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Mechanical unfolding of single oligorotaxanes using atomic force microscopy

Abstract
Nature leads the way in the design of biological molecular machines with specific functions in our bodies. But chemists are in hot pursuit, synthesizing highly sophisticated artificial molecular machines with well-defined folded architectures. Mechanically interlocked molecules, such as rotaxane, catenane or molecular knots, are very attractive for the design of molecular machine prototypes, enabling the controlled submolecular movement of their components. Using atomic force microscopy (AFM), we investigated here the mechanochemical properties of oligorotaxanes. These molecules are made of a flexible poly(ethylene oxide) thread containing electron-rich 1,5-dioxynapthalene units and encircled by multiple tetracationic molecular rings. Thanks to donor-acceptor interactions, they display a serpentine-like folded conformation in solution. Individual oligorotaxane foldamers were trapped between a substrate and an AFM tip and were mechanically unfolded by single-molecule force spectroscopy. Standard force experiments show the sequential breaking of intramolecular interactions. Real-time fluctuations between partially folded states were observed for the first time on a wholly synthetic molecule, evidencing the very fast (un)folding dynamics of those molecules. Pulling-relaxing experiments and dynamic force spectroscopy showed the remarkable ability of these synthetic oligorotaxanes to do work against a mechanical load and their higher performance compared to natural folding proteins.

Keywords
Molecular machines, atomic force microscopy, single-molecule force spectroscopy

1. Introduction

1.1. Molecular machines

Biological molecular machines are known to carry out specific tasks in our bodies. Muscles contraction, cargo transport inside the cell along actin filaments or flagella rotary motions are few examples of natural molecular machineries with a specific function [1]. For a few decades, the domain of artificial molecular machines has gained a lot of attention. This field was even recognized by the Nobel committee which attributed the 2016 Nobel prize in chemistry conjointly to Jean-Pierre Sauvage, Ben Feringa and J. Fraser Stoddart for *the design and synthesis of molecular machines*. In 1959, Richard P. Feynman gave a very inspiring insight into the promise of these man-made molecular machines, even before chemists were able to synthesize them. In his lecture *There's plenty of room at the bottom*, he considered the immense possibilities of exploring the world at the nanoscale and the advantages of building wholly synthetic molecular machines [2]. "*I can't see exactly what would happen*, Feynman said,

but I can hardly doubt that when we have some control of the arrangement of things on a small scale we will get an enormously greater range of possible properties that substances can have, and of different things that we can do".

In this contest for the control of molecular and sub-molecular motions at the nanoscale, mechanically interlocked molecules (MIMs) have gained a lot of attention [3]. These MIMs, such as rotaxanes and catenanes (Fig. 1), consist of several subcomponents interlaced together by mechanical bonds. As prototypes of artificial molecular machines, they enable the controlled movement and positioning of their component parts [4]. Under an external stimulus such as light irradiation or a change of solvent, each single machine is pulled out of its equilibrium state and is able to produce an effective work. The integration of those molecules into more complex architectures (such as polymer gels) can possibly lead to a macroscopic change of the material [5].

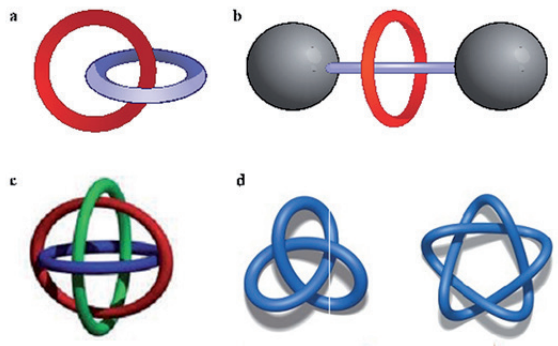

Figure 1. Mechanically interlocked molecules: (a) catenane, (b) rotaxane, (c) Borromean ring, (d) molecular knots. Adapted from reference [3].

1.2. Atomic force microscopy

Collecting information about the working processes of such molecules is crucial for the design of more efficient molecular devices. Atomic force microscopy (AFM)-based single-molecule force spectroscopy (SMFS) has proved to be an elegant technique to obtain exquisite and detailed information about the structure, dynamics and operation of functional complex molecules and machines, as well as probing intra- and intermolecular interactions [6,7]. During a force spectroscopy experiment, the molecule of interest is trapped between a microscopic probe (AFM tip) and a substrate, its restoring force being measured during the mechanical stretching. AFM-based force spectroscopy has been widely used to unravel the behavior of natural biomolecules under mechanical load [6], to measure the binding interactions between molecular partners [8], to elucidate the mechanochemistry of biological machines [9], and to investigate the mechanochemical properties of mechanically interlocked molecules [10-12].

Beyond standard force spectroscopy, dynamic force spectroscopy focuses on the influence of the loading rate on the force required to break an interaction [13]. By increasing the loading rate, the interaction is pulled out-of-equilibrium and the rupture force increases. The relationship between the rupture force and the loading rate has been described by several models, namely Bell-Evans [14], Friddle-Noy-De Yoreo [15], and Dudko-Hummer-Szabo [16], and can provide an estimation of thermodynamic and kinetic parameters. Additionally, by performing subsequent controlled pulling-relaxing experiments on a trapped molecule, it is even possible to determine the free-energy difference (ΔG) between two conformations of the molecule [13].

2. Mechanical unfolding of oligorotaxanes

Recently, donor-acceptor oligorotaxanes were synthesized [17], at the intersection between MIMs and foldamers — synthetic molecules presenting a folded conformation. These oligorotaxanes are based on a flexible poly(ethylene oxide) (PEO) thread bearing 1,5-dioxynaphthalene (DNP) units, and encircled by several tetracationic cyclobis(paraquat-*p*-phenylene) boxes (also known as blue boxes or $CBPQT^{4+}$) (Fig. 2). The intramolecular interactions between DNP donors and 4,4'-bipyridinium ($BIPY^{2+}$) units in the blue boxes induce a packed serpentine-like conformation, as observed by X-ray crystallography and ^{1}H nuclear magnetic resonance studies [17,18].

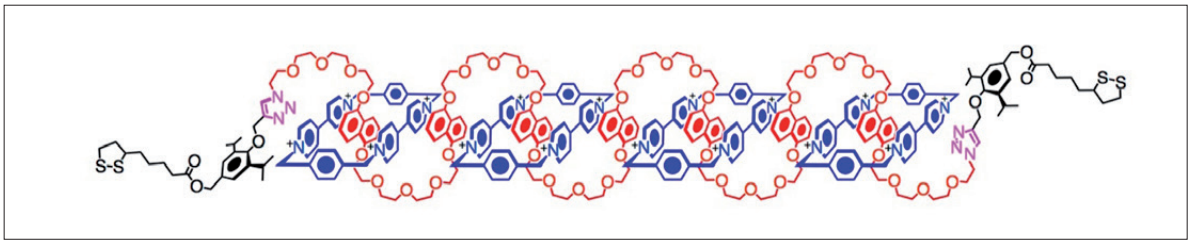

Figure 2. Chemical structure and serpentine-like conformation of an oligorotaxane, with the main PEO chain (in red) interlaced in the (blue) rings (counterions are not represented). Adapted from reference [17].

In this study in collaboration with the group of Prof. J. Fraser Stoddart, we have investigated the mechanochemical properties of those oligorotaxanes in solution and at the single-molecule level [19,20]. The molecules were prepared with 1,2-dithiolane rings at both end for their interfacing between a gold-coated AFM tip and a gold-coated silicon substrate. The molecules were grafted using our previous strategy [11] to obtain a low-density regime that prevails the attachment of individual molecules on the tip during the force experiments.

During a force spectroscopy experiment, we first approach the AFM tip to the surface in order to create Au-S interaction between the tip and the molecule. Then, the tip is retracted in a controlled manner (i.e. at a fixed loading rate) and the restoring force is monitored by the deflection of the tip-bearing cantilever (Fig. 3a). The obtained force curve is related to the behavior of the molecule during the stretching, as well as its initial conformation.

Oligorotaxanes were stretched in *N,N*-dimethylformamide (DMF) and present a characteristic force-distance sawtooth profile (Fig. 3b). This reproducible pattern consists of equally-separated rupture peaks, which we attribute to the sequential breaking of donor-acceptor intramolecular interactions (DNP-BIPY^{2+}). The length increment between successive peaks (ΔL_c) was measured using the worm-like chain (WLC) model, an entropic elasticity model describing the behavior of a flexible polymer. Two ΔL_c populations were observed: the first one at 1.2 ±

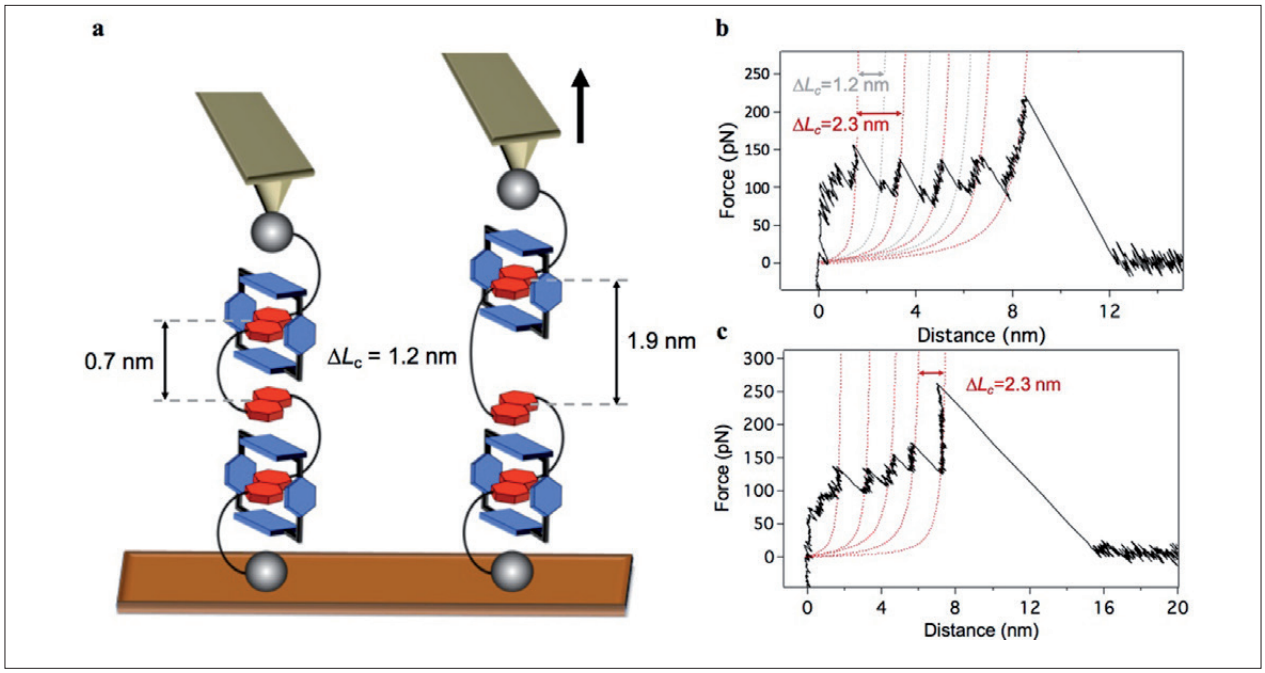

Figure 3. AFM mechanical stretching of individual oligorotaxanes: (a) scheme of the single-molecule force spectroscopy experiment showing the rupture of one donor-acceptor interaction and the characteristic length increase of 1.2 nm, (b) characteristic force-distance curve presenting a sawtooth pattern with equally-distant peaks (ΔL_c = 1.2 nm), (c) force-distance curve showing the rupture of intramolecular interactions by pairs (ΔL_c = 2.3 nm). Adapted from references [19,20].

0.1 nm and the second one at 2.3 ± 0.1 nm (as shown in Fig. 3b and c). Based on crystallographic data obtained previously [18], the distance between two subsequent DNP units increases from 0.7 nm in the folded state to 1.9 nm in the unfolded state (Fig. 3a). Hence, the length increment of 1.2 nm repeatedly observed in experimental force curve is in perfect agreement with the breaking of one DNP-BIPY^{2+} interaction, the second population representing the simultaneous breaking of two donor-acceptor interactions.

The most probable rupture force is about 90 pN for the stretching of the oligorotaxanes at 10^4 pN·s^{-1}. This value is consistent with the breaking of noncovalent interactions such as donor-acceptor π-interactions, hydrogen bonds or H-π interactions [21]. The last rupture peak displays a higher force (about 300 pN) that is characteristic of an S-S interaction with gold.
The stretching pattern is thus in agreement with the unfolding of the previously proposed serpentine-like (co)conformation of the oligorotaxanes in solution.

3. Real-time capture of (un)folding fluctuations

In order to determine the mechanical unfolding reversibility, we performed pulling-relaxing experiments. A single oligorotaxane was trapped between the substrate and the tip, the mechanical load being subsequently increased and decreased without breaking the interaction between the molecule and the tip. Pulling-relaxing curves are shown in Fig. 4 and illustrate the stochastic behavior of the molecule under mechanical load. The subsequent breaking and reformation of a single donor-acceptor interaction can take place without (Fig. 4a) or with (Fig. 4b) hysteresis (difference between the pulling and the relaxing curves). Hysteresis indicates that the work done to break one interaction is partially dissipated and is not fully recovered for the reformation of the interaction. Fig. 4c shows the breaking of two intramolecular interactions simultaneously (one arrow in pulling curve), followed by their sequential reformation (two arrows in relaxing curve). Such force curves evidence the reversibility of the mechanical unfolding, the molecule being able to reform the broken interaction as soon as the mechanical load is lowered.

Also, we sometimes noticed rapid fluctuations between partially folded and unfolded states both during pulling and relaxing movements (Fig. 4d and e). Such fluctuations are representative of the *hopping* of the molecule between two (co) conformations. Similar patterns have previously been observed on biomolecules and are the hallmark of an experiment performed near the thermodynamic equilibrium [22-24]. We show here the first example of small synthetic molecules undergoing such back-and-forth (un)folding transitions at high loading rates. A scrupulous analysis of the *hopping* phenomenon showed that a single oligorotaxane is able to exert a force of up to 50 pN under an external load of 60 pN in average. As a comparison, the maximum mechanical load that proteins can accommodate during such fluctuations is typically about 10 pN. At high loading rate (10^5 pN·s^{-1}), we observed more than 4,300 fluctuations per second between both states, about 300 times faster than calmodulin (a folding protein presenting similar fluctuations between folded and unfolded states) [23]. More importantly, the transition step occurs in less than 10 s, making the oligorotaxane the fastest folding system studied at high loading rate.

We believe that this robust behavior originates from the mechanically interlocked structure, maintaining both donor and acceptor units in a close neighborhood after being mechanically separated. Compared to larger biological specimens, the refolding of the oligorotaxane is associated with a low entropic penalty. The mechanically interlocked structure constrains the blue boxes around the DNP stations and maintains a pre-oriented conformation, facilitating the reformation of the broken interactions.

4. High performance of artificial molecules

Dynamic force spectroscopy experiments were performed on single oligorotaxanes, which were mechanically stretched at 11 different loading

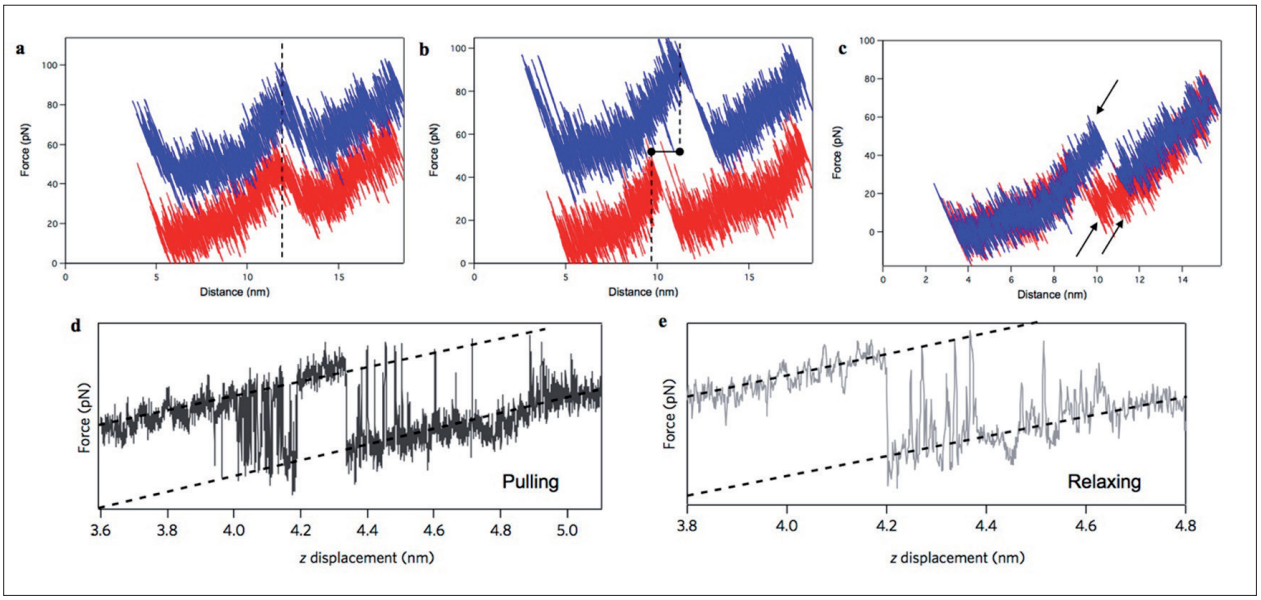

Figure 4. Pulling(blue)-relaxing(red) experiments on oligorotaxanes: breaking and reformation of a single DNP-BIPY^{2+} interaction without (a) and with (b) hysteresis (pulling curve is offset for more clarity), (c) breaking of two intramolecular interactions followed by their subsequent reformation, (d) pulling and (e) relaxing curve presenting many fluctuations between locally folded and unfolded states. Adapted from references [19,20].

rates varying from 10^2 to 10^6 pN·s^{-1} [19]. At each loading rate, the distribution of the rupture force was adjusted to provide the most probable rupture force (F). The evolution of F with the loading rate is shown in Fig. 5 (red marks). The quasi-linearity observed in this graph indicates a rate-independent rupture force in this range of loading rate, evidencing experiments performed in the near-equilibrium regime. In comparison, similar mechanical stretching of biomolecules were described by the Friddle-Noy-De Yoreo model (non-linear fit) [15] and showed the distinction between their near-equilibrium and out-of-equilibrium regimes in similar range of loading rates (Fig. 5, gray marks). The mechanical stretching of oligorotaxanes seems to be always performed in the near-equilibrium regime, at least up to 10^6 pN·s^{-1}. The kinetic (i.e. out-of-equilibrium) regime is not reached in these conditions, evidencing the high dynamics of such interlocked molecules. Furthermore, the equilibrium force (rupture force in the near-equilibrium regime) is higher than 100 pN, in comparison with the low equilibrium force (< 50 pN) experienced by biomolecules studied in similar conditions [15]. Again, the performance of those synthetic molecules surpasses the one of natural folding proteins, probably due to their specific chemical structure.

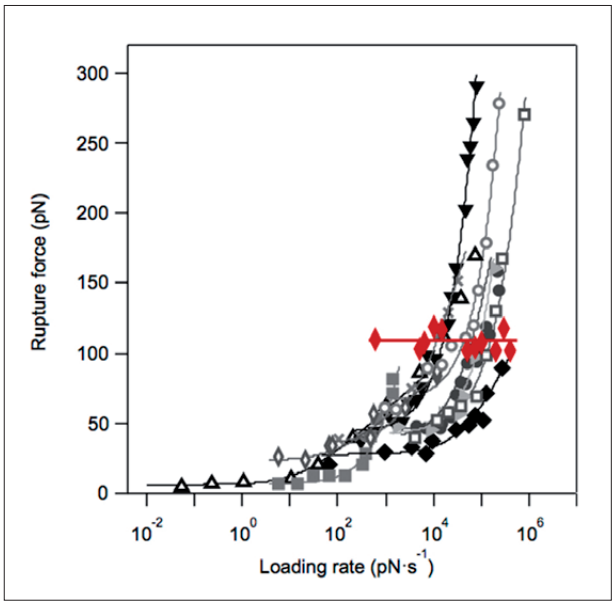

Figure 5. Dynamic force spectrum of the mechanical unfolding of single oligorotaxanes in DMF at 11 loading rates (in red) superimposed with force spectra of 10 data sets (gray scale) taken from the literature and fit by the Friddle-Noy-De Yoreo model (reference [15]). The near-equilibrium regime is maintained for the oligorotaxane, even at high loading rates, evidencing the high dynamics of this artificial interlocked molecule compared to biomolecules. Adapted from reference [19].

In an attempt to evaluate the energy required to break one DNP-BIPY^{2+} interaction, we designed pulling-relaxing experiments probing only one donor-acceptor interaction. We compared the distribution of work (area under the force curve) done to break this interaction with the distribution

of work performed by the molecule to reform it at 10^3 pN·s^{-1}. Using the Crooks fluctuation theorem (CFT) [25] relating the free-energy difference between two conformations with the forward and reverse works measured during pulling-relaxing experiments, we determined a ΔG (DNP-BIPY^{2+}) of 6 ± 1 kcal·mol^{-1}. This value is close to the one obtained previously by calculations [3] and is in agreement with the breaking of one interaction inside the oligorotaxane in a serpentine-like folded (co) conformation.

5. Conclusions and perspectives

In summary, we reported here the single-molecule investigation of oligorotaxanes using AFM-based force spectroscopy. Standard pulling experiments evidenced the sequential mechanical breaking of intramolecular donor-acceptor interactions supporting the serpentine-like folded structure. For the first time, we were able to capture real-time fluctuations of a single synthetic molecule between different (co) conformations. The transitions between partially folded states are at least as fast as that observed in proteins but remarkably more robust. Pulling-relaxing experiments showed the reversibility of the mechanical unfolding and revealed the stochastic nature of the process. Finally, dynamic force spectroscopy performed on these molecules illustrated their high dynamics compared to folding proteins, probably due to the specific nature and superstructure of the oligorotaxanes.

Taking inspiration from nature's molecular machines, chemists are now able to design artificial molecules with well-defined and stable folded architecture. Our results show that such synthetic molecules could eventually surpass the performance of their natural counterparts.

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