

A Polymeric Molecular “Handle” for Multiple AFM-Based Single-Molecule Force Measurements**

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Single-molecule force spectroscopy (SMFS) is a powerful tool for detecting and localizing single molecular recognition events, and for exploring the energy landscape of molecular interactions. In particular, this technique has been used recently to study single biological binding interactions at a cell surface.^[1,2] It is now recognized that many binding events at a cell surface are multivalent in nature.^[3] Herein, we describe a methodology for measuring multivalent interactions at a surface.

To study the mechanical resistance of a bond or of a molecular complex by the AFM-based SMFS methodology, synthetic “handles” or linkers must be introduced into the chain to apply the load to the bond. The linker reduces the effect of nonspecific interactions of cantilever and substrate on the unbinding event, and also avoids direct coupling of the fluctuations of the cantilever onto the examined molecular unit.^[2,4] Hetero-bis-functionalized linkers (based on polymeric chains) and appropriate surface derivatizations have been developed.^[2]

The SMFS methodology has also been applied to study protein folding using an experimental scheme developed to this end.^[5] Protein folding/unfolding studies are most commonly performed on multimodular constructs, in which protein domains serve as handles.^[6,7] This approach ensures two main methodological advantages. The first is experimental: no AFM probe chemical derivatization is needed to pick up and stretch one molecule at a time, as the protein modules serve as handles for the pressure-induced probe–protein bond. Each protein molecule is attached to a gold substrate through one terminal cysteine, and then anchored to the probe at a nonspecific point so that tension can be applied to the modules between these attachment points.

The second advantage is a great simplification in the interpretation of the data. The different modules of such a protein unfold one at a time. Force curves with a readily recognizable series of peaks are obtained, where each peak corresponds to the unfolding of a single protein module. Additionally, if well-characterized protein modules are inserted together with unknown ones in engineered constructs, these can also act as an internal standard.

This force spectroscopy approach, which has so far been employed for protein unfolding studies, is particularly effective but, as it is based on molecular biology, it has so far remained confined to the protein field. Herein, we report a novel approach to SMFS experiments that makes it possible to apply the above-mentioned scheme to any kind of molecular interaction.

We tailored a polymeric linker that can replicate any desired chemical interaction many times along its linear chain. A force curve with multiple peaks is thus obtained on each stretching run. The same linker can be tailored to serve as an internal gauge based on the relative distance of the peaks. The polymer chain with equally spaced side groups sketched in Figure 1 can fulfill these requirements. In the first implementation of our strategy (see Figure 1), we endeavored to characterize the force-induced rupture of the Ni-(His)_n-NTA complex, which is widely used in protein purification^[8,9] but still lacks full comprehension of its structure and behavior.

We synthesized a carrier polymer with a PEG backbone and side chains located 20 nm apart (polydispersity index, PDI = 1.15). The side chains are derivatized with a NTA group. The polymer is spread and adsorbed through multiple Ni-(His)_n-NTA bonds on a surface previously coated with a peptide monolayer exposing histidine tracts.^[10] The chains and the AFM probe are then engaged in a pressure-triggered bond, as in the case of multimodular proteins.^[5] Subsequently, the probe is retracted from contact with the surface and the

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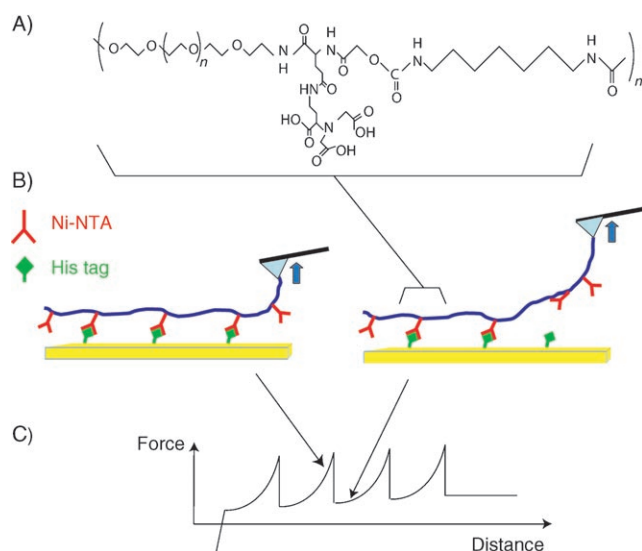


Figure 1. Polyethylene glycol (PEG)-based comblike polymer exposing nitrilotriacetate (NTA) groups with 20 nm separation. A) Chemical formula of the polymer. B) Representation of the force spectroscopy experiment in which the polymer is pulled away from the surface, thus breaking the specific bonds formed and leading to a force profile, such as that shown in (C). The peaks are separated by a distance corresponding to the linker elongation after the rupture of the corresponding bonds.

multiple coordination bonds along the chain are ruptured in sequence.

Force–extension curves show a series of evenly spaced peaks attributable to the rupture of Ni-NTA complexes (see Figure 2) following a single, often large peak arising from the nonspecific interaction force between the AFM probe and the peptide-coated surface. Each bond-rupture peak is characterized by a mild rise of the force as a result of the entropic stretching of the polymer portion separating two neighboring binding groups,^[11] then by a sudden fall in the force that results from bond rupture.

The distribution of the measured rupture forces (Figure 3A) reaches a maximum at approximately 110 pN. A shoulder at around 230 pN (see the Supporting Information for a more detailed analysis) may be caused by the rupturing of two bonds at exactly the same time (from the same or different chains). More likely, it could be because of a possible structural diversity of the Ni-(His)_n-NTA coordination bond that can lead to distinct distributions of rupture forces.

The distribution of the interpeak distances is reported in Figure 3B. The distribution maximum is found at around 14 nm, that is, shorter than the 20-nm designed spacing of side groups along the polymer main chain (expected to serve as an internal control for recognition). This difference arises from the incomplete extension of the polymer chain at the measured rupture forces. As reported in the literature,^[12] at about 100 pN the PEG chain in water reaches about 70 % of its full extension, in very good agreement with the maximum of our distribution. Other maxima in the reported length distribution are attributed to uncoordinated sites along the chain, or missing functionalization.

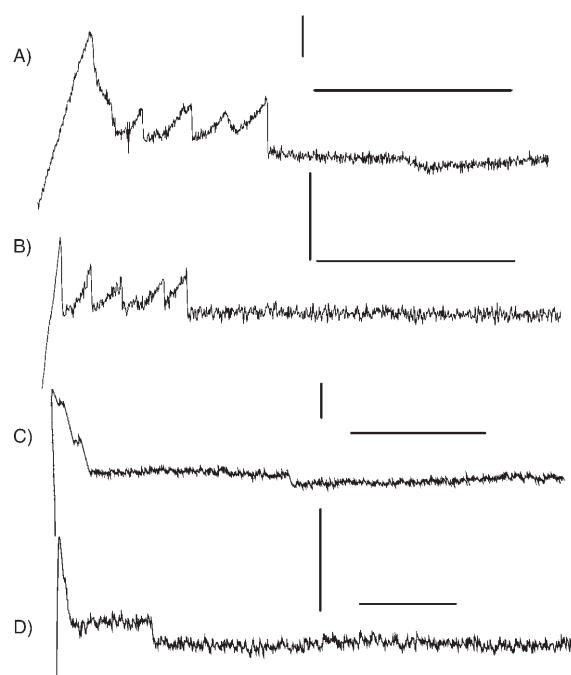


Figure 2. Force–extension curves obtained upon pulling the comblike polymer away from the surface. A,B) The evenly separated peaks, approximately 20 nm from one another, represent the rupture of the specific Ni-(His)_n-NTA bonds. C,D) The force curves obtained, as control experiments, in the presence of imidazole and ethylenediaminetetraacetic acid (EDTA) display only a desorption plateau because of the absence of any Ni-(His)_n-NTA bond. Scale bars: 250 pN (vertical); 100 nm (horizontal).

The measured force does not always return to zero after each bond rupture. This effect could be a consequence of the incomplete relaxation of the cantilever when the length of the polymer chain is too short, even right after a bond-rupture event (supported by numerical simulations: see Figure S1 in the Supporting Information). It could also be a result of the force-induced chain desorption of the polymer backbone from the substrate coupled to the rupture events taking place along the chain. In fact, on pulling the polymer adsorbed on bare gold (without peptides exposing the His tag), only desorption plateaus were obtained and additionally the length of the plateaus was compatible to the polymer length (see Figure S2).

A similar result was obtained when the peptide-coated surface was treated with imidazole (a competitor of histidine for coordination to Ni-NTA) prior to incubation with the polymer or when EDTA was used to scavenge Ni^{II} ions before the spreading of the polymer (see Figure 2). Very few bond-rupture force peaks were observed when the experiments were performed in the absence of Ni^{II}. This is not surprising, as it is extremely difficult to avoid trace metal contaminants, which can be responsible for the formation of those very few coordination bonds.

The measured rupture forces that were reported previously in the literature for the rupture of the Ni-(His)_n-NTA bond were obtained under rather different experimental conditions,^[13–16] with different geometries and linkers. In a

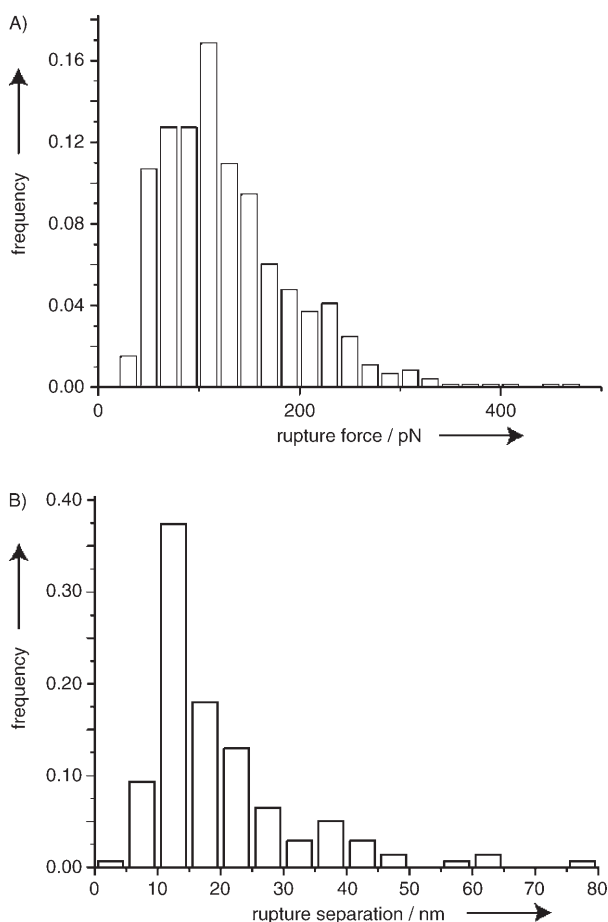


Figure 3. A) Rupture force distribution displaying a peak around 110 pN with a tail and a shoulder at 230 pN. B) Distribution of the observed separation between rupture events.

recent review, Beyer and Clausen-Schaumann examined the different Ni-(His)_n-NTA bonds reported to date.^[17] The bond-rupture force strongly depends on the force-loading rate,^[18–20] which, in turn, is a function of the rigidity of the linker that is used to load the mechanical tension. At the moment our rupture-force values seem to be in better agreement with those measured by Schmitt and co-workers.^[15] On the other hand, a solution of the still controversial assignment of the mechanical resistance of this coordination bond cannot be reached without a better understanding of its possible structural diversity. Further studies are needed to clarify this point.

Herein, we have described a strategy that makes use of a comblike polymer as a linker for AFM-based SMFS, to replicate and evenly space several bonds or molecular complexes along a molecular chain. This approach makes it possible to measure the force for the rupture of a sequence of specific chemical bonds after engaging a nonspecific pressure-triggered bond between the probe and the polymer chain. The constant distance between the peaks in the resulting force curves is also an ideal internal standard for the interpretation of the data. Furthermore, the optimum length of the handle can be matched on each run because the ruptures take place in correspondence with a progressively increasing handle

length. As recently reported, because of the interplay between the length of the linker and the mechanical noise and thermal fluctuations, the optimal length for the linker also depends on the mechanical properties of the investigated molecular complex.^[4]

Our strategy based on functionalized comblike polymers should prove general for the study of the rupture forces and dynamics of any bond or molecular complex, such as individual or multiple instances of ligand–receptor interactions. As an example, by varying the spacer length we will be able to investigate the influence of the concentration of different surface receptors (such as those regulating cellular adhesion and cell–cell interaction) on their binding to ligands and on the possible cross-talk among these events.

Experimental Section

AFM experiments: The peptide Cys-(Gly)₆-(His)₆ was synthesized by Tecnogen (Caserta, Italy). A solution of peptide (1 mg mL^{−1}) was spotted onto a freshly exposed ultraflat gold surface prepared by template stripping^[21] to form a monolayer, and then treated with a solution containing Ni²⁺ (10 mM Tris, pH 7.4; 100 μM NiCl₂). The polymer solution (10 μL) was then deposited on the functionalized surface to form coordination bonds. The surface was thoroughly rinsed with Milli-Q water (Millipore, USA) and mounted in the AFM fluid cell. Force spectroscopy was performed in Tris buffer (10 mM, pH 7.4) with a NanoScope IIIa instrument (Veeco, Santa Barbara, USA) equipped with a PicoForce unit. Force curves were analyzed with Hooke, a custom force spectroscopy analysis program (to be published online).

Linker synthesis: Details of the synthesis of the comblike polymer are reported in the Supporting Information.

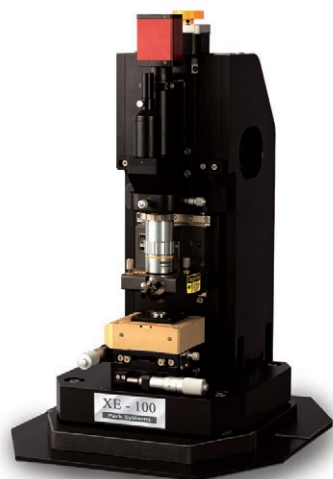
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