

*Invited paper*

Piotr E. MARSZAŁEK

## SHORT REVIEW: PROBING MECHANICAL PROPERTIES OF INDIVIDUAL MOLECULES WITH ATOMIC FORCE SPECTROSCOPY

**ABSTRACT** *In this short review I will first concisely describe the principles of single-molecule force spectroscopy (SMFS) for measuring the mechanical properties of individual polymeric molecules, as implemented on an Atomic Force Microscope (AFM) platform. Next, I will review a selected number of the most striking, in my opinion, discoveries and observations accumulated in this field of research that now spans over 25 years of dynamic growth. This selection will be limited to biomolecular systems such as DNA and polysaccharides (sugars) that for the last two decades were an important part of my own research. The mechanical properties of single protein molecules are described by the author or other researchers in numerous original or review papers that can be found in the world literature.*

**Keywords:** *Atomic Force Microscopy, Single Molecule Force Spectroscopy, Nanomechanics, Polymer elasticity, Biopolymers*

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### 1. INTRODUCTION

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The eighties of the last century witnessed important technical and methodological discoveries and developments that laid solid foundations for manipulating, probing and characterizing small objects at the nanoscale ( $\sim 10^{-9}$  m) and even single individual molecules. In 1987, Arthur Ashkin and co-workers focused a laser light through a microscope objective and created an optical trap (OT, or laser tweezers) that allowed dielectric objects (or even live bacteria) as small as 1 micron to be captured and controlled by this optical trap in 3 dimensions [1, 2] (A. Ashkin

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**Professor Piotr E. MARSZAŁEK**

pemar@duke.edu

Department of Mechanical Engineering and Materials Science, Duke University, Durham, North Carolina, 27708, USA

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received a Nobel prize in Physics in 2018 for his discovery). In 1981, the Scanning Tunneling Microscope (STM) was invented by Gerd Binnig and Heinrich Röhler, who shared a Nobel prize in 1986 for creating an instrument able to image conducting surfaces and their electrical properties at an atomic resolution using the electron tunneling current between the sharp metallic tip and the sample. In 1986, based on the STM concept, a new scanning instrument called the Atomic Force Microscope (AFM) was invented by Binnig, Quate and Gerber [3], which was capable of visualizing the topographical features of surfaces, based on mechanical deformations of its elastic probe terminated with a sharp point-like tip (the AFM cantilever tip). Unlike STM that could probe only conducting materials, AFM could, in principle, image all sorts of materials, conductors and insulators alike, because its operational principles do not involve measuring electrical currents but measuring the mechanical bending of a small lever probe interacting with the surface being imaged.

In the mid-nineties, both optical traps (laser tweezers) and AFM were used for the first time to investigate the mechanical properties of *individual* biomolecules such as DNA, proteins and polysaccharides. These polymeric molecules were attached at one end to a microscopic bead (or a surface) that could be controlled and moved with high precision using piezoelectric actuators, while the other end of the molecule was attached to a second dielectric bead, kept in an optical trap or attached to the tip of an AFM so these molecules could be stretched, while their length and tension could be simultaneously measured. These initial measurements immediately indicated that biopolymers are not simple entropic springs, as predicted by models of polymer elasticity, but have complex elasticity profiles. Measurements of their length at a given force by OT or AFM can yield many valuable insights into their structure-function-behavior relationships of particular significance to biology. These developments started a new field of single-molecule force spectroscopy that is the subject of this short review.

## 2. AFM-BASED SINGLE MOLECULE FORCE SPECTROSCOPY

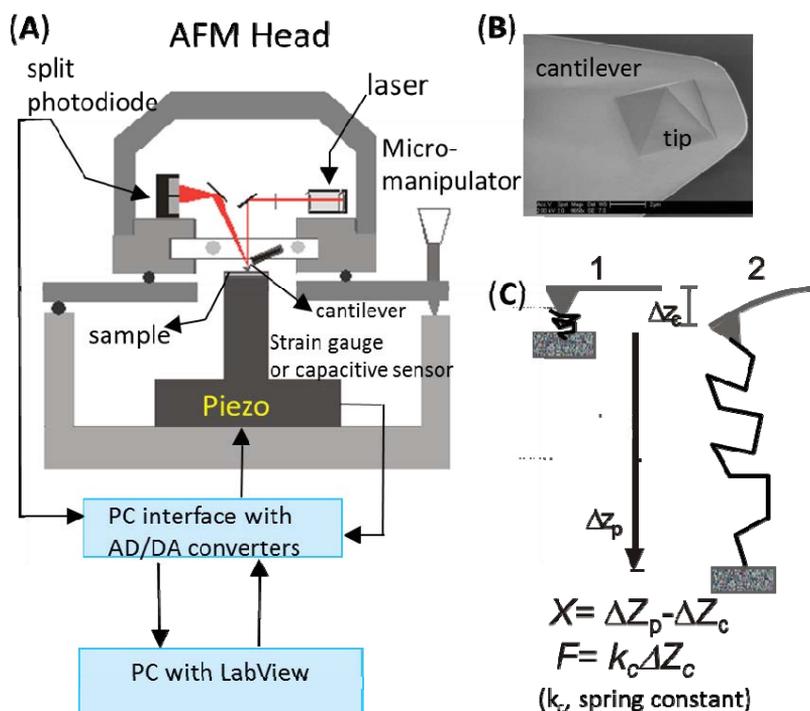
### 2.1. Single molecule force spectroscopy

In a nutshell, single molecule force spectroscopy involves the gradual stretching (or relaxing) of a molecule while its tension (the applied force) and length (extension) are simultaneously measured [4–8]. Single molecule force spectroscopy can be implemented on a number of physical instruments/platforms including optical traps, AFM or magnetic tweezers where one end of a molecule is attached to a paramagnetic bead and an external nonuniform magnetic field exerts a force on the bead, stretching the molecule (which is attached to a surface at the other end) [9, 10]. In this review, the implementation of single molecule force spectroscopy on the atomic force microscope will be described, as this platform has been exploited by the author. The principle of single molecule force spectroscopy implemented on an AFM instrument is illustrated in Figure 1.

## 2.2. The atomic force microscope

AFM instruments (Figure 1A) used for force spectroscopy measurements are composed of four main parts: a) a piezoelectric stage for moving the sample (typically the movement is executed vertically), b) an AFM cantilever that serves as a force sensor, c) a diode laser that is focused on the back of the cantilever which is then reflected and projected onto d) a position sensing split photodiode. An AFM instrument needs to be equipped with a very high-resolution piezoelectric actuator that moves a sample up and down with sub nanometer precision, which may be achieved when the actuator is equipped with an integrated position sensor measuring the actual expansion of the piezoelectric stack, such as a capacitive or a strain-gauge sensor. The piezoelectric actuator is powered by a stable, low-noise voltage source (e.g. 0-100 V) that is controlled by a computer through a digital to analog converter (AD/DA converter with at least 16-bit resolution is required for sub nm precision).

The piezo expands (or shrinks) according to the applied voltage (controlled by the computer), while its actual trajectory (position) is monitored by reading the electrical signal from the integrated sensor (capacitive or strain-gauge in a Wheatstone bridge configuration). The sensor signal is converted by an analog to digital converter

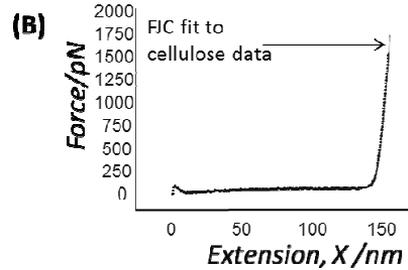
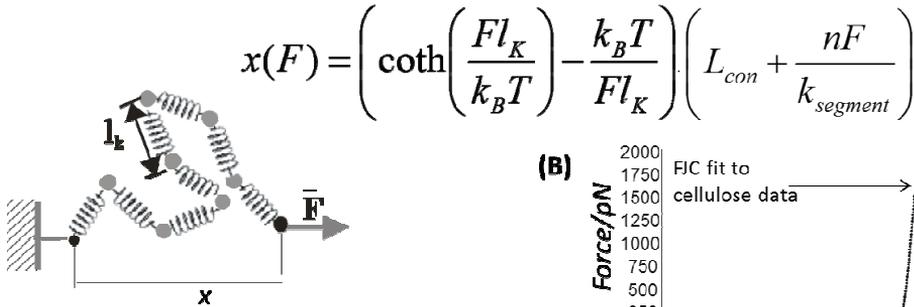
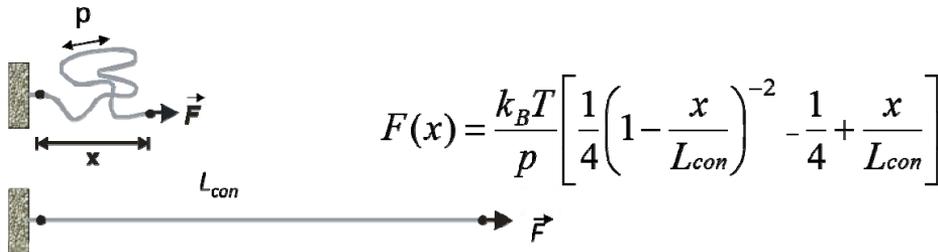


**Fig. 1.** Schematic of an AFM-based molecular puller (A), Scanning Electron Microscope image of an AFM cantilever with integrated pyramidal tip (B), Schematic of a stretching measurement on a single polymer chain (C)

(ADC) and is recorded by the computer. Through a feedback system, the piezo actuator can be made to move linearly with the applied voltage (on their own, piezoelectric materials are nonlinear and display significant hysteresis). A sample consisting of a solution of biopolymer to be studied is deposited on a substrate such as a gold-coated glass coverslip and is mounted on the piezoelectric actuator. The force measuring sensor of an AFM is a micromachined “diving-board” or a “V-shaped” cantilever with typical dimensions 100 x 20 x 0.5 micrometers (length x width x thickness) (Figure 1B) protruding from a larger glass chip (a few mm by few mm rectangularly shaped chip) that allows the cantilever to be securely mounted in the instrument. Typically, cantilevers with integrated tips (Figure 1B) are made from silicon or silicon nitride using lithographic processes and chemical etching. Because the thickness of each cantilever may be significantly different even in the same batch due to a somewhat limited control over the chemical processes used during manufacturing, it is necessary to directly calibrate the stiffness of each cantilever before force spectroscopy measurements (to determine its spring constant,  $k_c$ ). This is typically achieved by measuring the random, Brownian motion of the cantilever in solution to determine the average amplitude of its oscillations,  $\langle(\Delta Z_c)^2\rangle$  and using the energy equipartition theorem that posits that the elastic energy stored in the cantilever  $\langle U \rangle = \frac{1}{2} k_c \langle(\Delta Z_c)^2\rangle$  is at equilibrium equal to the thermal energy,  $E = 1/2 k_B T$ , where  $k_B$  is the Boltzmann constant [11]. When a molecule attached at one end to a substrate (glass, gold) and at the other end to the AFM cantilever tip is stretched by moving the piezo down (Figure 1,  $\Delta Z_p$ ), the tension that builds up in the molecule exerts a downward force on the cantilever causing its bending (deflection,  $\Delta Z_c$ ). The amount of this bending multiplied by the spring constant of the cantilever,  $k_c$  measures the force,  $F$  exerted by the molecule (typically in the range of a few to a few thousand pN, 1 pN =  $10^{-12}$  N, Figure 1C) whose length (extension)  $X$  is determined by the difference between piezo travel and cantilever deflection (Figure 1C). The standard way to measure the bending of the cantilever in AFMs (and thus to measure the force) is by projecting a laser beam from a diode laser on the back-side of the cantilever. This laser beam reflected by the cantilever is then projected onto a split quadrant photodiode. When the cantilever bends, the angle of the reflective surface relative to the laser beam changes and the reflected beam illuminates different parts of the photodiode as compared to the relaxed cantilever. Thus, measuring the differential photodiode current, which is zero, when the laser beam equally illuminates all quadrants of the photodiode, and different than zero when the laser spot moves on the photodiode in response to cantilever bending, allows, after simple calibration, bending of the cantilever and thus force to be measured. In practice, the determination of the cantilever spring constant is somewhat imprecise, thus the magnitude of the force measured by AFM has an uncertainty not smaller than 10-20%, while the length of molecules is determined with sub nanometer precision.

### 2.3. Attaching molecules for force spectroscopy measurements

In order to be stretched by an AFM, a molecule needs to be securely attached at two points, to a substrate and to the force sensor. In the simplest approach, the natural

**(A) The freely jointed chain with segment elasticity: ssDNA, sugars****(C) The worm-like chain: dsDNA, proteins**

**Fig. 2.** Models of polymer elasticity (A, C); Force extension relationship (B) of a cellulosic chain (measured by AFM) that can be adequately described by the extended FJC model (A)

tendency of biopolymers to stick to surfaces is exploited in the so-called non-specific attachment method. Molecules dissolved in a solvent (frequently water) sediment and spontaneously adsorb to the surface on which they are deposited. Then, the sample is brought to contact with the AFM tip (by means of a piezoelectric actuator) and the other ends of the adsorbed molecules have an opportunity to adsorb to the AFM tip. This process is random and a low-yield one, but after several attempts, a molecule (or sometimes more than one molecule) attaches itself to the tip allowing the stretching process to be executed. This attachment to the AFM tip is easily detected because only the molecules that stick to the tip exert a force on it causing its bending (the photodiode signal is generated) while all other molecules that did not attach do not produce any AFM signal. This nonspecific attachment works well for relatively long and homogenous molecules such as DNA and polysaccharides and some many proteins. As long as the molecular properties of a given polymer are homogenous, it is not that important which part of the molecule is stretched, as random fragments are representative of the whole molecule. However, for smaller biomolecules or the ones that have different properties along its length such as some proteins, it is very important to know which part of the molecule is bridged between the surface and the force sensor

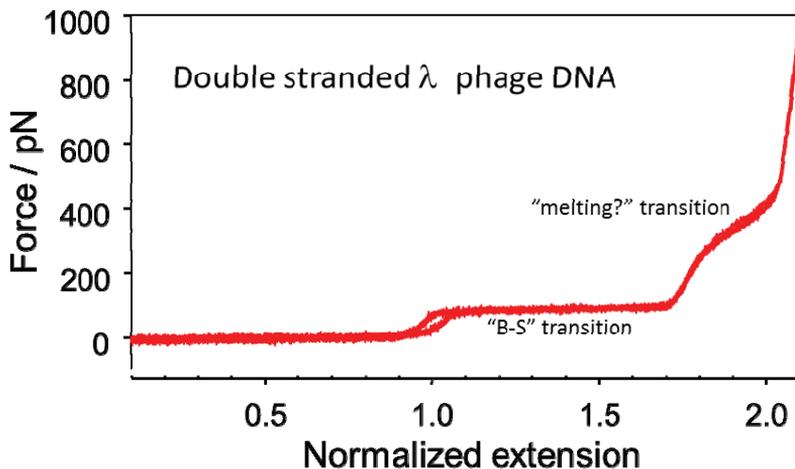
and thus more complex attachment methods may be necessary. A nonspecific attachment can be still used, by flanking the molecule of interest with molecular handles. The handles will then attach nonspecifically to the instrument and the whole molecule of interest will be measured during the stretching process as no part of this central molecule will be directly interacting with the surface and with the sensor because the applied force is transmitted to the molecule through the handles. Another option is to chemically modify molecules' termini and then use specific chemical reactions (that could be unique to both ends of the molecule and the surface and the tip), to couple the molecules to the instrument in a highly controlled and directional way [12].

## 2.4. Polymer elasticity

From the mechanics point of view, polymers are considered to behave as "entropic springs". As polymers are composed of a large number of units connected by chemical bonds, their configurational space is very large because the units (monomers) typically may assume various orientation relative to each other and in solution and when at thermal equilibrium the polymer chain maximizes the configurational entropy, by assuming a "random coil" like structure. Thus, to stretch a polymeric molecule by separating its ends, an external force is needed to carry out the mechanical work on the system to compensate for the decreasing entropy of the chain being stretched (a similar mechanism is responsible for rubber elasticity). Two models and their variations are widely used to describe this entropic elasticity of polymers. The freely jointed chain model (FJC) assumes that rigid (inextensible) units in the polymer are connected in such a way that they may assume arbitrary orientations relative to their neighbors without any restrictions (Figure 2A). This model may be further expanded (and thus can be more realistic chemically and physically) by considering that there are certain restrictions on the rotation of neighboring bonds and, also extended FJC models, may include the so called enthalpic segment elasticity, which reflects the elasticity of the chemical bonds themselves, which extend (they are not perfectly rigid) when the chain is stretched by an external force (Figure 2A). The worm-like chain model considers a polymer chain as a continuous tube rather than an assembly of individual discrete bonds. The tube may be aligned with the direction of the applied force, like the FJC chain, but also segments of the tube will elastically bend under the influence of the force, contributing bending energy to the system Hamiltonian (Figure 2C). The elasticity of some polymers, such as polysaccharides (e.g. cellulose) is typically described well by the (extensible) FJC model (Figure 2B), while molecules such as double stranded DNA (the double helix) or proteins, which are chains composed of amino acid units, are better described by the WLC model (in a certain force range only, as will be discussed later). While various experimental approaches such as using light scattering that study the dynamic behavior of polymers and their dimensions by averaging over very large population of molecules (e.g. on the order of 1 mole that is  $\sim 10^{23}$  molecules) can test the FJC and WLC models of polymers, direct measurements of polymer elasticity and thus confronting models with physical measurements on individual molecules became possible with the advent of single molecule force spectroscopy.

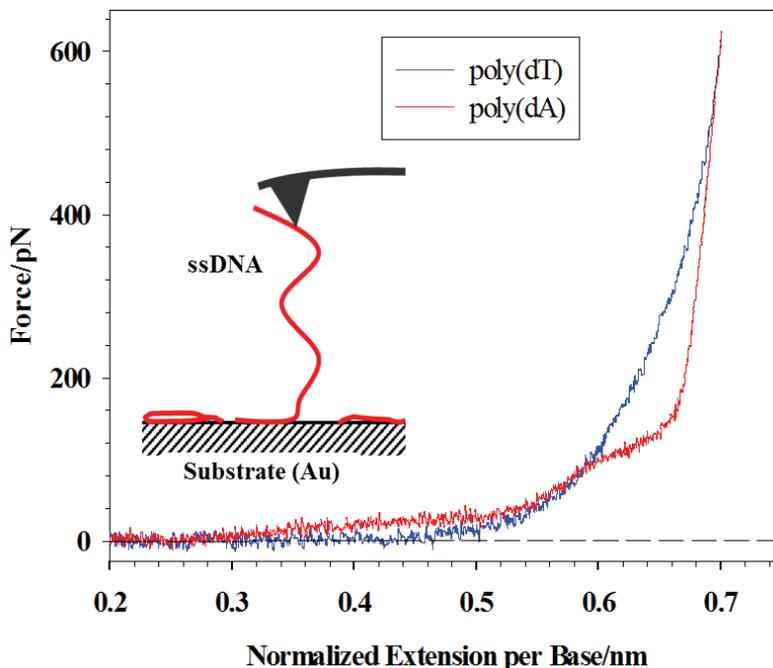
### 3. DNA ELASTICITY

DNA is a long polymeric molecule composed of four chemical bases (AGTC) that are connected through a sugar backbone. In its canonical form, DNA is composed of two chains with helical structures wrapped around each other. The molecule's chemical sequence contains all the hereditary information and codes instructions for making proteins by cellular machinery, which fulfill many fundamental functions in living organisms. DNA polymers in human cells are approximately 2 m long but they are packed in a very small cell nucleus with a radius of a few micrometers. Thus, for DNA to be packed in such a small volume, it needs to be highly organized structurally and bent sharply many times. For these and other reasons, DNA's mechanics and its elasticity are of critical importance to DNA functions. It is then not surprising that DNA was one of the first polymeric molecules subjected to single molecule force spectroscopy measurements when this technique became available. Early measurements were carried out by a magnetic tweezer-like apparatus that allowed only small stretching forces of some 10-20 pN to be applied to DNA and the conclusion from those measurements was that the FJC model does not describe DNA elasticity very accurately [9], suggesting that the DNA has a natural curvature and that its elasticity may be better described by the WLC model [13]. However, the measurements carried out on long DNA from a virus, the so-called lambda phage DNA (over 15 micrometers in length) using optical tweezers, reported by Carlos Bustamante and co-workers in 1996, revealed a highly unusual elastic profile of this DNA that could not be fitted with a WLC model, when the stretching force exceeded 65 pN. At this force, the extensibility of DNA



**Fig. 3. Force-extension profile of double stranded DNA as measured by AFM**

The graph shows two superimposed force curves, one corresponding to DNA stretching and one to DNA relaxation. In this case the traces overlap very well, but DNA commonly displays a significant hysteresis in the elasticity profile after being overstretched. This specific recording was obtained in author's laboratory.



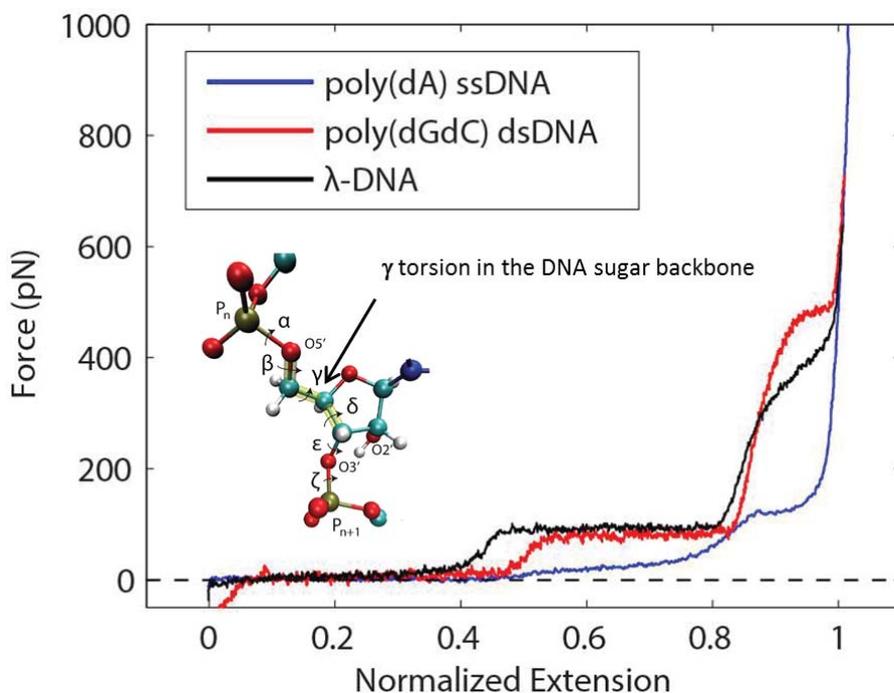
**Fig. 4. Force-extension curves obtained on single stranded DNA molecules with an AFM instrument (author's laboratory); Adapted from reference [17].**

increased abruptly, and the molecule extended additionally by about 70% at an almost constant force, as compared to its original contour length [14]. A similar result was independently reported in 1996 by François Caron and co-workers who used etched optic fibers as nanomechanical force probes to stretch DNA [15]. This extension of double-stranded DNA (dsDNA) beyond its equilibrium length was coined as the DNA overstretching or B-S transition (Figure 3, “B-S” transition). Although, the mechanism of the process underlying the B-S transition is not yet completely understood, it is generally assumed that it involves a forced unwinding of the double helix. Interestingly, force spectroscopy measurements carried out on single stranded DNA by laser tweezers in the Bustamante study, showed that the elasticity of a single DNA polymer chain closely follows the extended FJC model (that includes the segment elasticity) without displaying any overstretching transitions.

In 1999, Hermann Gaub and co-workers reported their use of AFM to measure the elasticity of DNA molecules with various sequences [16]. Because AFM uses stiffer force sensors as compared to laser tweezers, it can apply significantly greater forces to molecules as compared to laser tweezers. AFM measurements allowed the investigation of DNA elasticity at forces much greater than the 65 pN that triggers the B-S transition in laser tweezers measurements. Interestingly, at forces between 150-300 pN, the AFM recorded another abrupt change in DNA elasticity that resulted in the second force plateau that indicated the transition of DNA to even a longer structure (Figure 3, “melting(?) transition”). This structural rearrangement was considered to be related to the forced separation of the double helix into two separate and parallel chains and is

known in the literature as the forced “melting transition” [16]. The interpretation of this transition was examined a few years later (in 2007) by the author of this review and his co-workers and the conclusion was made that this transition is largely driven by other events in the DNA structure and not strand separation (melting). And for this reason a question mark is placed in Figure 3 by the word “melting” when it marks this second transition.

Around 2005, motivated by using AFM-force spectroscopy to examine structural DNA damage due to UV or gamma radiation that may cause mutations, we began to systematically examine the mechanics of various single and double stranded DNA. We started with the simplest homopolymeric sequences that included only one type of DNA base, such as “A” (A stands for adenine) in poly(dA) or “C” (that stands for cytosine) in poly(dC) or “T” that stands for thymine in poly(dT) (the letter d before A or C or T indicates that the polymer has the DNA backbone and not the RNA backbone). Careful AFM measurements of single stranded poly(dT) showed that this polymer indeed behaves elastically like a simple FJC (Figure 4, blue trace) similarly to the results obtained by Bustamante et al in 1996 using single-stranded DNA derived from double



**Fig. 5. Comparison of normalized force extension curves of poly(dA) with dsDNA (B-type helix).** Normalized force–extension curves of poly(dA) (blue),  $\lambda$ -phage DNA (black) and of poly(dGdC)poly(dCdG) dsDNA (red). The low-force and high-force plateaus occur at similar extensions for all DNA molecules suggesting common overstretching mechanisms. The inset shows a fragment of the DNA backbone with the sugar ring and gamma torsion that involves the rotation around the C4-C5 bond is marked by an arrow. Adapted from reference [18].

stranded lambda phage DNA. However, single stranded poly(dA) when stretched in the AFM instrument unexpectedly revealed two forced overstretching transitions that are similar to the transitions occurring in the canonical double-stranded DNA examined earlier [14, 16] (Figure 4, red trace). However, in poly(dA), these transitions understandably occur at lower forces (as compared to dsDNA), one at the force of approximately 20 pN and this transition, like the B-S transition in dsDNA overstretched poly(dA) by approximately 60-70%. The second transition occurs at approximately 100 pN and is shorter than the low force transition (Figure 4). Our measurements clearly showed that the elasticity of single-stranded DNA (ssDNA) may be significantly more complex as compared to the earlier observations, and actually may resemble the elasticity of double stranded DNA. We note that a DNA polymer composed of adenines poly(dA) has a known tendency to form a single stranded helix. Thus, our observations suggest that, as in double stranded DNA, the low force transition likely involves helix unwinding. Because the structure of a single helix cannot be stabilized by the classical Watson-Crick type base to base interactions (base pairing) that are present in dsDNA and must involve two helices presenting opposing bases to be paired, it must be stabilized by other forces. Those forces are generated when bases stack on top of each other (like steps in a helical staircase) and are known as base-stacking interactions. These stacking forces were always postulated to contribute to the stability of dsDNA but were difficult to measure independently of base-pairing. Thus, our AFM measurements on single stranded poly (dA), published in Physical Review Letters in 2007 [17], were the first to capture the mechanical signature of base stacking interactions in DNA because in poly(dA) these base stacking interactions appear in the absence of base pairing. In contrast to poly(dA), poly(dT) does not generate a regular helical structure, but rather a random coil structure in which T bases are not stacked. For that reason, the elasticity of poly(dT) is quite simple and can be reproduced by an FJC model. The origin of the second overstretching transition that we captured for poly(dA) at about 100 pN remained unclear until 2013. Computer modeling of poly(dA) and some additional experiments in which we examined the effect of various solution conditions, such as salt contents, suggested that the origin of this transition must be related to forced rotations of some bonds within the backbone of single-stranded DNA. In a nutshell, we demonstrated that specifically the so-called gamma torsions that involves rotations of chemical groups in DNA around the bond involving sugar atoms C4 and C5 must undergo a transition to different orientation under the applied force that abruptly extends the polymer backbone, giving rise to a change in the elasticity profiles captured as a plateau at ~100 pN. Interestingly, when the elasticity profile (the force-extension curve) of poly(dA) is compared to those of double stranded DNA (dsDNA) on the same graph by appropriately normalizing the extension, it is clear that the overstretchings produced in poly(dA) match the respective transitions in dsDNA (although they occur at lower forces because only one strand is subjected to the applied force, versus two strands in parallel, in dsDNA) (Figure 5). Since the transition that occurs at 100 pN in poly(dA) cannot involve DNA strand separation (there is only one strand) it is unlikely that the very similar transition that occurs in dsDNA is actually caused by strand separation (melting) as suggested in reference [16]. Alternatively, we suggested that, similarly to poly(dA), this “melting” transition in dsDNA is really caused by flipping of the gamma torsions after strands are separated at high forces. Thus, our measurements and modeling provided the basis for a unified model of DNA

elasticity that explains the behavior of both single- and double-stranded DNA [18]. The deviations of DNA elasticity from the FJC and WLC models, are explained by considering the helix unwinding at lower forces (long ~70% plateau) followed by the flip of gamma torsions at higher forces that produces a shorter plateau. It is important to note that this short review covers only some fundamental aspects of DNA elasticity and does not review a large number of observations and papers reporting other striking discoveries that used single-molecule force spectroscopy as a tool to examine fundamental biochemical processes on DNA such as transcription and replication (the reader is referred to many reviews on the subject, e.g. [19]).

## 4. POLYSACCHARIDES ELASTICITY

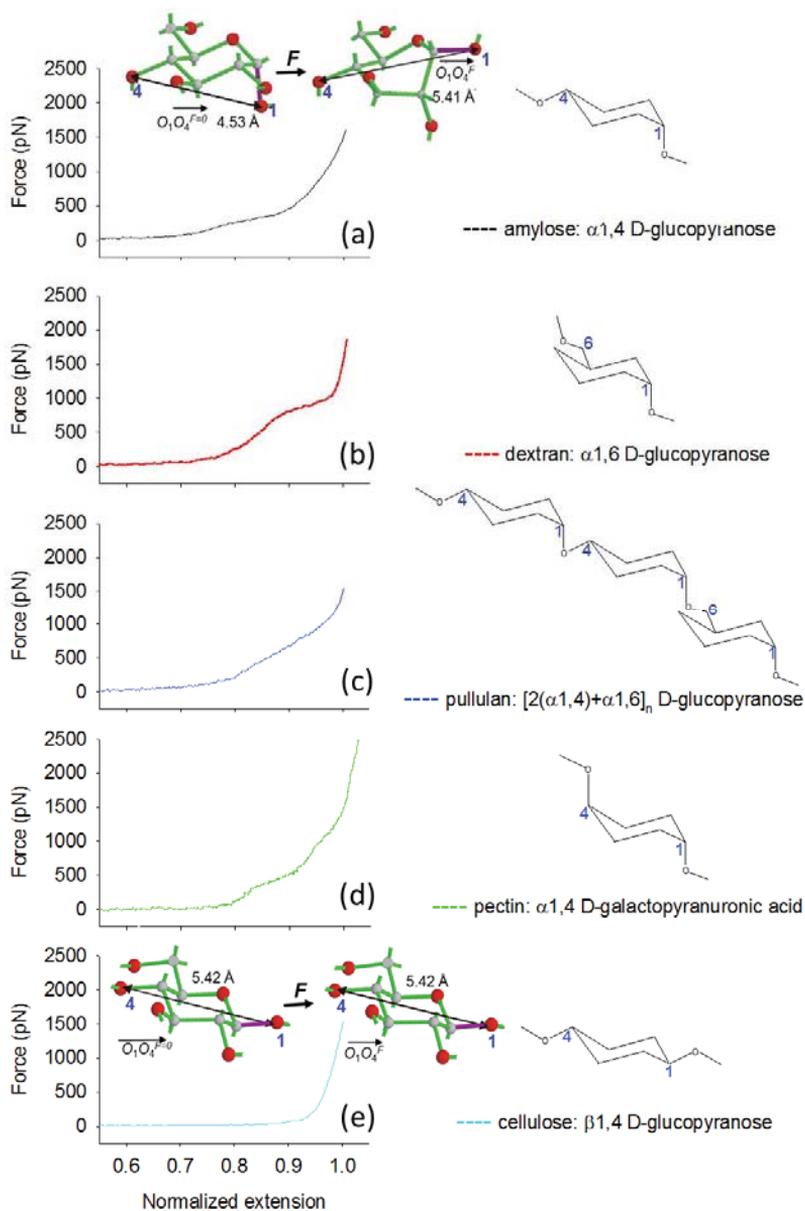
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Polysaccharides are very long linear or branched chains of various sugars (such as glucose, mannose, galactose, etc.) that are ring-like structures composed of carbon, oxygen and hydrogen atoms with typically 5 to 6 carbon atoms per ring. Consecutive sugar rings (e.g. R1 and R2) are connected by C-O bonds (R1-C-O-C-R2) that are called the glycosidic bonds. Sugar rings may be in different forms such as alpha or beta, which refers to the orientation of the C-O bond relative to the ring plane, with the alpha orientation projecting the C-O bond perpendicularly to the ring plane, while the beta form has the C-O bond almost parallel to the ring plane (Figure 6 a, e). These differences will play important roles in polysaccharide elasticity as discussed later. Polysaccharides are ubiquitous in plants and bacteria where they play structural roles (e.g. cellulose chains give the characteristic rigidity to cellular walls and wood structure. Polysaccharides are also exploited to store energy, e.g. starch in plants such as potato or corn, or glycogen in animal systems (also in humans, glycogen is used to store energy in long and highly branched glucose chains, that can be quickly degraded to release energy on demand). Cellulose, for example, is the most abundant biomaterial, contributing most of the biomass on earth. Because polysaccharides are long, have interesting internal structures and play mechanical structural roles, they were investigated by single molecule force spectroscopy as early as in 1997.

### 4.1. Dextran elasticity

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The first AFM stretching measurements of polysaccharides were executed on dextran (Figure 6b) and these measurements were reported by H. Gaub and co-workers in a seminal article [20]. This study appeared shortly after the overstretching transition of DNA was reported in 1996 and demonstrated that very interesting overstretching transitions may be observed in biopolymers not only at relatively low forces (<100 pN) but also at forces as high as ~1000 pN. Interestingly, dextran, which is composed of  $\alpha$ -D-glucose units connected by 1, 6 linkages (Figure 6 b) behaved as a simple FJC polymer up to very high forces exceeding 500 pN. Thus, if the measurements were carried out by laser tweezers, which can apply stretching forces of up to 100 pN or so, the conclusion would have been that dextran is a very simple polymer (considering its



**Fig. 6. Force-extension curves of selected polysaccharides**

Structure of sugar monomers and the type of glycosidic linkages in the polymer backbone are shown in the right panel. The inset in (a) shows a forced conformational transition of the  $\alpha$ -D-glucopyranose ring from its low energy “chair” conformation to its high energy “boat” conformation, while the inset in (e) indicates that  $\beta$ -D-glucopyranose ring stays in the chair conformation under force because this conformation provides already the maximum separation for consecutive glycosidic bonds and the transition to another conformation would reduce that separation, thus is prohibited during stretching. Adapted from references [21] and [24].

elasticity) and its elasticity is primarily of entropic origin. In AFM measurements however, a very interesting and pronounced deviation from the FJC elasticity in the form of a pronounced force plateau, was captured at forces of around 700–800 pN [20] (Figure 6b) that overstretched dextran by about 20% at almost constant force. Interestingly, and unlike DNA, the transition in dextran was fully reversible without any evidence of hysteresis, in repeated cyclic stretch and relax measurements on the same molecule. Computer simulations of the AFM stretching process of dextran were carried out using the so called Steered Molecular Dynamics (SMD) protocol in which an external force is applied to the molecule's end and pulls this end in a fashion similar to that executed by the AFM cantilever, while the other end of the molecule is fixed in space so the molecule undergoes stretching and not translation. These simulations suggested that dextran overstretching at ~700 pN is caused by forced rotations of specific bonds within the dextran backbone that are attached to each glucopyranose ring at position 5 (the C5-C6 bonds). This rotation was believed to cause the distance between the consecutive oxygen atoms, O1 and O6, in the dextran polymer backbone (Figure 2b) to increase abruptly at this critical force, resulting in an extra extension of the whole chain, captured by AFM as a force plateau.

#### 4.2. The elasticity of cellulose and amylose and force-induced chair-boat transitions

Amylose is a major component of the common starch (the other being amylopectin, which chemically is very similar to amylose but unlike amylose amylopectin is highly branched) that fills intracellular granules in many plant cells, e.g. in potato, where very long chains of amylose composed of thousands of glucose units are densely packed. In amylose, consecutive  $\alpha$ -D-glucopyranose rings are connected through alpha 1, 4 glycosidic linkages that connect carbon atom 1 on the first ring to carbon atom number 4 on the next ring through an oxygen atom. Amylose is chemically and structurally similar to cellulose with one significant difference related to the orientation of the glycosidic bond C1-O1, being alpha in amylose (axial, or perpendicular orientation to the ring plane, Figure 6a), and beta in cellulose (C1-O1 equatorial, or parallel to the plane of the ring, Figure 6e). When cellulose is dissolved (which is very difficult, and therefore easier to examine a soluble cellulose derivative such as methyl-cellulose) in water and deposited on a clean glass surface, individual chains of cellulose adsorb to the glass and can be picked up by the AFM tip for stretching measurements. The force-extension curves of individual cellulose chains are quite simple (Figure 6e) and can be fitted well with the extended FJC model up to the highest forces measured (> 1000 pN). In contrast to cellulose, force-extension curves obtained for amylose chains in water do not conform to the FJC model, but display a pronounced force plateau that occurs at around 300 pN and similar to dextran overstretched the amylose by some 20% (albeit at a significantly lower force, 300 pN versus 700–800 pN). This was an intriguing and unexpected observation on amylose elasticity, as in contrast to dextran, with its unique linkage involving a rotatable C5-C6 bond, amylose backbone bonds were not expected to significantly resist rotation during stretching, thus the forced rotation of bonds was unlikely to contribute to this plateau feature. Clearly, rotations of backbone bonds in structurally similar cellulose did not

produce and forced overstretching transitions during cellulose stretching. This comparison between amylose and cellulose pointed to a possible role in the chain elasticity of alpha bonds (in amylose) as compared to beta bonds (in cellulose) and to the mechanical behavior of the glucopyranose ring itself. It has been well known that similar to cyclohexane, the glucopyranose ring which is not flat, may exist in various geometrical forms that correspond to low and high energy conformers. The lowest energy conformation of the pyranose ring known to chemists is the so-called chair conformation (Figure 6a, inset). But the pyranose ring may also exist (although temporarily) in one of higher energy states corresponding to a “twist” or “boat-like” structure. These various structures may be populated by both alpha and beta D-glucopyranose rings. However, a careful examination of the distance between the O1 and O4 atoms of the glucopyranose ring, that determines the length of the backbone chain, indicates that this distance will increase when the alpha-D-glucopyranose flips from its chair conformation to the boat-like structure but will decrease when beta-D-glucopyranose ring experiences this transition. This logic is supported by quantum chemical calculations of the O1-O4 distances in various conformations of alpha-D-glucopyranose and beta-D-glucopyranose rings and additionally supported by AFM experiments on chemically disrupted rings structures in amylose treated with periodate and led to the conclusion that the force plateau captured for amylose in force spectroscopy measurements represents a massive force-induced transition of alpha-D-glucopyranose rings to their high energy, extended boat-like structures. This study carried out by the author and colleagues was published in 1998 [21]. It was the first demonstration of mechanically induced chair-boat conformational transitions in chemical systems. Similar results were also obtained for amylose and cellulose by Hongbin Li et al. [22]. Typically, in bulk systems, glucopyranose rings may flip spontaneously to a high energy boat-like structures, but because their lifetime is very short it is difficult to observe glucose in that state. However, by applying an external stretching force to an amylose chain, we demonstrated that all rings in the chain may be flipped at will to the high energy boat conformation, and the rings may be kept in this structure, away from the equilibrium state, for as long as the force is applied. This proved to be a highly unusual and unique way of controlling conformations of chemical units by force. Also, by focusing on the O1-O4 distance in various conformers of alpha and beta D-glucopyranose structures, it is straightforward to explain why cellulose does not display any force plateaus in its elasticity profile. This is because the length of the O1-O4 distance is already at a maximum in the lowest energy chair conformation for beta-glucose, thus any transition to a boat-like structure during mechanical stretching is inhibited as it would lead to chain contraction so the only effect of cellulose stretching is the entropic alignment of the rings with the stretching direction and a slight deformation of the rings themselves (segment elasticity). Such behavior can be well described within the FJC framework.

### 4.3. Elasticity of other polysaccharides

Because glycosidic linkages may connect various carbon atoms on consecutive rings (e.g. 1-2, 1-3, 1-4, 1-6) in both alpha and beta configurations and consecutive sugars may not be of the same (not only glucose, but galactose, mannose and their

various combinations) nature that generated a massive amount of different polysaccharides (homo and hetero polymers) that can be purified from plants or other organisms. Those polysaccharides offer many possibilities to investigate the mechanical behavior of various bonds in their alpha and beta configurations attached to different sugars. For example, pectin (Figure 6d) is an interesting polysaccharide present in many fruits, which connects consecutive galactouronic acid sugar rings uniquely using two axial linkages, at C1 and also at C4. Stretching pectin molecules by AFM (after dissolving pectin in water), captured very interesting elasticity profiles that displayed two characteristic force plateaus, suggesting two force-induced conformational transitions involving the sugar ring and its linkages. Inspecting the pectin ring suggests that the two axial bonds may mechanically work as “atomic levers”, exerting a torque on the ring that may flip it to various high energy structures that could increase the length of the polymer backbone. Quantum chemical calculations of the galactose ring suggested that the greatest extension may be produced when the rings flip from the lowest energy chair conformation to its “inverted” chair conformation with an intermediate state being a “boat-like structure”. This scenario considers two consecutive forced transitions that may produce two force plateaus in the force-extension curve [23]. Comparing force extension curves of cellulose, amylose, dextran and pectin it was tempting to connect the number of forced plateaus captured by AFM with the number of alpha (axial) linkages per sugar ring: one plateau is expected for amylose and dextran, two for pectin and none for cellulose [23]. AFM measurements performed on various polysaccharides proved very sensitive to variations in their composition and the orientation of the glycosidic bonds, thus it became possible to identify (“fingerprint”) these molecules by simple mechanical stretching and recording their characteristic force-extension curves (Figure 6a-e) [24]. The interested reader is referred to other papers and reviews that provide more mechanistic insights into polysaccharides mechanochemistry (e.g. [8, 24–35]).

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## 5. CONCLUSION

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Single molecule force spectroscopy is superb in examining the elasticity of biopolymers, their structural stability and force-induced structural rearrangements at near atomic resolution. Unlike traditional spectroscopic techniques that are limited to investigating molecules near their equilibrium states, single molecule force spectroscopy may apply small and large forces to the examined molecules and drive them away from their equilibrium structures to probe their properties in high energy states that are of interest not only to basic science but also may be critically important to many molecular functions in living organisms when these molecules interact with other molecules or are subjected to external forces and are temporarily driven to high energy states. This review covered only a small fraction of a large body of literature on single molecule force spectroscopy on DNA and sugars and did not include equally interesting and important works on protein mechanics and mechanical unfolding and refolding. The primary goal for me has been to interest the reader to seek independently further information about this exciting field whose contributions to life sciences and polymer physics are expected to grow continuously as the roles of molecular mechanics at the

nanoscale, in living organisms, are being continuously recognized and their significance appreciated.

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## REFERENCES

1. Ashkin A. and Dziedzic J.M.: "Optical trapping and manipulation of viruses and bacteria", *Science*, 235 (4795), 1987, pp. 1517–1520.
2. Ashkin A., Dziedzic J.M., and Yamane T.: "Optical trapping and manipulation of single cells using infrared-laser beams", *Nature*, 330 (6150), 1987, pp.769–771.
3. Binnig G., Quate C.F., & Gerber C.: "Atomic force microscope", *Phys Rev Lett*, 56 (9), 1986, pp. 930–933
4. Hansma P.K.: "Molecular Mechanics of Single Molecules", *Structure*, 14 (3), 2006, pp. 390–391.
5. Puchner E.M. and Gaub H.E. "Force and function: probing proteins with AFM-based force spectroscopy", *Curr. Opin. Struct. Biol.*, 19 (5), 2009, pp. 605–614.
6. Fisher T.E., Marszalek P.E., and Fernandez J.M.: "Stretching single molecules into novel conformations using the atomic force microscope", *Nature Structural & Molecular Biology*, 7 (9), 2000, pp. 719–724.
7. Dufrene Y.F., *et al.*: "Five challenges to bringing single-molecule force spectroscopy into living cells", *Nat. Meth.* 8 (2), 2011, pp. 123–127.
8. Marszalek P.E. and Dufrene Y.F.: "Stretching single polysaccharides and proteins using atomic force microscopy", *Chem. Soc. Rev.*, 41 (9), 2012, pp. 3523–3534.
9. Smith S.B., Finzi L., and Bustamante C.: "Direct Mechanical Measurements of the Elasticity of Single DNA-Molecules by Using Magnetic Beads", *Science*, 258 (5085), 1992, pp. 1122–1126.
10. Popa I., *et al.*: "A HaloTag Anchored Ruler for Week-Long Studies of Protein Dynamics", *Journal of the American Chemical Society*, 138 (33), 2016, pp. 10546–10553.
11. Florin E.L., *et al.*: "Sensing Specific Molecular-Interactions with the Atomic-Force Microscope", *Biosensors & Bioelectronics*, 10 (9-10), 1995, pp. 895–901.
12. Walder R., *et al.*: "Rapid Characterization of a Mechanically Labile  $\alpha$ -helical Protein Enabled by Efficient Site-Specific Bioconjugation", *Journal of the American Chemical Society*, 2017.
13. Bustamante C.: "Entropic elasticity of [lambda]-phage DNA", *Science* 265, 1994, pp. 1599–1600.
14. Smith S.B., Cui Y.J., and Bustamante C.: "Overstretching B-DNA: The elastic response of individual double-stranded and single-stranded DNA molecules", *Science*, 271 (5250), 1996, pp. 795–799.
15. Cluzel P., *et al.*: "DNA: An extensible molecule", *Science*, 271 (5250), 1996, pp. 792–794.
16. Rief M., Clausen-Schaumann H., and Gaub H.E.: "Sequence-dependent mechanics of single DNA molecules", *Nat Struct Biol*, 6 (4), 1999, pp. 346–349.
17. Ke C., Humeniuk M., S-Gracz H., and Marszalek P.E.: "Direct Measurements of Base Stacking Interactions in DNA by Single-Molecule Atomic-Force Spectroscopy", *Physical Review Letters*, 99 (1), 2007, pp. 018302–018304.
18. Scholl Z.N., *et al.*: "Origin of Overstretching Transitions in Single-Stranded Nucleic Acids", *Physical Review Letters*, 111 (18), 2013, p. 188302.
19. Bustamante C., Bryant Z., & Smith S.B.: "Ten years of tension: single-molecule DNA mechanics", *Nature* 421 (6921), 2003, pp. 423–427.
20. Rief M., Oesterhelt F., Heymann B., and Gaub H.E.: "Single Molecule Force Spectroscopy on Polysaccharides by Atomic Force Microscopy", *Science*, 275 (5304), 1997, pp. 1295–1297.
21. Marszalek P.E., Oberhauser A.F., Pang Y.P., and Fernandez J.M.: "Polysaccharide elasticity governed by chair-boat transitions of the glucopyranose ring", *Nature*, 396 (6712), 1998, pp. 661–664.
22. Li H.B., *et al.*: "Single-molecule force spectroscopy on polysaccharides by AFM – nanomechanical fingerprint of alpha-(1,4)-linked polysaccharides", *Chem. Phys. Lett.*, 305 (3-4), 1999, pp. 197–201.

23. Marszalek P.E., *et al.*: “Atomic levers control pyranose ring conformations”, *Proc. Natl. Acad. Sci. USA*, 96 (14), 1999, pp. 7894–7898.
24. Marszalek P.E., Li H.B., and Fernandez J.M.: “Fingerprinting polysaccharides with single-molecule atomic force microscopy”, *Nature Biotechnology*, 19 (3), 2001, pp. 258–262.
25. Lee G., Nowak W., Jaroniec J., Zhang Q., and Marszalek P.E.: “Nanomechanical control of glucopyranose rotamers”, *Journal of the American Chemical Society*, 126 (20), 2004, pp. 6218–6219.
26. Lee G., Nowak W., Jaroniec J., Zhang Q., and Marszalek P.E.: “Molecular Dynamics Simulations of Forced Conformational Transitions in 1,6-Linked Polysaccharides”, *Biophysical Journal*, 87 (3), 2004, pp. 1456–1465.
27. Lee W., Zeng X., Yang W., and Marszalek P.E.: “Mechanics of Polysaccharides”, *Molecular Manipulation with Atomic Force Microscopy*, ed.: Anne-Sophie Duwez NW (CRC Press, Boca Raton London New York), 2012.
28. Lu Z., Hu H., Yang W., and Marszalek P.E.: “Simulating Force-Induced Conformational Transitions in Polysaccharides with the SMD Replica Exchange Method”, *Biophys. J.*, 91 (6), 2006, pp. L57–59.
29. Lu Z., Nowak W., Lee G., Marszalek P.E., and Yang W.: “Elastic Properties of Single Amylose Chains in Water: A Quantum Mechanical and AFM Study”, *Journal of the American Chemical Society*, 126 (29), 2004, pp. 9033–9041.
30. Marszalek P.E., Li H.B., Oberhauser A.F., and Fernandez J.M.: “Chair-boat transitions in single polysaccharide molecules observed with force-ramp AFM”, *Proc. Natl. Acad. Sci. USA*, 99 (7), 2002, pp. 4278–4283.
31. Scholl Z.N., Li Q., and Marszalek P.E.: “Single molecule mechanical manipulation for studying biological properties of proteins, DNA, and sugars”, *WIREs Nanomedicine and Nanobiotechnology*, 2, 2013.
32. Zhang Q., Lu Z., Hu H., Yang W., and Marszalek P.E.: “Direct Detection of the Formation of V-Amylose Helix by Single Molecule Force Spectroscopy”. *J. Am. Chem. Soc.*, 128 (29), 2006, pp. 9387–9393.
33. Zhang Q. and Marszalek P.E.: “Identification of Sugar Isomers by Single-Molecule Force Spectroscopy”, *J. Am. Chem. Soc.*, 128 (17), 2006, pp. 5596–5597.
34. Zhang Q. and Marszalek P.E.: “Solvent effects on the elasticity of polysaccharide molecules in disordered and ordered states by single-molecule force spectroscopy”, *Polymer*, 47 (7), 2006, pp. 2526–2532.
35. Zhang Q.M., Lee G.R., and Marszalek P.E.: “Atomic cranks and levers control sugar ring conformations”, *Journal of Physics-Condensed Matter*, 17 (18), 2005, pp. S1427-S1442.

KRÓTKI ARTYKUŁ PRZEGLĄDOWY: BADANIA  
MECHANICZNYCH WŁAŚCIWOŚCI POJEDYNCZYCH  
CZĄSTECZEK PRZY POMOCY SPEKTROSKOPII SIŁ  
ATOMOWYCH

Piotr E. MARSZAŁEK

**STRESZCZENIE** *W niniejszym krótkim artykule przeglądownym, na początku zwięźle opiszę zasady spektroskopii siłowej do badania mechanicznych właściwości pojedynczych cząsteczek przy użyciu mikroskopu sił atomowych. Następnie, omówię najważniejsze, moim zdaniem, odkrycia i obserwacje w tej tematyce, która rozwija się niezwykle dynamicznie przez ostatnie ponad 25 lat. W tej krótkiej pracy skupię się wyłącznie na omówieniu zastosowania spektroskopii siłowej do analizy właściwości elastycznych biopolimerów, takich jak DNA i polisacharydy, których badaniom poświęciłem istotną część mojej pracy naukowej w ostatnich dwóch dekadach. Omówienie mechaniki pojedynczych cząsteczek białek czytelnik może znaleźć w innych oryginalnych lub przeglądowych pracach autora jak również innych badaczy, które dostępne są w literaturze światowej.*

**Słowa kluczowe:** *Mikroskopia Sił Atomowych, Spektroskopia siłowa pojedynczych cząsteczek, biopolimery, nanomechanika*



Piotr E. Marszałek received his Master's degree in Physics (biophysical specialization) from the University of Warsaw in 1985 and his PhD in Technical Sciences from the Electrotechnical Research Institute, Warsaw Poland in 1991. He was a Fulbright Fellow in 1989-90 in the Department of Biochemistry, University of Minnesota, St. Paul, Minnesota, USA where he also did his first postdoctoral work in 1992-93. Between 1993- and 2002 he worked at the Mayo Clinic and Foundation, Rochester, Minnesota, first as a postdoctoral associate and since 1996 as an Associate Consultant and since 1998 also as an Assistant Professor of Biophysics. In 2002 he moved to Duke University, Durham, North Carolina, USA where he has been working in the Department of Mechanical Engineering and Materials Science, first as Associate Professor, and since 2009 as a full, tenured professor. At Duke University, he also holds a secondary appointment as full professor in the Department of Biomedical Engineering. He authored 105 papers that accumulated over 9600 citations, per Google Scholar.