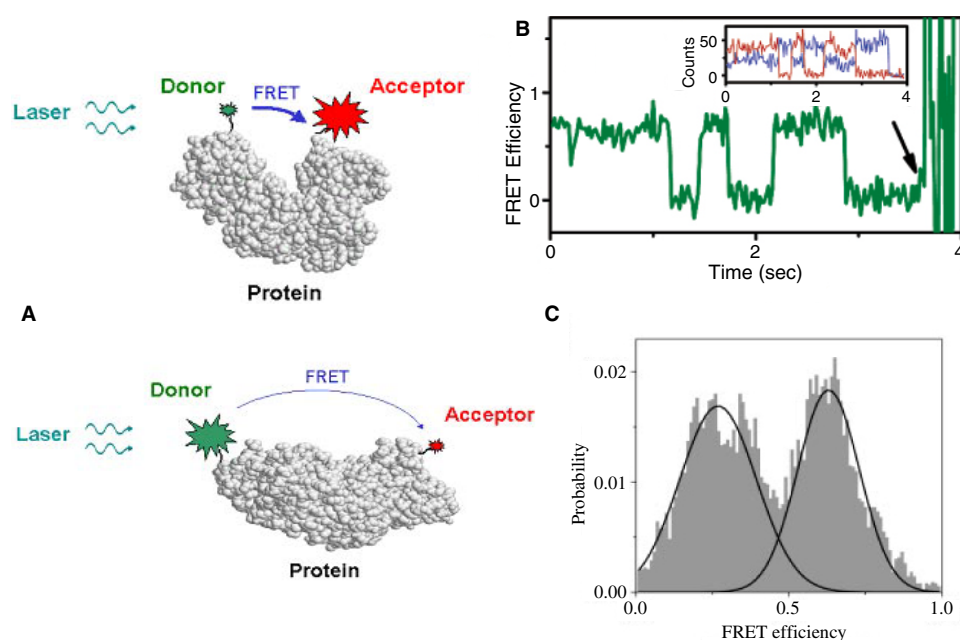
Coming back to our previous example, measurements taken over many proteins might show a three-modal distribution indicating three possible conformations of the molecules ( $N$ ,  $I$ ,  $U$ ). The fraction of molecules observed in each of these three states (i.e. the statistical weight of each *mode*) would be a function of the temperature and/or denaturant concentration. Below/above the melting temperature nearly all molecules are folded/unfolded. In the vicinity of the melting transition, intermediate conformations would be observable together with some native and unfolded conformations as well. Single-molecule techniques also offer the possibility to measure kinetic or time-dependent processes by following the trajectories of individual molecules. The signal would execute transitions as the conformation of the protein changes, providing direct evidence of the existence of an intermediate state as well as information about the kinetic rates between the three states. Sometimes the protein would execute transitions from the unfolded to the intermediate state ( $U \rightarrow I$ ) to later jump to the native state ( $I \rightarrow N$ ). In this case the molten globule is an intermediate *on-pathway* to the native state. In another scenario the protein directly folds into the native state without first collapsing into the intermediate ( $U \rightarrow N$ ). In this case the molten globule would be an intermediate state *off-pathway* to the native state. The statistics of the residence times of the protein in each state provides extremely valuable information about the folding process. All this information is usually masked in bulk measurements where results are averaged over molecules and time. SMEs complement standard spectroscopy and microscopy techniques in molecular biology and biochemistry and therefore have to be viewed as a new source of valuable information to interpret biomolecular processes.

### 3. From physics to biology and back

It is fair to say that single-molecule techniques have been largely developed by physicists. These new kinds of measurements are providing lots of quantitative information about molecular processes that have to be analysed using statistical methods. This is very attractive to the physicist who can propose experiments to test new theories or to investigate theories and analyse models to interpret the experimental results. This statement can be illustrated with the following example. It is worth mentioning that one among the first single-molecule pulling experiments revealed that the elastic response of individual double-stranded DNA (dsDNA) molecules is excellently described by the worm-like chain model introduced in polymer theory by Kratky and Porod in 1949 [33] (see later in section 5.1.1). By pulling an individual molecule it was possible to experimentally measure the force as a function of the molecular extension, also called the force–extension curve (FEC) [34, 35]. These results verified the prediction of the worm-like chain model for the FEC and provided the first direct mechanical estimate of the persistence length of individual DNA molecules (roughly speaking the persistence length is the distance along the contour length of the molecule where the molecule keeps a straight direction due to its bending rigidity), which was in agreement with previous light scattering measurements. By stretching the molecule above 60 pN ( $1 \text{ pN} = 10^{-12} \text{ N}$ ) it was also possible to observe a plateau in the FEC at 65 pN (see figure 3(C)). This is characteristic of a first-order transition and interpreted as a structural change in the DNA molecule that gets overstretched as the DNA double helix unwinds and bases get tilted in the direction where the force is applied. The FEC of DNA (or any other polymer) in single-molecule pulling experiments is similar to the magnetization–field curve in magnets, the load–deformation curves in plastic materials or the polarization–voltage curves in dielectrics, showing the physical flavour of such experiments.

A particular area of physics that can greatly benefit from knowledge emerging from SMEs is statistical physics. SMEs allow one to measure forces in the range of a few piconewtons (a piconewton is approximately a trillionth of the weight of an apple) and have spatial resolution in the range of a nanometre. These are the ranges of forces and extensions typically involved in many biomolecular reactions where high energy bonds (such as ATP) are hydrolysed and the energy released is subsequently used to perform mechanical work. Work values typically encountered in such reactions are of the order of a few  $k_B T$  units. At room temperature  $T \simeq 300 \text{ K}$  and therefore  $1k_B T \simeq 4 \text{ pN} \times \text{nm} \simeq 0.6 \text{ kcal mol}^{-1} \simeq 2.5 \text{ kJ mol}^{-1}$  (in the biophysics or molecular biology community it is common to refer to energies in  $k_B T$  or  $\text{kcal mol}^{-1}$  units whereas  $\text{kJ mol}^{-1}$  is preferred among the chemists). Most biomolecular reactions take place in an aqueous environment in the presence of water molecules. As the energy of the biochemical reaction is not much different from the average kinetic energy carried by one water molecule, such processes take place in a highly noisy environment. Therefore, we can imagine a molecular enzyme acting on a substrate carrying out a specific molecular reaction impinged by hundreds of water molecules each nanosecond, each of these molecules carrying enough kinetic energy to interfere in the process. Under such conditions we expect strong Brownian fluctuations in the behaviour of the enzyme, which show up as rare and large deviations of its motion from the average behaviour [36]. What if one water molecule carrying ten times the average kinetic energy collides with the enzyme while a particular biochemical reaction takes place? This happens from time to time and such large deviations must influence the behaviour of the motor. It is surprising to know that most of these work producing molecular machines have a very large efficiency, where a large fraction of the energy consumed (typically around 20–90%) is used as mechanical work; part of the rest of the energy gets lost in the form of heat released into the aqueous environment.





**Figure 1.** Single-molecule detection using FRET. (A) A conformational change in a protein changes the relative intensities of light emitted by the donor and the acceptor. (B) Typical FRET efficiency trajectories and donor/acceptor intensities  $I_D$ ,  $I_A$  (inset) for individual proteins trapped in vesicles. These show multiple folding/unfolding events close to the midpoint folding transition. The arrow indicates when photobleaching occurs. (C) Probability distributions of FRET efficiencies showing the existence of two families of proteins (folded and unfolded). The average FRET efficiency is around 0.6 and agrees well with values obtained in bulk assays. Figures taken from [31, 32].

Fluctuations are well known to be the cause of some mutations that occur during the replication processes of DNA, when a new strand is synthesized from the parental strand and the genetic information is transmitted to a new generation of cells. During the replication process many proteins interact with the DNA and participate in the process by self-assembling into a large complex. The replicating machinery is immersed in water and subjected to strong Brownian fluctuations. Under such harsh conditions mutations occur frequently during the replicating process. However, what is most surprising from the replication process is not the fact that mutations are common but that mutation rates are regulated inside the cell. Mutations are necessary as evolution takes advantage of them to produce better adapted individuals. However, mutation levels during the replication process are kept under such tight control that mismatches occur as rarely as one every  $10^9$  replicated base pairs. To avoid the damaging effects of noise fluctuations, cells are endowed with a complex machinery of repair that is active during the crucial steps of the replicating process and important for the maintenance of the genome. Large fluctuations are expected to occur whenever a large number of fast moving molecules clash simultaneously with the enzyme. Will these large deviations affect the performance of the enzyme? In what way will they alter its function? Even more interesting, are these deviations an integral part of the function and efficiency of the enzyme? Such questions are just a few among many others that biophysicists and statistical physicists are ready to confront.

The possibility to measure such tiny energies brings us close to what we can distinguish as an emerging area of science, i.e. how to understand and design molecular motors that operate in the nanoscale and efficiently use chemical energy from naturally available (or synthesized)



sources to perform specifically designed functions. We might call this thermodynamics of small systems, as the main goal of this discipline is to understand the performance of these small machines in a noisy environment. Such a term was coined by Hill many years ago when referring to thermodynamic equilibrium properties of ensembles of small size systems where the equations of state depend on the ensemble or collectivity, i.e. the conditions or parameters that are kept fixed in the ensemble [37]. However, the most important aspect of molecular machines is that they operate far from equilibrium by hydrolysing sizable amounts of energy and taking advantage of large and rare deviations. The relation between the nonequilibrium properties of small machines and their thermodynamic properties is shaping a new discipline in statistical physics, the so called *nonequilibrium thermodynamics of small systems*. The main facts behind this new discipline have been discussed in [28] at a very introductory level.

A related aspect of great interest to the statistical physicist is how to use SMEs to test the basic foundations of statistical mechanics. This line of thought has seen important progress in recent years and we foresee that the exploration will continue and improve as more precise and quantitative measurements are becoming available. Examples are the study of the nonequilibrium work relations or fluctuation theorems under various conditions. Biological systems have been particularly useful in this regard. The experimental access to small energies is interesting in non-linear systems, i.e. systems that do not respond in a linear way to an applied external perturbation. Systems of this type abound in molecular biology, where conformational changes and macromolecular interactions are of the all-or-none type. This is also referred to as the *lock and key* interaction mechanism among molecular biologists, *substrate–enzyme* reactions in biochemistry or *activated behaviour* in the language of the physicist. In these types of interactions, a tiny variation of the external conditions can cause a big change in the outcome of the reaction. This fact is behind the high sensitivity of protein structures to single amino acid mutations or the strong dependence of some enzymatic reactions to a small amount of some specific substances (e.g. activators or repressors). In proteins, although not all single amino acid changes lead to new folded structures, a few of them in some specific parts of the chain can have dramatic effects in the structure, with lethal consequences at the level of the cellular functions regulated by that protein. In physical systems such stability conditions are not so usually encountered. Under the influence of small perturbations physical systems usually respond in a smooth way, although, of course, counterexamples also abound. The main difference between physical and biological systems is that structure and function are intimately related in the latter. As a result of evolution over millions of years a new kind of interactions and interrelationships have emerged between different parts of living matter (evolutionary constraints), which are not expected to be common in other physical or chemical systems.

The idea of following individual biomolecules to understand processes that occur in a crowded environment like the cell pertains to the kind of abstractions typical of a physicist. How has been received this idea by the community of biologists? The response is not yet uniform among the rows of biologists who, depending on their area of specialization, can feel more akin to the new techniques. Molecular biologists and biochemists are probably the most receptive because single-molecule methods offer complementary tools to investigate problems of their interest. For example, many processes that occur inside the cell, such as DNA transcription and replication, molecular transport, virus infection, DNA condensation and ATP generation can be studied by using these new techniques. As we discussed in section 2, this approach provides new relevant information to the molecular biologist which is usually unavailable with traditional techniques. A common criticism of single-molecule methods is that molecular processes cannot be studied *in vivo* by following a molecule in its natural environment (e.g. DNA replicating inside the nucleus). This is indeed a limitation at present but

it must be said that the same occurs in traditional biochemical assays. The specific conditions found in the cell cannot be reproduced in the test tube, which contains only a tiny fraction of the total number of cell constituents. This limitation, however, is not seen as a drawback in the mind of a physicist, who is educated to explore simplified versions of complex and difficult problems. The true fact is that more and more molecular biologists are becoming steadily interested in adopting such techniques in their laboratories. This gives rise to an unprecedented excitement between physicists and biologists, who are joining efforts and expertise to accomplish common scientific goals.

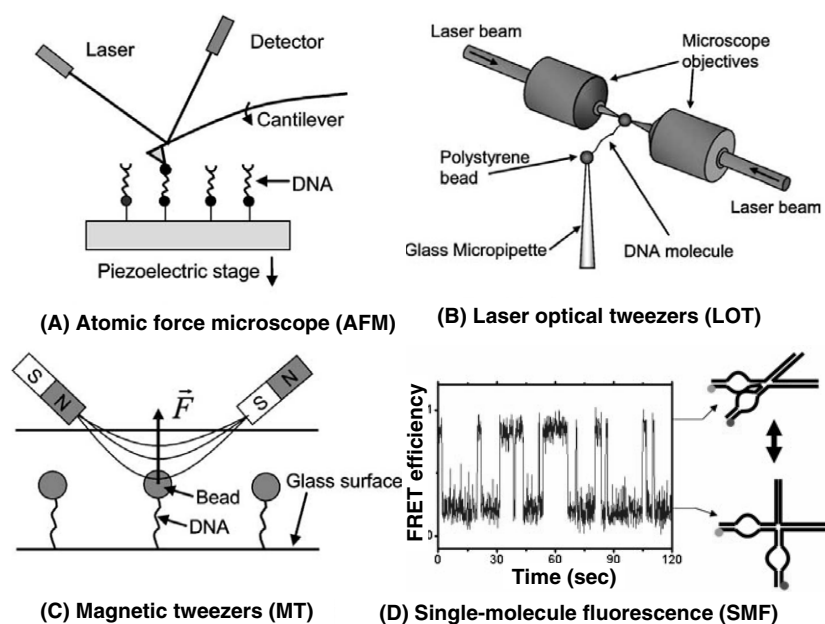
#### 4. Experimental techniques

In this section I succinctly describe the experimental techniques mostly used in SMEs. We have to distinguish between techniques required to manipulate individual molecules and techniques that allow us to detect and follow in real time (but not manipulate) individual molecules. In the first class we have atomic force microscopy (AFM), laser optical tweezers (LOTs), magnetic tweezers (MTs) and biomembrane force probe (BFP) to cite the most representative ones. Other techniques (such as glass microfibres) are not of widespread use in SMEs and we are not going to discuss them here. In the second class, and in addition to AFM (which is also an imaging technique), there are predominantly optical techniques such as single-molecule fluorescence (SMF), Raman spectroscopy, two-photon spectroscopy and semiconductor quantum dot emission, to cite the most common. Combination of manipulation and fluorescence techniques (e.g. laser tweezers with fluorescence) has already begun and will allow us to explore new phenomena with enhanced precision.

##### 4.1. Single-molecule manipulation techniques

In this section the different techniques to manipulate individual molecules and measure microscopic forces are outlined. AFMs are particularly useful because these can be used to sweep surfaces and take images of individual molecules. At the same time, AFMs can be used to apply mechanical force on individual molecules. LOTs cannot be used to take images of individual molecules but have the advantage that manipulation of individual molecules can be more easily controlled, for example in the study of molecular motors. The case of LOTs has been chosen to be discussed in detail to emphasize the difficulties and the methodology common to all techniques. All techniques discussed in this section have complementary forces and loading rate regimes, each technique enables some experiments that are not possible with the other. Most of the methods described to capture video images and other details of the experimental LOT set-up (such as the implementation of force-clamp methods) are also applied to the other techniques.

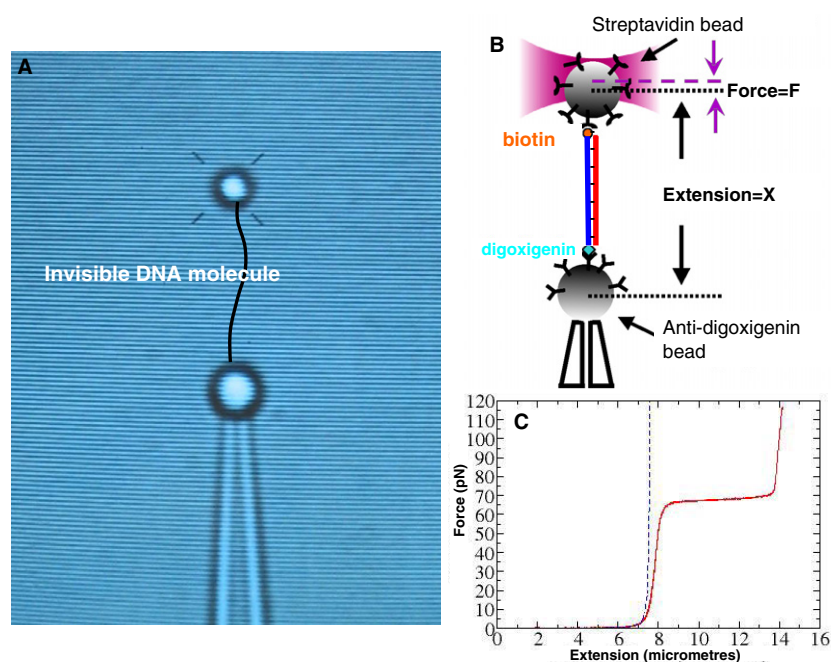
*4.1.1. Atomic-force microscopy (AFM).* Probably the best well known among these techniques is atomic force microscopy (AFM). The AFM is a descendant of the scanning tunnelling microscope invented by Binnig and Rohrer applied to obtain Angstrom resolution images of metallic surfaces using the quantum tunnel effect [38]. The AFM [39] is based on the principle that a very soft cantilever with a tip that is moved to the vicinity of a surface (metallic or insulating) can sense the roughness of the surface and deflect by an amount which is proportional to the proximity of the tip to the surface (figure 2(A)). The most important application of the AFM is imaging where it can work in various modes: the contact mode, tapping mode and the jumping mode [40]. For example, in the tapping mode the tip is made to



**Figure 2.** Experimental techniques: (A) atomic force microscope (AFM); (B) laser optical tweezers (LOTs); (C) magnetic tweezers; (D) single-molecule fluorescence (SMF) using FRET.

oscillate close to the sample surface. The amplitude of the oscillation is recorded and controlled by a feedback loop mechanism that keeps such amplitude constant. When passing over a bump the amplitude decreases so the distance between tip and surface is increased to keep the amplitude of oscillation constant. When passing over a depression the tip is moved to the surface. This mode has the advantage that the transverse motion of the tip along the surface is not influenced by shearing and frictional forces, thereby avoiding damage to the sample and noisy interference effects. A map of the distance of the tip from the sample provides an accurate topographic image of the surface. Other modes are preferable depending on the particular system; for example, the contact mode is useful to take images of biological samples in fluids [41]. The use of the AFM for biomolecular imaging has been reviewed in several excellent papers [42–48].

The AFM is also used to manipulate and exert mechanical force on individual molecules (figure 2(A)). As usual in single molecule techniques, elaborate chemistry is often required to treat the surface and the tip. The surface has to be coated with the molecules to be manipulated. The AFM tip also has to be coated with molecules that can bind (either specifically or non-specifically) to the molecules on the substrate. By moving the tip to the substrate a contact between the tip and one of the molecules adsorbed on the substrate is made. Retraction of the tip at constant speed allows us to measure the deflection of the tip in real time, providing a measure of the force acting on the molecule as a function of its extension, the so-called force–extension curve (FEC). The AFM covers forces in the (20 pN–10 nN) range depending on the stiffness of the cantilever. Typical values of the stiffness are in the range 10–1000 pN nm<sup>−1</sup>. Although AFM is a very versatile and powerful tool it has a few drawbacks for manipulating single molecules. The most important one is probably the presence of undesired interactions between tip and substrate (van der Waals, electrostatic and adhesion forces) and the non-specificity of the attachments that often occur between tip and substrate. When moving the tip to the



**Figure 3.** Pulling DNA using LOTs. (A) Image taken by a CCD camera of the fluid chamber where manipulation of the DNA molecule takes place. The DNA is tethered between two beads. The upper bead is trapped in the optical well whereas the lower bead is immobilized on the tip of a micropipette and held fixed by air suction. The presence of a DNA tether between the beads (invisible in the real image but illustrated here as a thick black line) is detected by the presence of a vertical force pointing downward acting on the trapped bead. (B) Experimental set-up in DNA pulling experiments where the 3' and 5' ends of one strand are attached to the beads through biotin–streptavidin and digoxigenin–anti-dig specific bonds. The force is then measured from the deviation of the upper bead respect to the centre of the trap. By measuring the force ( $F$ ) as a function of the extension ( $X$ ) it is possible to record the force–extension curve (FEC). (C) FEC in half  $\lambda$ -DNA (24 000 base pairs long). The blue-dashed line is the worm-like chain prediction from polymer theory. It describes very well the elastic behaviour of the DNA molecule up to forces  $\sim 5$  pN. Above 5 pN enthalpic corrections are important. Note the overstretching transition that occurs at 65 pN. See the more detailed discussion in section 5.1.1.

substrate it is easy to attach many molecules at a time. Moreover, it is difficult to control the specific location of the attachment between the tip and the molecule. Single-molecule markers (e.g. polypeptides) and functionalization strategies have been specifically developed to overcome these limitations.

Spatial and force resolution in the AFM are limited by thermal fluctuations. When the cantilever stage is held at a constant position the force acting on the tip and the extension between tip and substrate fluctuate. The respective fluctuations are given by  $\langle \delta x^2 \rangle = k_B T / k$  and  $\langle \delta F^2 \rangle = k_B T k$  where  $k_B$  is the Boltzmann constant,  $T$  is the absolute temperature of the environment and  $k$  is the stiffness of the cantilever. At room temperature  $k_B T \simeq 4$  pN nm and therefore  $\sqrt{\langle \delta F^2 \rangle} \simeq 20$  pN,  $\sqrt{\langle \delta x^2 \rangle} \simeq 2$  Å if we take  $k \simeq 100$  pN nm $^{-1}$ . This shows that the signal-to-noise ratio for the force is small for force values of just a few tens of piconewtons. This is the range of forces characteristic of weak interactions, therefore showing the limitations of AFMs to study the mechanochemistry of weak interactions in the lower piconewton regime. In contrast, AFMs are ideal to investigate strong to covalent interactions. They have mostly

been used to probe relatively strong intermolecular and intramolecular interactions, e.g. pulling experiments in biopolymers such as polysaccharides, proteins and nucleic acids.

**4.1.2. Laser optical tweezers (LOTs).** The principle of LOTs is based on the optical gradient force generated by a focused beam of light acting on an object with an index of refraction higher than that of the surrounding medium. Discovered by Ashkin in 1970 [49, 50], the principle was developed later to trap dielectric particles by Ashkin and collaborators at the Bell Laboratories [51]. The application of gradient force by light radiation pressure has been used to trap neutral atoms [52], eukaryotic cells [53] and viruses and bacteria [54]. A good review on the origins of optical trapping can be found in [55]. In the basic experimental LOT set-up a near-infrared laser is collimated by a high numerical aperture water immersion lens. A micron-sized polystyrene or silica bead is then trapped in the focus of the laser by exerting forces in the range 0.1–100 pN depending on the size of the bead and the power of the laser. Typical bead sizes are on the order of 1–3  $\mu\text{m}$  and laser powers of a few hundred milliwatts to avoid the heating of the bead and undesired heat convection effects close to the bead that could either damage the sample or affect the measurements. To a very good approximation the trapping potential is harmonic, therefore forces acting on the bead are directly proportional to the distance between the bead and the centre of the trap,  $F = kx$ , where  $k$  is the stiffness constant of the trap. To determine the stiffness of the trap, noise measurements or Stokes force calibration are often used. Typical values of the stiffness of the trap are  $10^2$ – $10^4$  times smaller than AFM tips, therefore force resolution is at least 10 times better, on the order of 0.1 pN. Major improvement in this basic set-up is obtained by using dual counter-propagating laser beams passing through two identical objectives [56] (figure 2(B)). There are several advantages in this more complex set-up. First, the axial scattering force is reduced. Second, the trapping forces that can be reached (up to 200 pN) are higher than in the one-beam set-up. Finally, continued force calibration is not required because the force is directly measured from the total amount of light deflected by the bead, which is collected by position sensitive detectors (PSDs) located at the two opposite sides of the laser beams. The force is given by  $F = S/(Lc)$ , where  $S$  is the radiation pressure flux,  $c$  is the speed of light and  $L$  is the distance that separates the beads (located between the two objectives) and one of the objectives. This formula is valid for any size, shape and refractive index of the bead. In this way there is no need for calibration of the trap every time a new bead is captured.

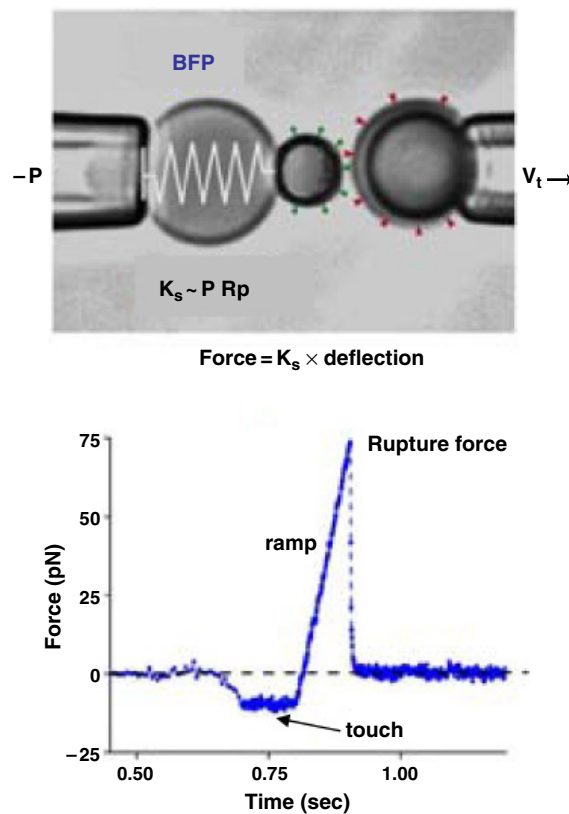
Manipulation of individual molecules is carried out inside a fluids chamber made out of two glass surfaces separated by a parafilm of 200  $\mu\text{m}$  width. A fluidics system is designed in such a way that water and chemicals can flow and be replaced at any time inside the chamber. A glass micropipette is inserted and fixed inside the chamber and used to hold another bead by air suction. The chamber is then mounted on a moving stage whose position is controlled by a piezo and positioned in front of the objective lens so that the laser can be focused inside the chamber. To manipulate individual molecules beads are coated with a chemical substance (e.g. avidin or streptavidin) that can bind specifically to its complementary molecule (biotin). The molecules of interest (e.g. DNA) are then biotinylated (i.e. labelled with biotin molecules) at their ends so they can bind to the avidin (streptavidin) coated beads through a strong non-covalent bond. To avoid double attachments between the two ends of a single molecule and the same bead it is customary to differently label the molecule at its two ends. One end is then labelled with biotin, the other with digoxigenin. Digoxigenin recognizes specifically its anti-dig antibody partner through the ‘lock and key’ interaction mechanism typical of antigen–antibody interactions. This is a weak bond so the biomolecule is often labelled with many dig molecules at one of its ends in order to increase the strength of the attachment. After incubation of the molecules with the beads (for instance the streptavidin beads), other beads coated with



anti-dig flow inside the chamber. One bead is captured with the laser trap and moved to the tip of the micropipette, where it is held fixed by air suction. Incubated beads then flow inside the chamber and one bead is captured in the trap. By moving the chamber relative to the trap the two beads approach each other until a connection between the digoxigenin end of the molecule and the bead in the micropipette is established. The tether is then pulled by moving the chamber at a given speed and the FEC measured; see figure 3.

The extension of the molecule can be monitored by using a CCD video camera that uses a framegrabber to take pictures of the two beads and operates at a few tens of Hertz. Because spatial resolution is strongly limited by the pixel resolution (about 10 nm), other methods have to be implemented to resolve the position of the bead with higher accuracy. A standard procedure is to use a light lever or reference beam where a low power light beam passing through a small lens in the frame of the chamber is collected by using additional position sensitive detectors. By recording the position of the chamber it is possible to determine the extension of the tether down to a few nanometres of precision. Spatial resolution is however hampered by strong drift effects in the optical components and the manipulation chamber. Depending on the experimental set-up the spatial resolution can reach the nanometre level only in carefully isolated environments (absence of air currents, mechanical and acoustic vibrations and temperature oscillations). Force-clamp (also called force-feedback) methods that use acoustic-optic deflectors and incorporate a piezoelectric stage with capacitive position sensing are providing better and more versatile instruments [57, 58]. LOTs have been widely used to investigate nucleic acids and molecular motors.

**4.1.3. Magnetic tweezers (MTs).** Magnetic tweezer (MT) design is based on the principle that a magnetized bead experiences a force when immersed in a magnetic field gradient  $F = -\mu \nabla B$ . The basic set-up is shown in figure 2(C). A bead is trapped in the magnetic field gradient generated by two strong magnets. Molecules are attached to the surface of the magnetic bead and to a glass surface. A microscopic objective with a CCD camera is used to determine the position of the bead. Molecules are pulled by moving the translation stage that supports the magnets. MTs have several advantages compared to AFM and LOTs [59]. First, sensitivity to very low forces can be easily achieved due to the low value of the stiffness of the magnetic trap. The typical range of forces is  $10^{-2}$ –10 pN, where the maximum value of the force depends on the size of the magnetic bead. Second, in the passive mode (when the magnet stage is kept fixed) the force acting on the bead can be kept constant because the spatial region occupied by the bead is small enough for the magnetic field gradient to be considered uniform. Therefore, although the bead position fluctuates the force is always constant. A constant force can be also achieved in the AFM and LOT set-ups by implementing force-feedback control mechanisms (see section 4.1.2). The main drawback of feedback loops is their working frequency, typically limited to a few kilohertz, which does not allow us to detect dynamical processes faster than milliseconds. Third, magnetic traps allow us to twist molecules by rotating the magnets. Modifications of the basic set-up by using a third bead to create a single chemical bond swivel allow us also to measure torques [60]. MTs are calibrated in flow fields using the Stokes law or measuring Brownian motion in the direction transverse to the application of the force,  $k = K_B T / \langle \delta x^2 \rangle$ , where  $x$  denotes the transverse coordinate. Typical values of the magnetic trap stiffness are  $10^{-4}$  pN nm $^{-1}$ , thereby one million times smaller than in AFMs and a thousand times smaller than in LOTs. Force-extension curves (FECs) can be recorded in real time by moving the stage and measuring the transverse fluctuations  $\langle \delta x^2 \rangle$ . Force is measured by using the expression  $F = k_B T l / \langle \delta x^2 \rangle$ , where  $l$  is the extension of the molecule. The value of  $l$  is determined using depth imaging techniques that provide excellent force-position measurements. The smallness of the stiffness in MTs induces large fluctuations



**Figure 4.** BFP technique. Upper figure, a red blood cell acts as a force transducer (symbolized as a spring in the figure) by transforming the pressure suction applied on the pipette ( $-P$ ) into the elastic stiffness of the cell membrane  $\kappa_s \sim P R_p$ , where  $R_p$  is the pipette radius. A bead covered with ligands is then attached to the cell (left bead). Another bead covered with receptors is then immobilized on the tip of another pipette (right bead). A ligand–receptor bond can be formed by touching the beads. By retracting the right pipette at speed  $v_t$  the formed bond dissociates at a given force value  $F$ , measured from the formula  $F = \kappa_s x$ , where  $x$  is the deflection of the left bead. Lower figure, typical force–time curve when probing ligand–receptor interactions. Figure taken from [62].

in the extension of the molecule, on the order of 20 nm. MTs have been extensively used to investigate elastic and torsional properties of DNA molecules.

**4.1.4. Biomembrane force probe (BFP).** Finally, we mention the biomembrane force probe technique developed by Evans and collaborators [61]. The basic experimental set-up is shown in figure 4. In this set-up a biotinylated red blood cell is pressurized by micropipette suction into a spherical shape. The tip is made of a streptavidin coated bead (left bead in figure 4) functionalized with some molecules (e.g. ligands). The other bead is functionalized with complementary molecules (e.g. receptors) and kept fixed by air suction on the tip of the other micropipette (right). The blood cell acts like a spring so the force can be measured by calibrating the stiffness of the cell. This is directly related to the membrane tension and can be controlled by fine tuning of the pressurization of the micropipette (left). Typical stiffness values are in the range  $0.1\text{--}1 \text{ pN nm}^{-1}$ . By moving the micropipette (right) using a piezo translator



stage the two beads approach each other until they touch. The micropipette is further retracted and ligand–receptor interactions detected as rupture events. A CCD video camera records the extension between the two beads with a resolution on the order of 10 nm. The BFP has been mainly used to study ligand–receptor interactions.

#### 4.2. Single-molecule fluorescence techniques

Single-molecule fluorescence (SMF) is based on the detection of light emitted by fluorophores that have been attached to the molecule under investigation. Fluorophores are excited from their ground state by absorbing light from an external light source. After internal conversion and vibrational relaxation they emit fluorescent light in  $10^{-9}$ – $10^{-7}$  s. Detection of single molecules is possible by exciting a very small volume with light and observing the emitted signal. Typical volumes are on the order of the femtolitre ( $10^{-15}$  l), corresponding to a water drop of diameter on the order of a micron. In diluted solutions (on the order of nanomolar concentration) the typical number of photons intercepted by a molecule are estimated to be on the order of a million photons per second, giving a number of photons emitted in such a volume of a thousand photons per second. The emitted light can be detected using sensitive photodetectors such as avalanche photodiodes and photomultipliers. The main advantage of SMF is its high time resolution. This covers from the range of microseconds for individual events up to picoseconds when identical experiments are repeated many times and lots of statistics are collected. SMF is a non-invasive technique that can be used to study biological samples *in vivo*. In general, the spatial resolution in an optical system is limited by the Rayleigh criterion (typically around 200 nm). However, the limit imposed by the diffraction of light can be overcome by using other methods such as FIONA, a method based on centroid localization, where nanometre precision can be achieved when a sufficient number of photons is collected [63–65]. Other methods include SMF polarization measurements using total internal reflection fluorescence (TIRF) that allow us to determine the orientation of individual molecules with tens of millisecond resolution [66]. With these methods it has been possible to determine that myosin V, a two-headed molecular motor that translocates along actin filaments, walks in a hand-over-hand way by alternating the role of the lead and trail heads during its motion [66, 67] (see section 5.3).

A powerful SMF technique to detect conformational changes within 10 nm is fluorescence resonance energy transfer (FRET), discovered by Förster (sometimes called Förster resonance energy transfer) and discussed already in section 2. FRET is based on a quantum interaction mechanism between two fluorescent dyes that can transfer energy when they are kept close enough (typically within a distance of 10 nm) and one of them (the so called donor) is excited by light. When the donor is excited by an external source of light the other dye (the so called acceptor) emits part of the light at another wavelength through a non-radiative resonance energy transfer mechanism between donor and acceptor. The efficiency of energy transfer depends on the distance between donor and acceptor according to the formula  $E = E_0/(1 + (R/R_0)^6)$ , where  $R_0$  is the Förster radius or the value of the distance above which the efficiency goes below 50%. The value of  $R_0$  is a function of the fluorescence and absorption spectrum of donor and acceptor and the orientation between the electric dipoles of both molecules. The Förster efficiency formula gives a spectroscopic ruler to determine distances between the two dyes, thereby allowing us to identify conformational transitions in the biomolecule (figure 2(D)).

Difficulties associated with SMF are the expertise required to chemically attach fluorophores in biomolecules, which often consists of a ‘try and repeat’ procedure. FRET has the added complication that two dyes have to be chemically attached to the same molecule at specific locations. Often it is not possible to know the dipolar orientation of the dyes, which makes it difficult to determine their distance using the spectroscopic ruler. Combination of

FRET with other techniques (e.g. electron microscopy or x-ray diffraction) helps to identify and characterize conformational changes. A widespread problem in SMF is photobleaching of fluorophores. Photobleaching is a process by which excited fluorophores undergo a chemical transformation (e.g. after reacting with oxygen) and stop fluorescing. Methods are currently employed to reduce such an effect, which is still a main nuisance of SMF techniques.

SMF has been used to study molecular transport, protein folding and conformational transitions in enzymatic reactions. There is much current effort to combine SMF with force measurements. This would allow us to identify conformational changes with force jumps, thereby giving precious information about biomolecular function.

## 5. Systems

Single-molecule techniques have been applied to a great variety of systems. From polymers to living cells many system properties have been characterized and studied. In what follows I provide a general overview of a personal selection of these problems. My choice is unavoidably biased by my specific knowledge of some of these questions.

### 5.1. Nucleic acids

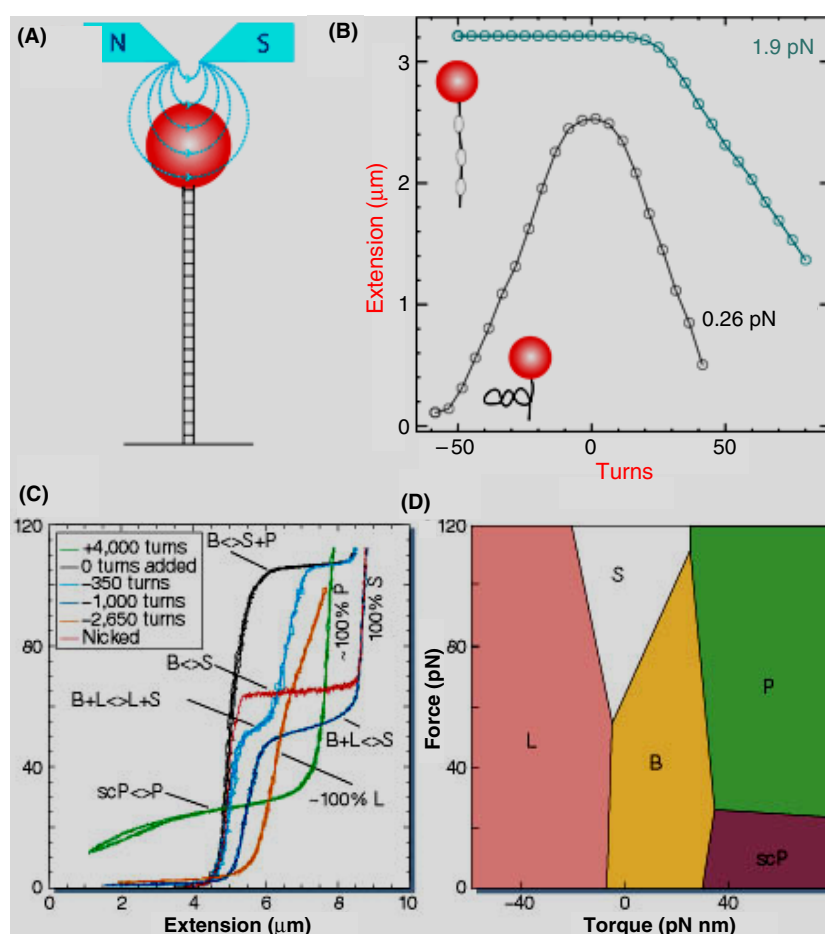
**5.1.1. DNA.** Historically, DNA is the most important player in molecular biology [68, 69]. Since the discovery of the double helix in 1953 [70], the structure of the DNA molecule has been studied under different conditions using crystallographic and bulk methods [71, 72]. DNA can be found in various structural forms and it is now recognized that the phase diagram of the molecule in the presence of force and torque is rich and complex. Pulling experiments in DNA [73] use glass microfibres [74], LOTs [75], MTs [76] or AFM [77]. Pioneering work in the study of the elastic properties of DNA was carried out by Finzi, Smith and Bustamante in 1992, when they visualized the motion of fluorescent DNA molecules attached to micron-sized beads acted on by magnetic and hydrodynamic forces [34]. B-form DNA molecules 48 kbp long (one base pair—bp—is about 3 Å long) of the  $\lambda$ -bacteriophage virus were stretched up to forces as high as 100 pN using AFM [77] and LOTs [75]. DNA shows a elastic response at low forces (below 5 pN) dominated by entropic effects, whereas at high forces (above 5 pN) enthalpic contributions start to be important. Pulling experiments in DNA confirmed that B-DNA is an elastic molecule whose force–extension behaviour can be well described using the worm-like chain model [35, 78–81] introduced in polymer theory by Kratky and Porod [33]<sup>1</sup>. Above 5 pN the FEC is well described by the phenomenological extensible worm-like chain where the contour length  $L$  changes as a function of the applied force  $F$  by  $\Delta L = LF/Y$  where  $Y$  is the Young elastic modulus ( $Y \simeq 1$  nN). In torsionally unconstrained DNA one end is immobilized in one bead and the other end of either one of the two strands (3' or 5') is immobilized to another bead or surface (depending on the experimental set-up). For torsionally unconstrained DNA a transition is identified at an applied force of 65 pN, where the B-DNA overstretches into a new form (the so called S-form DNA or S-DNA), where the new extension of the molecule is approximately 1.7 times its original contour length. In the S form the double helix (characteristic of the B form) unwinds and all base pairs tilt along the force direction. The overstretching transition has a force plateau characteristic of first-order phase transitions (see figure 3(C)). Curiously enough this extended form of DNA was anticipated 50 years ago from the measurement of the optical properties of fibres under tension [83]. A first order transition is also found when DNA melts when heated up above 65 °C. Although it

<sup>1</sup> The solution of the model under the action of external force is equivalent to the solution of the classical Heisenberg ferromagnetic chain in a magnetic field [82].

has been suggested that the S form is force-induced melted DNA [84–86] the current evidence suggests that S-DNA keeps the Watson–Crick base pairs intact in the absence of nicks along the DNA phosphodiester backbone. Noticeable salt dependence effects have been reported for the elastic properties and overstretching transition in DNA due to its large electrostatic charge [87]. Various statistical models have been introduced in the literature that investigate several aspects of DNA such as thermal denaturation [88–96] in the presence of force and torque [97, 98], bubble formation [99, 100] and the overstretching transition [101–106]. The response of DNA to mechanical force is similar to that observed in other biopolymers such as peptides and polysaccharides. These show an elastic response at low forces which is dominated by entropic effects, whereas at high forces enthalpic contributions and structural transitions are often observed [107–111].

Current experiments can now exert force and torque at the same time on torsionally constrained DNA. In torsionally constrained DNA both strands are immobilized at one end of the molecule. MTs allow us to rotate a magnetized bead attached to the end of a DNA molecule that is attached to a glass coverslip through its other end [76]. DNA is a coiled molecule of 2 nm diameter covering one helical turn every 10.5 bps equal to 3.4 nm, also called helical pitch. By exerting torque it is possible to change the helical pitch of the molecule, leading to a supercoiled molecule. The topological properties of a closed DNA molecule are determined by the so-called linking number  $Lk$ , which is equal to the number of crossings between the two strands. In a closed DNA molecule (such as circular DNA from bacteria)  $Lk$  is a topological invariant equal to the sum of twist ( $Tw$ ) and writhe ( $Wr$ ),  $Lk = Tw + Wr$ . The twist is the number of helical turns whereas the writhe is the number of loops occurring along the DNA molecule. The amount of supercoiling, usually termed as superhelical density  $\sigma$ , is measured as  $\sigma = \frac{Lk - Lk_0}{Lk_0}$  where  $Lk_0$  is the linking number of torsionally relaxed DNA. Supercoiling is a biologically important property of DNA. Supercoiling plays an active part in the regulation of the genome in both eukaryotes and bacteria (inside cells DNA is negatively supercoiled,  $\sigma \sim -0.06$ ) and is controlled by a family of enzymes called topoisomerases (see below in section 5.3). These are involved in packaging, transcription, replication, repair and recombination of genomic DNA. Under the application of constant force the extension of the DNA molecule changes as the bead is rotated and the twist increases or decreases (figures 5(A) and (B)). Under twist various structural transitions are observed [112, 113]. At very high forces a new form of DNA (called the P-form) is found when the DNA is overtwisted and the extension of the molecule decreases [114]. In this new form bases are extruded from the inside of the backbone in a form that is reminiscent of the triple-strand model for the structure of DNA proposed by Pauling in the 1950s (hence the name P for the phase) [115]. At low forces the DNA forms plectonemic supercoils (the coils often observed in telephone cords) if overtwisted and denatured bubbles if undertwisted. All these behaviours have been extensively studied and modelled [116–121]. They have resulted in a complex force–torque phase diagram of DNA [60, 11] (see figures 5(C) and (D)). Conformational fluctuations have been shown also to be important in regulatory mechanisms such as protein–DNA interactions [122, 123].

In another class of experiments the 3' and 5' ends on one end of the DNA molecule are immobilized into a bead and a surface. By pulling apart the bead from the surface the Watson–Crick base pairs connecting the two strands along the phosphate backbone break and the DNA molecule opens like a zipper. Unzipping is the process where hydrogen bonds between complementary bases fall apart and the bases that are buried inside the helix become exposed to the solvent. It naturally occurs in various biomolecular processes. For example, the initiation process during DNA replication is led by the exposure of specific DNA segments of the genome to the replicating machinery (a set of various multimeric protein complexes). The replication of DNA is then determined by the advance of the replication fork, which



**Figure 5.** Force–torque DNA measurements. (A) Experimental set-up with MTs. DNA can be twisted by exerting a torque through the rotation of two magnets. (B) Extension–twist curves showed a marked different behaviour depending on the value of the constant applied force. If undertwisted the molecule forms bubbles at high forces, whereas it forms plectonemes at low forces. (C) Various FECs obtained by pulling on twisted DNA using LOTs. The different plateaus indicate different structural transitions. The possible DNA forms are: normal B-form DNA, overstretched (S-DNA), the highly overwound DNA Pauling (P) form, supercoiled and shortened Pauling (scP) form and the underwound and denatured (L) form. (D) Phase diagram indicating all possible phases. Pictures (A) and (B) were taken from [124]. Pictures (C) and (D) were taken from [11].

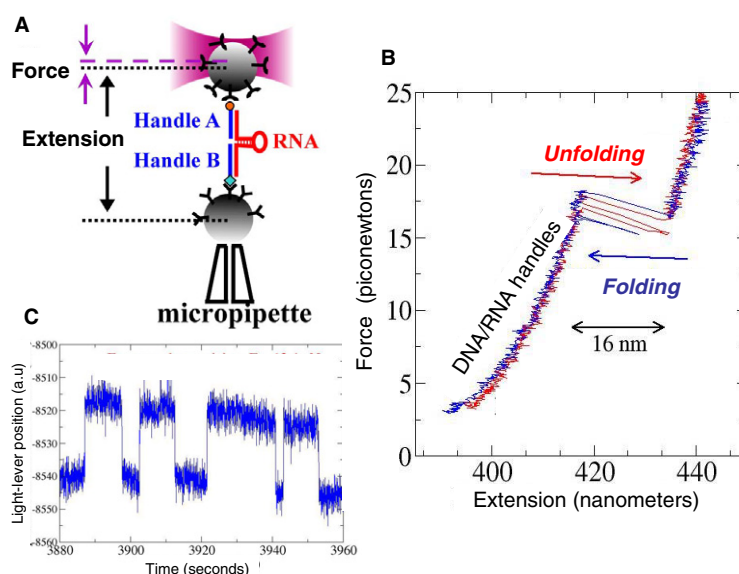
proceeds by untwisting and unzipping the DNA structure. Typical unzipping forces are on the order of 15 pN and are sequence dependent [125, 126, 77, 127, 128]. Below such force (e.g. around 10 pN) the molecule does not unzip. Above that force (e.g. at 17 pN) unzipping proceeds fast with a signal that is a fingerprint of the DNA sequence. During the unzipping process, the recorded force/extension signal strongly depends on the particular DNA sequence. By unzipping the molecule a repeated number of times a characteristic force pattern emerges except for thermal fluctuations. This fact makes force unzipping a promising technique for DNA sequencing [129–132]. Similar experiments are also being conducted with RNA and will be discussed below in section 5.1.2. Force unzipping has been investigated using various statistical models [133–136], which predict re-entrance of the transition due to excluded volume

effects between the two strands [137–139]. At present, various technical precision limitations and noise fluctuations due to the softness of the single stranded DNA (ssDNA) handles limit the reliability of the sequencing procedure. Although at present it is possible to resolve the unzipping of a DNA patch containing a few tens of base pairs, the detection of the opening of just one base pair is an experimental challenge. Improved detection resolution using two traps and combination of LOTs with SMF [17] are expected to provide far more accurate results. Other sequencing strategies are SMF measurements of DNA polymerase activity during the synthesis of the complementary strand [140].

**5.1.2. RNA.** RNA is a very important player in molecular biology. It participates in most processes where the genomic information that is kept inside the nucleus must be exported to the cytoplasm of the cell, where it is translated into proteins that are synthesized in the ribosome. The ribosome, one of the largest biological machines in the cell, contains RNA as part of its structure as well as ribosomal proteins. RNA is considered a relic of the past [141], where the precursors of the first living cells (more than 2 billion years ago) used the chemistry of RNA long before proteins took over most of the essential functions of ancestral prokaryotic cells. The RNA world refers to a hypothetical scenario where the majority of important living functions were carried out by ancient RNA molecules [142]. There are many functions where RNA is essential (e.g. in enzymatic and regulatory processes). Every few years new biological roles of the RNA are discovered. RNA and DNA are chemically very similar molecules. The main difference of RNA with respect to DNA is the presence of the highly reactive OH group in the sugar (ribose) and the replacement of thymine by uracil (adenine pairs with uracil) in RNA. Structurally they are also very different: RNA is found in nature in single-stranded form whereas DNA is found in double-stranded form.

The elastic properties of synthesized double-stranded RNA and DNA are very similar [143], but the single-strand nature of RNA makes the difference. RNA is a more complex molecule than DNA. While complementarity is strict in DNA, RNA allows for additional non-Watson–Crick base pairs (such as GU or GA) between the bases. Consequently, it allows for more base pair interactions and a larger number of possible structures. Single-stranded RNA molecules form complex secondary structures of stems, junctions, loops, bulges and other motifs. The main thermodynamic stability of the molecule is derived from the complementarity of the bases and base stacking interactions. However, secondary RNA structures fold into more complex three-dimensional structures stabilized by specific interactions between different hydroxyl groups of the bases that bind divalent metal cations (e.g. magnesium). Tertiary interactions produce other sorts of structural elements such as pseudoknots or kissing loops. In RNA the contribution to the free energy of the native state due to the tertiary structure is a perturbation to the main contribution due to the secondary structure. In addition, the chemistry of RNA is much simpler than that of proteins (there are four different nucleotides in RNA as compared to the 20 amino acids in proteins). This fact makes RNA easier to study at both theoretical and experimental level. RNA research is very attractive to the biophysicist not only because RNA is less complex than proteins, but also because RNA shows all important properties exhibited by proteins. RNA molecules can be unfolded under the action of mechanical force [144–146]. Thermal and force denaturation experiments reveal that RNA folds into a native three-dimensional structure in the same way as proteins do. Understanding how RNA folds will help to better understand the corresponding process in proteins [147–149]. The problem of RNA folding has also motivated theoretical insight from the theory of disordered systems in statistical physics [150–155].

Glass microfibres, AFM and LOTs have been used to stretch and unzip RNA molecules. The latter is now the most accurate technique to resolve forces on the order of the piconewton



**Figure 6.** RNA pulling experiments using LOTs. (A) Experimental set-up. An RNA molecule is inserted between two hybrid RNA/DNA handles. The force ( $F$ ) and extension ( $X$ ) of the molecular assembly are measured as the micropipette is moved. (B) FEC of a 20 bp RNA hairpin in a pulling cycle. During the stretching part of the cycle (red curve) the elastic response of the handles is followed by the sudden release of extension and a drop in the force corresponding to the unfolding of the RNA molecule. During the relaxation part of the cycle (blue curve) the RNA molecule folds back again. (C) Hopping experiments. If the force is held constant the RNA molecule hops between the folded and unfolded conformations. The vertical axis represents the position of a reference laser beam for the chamber which is related to the molecular extension.

and distances on the order of a few nanometres that are observed in the unfolding of small RNA hairpins. The first RNA pulling experiments were carried out in the Bustamante laboratory [156], where the P5ab RNA hairpin (a derivative of the L121 *Tetrahymena* ribozyme) was studied. In such experiments an RNA hairpin is stretched in a force-ramping experiment and the FEC recorded. The molecule is found to unzip at forces around 15 pN, resulting in a denatured single-stranded RNA form (ssRNA). The RNA molecule also hops between the folded and the unfolded states at a critical value of the extension or force applied on the molecule where the folded and unfolded conformations are equally populated. Pulling RNA (or DNA) hairpins has additional complications as compared to the case of stretching dsDNA. The hairpin being a few tens of base pairs long, the gain in extension after the molecule unfolds is between 10 and 50 nm. Such extension is too short to manipulate the RNA molecule using micron-sized beads. To pull on the RNA hairpin, two hybrid RNA/DNA handles are synthesized and annealed to the RNA molecule at its flanking sides. The handles are typically a few hundred base pairs long so the whole construct is approximately a few hundred nanometres long when fully extended. The experimental set-up and a typical FEC is shown in figures 6(A) and (B). Upon increasing the force the RNA hairpin unfolds, the extension of the molecule increases and the force drops in response to the retraction of the trapped bead that follows the sudden gain in molecular extension. If the force is relaxed back then the hairpin refolds again, showing a sudden increase in the force upon formation of the hairpin. Several thermodynamic and kinetic properties can be investigated in these experiments. By pulling slowly enough (the lowest value of the pulling speed being limited



by low-frequency drift effects in the instrument) one can infer the mechanical work necessary to unfold the hairpin which is equal to the area below the FEC. After subtraction of the reversible work required to stretch the hybrid handles and the unfolded ssRNA, the free energy difference between the folded and unfolded states at zero force and room temperature can be inferred [156]. The value of the folding free energy obtained for RNA secondary structures agrees well with theoretical estimates by Mfold [157, 158]. By repeatedly pulling–relaxing the molecule many times, hysteresis is often observed in the FECs. The average unfolding force is always larger than the average refolding force. Kinetic properties can be studied by pulling the hairpin at different rates and measuring the breakage or dissociation force distribution during unfolding. Combined with the measurement of the refolding force distribution along the retracting part of the cycle it is possible to identify the location of the transition state and the free-energy landscape of the molecule as a function of its extension [159]. Pulling experiments using LOTs are usually carried out near equilibrium conditions. AFMs allow us to perform dynamic force spectroscopy measurements of RNA dissociation far from equilibrium by exploring a few orders of magnitude of loading rates [160]. These studies reveal that the average dissociation force increases logarithmically with the pulling speed as has been found in the study of intermolecular protein–protein interactions (see section 5.2.1). Similar experiments have been carried out to investigate the kinetics of short DNA hairpins using SMF [161, 162] or AFM [163–167], finding slower kinetics of unfolding/refolding depending on the length of the sequence as predicted by some theoretical models [133, 168]. Mechanical unfolding of single RNA molecules through nanopores has also been proposed as a method to determine the secondary structure [169]. The unzipping of RNA hairpins has motivated several theoretical studies of thermodynamic [170–172] and kinetic properties [173–177].

Other related experiments provide additional insight on the kinetics of unfolding of the molecule. For example, if a constant force that is close to a critical value is maintained by a force feedback mechanism then the RNA molecule hops between the folded and unfolded states (figure 6(C)) (the mechanism being similar to the opening and closure activity that is observed in single ion channels [178]). Hopping can be investigated in two different modes: (1) the passive mode where the position of the micropipette and the trap are kept fixed; (2) the force-feedback mode where the force is maintained constant by using a piezo controller that corrects the position of the micropipette every time there is a change in the force (see section 4.1.2). Working in the vicinity of the critical extension or force (where the molecule hops between the folded and unfolded conformations) it is found that the molecule follows exponential kinetics. The probability distribution of the residence times in the folded (unfolded) conformations is well described by an exponential function whose width is equal to the inverse of the kinetic rates of unfolding (folding). The dependence of these rates on the applied force gives accurate information about the height and position of the kinetic barrier. The hopping kinetics of RNA hairpins has been modelled by Cocco and collaborators who have introduced a one-dimensional representation of the possible configurations of the molecule in terms of the number of sequentially open base pairs starting from the beginning of the fork [179, 180]. Models for the experimental set-up where the distance between the micropipette and the centre of the trap are the appropriate control parameter have been considered in [181] whereas a detailed analysis of the influence of the experimental set-up (length of the handles, stiffness of the trap, bandwidth of data collection, time delay of the force-feedback mechanism) on the measurement of the intrinsic molecular kinetic rates of the RNA molecule in the different modes (passive or force feedback) has been carried out in [182, 183]. Passive force clamp methods operating without force feedback have also been implemented in dual laser traps by taking advantage of the anharmonic region of the trapping potential. Studies of the hopping kinetics

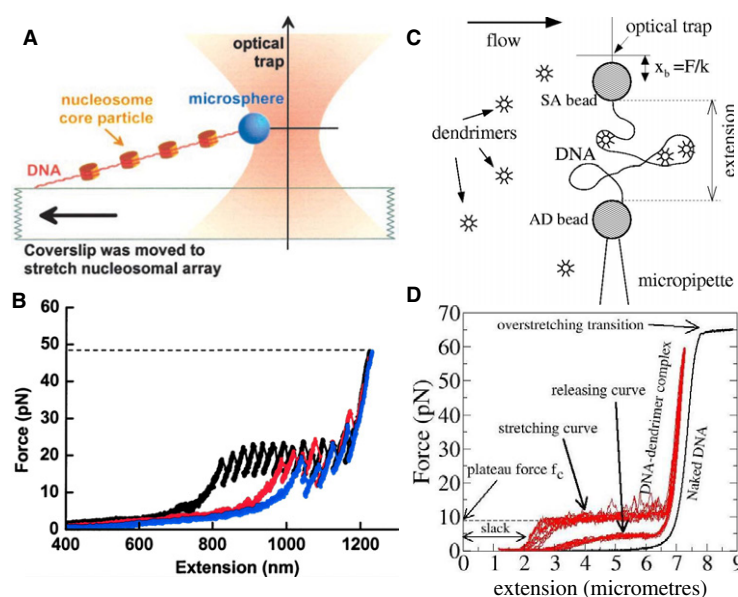


of a tetraloop DNA hairpin show that the hopping frequency increases with the stiffness of the trap [184, 185].

Two-state behaviour is usually found during the unfolding and refolding of short RNA hairpins [156, 186]. Experiments have been carried out in more complex molecules such as the full *Tetrahymena thermophila* ribozyme L21 (containing approximately 400 nucleotides) using FRET [187] or LOTs [188]. LOTs also made possible the mechanical unfolding of the *Escherichia coli* 1540 bp long 16S ribosomal RNA by the group of Chatenay [189]. The resulting FECs reveal a series of complex rips that correspond to the opening of different domains of the molecule. Other studies have investigated specific RNA motifs such as internal bulges [189], the transactivation response region (TAR) RNA derived from the human immunodeficiency virus (HIV) [190] and three-helix junction RNA molecules [191–193]. These experiments show that force unfolding proceeds through the successive opening of domains, whereas force refolding is a much more complex process, where various folding pathways and trapped intermediates are often observed [194–197]. The kinetic behaviour of RNA molecules shares many resemblances to what has been observed in proteins (see section 5.2.2), often showing misfolded structures [198], reinforcing the observation that the free-energy landscape underlying the folding dynamics is more rugged in RNA than in proteins [199–201].

**5.1.3. DNA condensation.** The nuclear genome is not isolated but surrounded by many different proteins engaged in its maintenance and regulation [202, 203]. Proteins are also responsible for the compaction of eukaryotic DNA inside the nucleus of the cell [204]. The nuclear DNA is condensed with proteins into a huge molecular complex called chromatin. Linear compaction defined as the ratio between the length of fully extended DNA to the length of the condensed DNA reaches values on the order of  $10^4$ – $10^5$ . The basic unit of the condensed DNA is the nucleosome core particle, a flat disc of 11 nm diameter with 146 bps of supercoiled DNA wrapped around a histone octamer formed by pairs of histones H2A, H2B, H3 and H4. The main force stabilizing the nucleosome particle is the electrostatic attraction between the negatively charged phosphate backbone of DNA and the protonated (positively charged) arginine and lysine lateral chains of histones. Nucleosome particles are destabilized and readily dissociate at low salt concentrations [205]. Nucleosomes are connected to each other by segments of variable length of linker DNA (around 60 bps) forming a *beads in a string* structure. The histone H1 stabilizes the structure of the nucleosome by fixing the entry and exit angle of the wrapping DNA. Chromatin organizes into different structures at different length scales [206]. The nucleosome is the minimal unit in such organization, also called the 10 nm fibre. At physiological salt values nucleosomes form a complex and dense structure recognized as the 30 nm fibre, formed by the folding of nucleosomes into an as yet unknown three-dimensional structure [207–209].

There are few experimental single-molecule studies of DNA condensation. The first study used LOTs to pull chicken erythrocyte chromatin fibres [210]. It showed irreversible force–extension cycles above 20 pN, interpreted as due to the mechanical removal of histone cores from native chromatin. SMF and AFM imaging have explored the assembly kinetics of chromatin from *Xenopus* eggs and *Drosophila* embryos [211]. None of these preliminary studies could identify the dynamics of individual nucleosomes. Subsequent studies analysed the condensation of reconstituted chromatin fibres of  $\lambda$ -phage DNA suspended between two beads and exposed to *Xenopus laevis* egg extract [212, 213]. The fibre condensed under a constant frictional Stokes force. The rate of condensation decreased considerably under applied force and no condensation events were observed at forces exceeding 10 pN. Strong inhibition of chromatin assembly in chromatin fibres above 10 pN has also been observed in



**Figure 7.** FECs in DNA condensation using LOTs. (A) Mechanical disruption of nucleosomal arrays. Experimental set-up. (B) Nucleosomal arrays repeatedly stretched three times (from left to right: first (black), second (red), third (blue)) up to a maximum force of 50 pN. The unwrapping of individual nucleosomes is observed as jumps in the FEC. As the array is repeatedly pulled the number of remaining nucleosomes in the fibre decreases. Figure taken from [215]. (C) Condensation of DNA molecules with polyaminoamide (PAMAM) dendrimers (experimental set-up). (D) FECs of DNA fibres (red curves) condensed with dendrimers of generation G8 (diameter of particles around 10 nm) [217]. The rightmost (black) curve is the FEC of naked DNA before condensation. The condensation/decondensation (red) curves show hysteresis between the stretch and release parts of the cycle, the presence of a slack (due to the presence of uncondensed segments in the fibre) and a decondensing force plateau around 10 pN.

MT studies [214]. After the fibre had condensed subsequent FECs revealed a series of force jumps between 15 and 40 pN, attributed to the release of the wrapped histone octamer. After all releasing events had taken place the FEC characteristic of naked DNA was recovered. However, due to the large amount of different types of proteins in the cell extract, it is difficult to tell which jumps correspond to other bound proteins and which jumps are due to the unravelling of the histone octamer. Similar experiments have been carried out in the study of nucleosomal arrays of reconstituted pure histones [215]. FECs reveal a series of disruptive events attributed to the unwrapping of individual nucleosomes containing 80 bp of dsDNA (figure 7(A)). Single-molecule force measurements to test higher order chromatin structures have been applied to investigate the protein scaffold of mitotic chromosomes [216].

Recent studies of DNA condensation have considered synthetic condensing agents much simpler than protein histones such as shell cross-linked nanospheres [218] and dendrimers [219]. Dendrimers are synthetic branched polymers that are synthesized via an initiator core terminating (after a repeated series of steps) with amino  $\text{NH}_2$  groups on the surface [220, 221]. Questions such as the importance of charge, shape and size in the condensed state have been recently considered by studying PAMAM (polyaminoamide) dendrimers condensed with  $\lambda$ -phage DNA using LOTs [217]. FECs in dendrimers show a force plateau around 10 pN, characteristic of a decondensation transition between a condensed and an extended phase (figure 7(B)). These experiments also reveal that residual inter-dendrimer

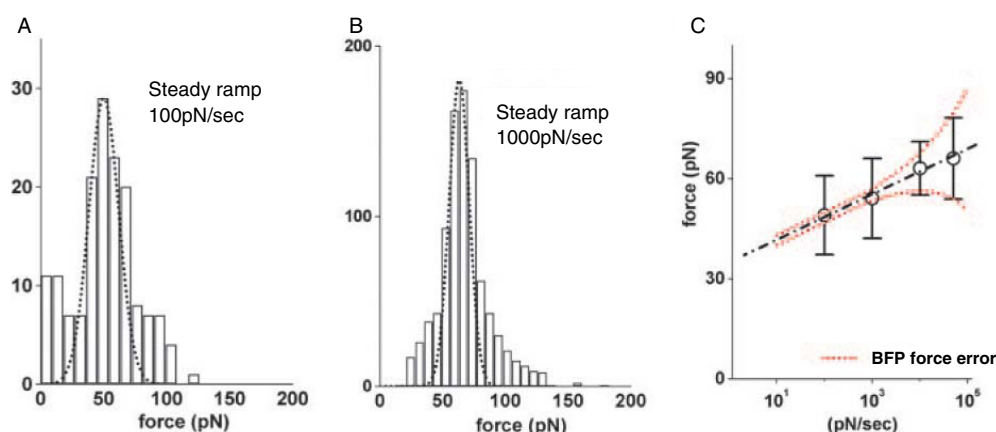
electrostatic interactions keep the structure of the condensed state as revealed by the salt dependence of the force value from the *plateaux*, similar to what has been found in the condensation of DNA by other polycationic complexes such as eukaryotic condensin and trivalent cations like hexaammine cobalt (CoHex) and spermidine [222–224]. The mechanism by which nucleosomes form and arrange into a compact globular structure has been the subject of many theoretical and numerical studies [225–237].

## 5.2. Proteins

Many single-molecule studies have investigated in detail the mechanochemistry of some relevant proteins. Two categories of protein systems must be distinguished: (1) those where an energy source (e.g. from ATP or GTP hydrolysis) is not required; (2) those where catalytic functions driven by high energy phosphate compounds (e.g. ATP or GTP hydrolysis) are necessary. The first class of proteins is closer in spirit to manipulation experiments of single nucleic acids, in which molecular interactions are probed either by mechanical force or observed using optical imaging techniques. The second class of proteins includes the study of many molecular motors and enzymes. Due to the large number and the importance of the studies on ATP-dependent motors a whole section is devoted below to them.

**5.2.1. Protein–protein interactions.** Proteins have important regulatory functions as demonstrated by Jacob and Monod in the early 1960s with the discovery of the Lac repressor. Proteins can participate in chemical signalling at the intra- and intermolecular levels. The successful development of an organism relies on the coordination of myriads of signals transmitted among a large number of proteins all participating in a structurally complex and highly dynamic web of interactions. Developmental processes such as cellular association, differentiation, patterning and reproduction are all controlled mainly by proteins.

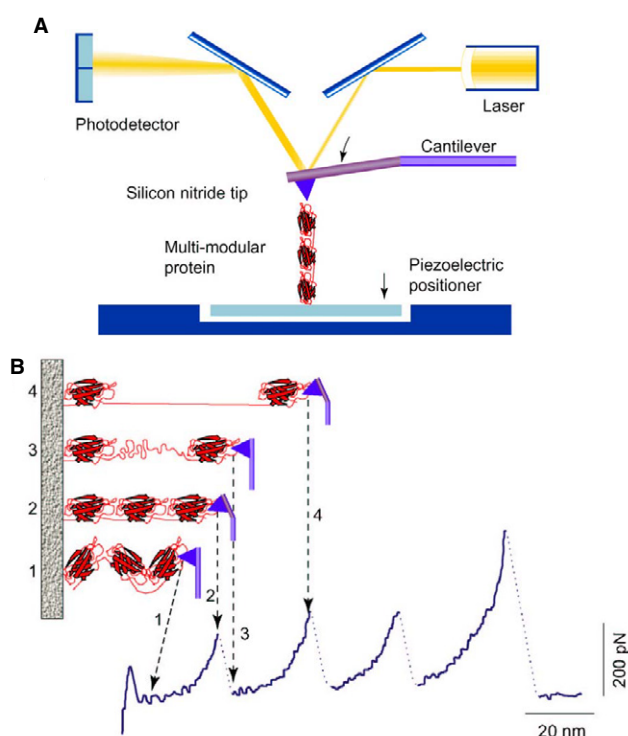
A large number of studies have focused in the study of protein–protein interactions of the ligand–receptor type [238]. These interactions are governed by what in biological terms has become known as the ‘lock and key’ mechanism. Proteins can interact and *recognize* each other by assembling into larger complexes by mutual complementarity and fit of their molecular surfaces. Allostery refers to the regulation of such interaction by changes in either one or both proteins. Protein–protein interactions are studied in dynamic force spectroscopy using AFM, BFP and LOTs. The first type of measurements is the most common. Samples are prepared by coating a substrate with ligand molecules and the tip of an AFM with receptor molecules. The substrate is then moved towards the tip until a few contacts between ligand and receptor molecules are formed. Ideally, one would like to have just one molecular contact between the tip and the substrate. In general this is not possible and the concentration of the proteins has to be carefully tuned to ensure that just one connection is often established between tip and substrate (implying that the large majority of contact trials are unsuccessful and a connection is rarely established). Upon retraction of the tip the extension of the protein–protein contact increases and the force increases up to a value where the contact breaks. The rupture of the contact is stochastic, therefore upon repetition of the experiment several times (each time a new contact has to be sought) the value of the rupture force is always different, the overall rupture process being described by a breakage force distribution. The rupture force distribution depends in a non-trivial way on the retraction rate of the tip. Typically, the larger the retraction rate the higher the average rupture force, which grows approximately as the logarithm of the loading rate. Kramer theories for chemical reactions [239–241], extended by Bell in the 1970s to include the effect of mechanical force on bond dissociation [242], have been adapted by Evans and Ritchie [243–245] to interpret the observed rate dependences.



**Figure 8.** Mechanical strength of *trans*-bonded cadherin fragments. (A), (B) Force histograms in steady ramp protocols at 100 and 1000  $\text{pN s}^{-1}$ . Force distributions shift to larger forces as the loading rate increases. (C) Logarithmic rate dependence of the average rupture force over nearly four orders of magnitude in loading rates. The dotted line corresponds to the measurement error in force. The error increases with the loading rate because of viscous corrections appearing due to probe damping. Figure taken from [257].

Dynamic force spectroscopy [61, 62] has become nowadays a standard method to probe the strength of molecular bonds. It has been applied to the study of biotin–avidin and biotin–streptavidin interactions [246–248], antigen–antibody interactions [249–251], P-selectin/ligand interactions [252, 253], adhesion forces in lipid bilayers [254], substrate–protein adsorption forces [255], cadherin mediated intermolecular interactions [256, 257], carbohydrate–protein bonds [258], proteoglycans [259, 260] and antibody–peptide interactions [261] just to cite a few examples. Typical rupture force distributions are shown in figure 8. The problem of bond rupture has been extended to multiple bonds in several configurations (series, parallel, zipper) [62, 262, 263]. An annoyance inherent to dynamic force spectroscopy in ligand–receptor studies is that after a rupture event occurs a contact has to be established again. As discussed below, this is different from what happens when studying intramolecular interactions, where the initial set of bonds can be reformed again by moving back the surface to the tip.

**5.2.2. Protein folding.** SME allow us to investigate intramolecular interactions in proteins. Pioneering studies have been carried out by pulling the muscle protein titin. The sarcomere is a repeating unit found in fibres of muscle cells responsible for contractile motion. The sarcomere is a highly complex structure  $2.5 \mu\text{m}$  long made out of thick (myosin) and thin (actin) filaments and a *third filament* (titin). Titin connects the Z-disk to the M-line in the sarcomere, and is a huge modular protein responsible for the passive elastic properties of the muscle. Titin is formed by tandem pseudorepeats of many different protein domains such as the immunoglobulin (Ig) and the fibronectin type III (fnIII) domains. The characterization of the mechanical properties of modular proteins like this one is very important because they are present in the cytoskeleton and the extracellular matrix of all eukaryotic cells. AFM and LOT pulling experiments in proteins were first carried out in titin [264–266] by the groups of Gaub in Munich and Bustamante in Berkeley. Upon application of force titin unravels in a series of force jumps, one jump corresponding to the unfolding of an individual module. A FEC reveals a characteristic sawtooth pattern as shown in figure 9. Due to the heterogeneous structure of the module sequence in modular proteins (such as titin) it is difficult to identify which specific module corresponds to which unfolding event. Considerable progress has been



**Figure 9.** Pulling multi-modular proteins with AFM. (A) Experimental set-up. A laser beam is deflected by the cantilever tip of the AFM. The amount of the deflection is detected in a position sensitive detector (PSD). (B) The characteristic *saw-tooth pattern* of force obtained by pulling on a recombinant construct of Ig27 domains from titin. Figure taken from [4].

later achieved in the group of Fernandez using protein engineering techniques to construct homomeric polypeptides, i.e. tandems of identical repeats of a protein such as the I27 module of titin [267, 268, 13] (similar constructs have been synthesized in the T4 lysozyme [269]). The study of polypeptides of I27 allows us to carry out detailed studies of the mechanical stability and unfolding/refolding kinetics of a single molecule.

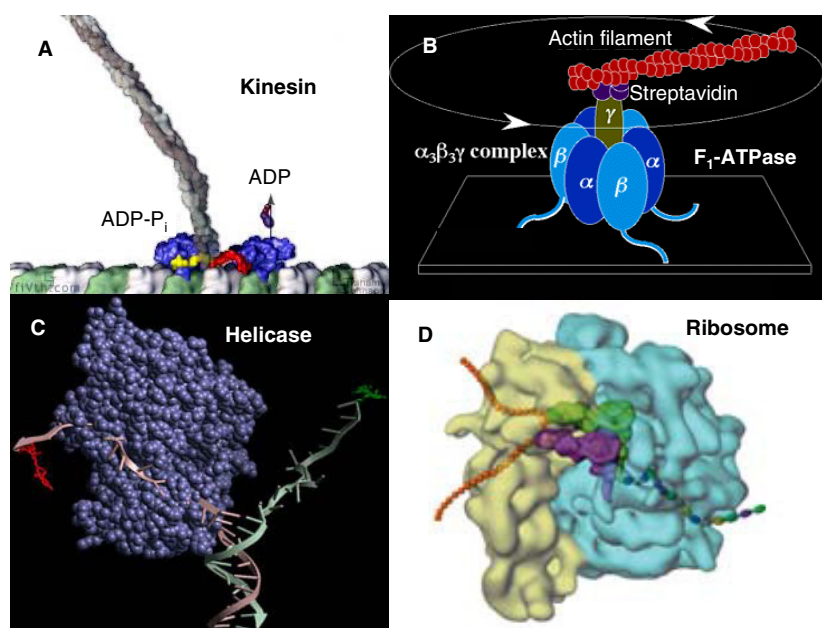
Similar studies have been conducted in other modular proteins such as tenascin [270], triple helical coiled coils of spectrin [271], bacteriorhodopsin [272] and the cellular adhesion molecule Mel-CAM [273]. In all cases the unfolding kinetics is well described by a two-state process, where the distribution of ripping forces and FECs depends on the loading rate. The distributions are also influenced by the polymer spacers and the instrument limitations [274, 275]. These studies also provide insight into the role of the different structural elements of the protein in its mechanical stability, such as  $\beta$ -sheets and  $\alpha$ -helices, the main building blocks of the secondary structure in proteins. Experimentally it has also been found that as a rule  $\beta$ -sheets are more robust elements than  $\alpha$ -helices [276]. The geometry of the pulling, whether unzipping or shearing  $\beta$ -strands, and the point of application of the force also determines the final mechanical stability of the protein [13, 14]. The influence of specific solvent conditions on protein stability can also be studied using the AFM. In this line interesting studies have been carried out by the Discher's group on the mechanical stability of a vascular cell adhesion molecule (VCAM-1), a tandem of seven Ig domains that are stabilized by disulfide bonds and that can be destabilized in the presence of reducing agents [277].

Simultaneous force experiments and reduction of disulfide bonds (to SH) can be studied on single molecules [277–279]. Finally, the study of force unfolding kinetics in proteins has motivated various theoretical and numerical studies [280–282].

Protein folding, the process by which a molecule folds into a three-dimensional functionally active structure, is still a major unsolved problem in modern biophysics. The dynamics of such a process has many aspects and complications, for example many proteins need to be assisted by some other proteins (chaperons) to become efficiently and quickly folded [283]. A fascinating aspect of SME is the possibility to investigate folding under applied force in a single molecule, i.e. the equivalent of protein folding in bulk experiments but with force being the externally controlled variable. Upon pulling the protein is unfolded, while upon retraction of the positioner the protein can fold back again into its native structure. The process should be similar to that observed in RNA molecules. However, there are two inconveniences or difficulties that make the study of force-folding of proteins with AFM more difficult than RNA folding using LOTs. The first difficulty is found in the high value of the stiffness of the AFM tip (typically 100 times larger than that of LOTs, see section 4.1.1), which considerably increases thermal fluctuations in the force, making it difficult to control the value of the force during the refolding process. The second inconvenience is related to the experimental difficulties of unequivocally identifying individual molecules due to many non-specific interactions between tip and substrate. To overcome this second limitation the synthesis of polyproteins has been used in order to identify true unfolding events. The study of force-folding kinetics in proteins has been carried out by measuring the force-clamp relaxation (i.e. the force is maintained constant by using a feedback loop) of polyproteins that have been previously mechanically unfolded. However, it is unclear whether in such conditions the folding kinetics of individual modules might be affected by the folding kinetics of other modules. Recent AFM measurements by the group of Fernandez in polyubiquitin proteins (ubiquitin is an ubiquitous and highly conserved eukaryotic protein in charge of signalling for the proteosomal degradation of proteins, among other functions) using the force-clamp technique [284] have shown that the refolding kinetics is not a two-state process but a multiple-pathway dynamical process determined by the roughness of the free-energy landscape of the molecule [285, 286] (see however [287–290] for a discussion of these results and alternative interpretations). Similar observations have been reported in RNA pulling experiments [198]. These studies suggest the existence of intermediate states along the unfolding/folding pathway of proteins. Evidence for intermediates has been obtained in the study of the unfolding kinetics of titin with AFM [291–293]. Recently, LOTs have also been used to investigate the folding kinetics of RNaseH enzyme, revealing the presence of an intermediate conformation of the protein, probably the molten globule state observed in thermal denaturation experiments [294]. This work paves the way to investigate other proteins using LOTs. SMEs in proteins are expected to greatly contribute to our current understanding of protein folding from a statistical physics approach [295–297].

Fluorescence spectroscopy has also been used to investigate and detect the presence of intermediate states in proteins. Different methods are used to immobilize and observe proteins: passive adsorption, specific tethering, trapping inside polymer gels or vesicle encapsulation [18]. In this last technique proteins are encapsulated inside vesicle cells of size larger than the protein but smaller than the laser beam section, allowing for a mild immobilization of the protein. Haran and collaborators have shown that two-state folders, well characterized by bulk biochemistry methods, display exponential kinetics in their relaxation [31]. Adenylate kinase, an enzyme 214 amino acids long that catalyses the production of ATP from ADP, shows a heterogeneous and slow dynamics characterized by multiple folding pathways [32]. These results are in agreement with the conclusions obtained





**Figure 10.** Examples of molecular machines. (A) Kinesin walking along a microtubule and transporting a cargo (not shown). (B)  $F_1$ -ATP synthase is the proton pump responsible for synthesizing ATP in the mitochondria of eukaryotic cells. (C) Helicases are forerunners of the DNA polymerase that unwind DNA by transforming dsDNA into two strands of ssDNA. (D) The ribosome is among the largest molecular machines inside the cytoplasm of the cell and is in charge of manufacturing proteins.

in the aforementioned force-clamp AFM studies on polyubiquitin [285] and FRET studies on RNase P (a transfer RNA that contains a catalytic RNA subunit) [298].

### 5.3. Molecular motors

One of the great applications of SME is the possibility to follow individual molecular machines in real time while they carry out specific molecular tasks [299]. Molecular motors are proteins that typically use the energy extracted from available sources, such as chemical gradients or high energy phosphate compounds (e.g. ATP or GTP), to exert mechanical work (figure 10). For example, in ATP hydrolysis a molecule of ATP is broken into ADP and inorganic phosphate  $P_i$  ( $ATP \rightarrow ADP + P_i$ ) in a highly irreversible reaction that releases around  $7 \text{ kcal mol}^{-1} \simeq 12k_B T$ . The chemical process by which motors utilize the energy stored in the high energy bonds of the ATP molecules to perform mechanical work is based on two hypothesized mechanisms: (1) power stroke generation and (2) Brownian ratchet mechanism. In the first mechanism either the production of ADP or the release of the inorganic phosphate during the ATP hydrolysis cycle induces a conformational change in the substrate that is tightly coupled to the generation of force and motion in the motor. In the second mechanism, the motor diffuses reversibly along the substrate. Unidirectional movement is produced by rectification of thermal fluctuations induced by the conformational change in the protein caused by ATP hydrolysis. By steady repetition of a mechanochemical cycle (one or more ATP molecules are hydrolysed per cycle) the motor carries out important cellular functions. Motors are characterized by the so called processivity or number of turnover cycles the motor does before detaching from the substrate. Processivities of molecular motors can vary by several orders



of magnitude, depending on the type of motor and the presence of other regulating factors. For example, the muscle myosin II motors work in large assemblies, each myosin having a processivity around unity, meaning that each myosin performs one mechanochemical cycle on average before detaching from the substrate. At the other extreme of the scale there are DNA polymerases (DNAPols, enzymes involved in the replication of the DNA) in eukaryotes which show processivities that range from unity (adding approximately one nucleotide before detaching) up to several thousands or even millions. However, in the presence of sliding clamps (proteins with the shape of a doughnut that encircle the DNA and tightly bind DNAPols [300]) processivities go up to  $10^9$ .

Molecular motors are magnificent objects from the point of view of their efficiency. If we define the efficiency rate as the ratio between the useful work performed by the motor and the energy released in the hydrolysis of one ATP molecule in one mechanochemical cycle, then typical values for the efficiencies are around several tens per cent, reaching the value of 97% in some cases (like in the  $F_1$ -ATPase, see below). For example, out of the  $20 k_B T$  obtained from ATP hydrolysis, kinesin can exert a mechanical work of  $12 k_B T$  at every step, having an efficiency of around 60%. Such large efficiencies are rarely found in macroscopic systems (motors of cars have efficiencies in the range 20–30%), meaning that molecular motors have been designed by evolution to efficiently operate in a highly noisy environment [301]. Molecular motors are expected to be essential constituents of future nanodevices. Single-molecule devices that operate out of equilibrium and are capable of transforming externally supplied energy into mechanical work are currently being studied [302].

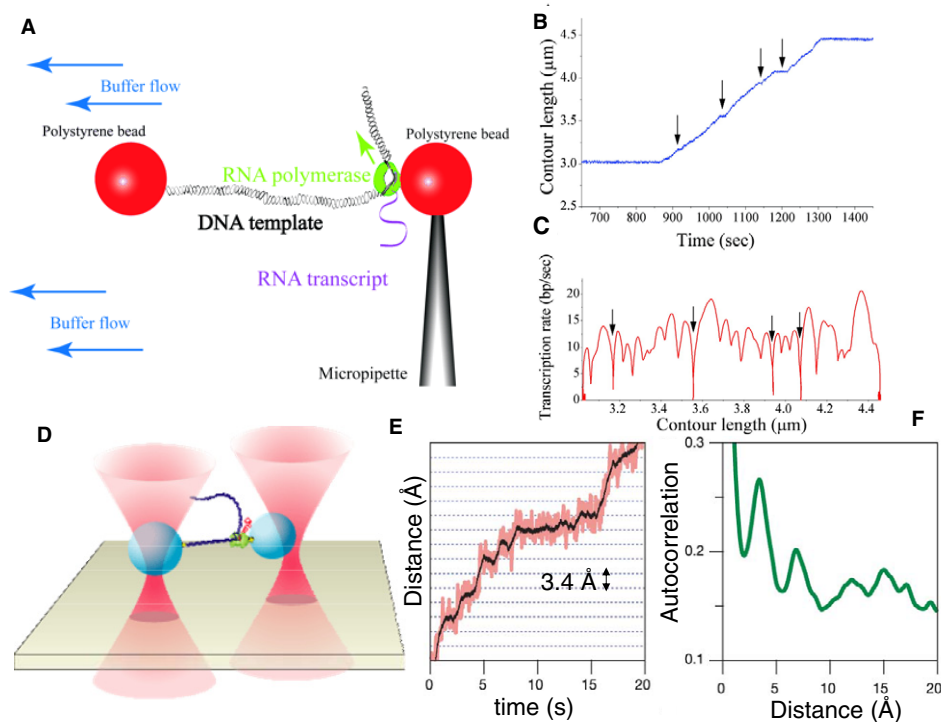
Two major classes of molecular motors have been experimentally studied using single-molecule techniques. One class corresponds to molecular motors involved in several transport processes inside the cell cytoplasm such as the aforementioned kinesin, dynein and myosin [303]. Single-molecule measurements in molecular motors were carried out by Block and co-workers, who studied a single kinesin on a microtubule rail. Kinesin and dynein are both microtubule associated proteins (MAPs) involved in the transport of organelles and vesicles along microtubules in the cytoplasm of the cell. They move in opposite directions with respect to the polarity of the microtubule. Using LOTs it is possible to attach one kinesin molecule to a bead captured in an optical trap and measure the force exerted on the bead while the kinesin walks along the microtubule [304, 305]. Kinesins are found to move in 8 nm steps at an average speed of  $2 \mu\text{m s}^{-1}$  [306]. Kinesins move in a hand-over-hand way by alternate exchange between the head of the molecule (that makes a strong bond with the  $\beta$ -tubulin domain of the microtubule while ATP is hydrolysed) and the other head, that detaches from the substrate [307]. The average velocity of kinesin has been shown to depend on the value and direction of the applied force [308, 309], with a stall force around 7 pN required to arrest the motion [310, 311]. Kinesin also shows backward steps during its motion, suggesting a Brownian ratchet mechanism [312, 311]. Depolymerizing kinesin motors of the cytoskeleton and extraction of membrane nanotubes by kinesins have also been investigated in SMF experiments [313, 314]. Dynein has also been studied and shown to display steps that are multiples of 8 nm [315], depending on the applied force. The force generation mechanism is still unknown [316].

Myosins are a large class of proteins responsible for muscular cell contraction that walk along actin filaments [317]. Two main types of myosins have been studied in SMEs: monomeric (single-head) and dimeric (two-head) motors. Examples of single-head myosins are myosin I, myosin II and myosin IXb [318–320]. Examples of two-headed myosins are myosin V and myosin VI [321, 322, 66]. LOT and SMF measurements have shown that myosins move along actin in steps of average size about 10 nm in single-head myosins [323, 324] and 36 nm for the two-headed myosin V [67].

Another motor that has been extensively studied using SMF techniques is the protein machine  $F_0$ ,  $F_1$ -ATP synthase, a proton pump that synthesizes ATP in the mitochondria, the *power plant* of eukaryotic cells. The  $F_0$ ,  $F_1$ -ATP synthase dissociates into two parts,  $F_0$  (the proton channel) and  $F_1$  (often called  $F_1$ -ATPase). The latter is a complex made out of a shaft containing two different types of three tubular subunits each (called  $\alpha$  and  $\beta$ ) and a central subunit called  $\gamma$ . By engineering appropriate mutations in the complex, Kinoshita and co-workers [325] immobilized the shaft to a surface and attached the central  $\gamma$  subunit to a fluorescent actin filament (figure 10(B)). In the presence of ATP the actin filament was observed to rotate in steps of  $120^\circ$  [326–328], which were later resolved into substeps of  $30^\circ$  and  $90^\circ$ , each substep corresponding to different phases of the mechanochemical cycle of ATP synthesis [329].  $F_1$ -ATPase motors have also the capability to be integrated with nanoelectrochemical systems to produce functional nanomechanical devices [330].

A second class of motors is those that interact with the DNA and participate in maintenance tasks of the genome such as transcription and replication. Examples are DNA polymerases (DNAPols), DNA translocases, RNA polymerases (RNAPols) and DNA topoisomerases. DNAPols have been studied using LOTs and MTs. Studies have been carried out in [331] with the T7 DNAPol (belonging to the bacteriophage T7 and characterized by having a high processivity of several thousand base pairs) and in the Bensimon group [332], who studied the sequenase (a mutant of T7 DNAPol lacking the exonuclease site) and the Klenow DNAPol (a fragment of *Escherichia coli* DNAPol I lacking exonuclease activity). In these experiments a ssDNA containing a DNA primer required for the initiation of replication is tethered between one immobilized surface and one detector bead that measures the force acting on the molecule. After flowing DNAPol, ATP and other nucleotides (NTP) inside the chamber the tether extension of the molecule is recorded at a constant applied force as a function of time as the ssDNA is converted into dsDNA. From these measurements it is possible to recover the number of replicated nucleotides as a function of time and the speed of the motor. In all cases the replicating activity was found to decrease, with the applied force ceasing at around 40 pN in the case of the T7 DNAPol, above which exonuclease activity was observed. Models of the mechanochemical cycle of DNAPol have been proposed for T7, which qualitatively reproduce the dependence of the net replication rate as a function of the opposing load [333, 334]. Another class of enzymes that move in specific directions along dsDNA are translocases. These are regulatory enzymes important during transport and replication processes, which sometimes show sequence dependent bidirectional motion. FtsK, a translocase of *Escherichia coli* involved in chromosome segregation and cellular division, has been shown to move at an average speed of  $5 \text{ kbp s}^{-1}$  and against loads up to 60 pN [335, 336].

A related experimental set-up has been applied to investigate the transcription dynamics of RNAPol. This is a processive enzyme that synthesizes an ssRNA strand by translocating along dsDNA without the need of a primer [339]. The newly synthesized strand, messenger RNA, codes for the amino acid sequence of proteins, which are synthesized in the ribosome [340]. Transcription consists of three main steps: initiation of the transcribing complex, elongation and termination [341]. Pioneering SMEs on transcription were carried out in the T7 RNAPol by the groups of Block, Gelles and Landick using LOTs [342, 343]. Under an applied force the rate of RNA transcription did not change much but the process stalled at around 20–30 pN. Similar results have been found in studies of the transcription dynamics of *Escherichia coli* RNAPol under assisting or opposing force [344, 337] (see figures 11(A)–(C)). The recent development of an ultra-stable trap with Angstrom-level resolution has shown discrete steps of average around  $3.5 \text{ \AA}$  for the step size of the enzyme indicative of a mechanochemical cycle of one base pair at a time [345, 338] (figures 11(D)–(F)). The dynamics of RNAPol displays a complex behaviour sensitive to DNA sequence with pauses (temporary halts to transcription)



**Figure 11.** RNAPol motion in *E. coli* using LOTs. (A) Experimental set-up in force-flow measurements. LOTs are used to trap beads but forces are applied on the RNAPol–DNA molecular complex using the Stokes drag force acting on the left bead immersed in the flow. In this scheme force assists RNA transcription as the DNA tether between beads increases in length as a function of time. (B) The contour length of the DNA tether as a function of time (blue (online) curve) and (C) the transcription rate (red (online) curve) as a function of the contour length. Pauses (temporary arrests of transcription) are shown as vertical arrows. (D) Experimental set-up in ultrastable LOTs. The right trapped bead is located in the region of the trap where the stiffness vanishes. This creates a force-clamp where only the right bead moves, determining the extension of the complex. (E) Motion of RNAPol resolved in discrete steps of 3.4 Å. (F) Average autocorrelation function obtained from the position histograms showing peaks at distances of multiples of 3.4 Å. Pictures (A)–(C) taken from [337]. Pictures (D)–(F) taken from [338].

and arrests (permanent halts). Many aspects of the elongation process in RNAPol are not well understood. For example, the distribution of residence times for pauses shows strong statistical variations in their frequency and duration [346]. Many of these features are also found in DNA replication and are expected to be important in the regulation of gene expression [347].

Another type of DNA–protein motor that has attracted considerable attention recently is virus packaging motors. The packaging motor of bacteriophage virus  $\phi 29$  encapsulates DNA inside the heads of the virus. It has been shown to be a highly processive motor, capable of working against loads of up to approximately 50 pN [348]. These experiments have stimulated very interesting theoretical activity in the packaging problem [349–351].

SME have also been applied to the study of topoisomerases. These are a large class of enzymes present in both prokaryotes and eukaryotes and are very important in the maintenance of the genome. Topoisomerases act on the topology of DNA, their main task being to relax and introduce supercoils in DNA by cutting the phosphate backbone of ssDNA and dsDNA. For example, relaxing stress of supercoiled DNA is important for transcription, replication and

recombination. There are two major families of topoisomerases: type I and type II. Type I topoisomerases relax DNA supercoils by changing the linking number (for a definition see section 5.1.1) by one unit ( $\Delta L_k = -1$ ) without ATP consumption. Type II topoisomerases change the linking number by two, being capable of introducing supercoils in DNA,  $\Delta L_k = \pm 2$ , therefore consuming ATP. SMEs using MTs are ideal to study the action of topoisomerases. By twisting the bead attached to a DNA molecule it is possible to build up torsional stress in the molecule and follow the subsequent relaxation upon addition of topoisomerase in the buffer. Examples include the study of topoisomerase II in *Drosophila melanogaster* [352, 353] and the observed torque dependence of kinetics in eukaryotic topoisomerase I [124, 354]. Also, DNA gyrase, a topoisomerase II of prokaryotes known to introduce negative supercoils, has been studied [355] using the bead tracking method [60]. Related studies have been conducted with *Escherichia coli* topoisomerase IV, an ATP-dependent enzyme that removes positive (but not negative) supercoils [356]. Most of these experiments have been carried out using MTs [357] or SMF where limited spatial resolution hinders time-dependent aspects of the kinetics such as pauses or stalls; see however [355].

Recent studies on helicases, however, have already identified such effects. Helicases are yet another class of DNA–protein motors crucial during DNA replication that have been studied with SME. Helicases are forerunners of the DNAPol that unwind DNA by transforming dsDNA into two strands of ssDNA, a necessary step for the advance of the replication fork during DNA replication. DNA unwinding has been studied in the RecBCD helicase/nuclease of *E. coli* [358, 359] and in the Rep helicase using SMF assays [360]. RNA helicases also play an important part in the infection cycle of many viruses by making two strands of ssRNA (available to produce new virus copies) from a double-stranded RNA (dsRNA) synthesized inside the infected cell. Recent studies of the NS3 helicase, part of the protein machinery of hepatitis C virus, has been recently studied using LOTs, showing a great richness of kinetic effects including pauses, arrests or even reversal of the motor followed by rewinding of the previously unwound dsRNA [361]. Finally, we mention the study of the molecular complex of the ribosome, an experimental challenge to the biophysicist using single-molecule methods [362–364].

Molecular motors have inspired a large number of theoretical studies in statistical physics [365–376]. All motors studied up to now show generic properties such as ATP and load dependence of their average velocity. For example, the average speed as a function of ATP concentration follows the Michaelis–Menten kinetics. Also, there is some evidence that kinetic phenomena such as pauses, arrests and backtracking motion are generic features of motors. One wonders whether there exists a relationship between these dynamical features of motors and their astonishing mechanical efficiency.

## 6. Tests of nonequilibrium theories in statistical physics

Recently there has been a lot of interest in applying single-molecule techniques to explore physical theories in systems out of equilibrium. The use of new micromanipulation tools in the exploration of the behaviour of tiny objects (such as biomolecules and motors) embedded in a thermal environment opens the possibility to investigate how these systems exchange energy with their environment. This question is of great interest both at a fundamental and a practical level. From a fundamental point of view, the comprehension of how biomolecules operating very far from equilibrium are so efficient (see the discussion in section 3) raises the question of whether such tiny systems exploit rare and large deviations from their average behaviour by rectifying thermal fluctuations from the bath. From a practical point of view, this might help in the design of efficient nanomotors in the future. The study of such questions

is steadily becoming an active area of research, nowadays referred to as *Nonequilibrium thermodynamics of small systems* [28, 29]. Such discipline is becoming quite popular among statistical physicists, who recognize there are new aspects of thermodynamics where large Brownian fluctuations are of pivotal importance as compared to fluctuations in macroscopic (or large) systems [36]. In macroscopic systems, fluctuations represent just small deviations with respect to the average behaviour. For example, an ideal gas of  $N$  molecules in thermal contact with a bath at temperature  $T$  has an average total kinetic energy of  $(3/2)Nk_B T$ . However, the total energy is not a conserved quantity but fluctuates, its spectrum being a Gaussian distribution of variance  $(3/2)N(k_B T)^2$ . Therefore, relative deviations of the energy are on the order  $1/\sqrt{N}$  with respect to the average value. For macroscopic systems such deviations are very small: for  $N = 10^{12}$  (this is the typical number of molecules in a 1 ml test tube at nanomolar concentrations), then relative deviations are on the order of  $10^{-6}$ , hence experimentally unobservable by calorimetry methods. For a few molecules,  $N \sim \mathcal{O}(1)$  relative deviations are large enough for fluctuations to be measurable. SMEs, by allowing us to study molecules one at a time, grant access to such large deviations that are inaccessible in bulk experiments, which use a macroscopic number of molecules. As a rule of thumb we can say that in nonequilibrium processes in small systems the typical amount of energy exchanged with the environment is a few times  $k_B T$ , maybe from 1 to 1000 but not much more. As often happens when establishing the limits of validity of certain regimes, there is not a well defined frontier separating the small-size regime from the large-size regime.

The name thermodynamics of small systems was coined by Hill [37], who showed the importance of the statistical ensemble in thermodynamic relations. A main result of statistical mechanics is the independence of the equation of state on the statistical ensemble in the thermodynamic limit. Such independence breaks down in small systems due to the contribution of fluctuations which depend on the type of statistical ensemble considered (e.g. for the case of a stretched polymer [377]). The search for a new thermodynamic description of small systems has given rise to microcanonical ensemble theory of phase transitions [378] and new classical statistics such as that embodied in Tsallis entropy [379] and Beck's theory [380] (for a review see [381]). From the current point of view, the most important aspect of biomolecular complexes is that they operate far from equilibrium, yet the possible relationship between nonequilibrium behaviour and biological function is still unknown. The combination of small size and nonequilibrium behaviour appears as the playground for the striking behaviour observed in molecular complexes inside the living cell.

Since the beginning of the 1990s some exact results in statistical mechanics have provided a mathematical description of energy fluctuations (in the form of heat and work) for nonequilibrium systems. This new class of results goes under the name of fluctuation theorems (FTs) and provides a solid theoretical basis to quantify energy fluctuations in nonequilibrium systems [382, 383, 29]. FTs describe energy fluctuations in systems while they execute transitions between different types of states. For these fluctuations to be observable the system has to be small enough and/or operate over short periods of time, otherwise the measured properties approach the macroscopic limit where fluctuations get masked by the dominant average behaviour. Most fluctuation theorems are of the form

$$\frac{P(+S)}{P(-S)} = \exp\left(\frac{S}{k_B}\right), \quad (1)$$

where  $S$  has the dimensions of an entropy that may represent heat and/or work produced during a given time interval. The precise mathematical form of relations such as (1) (for instance, the precise definition of  $S$  or whether they are valid at finite time intervals or just in the limit where the time interval goes to infinity) depends on the particular nonequilibrium



conditions (e.g. whether the systems start in an equilibrium Gibbs state, or whether the system is in a nonequilibrium steady state, or whether the system executes transitions between steady states, etc).

Various categories of FTs have been introduced and experimentally validated [29]. The differences between various FTs can be illustrated by introducing the concept of the control parameter. The control parameter (let us say  $\lambda$ ) is a value or a set of values that, once specified, fully characterizes a given stationary state of the system (either equilibrium or nonequilibrium). Upon variation of the control parameter a system that is initially in a well defined state will evolve toward a new state. In general, if the control parameter is varied with time according to a given protocol,  $\{\lambda(t); 0 \leq t \leq t_f\}$  the system will evolve along a given trajectory or path. If, after time  $t_f$ , the value of the control parameter is kept fixed at the value  $\lambda(t_f)$  then the system will eventually settle into a new stationary state. Along a given path the system will exchange energy with its environment in the form of heat and work. The values of the heat and work will depend on the path followed by the system. Upon repetition of the same experiment an infinite number of times (the protocol  $\lambda(t)$  being the same for all experiments), there will be a heat/work distribution characterizing the protocol  $\lambda(t)$ . Generally speaking, FTs relate the amounts of work or heat exchanged between the system and its environment for a given nonequilibrium process and its corresponding time-reversed process. The time-reversed process is defined as follows. Let us consider a given nonequilibrium process (we call it forward, denoted by F) characterized by the protocol  $\lambda_F(t)$  of duration  $t_f$ . In the reverse process (denoted by R) the system starts at  $t = 0$  in a stationary state at the value  $\lambda_F(t_f)$  and the control parameter is varied for the same duration  $t_f$  as in the forward process according to the protocol  $\lambda_R(t) = \lambda_F(t_f - t)$ . FTs depend on the type of initial state and the particular type of dynamics (deterministic versus stochastic) or thermostatted conditions.

Despite the fact that most of these theorems are treated as distinct they are in fact closely related [29]. The main hypothesis for all theorems is the validity of some form of microscopic reversibility or local detailed balance (see however [384–386]). Major classes of FTs include the transient FT (TFT) and the steady state FT (SSFT):

- The transient FT (TFT). In the TFT the system initially starts in an equilibrium (Boltzmann–Gibbs) state and is driven away from equilibrium by the action of time-dependent forces that derive from a time-dependent potential  $V_{\lambda(t)}$ . The potential depends on time through the value of the control parameter  $\lambda(t)$ . At any time during the process the system is in an unknown transient nonequilibrium state. If the value of  $\lambda$  is kept fixed then the system relaxes into a new equilibrium state. The TFT was introduced by Evans and Searles [387] in thermostatted systems and later extended by Crooks to Markov processes [388].
- The steady state FT (SSFT). In the SSFT the system is in a nonequilibrium steady state where it exchanges net heat and work with the environment. The existence of the SSFT was numerically anticipated by Evans and collaborators for thermostatted systems [389] and demonstrated for deterministic Anosov systems by Gallavotti and Cohen [390]. The entropy production  $\mathcal{S}$  by the system (equal to the heat exchanged by the system divided by the temperature of the environment) satisfies the relation (1) in the asymptotic limit of large times  $t \rightarrow \infty$  and for bounded energy fluctuations,  $\sigma = \frac{|\mathcal{S}|}{t} < \sigma^*$ , where  $\sigma^*$  is a model dependent quantity. Other classes of SSFTs include stochastic dynamics [391], Markov chains [392, 393] or the case where the system initially starts in a steady state [394] and executes transitions between different steady states [395, 396].

The first experimental tests of FTs were carried out by Ciliberto and co-workers for the Gallavotti–Cohen FT in Rayleigh–Bernard convection [397] and turbulent flows [398]. Later

on FTs were tested for beads trapped in an optical potential and moved through water at low Reynolds numbers. The motion of the bead is then well described by a Langevin equation that includes a friction (non-conservative) force, a confining (conservative) potential and a source of stochastic noise. Experiments have been carried out by Evans and collaborators who have tested the validity of the TFT [399, 400], and by Liphardt and collaborators for a bead executing transitions between different steady states [401]. The validity of the TFT has also been recently tested for non-Gaussian optical trap potentials [402].

Particularly relevant to the single molecule context is the FT by Crooks [388, 403] which relates the work distributions measured along the forward (F) and reverse (R) paths,

$$\frac{P_F(W)}{P_R(-W)} = \exp\left(\frac{W - \Delta G}{k_B T}\right), \quad (2)$$

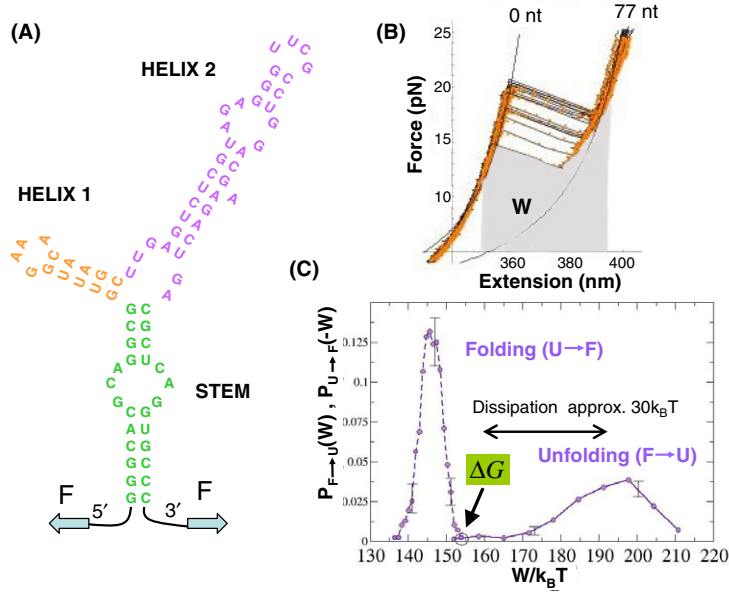
where  $P_F(W)$ ,  $P_R(-W)$  are the work distributions along the F and R processes respectively, and  $\Delta G$  is the free energy difference between the equilibrium states corresponding to the final value of the control parameter  $\lambda_f = \lambda(t_f)$  and the initial one  $\lambda_i = \lambda(0)$ . A particular result of (2) is the Jarzynski equality [404, 405] that is obtained from (2) by rewriting it as  $P_R(-W) = \exp\left(\frac{-W + \Delta G}{k_B T}\right) P_F(W)$  and integrating both sides of the equation between  $W = -\infty$  and  $W = \infty$ . Because of the normalization property of probability distributions, the left hand side is equal to unity and the Jarzynski equality reads

$$\left\langle \exp\left(-\frac{W}{k_B T}\right) \right\rangle_F = \exp\left(-\frac{\Delta G}{k_B T}\right) \quad \text{or} \quad \Delta G = -k_B T \log\left(\left\langle \exp\left(-\frac{W}{k_B T}\right) \right\rangle_F\right), \quad (3)$$

where  $\langle \dots \rangle_F$  denotes an average over an infinite number of trajectories all generated by a given forward protocol  $\lambda_F(t)$ . Relations similar to Jarzynski's equality can be traced back in the free-energy perturbation identity derived by Zwanzig [406] and a generalized fluctuation–dissipation relation proposed by Bochkov and Kuzovlev [407]. The Jarzynski equality and the FT by Crooks can be used to recover equilibrium free-energy differences between different molecular states by using nonequilibrium SME using LOTs [408–410]. In 2002, the Jarzynski equality was tested to pull the P5ab RNA hairpin, a derivative of the *Tetrahymena thermophila* L21 ribozyme [411]. However, in that case the molecule was pulled not too far from equilibrium. The Jarzynski equality and related identities for athermal systems have been recently put under scrutiny in other systems [412–414]. The Jarzynski equality and the FT by Crooks have inspired several theoretical papers discussing other related exact results [415–420], free-energy recovery from numerical simulations [421–426], bias and error estimates for free-energy differences [427–433] and applications either to single-molecule pulling experiments [186, 434, 435], enzyme kinetics [436, 437] or solvable models [438–443]. In addition, analytical studies on small system thermodynamics show that work/heat distributions display non-Gaussian tails describing large and rare deviations from the average and/or most probable behaviour [415, 416, 444–447]. These theoretical studies open the way to investigate the possible relevance of these large deviations in other nonequilibrium systems in condensed matter physics [397, 398, 448, 449].

The FT by Crooks can be applied and tested by measuring the unfolding and refolding work distributions in single-molecule pulling experiments. For example, let us consider the case of a molecule (e.g. a DNA or RNA hairpin or a protein) initially in thermal equilibrium in the folded (*F*) or native state. By applying mechanical force (e.g. using AFM or LOTs) the molecule can be mechanically unfolded and the conformation of the molecule changed from the native to the unfolded (*U*) state. The unfolding event is observed by the presence of a rip in the FEC of the molecule (figure 12(B)). During the unfolding process the tip of the cantilever





**Figure 12.** Recovery of folding free energies in a three-helix junction RNA molecule [193]. (A) Secondary structure of the junction containing one stem and two helices. (B) Typical force–extension curves during the unfolding process. The grey area corresponds to the work exerted on the molecule for one of the unfolding curves. (C) Work distributions for the unfolding or forward paths ( $F \rightarrow U$ ) and the refolding or reverse ( $U \rightarrow F$ ) paths obtained from 1200 pulls. According to the FT by Crooks (2) both distributions cross at  $W = \Delta G$ . After subtracting the free energy contribution coming from stretching the handles and the ssRNA these measurements provide a direct measure of the free energy of the native structure.

or the bead in the trap exerts a mechanical work on the molecule that is given by

$$W = \int_{x_0}^{x_f} F dx \quad (4)$$

where  $x_0, x_f$  are the initial and final extension of the molecule. In (4) we are assuming that the molecular extension  $x$  is the externally controlled parameter (i.e.  $\lambda \equiv x$ ), which is not necessarily the case. However the corrections introduced by using (4) are shown to be often small. The work (4) done upon the molecule along a given path corresponds to the area below the FEC that is limited by the initial and final extensions,  $x_0$  and  $x_f$  (grey shaded area in figure 12(B)). Because the unfolding of the molecule is a stochastic (i.e. random) process, the value of the force at which the molecule unfolds changes from experiment to experiment and so does the value of the mechanical work required to unfold the molecule. Upon repetition of the experiment many times a distribution of unfolding work values for the molecule to go from the folded ( $F$ ) to the unfolded ( $U$ ) state is obtained,  $P_{F \rightarrow U}(W)$ . A related work distribution can be obtained if we reverse the pulling process by releasing the molecular extension at the same speed at which the molecule was previously pulled, to allow the molecule to go from the unfolded ( $U$ ) to the folded ( $F$ ) state. In that case the molecule refolds by performing mechanical work on the cantilever or the optical trap. Upon repetition of the folding process many times the work distribution,  $P_{U \rightarrow F}(W)$  can be also measured. The unfolding and refolding work distributions can then be measured in stretching/releasing cycles; an example is shown in figure 12(C).

The FT by Crooks has been tested in different types of RNA molecules and the method has been shown to be capable of recovering free energies under strong nonequilibrium conditions [193]. From (2) we observe that  $P_{F \rightarrow U}(\Delta G) = P_{U \rightarrow F}(-\Delta G)$  so the forward and reverse work probability distributions cross each other at  $W = \Delta G$ . By repeatedly measuring the irreversible mechanical work exerted upon the molecule during the unfolding process and the mechanical work delivered by the molecule to the LOT instrument during the refolding process, it has been possible to reconstruct the unfolding and refolding work probability distributions,  $P_{F \rightarrow U}(W)$  and  $P_{U \rightarrow F}(-W)$ , and extract the value of the work  $W = \Delta G$  at which both distributions cross each other (figure 12(C)). The work probability distributions were measured along the unfolding and refolding pathways for a three-way junction RNA molecule and found to strongly deviate from a Gaussian distribution [193] (figure 12(C)). These experimental results pave the way for other related studies, for example in molecular dynamics simulations [450].

These kinds of studies will expand in the future to cover more complex cases and other nonequilibrium situations such as the free-energy recovery of folding free energies in native states in proteins or free energies in misfolded structures and intermediate states in RNA molecules and proteins. Ultimately FTs, when combined with SMEs, will offer an excellent opportunity to characterize and understand the possible biological relevance of large deviations and extremal fluctuations in molecular systems.

## 7. Conclusions

In this review I have discussed the potential of SMEs to investigate various topics in molecular biophysics and statistical mechanics. After a brief discussion of the most widely used experimental techniques I have presented applications to various molecular systems. As stressed in the introduction, this review does not exhaust all relevant applications of SMEs. By focusing on the field of molecular biophysics I have just covered a small fraction of problems. Other areas such as cellular biophysics and condensed matter physics are progressively incorporating such techniques in the laboratories.

What is the future of SME? We can foresee two aspects of SME whose development will be crucial for the progress in the field: development of new and better instruments and development of new and better protocols of chemical synthesis.

A major contribution to the progress of the field will come from instrumentation design with enhanced spatial and temporal resolution. Recently, the development of an ultra-stable optical trap with Angstrom-level resolution has allowed for the direct observation of base-pair stepping during the transcriptional elongation of *E. coli* RNAPol [345, 338]. Future developments to improve spatial detection will certainly include optical tweezers with dual traps [451]. Combination of SMF techniques for imaging with force measurements also opens the way to more sensitive measurements. Single-molecule FRET can be combined with LOTs to measure forces and correlate them with conformational changes [452]. Also, total internal reflection fluorescence (TIRF) techniques capable of monitoring the position of a molecule along a vertical direction using a calibrated evanescent wave can be combined with AFM measurements [453]. Instruments that can manipulate molecules at different temperatures and forces will grant access to the potential energy surface of the molecule. Along this direction, AFM and LOTs that include various temperature controllers have been already developed [454–456]. Finally, LOTs with multiple traps offer interesting possibilities in the near future although it is not yet clear how to use them to manipulate molecular complexes in a controlled way [457]. Holographic tweezers also offer exciting possibilities [458]; however, we must first learn how to calibrate them in order to measure forces.

Also, the development of better protocols to synthesize molecular systems will facilitate the outcome of the experiments. SMEs may present a considerable difficulty regarding the preparation of the samples, especially in those cases where biological activity is required. It is commonly said that SMEs are 100% noise and 100% signal. Uncertainties in experimental conditions and sample preparation imply that experiments have to be repeated several times until good results are obtained. Usually, just a few molecules show the interesting behaviour one is looking for, the rest simply do not work. By synthesizing better complexes and having a good control on the chemistry it will be possible to carry out more efficient experiments and investigate new problems. A good example of potentially interesting SMEs, where the chemical synthesis of the molecular complex to manipulate is the rate-limiting step, is found in the study of intermolecular interactions, for example protein–protein interactions (section 5.2.1). Most studies on intermolecular interactions use the AFM. Unfortunately, with such a technique it is difficult to repeatedly form and dissociate the same set of molecular interactions. This experiment is more feasible using LOTs, where, in addition, non-specific interactions between substrate and the molecule are more efficiently avoided. The chemical synthesis of appropriate handles (e.g. carbon nanotubes) for protein complexes will facilitate such experiments. We can foresee future SMEs to investigate protein–protein aggregation processes that are crucial in many biological processes.

SMEs are fascinating but difficult at the same time. They require imagination and strenuous efforts. SMEs are redefining the shape and composition of modern research teams. These must include researchers with expertises and knowledge covering a wide range of topics. SMEs represent one of the most interdisciplinary fields at present, that will require the combined efforts of biologists, chemists and physicists. SMEs represent a good example of the new trends in modern science, that will reshape the way we are going to do research in this century.

## 8. List of abbreviations

ADP	Adenosine diphosphate
AFM	Atomic force microscopy
ATP	Adenosine triphosphate
BFP	Biomembrane force probe
dsDNA	Double-stranded DNA
dsRNA	Double-stranded RNA
DNA	Deoxyribonucleic acid
DNApol	DNA polymerase
FEC	Force–extension curve
FRET	Fluorescence resonance energy transfer
FT	Fluctuation theorem
MTs	Magnetic tweezers
LOTs	Laser optical tweezers
RNA	Ribonucleic acid
RNApol	RNA polymerase
SME	Single-molecule experiments
SMF	Single-molecule fluorescence
ssDNA	Single-stranded DNA
ssRNA	Single-stranded RNA

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