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Measuring the force generated by a single Tiny Synthetic Molecular Machine

Abstract
In order to function, living organisms use an amazing number of molecular machines. These biological molecular machines are able to rectify random thermal motion to generate directional forces and carry out macroscopic tasks, like muscle contraction or intracellular transport [1]. This ability has inspired chemists who synthesize molecular machines able to imitate the machinery of biological world [2-4]. Although some of these nanomachines are able to carry out mechanical tasks collectively, such as inducing the movement of much larger objects [5-7], the direct measurement of synthetic small molecules performing mechanical work at the single molecule level has yet to be realized. Here we demonstrate that biased Brownian motions of the components of a small synthetic molecule can be harnessed to generate significant forces. For that purpose, a hydrogen-bonded [2]rotaxane [3](a molecular ring threaded onto a molecular axle) was synthesized and attached to a tether to track the ring motion with the tip of an atomic force microscope (AFM). The tether was stretched by the AFM tip to apply mechanical force to the ring through single-molecule pulling-relaxing cycles. The ring was moved away from the most stable binding site along the thread and was found to shuttle back to this site against an external load of 30 pN, thus delivering mechanical work against the AFM cantilever.

Keywords
Molecular machine, atomic force microscopy, single molecule

In the molecular world, the dynamic behaviour of molecules is dominated by random thermal fluctuations. Nature "tame" Brownian motions by using specific architectures (like structural tracks) which restrict most of the degrees of freedom of the biomolecule [1]. Inspired by the fascinating mechanism by which biomolecules move in the face of thermal noise, chemists are designing synthetic molecular systems capable of mimicking the natural world [2-4]. Among these synthetic systems, rotaxanes are promising prototypical synthetic molecular machines. Rotaxanes (Figure 1) are molecules consisting of a molecular ring (a macrocycle) threaded onto a molecular axle capped with bulky end-stoppers. The thread bears one or more recognition sites called stations, onto which the ring can bind through intra-molecular bonds according to its affinities. The chemical environment also forces the ring to preferentially remain on one of the stations (where it can make the strongest bonds), this site is thermodynamically favoured [3].

Figure 1: Illustration of a [2]rotaxane with one thread (grey) and two stations (orange and red), one ring (blue) and two bulky end-stoppers at either end to prevent the ring from de-threading.

Although, such artificial molecular machines have been studied in solution by ensemble techniques, and

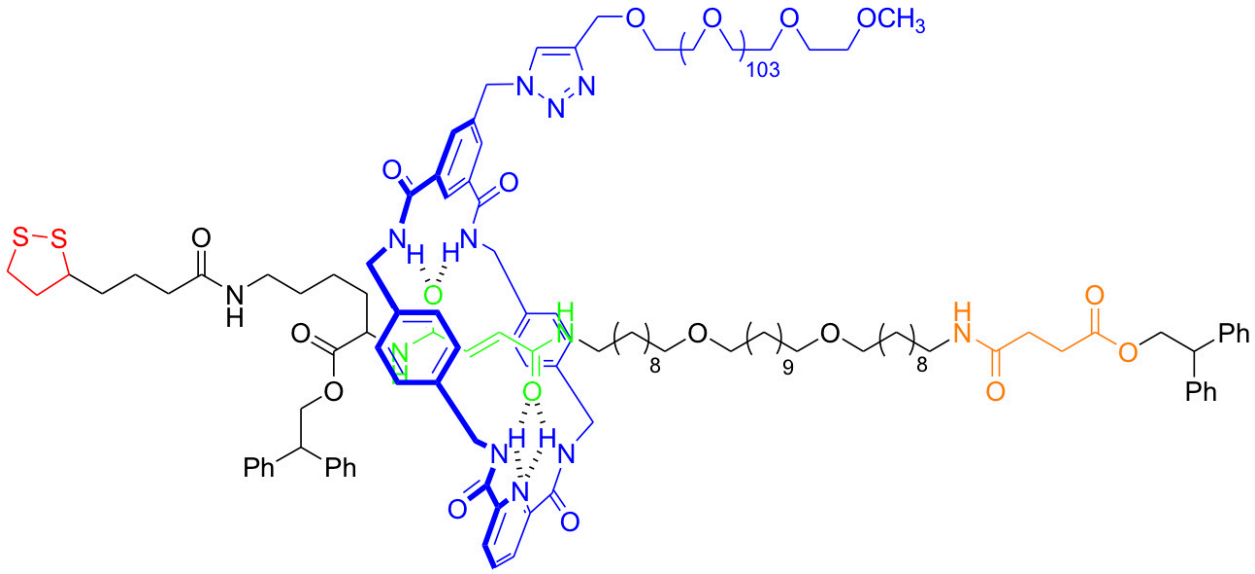

Figure 2: Chemical structure of the rotaxane molecule, synthesised by the group of D.A. Leigh, UK. The rotaxane consists of a benzylic amide molecular ring (in blue) mechanically locked onto an axle by bulky diphenylethyl ester groups situated at either end. The axle bears a *fumaramide* group (in green) and a succinic amide-ester group (in orange), either of which can act as a binding site for the ring through up to four intercomponent hydrogen bonds. The affinity of the ring for the fumaramide site is much higher than for the succinic amide-ester site, so that the fumaramide:succinic amide-ester occupancy ratio is higher than 95:5. Next to the fumaramide binding site, a disulfide group (in red) was introduced to enable the grafting of the molecule onto gold substrates. A 4600 M_n PEO tether (in blue) is attached to the ring in order to link the molecule to the AFM probe and track the motion of the ring along the axle.

used collectively to perform mechanical tasks [5-7], so far there have been no direct measurements of mechanical processes at the single-molecule level. In contrast, direct measurements made on single biological molecular machines have been realized with manipulation techniques like optical tweezers or force clamp AFM and allowed us to gather much of the exquisite and detailed information about how these biomolecular machines operate. Such measurements have shown that single-molecule biological machines are able to generate force against loads of 5-60 pN, and have highlighted many details about their mechanical properties [8,9]. AFM-based single molecule force spectroscopy is able to monitor mechanical forces in real time with sub-nanometre resolution and has provided unprecedented insights into many molecular-level processes [8-12]. Although single macromolecules, such as proteins and synthetic polymers, have been widely manipulated and studied by single molecule force spectroscopy [13,14], implementing this technique on smaller molecules remains a major challenge.

In 2006, Brough *et al.* [15] have pulled the molecular ring over the bulky end groups of a redox-active [2]rotaxane with an AFM tip. They compared the forces required to de-thread the ring of the oxidized and unoxidized molecules. However, this de-threading was an irreversible process, precluding the possibility of a relaxing step to complete the cycle required to directly probe the mechanical forces generated against an external load.

In the present work, in collaboration with Prof. C.-A. Fustin (UCL) and Prof. D.-A. Leigh (Univ. Manchester), we have succeeded in detecting sub-molecular movements in a tiny synthetic molecular machine and in measuring the force generated by this molecule against a load [16]. We have designed a hydrogen-bonded [2]rotaxane with a tether attached to the molecular ring (Figure 2) to track its motion by an AFM cantilever. The thread bears two binding sites, each of which can bind to the ring through up to four hydrogen bonds. The ring predominantly resides over one of the two sites, the occupancy ratio being higher than 95:5 [17]. Close to the thermodynamically favoured binding site, disulfide groups were introduced to enable the grafting of the rotaxane onto a gold surface. A polyethylene oxide chain (PEO), suitable for binding to an AFM tip, was attached to the ring and the resulting system was grafted onto substrates in a dilute distribution (isolated molecules).

We used the cantilever of an AFM microscope to catch the PEO tether, then apply a mechanical load to the ring of the rotaxane and follow its motion. The experiments were done in 1,1,2,2-tetrachloroethane

(TCE). The AFM tip was brought into contact with the rotaxane-PEO substrate to allow the tether to absorb onto the tip. During the tip retraction, the caught molecules were stretched in a controlled manner by moving the tip away from the substrate at a fixed pulling rate, and the force-extension profiles were measured (Figure 3a). This is just like fishing, it is very random: often no molecule had been caught by AFM tip but sometimes we catch the tether of a rotaxane, then we pull it. We attribute the main peak to extension of the polymer tether, and the superimposed small peak to the rupture of the hydrogen bonds that bind the ring to the fumaramide group which is the thermodynamically favoured binding site (Figure 3b). The pulling curves of simple PEO polymer chains were realized as a comparison and show no characteristic feature in the profile (Figure 3c).

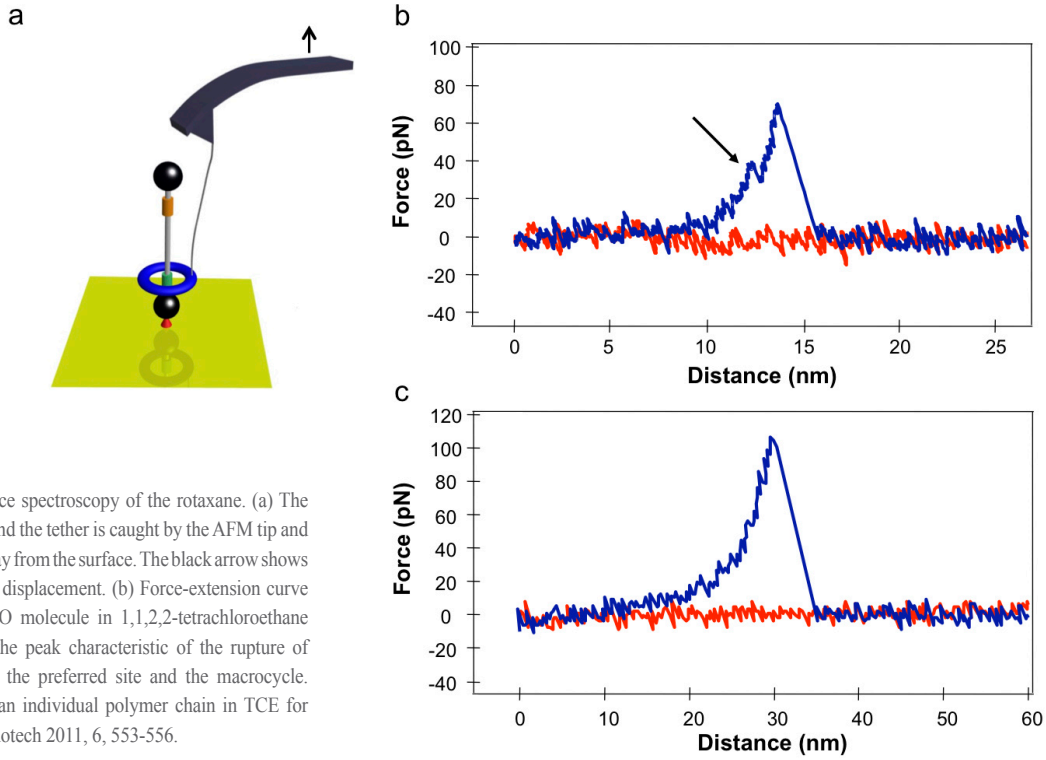

Figure 3: Single molecule force spectroscopy of the rotaxane. (a) The rotaxane is grafted onto gold and the tether is caught by the AFM tip and stretched by moving the tip away from the surface. The black arrow shows the direction of the cantilever displacement. (b) Force-extension curve of an individual rotaxane-PEO molecule in 1,1,2,2-tetrachloroethane (TCE). The arrow indicates the peak characteristic of the rupture of the hydrogen bonds between the preferred site and the macrocycle. (c) Force-extension curve of an individual polymer chain in TCE for comparison. From Nature Nanotech 2011, 6, 553-556.

Figures 4a and 4b show the interpretation of the events taking place when pulling on the rotaxane-PEO molecule with the AFM tip that could explain the observed force profile. During AFM pulling experiment, the polymer tether is progressively stretched by the tip which gives the characteristic parabolic profile in the force-extension curve due to the entropic response of the polymer chain (I). If the force exerted on the tether is higher than the force of the hydrogen bonds linking the ring to its preferred station, the H bonds break (II). The ring detaches and moves freely along the axle, away from its most stable binding site, the tension in the polymer tether is reduced and the force decreases (III) until the cantilever displacement again increases the tension in the tether. Further, the displacement of cantilever continues the stretching of tether until the force exceeds the interaction strength of the PEO chain with the tip, which leads to detachment (IV).

To further support the hypothesis that this peak is the signature of the breaking of the intercomponent hydrogen bonds, we fitted the pulling profiles using entropic elasticity models [18], such as the worm-like chain (WLC) model that predicts the relationship between the extension of a linear polymer and the entropic restoring force generated. The fits adequately described the shape of the force-extension curves (Figure 5). The force curves could be fitted using the same persistence length, i.e. 0.35 ± 0.05 nm, close to the theoretical persistence length of the PEO tether, (0.37 nm) [19]. The fits of the profile before and after the rupture peak give the increase in length (ΔL_c) of the molecule after the rupture of the intramolecular hydrogen bonds (Figure 5). The average ΔL_c obtained, 3.9 ± 0.5 nm, is consistent with the theoretical length of the fully extended thread, ~4.5 nm, providing further evidence that the small peak is due to the rupture of the interactions between the macrocycle and the fumarimide preferred binding site.

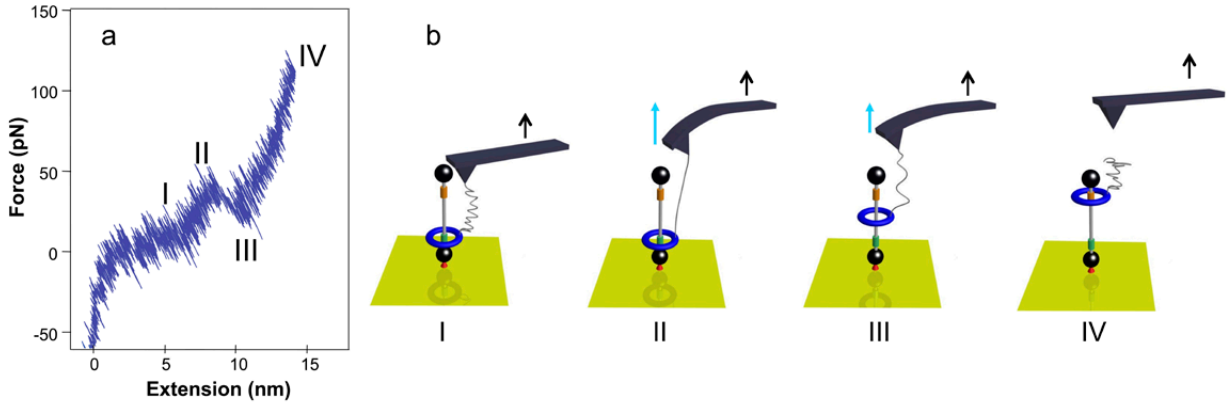

Figure 4: (a) High resolution force-extension curve for the rotaxane-tether molecule in TCE. (b) Interpretation of the sequence of events taking place when pulling on the rotaxane-polymer molecule. The black arrows show the direction of the cantilever displacement and the blue arrows show the direction of the force exerted on the molecular ring. Adapted from Nature Nanotech 2011, 6, 553-556.

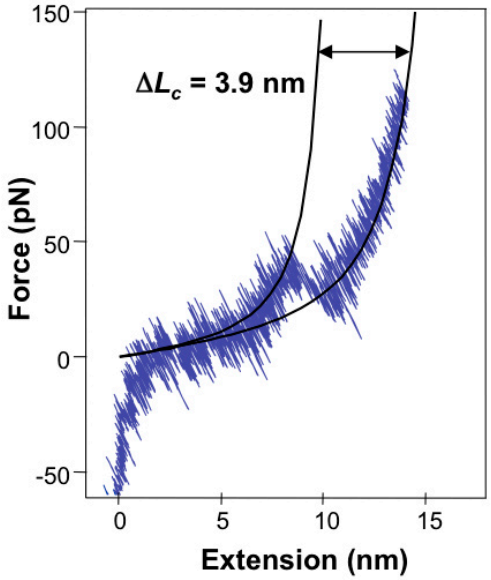

Figure 5: Force-extension curve (data as in Fig 4a) with worm-like chain fits to the data (thin solid lines) with an increase in length (ΔL_c) of the molecule after rupture of the hydrogen bonds of 3.9 nm. Adapted from Nature Nanotech 2011, 6, 553-556.

The detection of generated forces requires the analysis of pulling-relaxing cycles. Single-molecule pulling-relaxing cycles could be recorded when the stretching of the PEO tether was stopped before the force exerted by the tip exceeded the force required for detachment (Figure 6a). Somewhat unexpectedly, we observed that while relaxing the tension in the PEO tether, the force suddenly increases. An increase of tension while decreasing the tip-surface distance can be explained by the appearance of force acting on the tether at the other end (Figure 6b). This means that the ring has travelled back to the fumaramide site, in the opposite direction to the force exerted on it by the cantilever and that it is able to generate force when rebinding against the external load exerted by the cantilever. Figure 6c shows that in TCE the ring is able to travel against an external load of 30 pN. The mechanical work produced by this sub-molecular motion is about 6 kcal mol^{-1} (grey area).

The energy used by the rotaxane to perform work comes from the binding-energy difference ($\Delta\Delta G$) between the two hydrogen-bonding motifs and for a reversible process in the macroscopic world, the mechanical work equals the Gibbs free-energy change of the process. However, the laws of physics and chemistry that govern the macroscopic world are no longer necessarily valid at the molecular level. The principles of thermodynamics describe processes of energy exchange (work and heat) of macroscopic systems with their environment. In a general way, in macroscopic systems, the average behaviour is reproducible and the fluctuations are of little importance. When the dimensions of the system become nanoscopic, the fluctuations can give rise to significant deviations with regard to the average behaviour [20]. An individual molecule can, for example, extract energy (which comes from thermal energy) from its environment to perform a mechanical work higher than the energy available in the molecule. Thus, well-known concepts of physics and chemistry must be redefined or improved in order to reconcile the nanoscopic and macroscopic worlds. It is from this perspective that for several years, theoretical physicists have established fluctuation theorems. These theorems reconcile what we observe on a large scale and for a large number of molecules in equilibrium with the data obtained for a single molecule (or a small number of molecules) in non-equilibrium conditions. In single molecule force spectroscopy

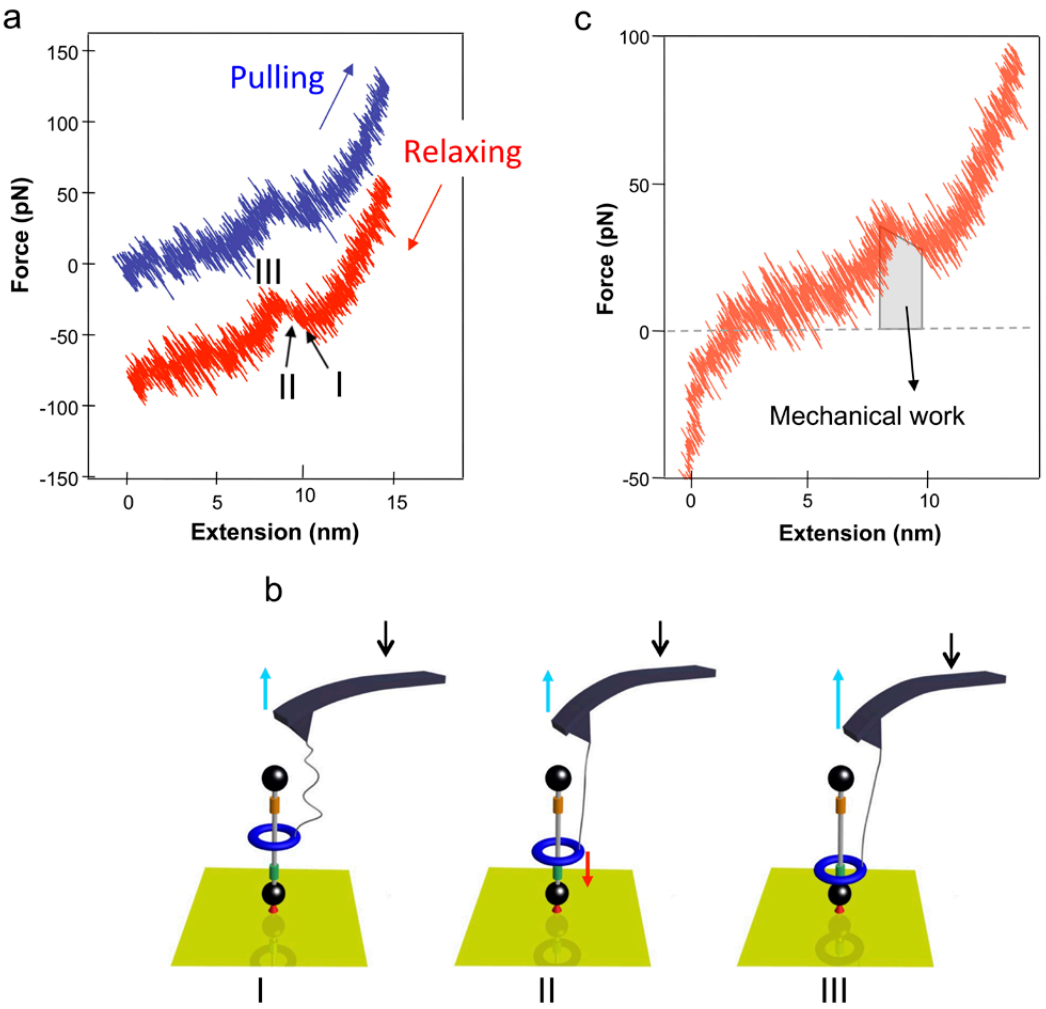

Figure 6: (a) Pulling (blue) and relaxing (red) curves for a single-rotaxane-PEO molecule in TCE. The relaxing trace is offset vertically for clarity. (b) Schematic of the relaxing experiment showing our interpretation of events. Black arrows show the direction of cantilever displacement. Blue arrows show the direction of the force exerted on the ring. (I) Progressive release of the tension in the PEO tether. (II) The force suddenly increases as a result of the ring shuttling in the opposite direction (red arrow) to the force exerted on it (blue arrow). (III) The ring has rebound to the fumaramide station. (c) Relaxing curve (data as in a) with the area under the trace representing the work done by the molecule as the ring shuttles back to its preferred binding site. Adapted from Nature Nanotech 2011, 6, 553-556.

experiments, the molecular system is driven away from thermal equilibrium by the action of an external perturbation and the distribution of work trajectories typically results in hysteresis because of the fluctuations. Recent developments in non-equilibrium statistical mechanics enable recovery of the reversible work, and thus the zero-force free energy, from the distribution of work trajectories in pulling–relaxing cycles. Fluctuation theorems relate the equilibrium free-energy change ΔG to non-equilibrium measurements of the work done on a single molecule. The Crooks fluctuation theorem [21] relates ΔG to the probability distributions of the non-equilibrium work measured during the forward and reverse changes that occur when a system is driven away from thermal equilibrium by the action of an external perturbation. It enables us to quantify the amount of hysteresis observed in the values of the irreversible work done during a rupture and rebinding process. The CFT predicts that: $(P_W/P_{WR}) = \exp((W\text{-}\Delta G)/k_BT)$ where ΔG is the free-energy change between the final and the initial states, and thus equal to the reversible work associated with this process. P_W is the probability distribution of the values of the work during the rupture process, and P_{WR} corresponds to the reverse process. The CFT states that although the probability distribution of the work performed on the molecule during the rupture process, and the work done by the molecule during the rebinding process depend on the pulling protocol, their ratio depends only on the equilibrium free-energy difference, ΔG. Thus the value of ΔG can be determined once the two distributions are known, regardless of the pulling speed.

We used the CFT [21] to estimate the free energy driving the macrocycle to bind to the fumaramide

binding site at zero force. We obtained a ΔG of 9.1 ± 2.3 k_BT (= 5.4 ± 1.3 kcal mol-1) (Figure 7). This value of ΔG from single-molecule measurements in TCE is in good agreement with the difference in energy between the molecular ring binding to the fumaramide and succinic amide-ester binding sites determined by ^{1}H NMR for bulk solutions of related rotaxane molecules [17, 22, 23]. The work value for a loading rate of 500 pN s^{-1} (Figure 6c) is close to this binding-energy difference between the two hydrogen-bonding motifs, indicating that under these conditions, the rotaxane is able to make use of almost all the energy available from hydrogen bonding to perform work along the direction of the applied load.

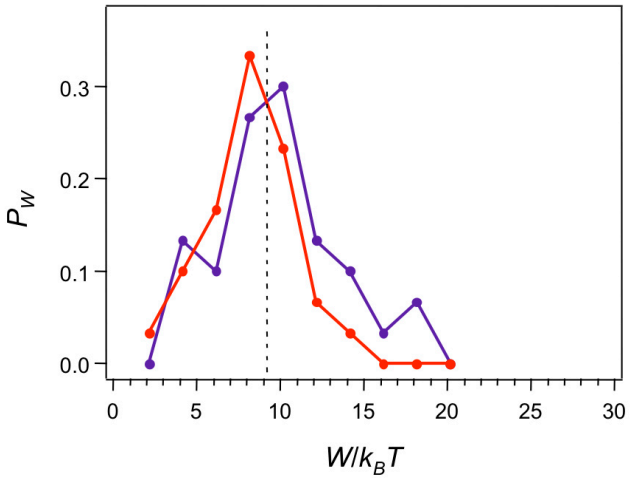

Figure 7: Work distributions for the rupture peak (blue) and rebinding peak (red) at a loading rate of 500 pN s^{-1}. The distributions show a crossing around ΔG = 9.1 ± 2.3 k_BT.

In summary, we reported for the very first time the direct measurements of the work performed by a single synthetic small molecule. Our results show that an individual synthetic molecule less than 5 nm long can generate directional forces of similar magnitude to those generated by natural molecular machines. The results also demonstrate that AFM-based single-molecule force spectroscopy, which has been widely used to investigate the mechanochemical behaviour of (bio)macromolecules, can be applied to a molecule that is considerably smaller. The direct observation of the dynamic behaviour of the components of single rotaxanes performing mechanical work paves the way for a detailed investigation of the mechanochemical properties of synthetic molecular machine systems (such as the stall force, thermodynamic efficiency under various sources of external energy, coupling constant, velocity under load, and so on), as has been conducted in the past 15 years for biological systems.

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