Chapter 5

Unequivocal Single-Molecule Force Spectroscopy of Intrinsically Disordered Proteins

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Abstract

Intrinsically disordered proteins (IDPs) are predicted to represent about one third of the eukaryotic proteome. The dynamic ensemble of conformations of this steadily growing class of proteins has remained hardly accessible for bulk biophysical techniques. However, single-molecule techniques provide a useful means of studying these proteins. Atomic force microscopy (AFM)-based single-molecule force spectroscopy (SMFS) is one of such techniques, which has certain peculiarities that make it an important methodology to analyze the biophysical properties of IDPs. However, several drawbacks inherent to this technique can complicate such analysis. We have developed a protein engineering strategy to overcome these drawbacks such that an unambiguous mechanical analysis of proteins, including IDPs, can be readily performed. Using this approach, we have recently characterized the rich conformational polymorphism of several IDPs. Here, we describe a simple protocol to perform the nanomechanical analysis of IDPs using this new strategy, a procedure that in principle can also be followed for the nanomechanical analysis of any protein.

Key words: Single-molecule force spectroscopy, Atomic force spectroscopy, Intrinsically disordered proteins, Conformational plasticity, Protein nanomechanics

Abbreviations

ΔL_c	Increase in contour length
AFM	Atomic force microscope
DTT	Dithiothreitol
FPLC	Fast protein liquid chromatography
F_u	Average unfolding force
IDP	Intrinsically disordered protein
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Lysogeny broth
MOPS	3-(*N*-Morpholino) propanesulfonic acid
MPTS	Mercaptopropyl trimethoxysilane
NTA	Nitrilotriacetic acid
OD_{595}	Optical density at 595 nm

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p	Persistence length
pFS	Plasmid for force spectroscopy
SDS	Sodium dodecyl sulfate
SDS-PAGE	Polyacrylamide gel electrophoresis in the presence of SDS
SMFS	Single-molecule force spectroscopy
WLC	Worm-like chain

1. Introduction

IDPs have not a well defined structure when isolated in solution. Instead, it has been recently shown that these proteins can adopt several conformations, some of which may be scarcely populated and fast fluctuating (1–4). Thus, the characterization of these conformers remains a challenging task for bulk biophysical techniques (5). As a single-molecule technique, SMFS provides a unique means of analyzing this conformational polymorphism, relating protein conformation to mechanical stability.

In SMFS, the protein of interest is stretched in order to measure its mechanical resistance, typically. This resistance is usually unique and characteristic in folded proteins. However, the different conformations of IDPs may also exhibit diverse mechanical properties. Hence, SMFS techniques provide a way to analyze the conformational plasticity of these proteins. In the most common SMFS technique used, AFM, a protein attached to a substrate and the tip of a cantilever (the force sensor) is stretched (usually in N-C direction) by a piezoelectric device and the resistance forces are measured (6). Several approaches have been developed to unambiguously identify and select single-molecule recordings. Most of these approaches are based on polyproteins, tandem repeats of proteins or protein modules, which are easily identified in SMFS force-extension recordings based on the periodicity of equally spaced peaks seen when the length-clamp mode of the AFM is used (6). In this so-called saw-tooth pattern each peak typically originates from the unfolding of an individual protein structure (6–11). The height of each force peak is used to calculate the mechanical stability of the protein (F_u, defined as the average unfolding force) while the distance between peaks reflects the length of the protein region that was previously hidden to the force. By determining the so-called increase in contour length of the molecule (ΔL_c, obtained after fitting the force-extension recordings to the worm-like chain (WLC) model of polymer elasticity, 7) the number of amino acids contained in the force-hidden region of the protein can be calculated and thus, the position of the mechanical barriers can be assigned allowing to infer the type of mechanical structures involved (9, 12–14).

The mechanical unfolding of proteins is a hierarchical process such that a less mechanostable structure will unfold prior a more mechanostable one. However, the proximal region of the force-extension recording is frequently contaminated with nonspecific interactions (15). This spurious contamination can sometimes mask the mechanical unfolding pattern of the protein under study, particularly if it has weak mechanostability and/or a complex ΔL_c pattern (as is the case for some IDPs, (16)).

To circumvent this particular drawback, we have developed a new family of vectors for general use in protein nanomechanics (pFS for *p*lasmid for *F*orce *S*pectroscopy, (15)) that contains a polypeptide with undetectable mechanical resistance by SMFS, as a spacer to bridge the proximal region of the force-extension recordings (pFS-1 version). A second version of the vector (pFS-2) that carries a multi-cloning site in a tolerant loop of a ubiquitin repeat (or the I27 module) has also been developed, which allows the sequence of the protein of interest to be lodged inside its fold, an approach we have termed "carrier-guest" strategy (15, 16). Using this approach, the protein of interest will always be stretched after the unfolding of the carrier protein, and thus far from the problematic proximal region of the recordings. This strategy has recently enabled the unambiguous mechanical analysis of a variety of IDPs (both amyloidogenic and non-amyloidogenic) at the single-molecule level (16). However, this strategy only guarantees that our SMFS data originate from stretching a single IDP molecule, which could still be involved in a series of interactions (with the AFM elements, substrate or cantilever tip, or other IDP molecules forming dimers or even oligomers). We have already described strategies to control each of these interactions (16). For instance for the case of amyloidogenic IDPs, since they have a tendency to oligomerize, we used an inhibitor of the oligomerization process to confirm (by comparison with the untreated sample) that our SMFS data originate from stretching single monomeric molecules (i.e., intramolecular interactions). Ideally, one could perform refolding experiments in a buffer devoid of IDPs in solution as a more general control that would rule out possible interactions between the IDP molecules.

As amyloidogenic IDPs (and in particular, neurotoxic proteins, which are causally related to neurodegenerative diseases) exhibit a well-known amyloidogenic behavior (i.e., formation of toxic oligomers and amyloid fibers), it is of great interest to determine the aggregation state of the sample in order to select the species of potential mechanical interest (i.e., monomers, soluble oligomers, insoluble aggregates, or fibrils). In its original configuration, our custom-made AFM setup was unable to allow the determination of the topography of a given sample (17). However, we have recently added imaging capabilities to this system by integrating commercial imaging hardware (Dulcinea control unit) and software (WSxM)

both from Nanotec Electrónica S.L., (http://www.nanotec.es; (18, 19)). This system is now capable of obtaining an AFM image of a region, in contact or dynamic mode, and then selecting the area of interest in order to record a force curve *quasi*-simultaneously with the image. However, since there are conflicting technical requirements between both modes of AFM, applying this new approach to the combined analysis of single molecules would require the development of a new functionalization protocol preserveing the integrity of the single molecules attached to the substrate (18).

Here, we describe a protocol for the use of the pFS-2 vector to analyze the mechanical properties of IDPs using the carrier-guest strategy (see Note 1). This protocol can also be applied in principle to the nanomechanical analysis of any protein (or protein region) using either the pFS-1 or pFS-2 vectors. We detail the steps involved, including the cloning, expression and purification procedures, as well as SMFS data acquisition and analysis.

2. Materials

The materials used for the cloning, expression, and purification of the recombinant proteins are all commercially available, and where relevant, the information of the provider is specified. The materials related to SMFS data acquisition and analysis are specific to our AFM setup, which was first described in ref. 17, and then added with imaging capacities in ref. 18. As such, our setup can perform *quasi*-simultaneous imaging-pulling analysis, although the protocol described here focuses on pulling. While the protocol we describe is based on our specific setup, the use of pFS-1/pFS-2 vectors, their mechanical properties and the criteria for nanomechanical analysis are independent of the AFM apparatus used (see Note 2).

2.1. Cloning, Expression, and Purification of IDPs in pFS-2

1. pFS-1 and pFS-2 vectors have been described elsewhere in detail (Fig. 1a, b; (15, 16)). In brief, these vectors contain a fragment of around 200 amino acids from the N2B polypeptide of human cardiac titin (UniProtKB/Swiss-Prot code Q8WZ42), which unfolds without detectable mechanical resistance and that therefore acts as a spacer, bridging the problematic proximal region of the force-extension recordings (Fig. 1c, d; (20)). In addition, they contain several human ubiquitin repeats (UniProtKB/Swiss-Prot code P0CG47), a protein with putative chaperone activity and well-characterized mechanical properties (21, 22). Both vectors contain a series of interdomain restriction sites (BamHI, XbaI, SalI, NotI, SpeI, BssHII, XhoI, and KpnI, from the N- to C-terminus, Fig. 1a, b) some of which can be used for the directional cloning of any protein of interest (NotI, SpeI, BssHII, and

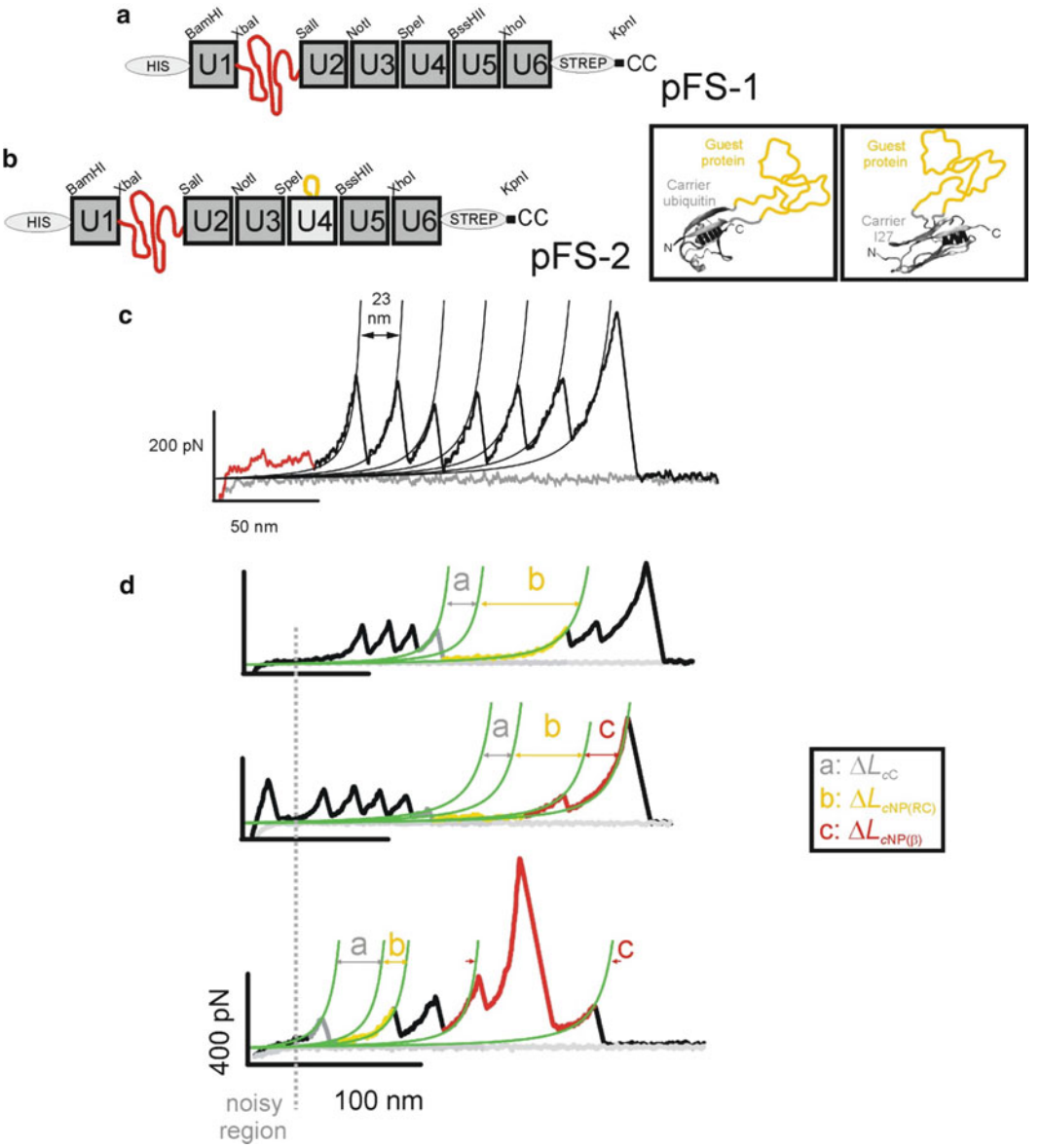

Fig. 1. Nanomechanical analysis of IDPs using pFS polyproteins. (**a**) Schematic representation of the pFS-1 polyprotein. The ubiquitin repeats are represented by *grey boxes* and the N2B fragment is represented as a non-folded polypeptide. (**b**) Schematic representation of pFS-2. The ubiquitin (or I27) repeat containing the multi-cloning site is located at position 4 of pFS. On the *right* is a representation of the carrier-guest construction using both carrier modules: ubiquitin (*left*, with the multi-cloning site located between residues T9 and G10) and I27 (*right*, with the multi-cloning site located between A42 and A43). The mechanical clamps of both carrier modules are indicated (6, 22), demonstrating that the guest IDP is "force-hidden" and that the carrier must unfold prior to stretching the grafted IDP. (**c**) Typical force-extension recording of the pFS-2 polyprotein. The extension gained by the stretching of the N2B fragment, at the beginning of the force-extension recording, serves as a spacer to avoid the usually contaminated proximal region of the force-extension recordings. The ubiquitin force peaks are shown in *black*. (**d**) Representative force-extension recordings of the pFS-2 + Sup35NM. By using this vector we can unambiguously resolve a variety of conformations adopted by Sup35NM (NP in the figure), ranging from mechanically undetectable conformations (putatively random coil, RC, b in the figure, *top trace*) to different mechanostable conformations that exhibit different degrees of mechanical stability (putatively β-structured, shown as C in the recordings). Modified from refs. 15 and 16.

XhoI). In pFS-2, a multi-cloning site is positioned inside the fold of either a ubiquitin repeat (containing AgeI, BsiWI, SmaI, and MluI sites, from the N- to C-terminus, located in loop AB between residues T9-G10, Fig. 1b; (15, 16)) or an I27 module (AgeI and SmaI restriction sites located between residues A42 and A43 in the CD loop, Fig. 1b; (16)) to clone proteins following the carrier-guest strategy. Both multicloning sites are located behind the resistance region of the modules (a.k.a. mechanical clamp). The pRSETA vector was used as the basic platform to construct these vectors (Invitrogen).

2. The cloning steps are performed in the *E. coli* XL1-Blue strain (Stratagene). The culture medium used is Lysogeny Broth (LB, 10 g/l Bacto Tryptone, 5 g/l Bacto Yeast extract, 10 g/l NaCl) with antibiotic added according to the plasmid vector's antibiotic resistance.
3. The recombinant protein is expressed in the *E. coli* C41(DE3) strain (23) by 1 mM IPTG using LB medium (see above).
4. The purification of recombinant proteins can be performed using many different approaches, although we use an FPLC apparatus (ÄKTA Purifier, GE Healthcare). Depending on the purity achieved at each step, several chromatography purification steps can be performed alternatively or sequentially:
   - Ni^{2+}-affinity chromatography: use Histrap HP FPLC columns (GE Healthcare).
     - Binding buffer: 50 mM sodium phosphate/500 mM NaCl/50 mM imidazole [pH 7.4].
     - Elution buffer: 50 mM sodium phosphate/500 mM NaCl/500 mM imidazole [pH 7.4].
   - Strep-tag affinity chromatography: use Streptrap HP FPLC columns (GE Healthcare).
     - Binding buffer: PBS (137 mM NaCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 2.7 mM KCl, [pH 7.4]).
     - Elution buffer: PBS/2.5 mM desthiobiotin.
   - Size exclusion chromatography: use HiLoad 16/60 Superdex TM 200 column (GE Healthcare).
     - Buffer: 100 mM Tris–HCl [pH 7.5]/1.25–1.5 M guanidinium chloride (this concentration does not denature ubiquitin or I27 domains allowing the removal of contaminants that may co-elute with the recombinant protein; (24, 25)).
5. All concentration and buffer-exchange steps are performed by ultrafiltration using Amicon 10K filters (Millipore).

2.2. Preparation of AFM Substrates

Two SMFS substrates have been used with pFS-based recombinant polyproteins: gold-coated substrates, which permit covalent attachment of pFS polyproteins through their C-terminal cysteine residues (26), and NTA-Ni^{2+} functionalized glass coverslips, which attach pFS polyproteins via their N-terminal His-tag (27). Although gold-coated coverslips can be custom-made by thermal deposition (13), commercially available gold substrates (Arrandee) yield acceptable results in SMFS. The following materials are used in the preparation of NTA-Ni^{2+} substrates:

1. Round microscope borosilicate glass coverslips, 14 mm diameter (Thermo Scientific).
2. An oven.
3. Solutions:
   - 20N KOH.
   - MilliQ water.
   - 2% 3MPTS (Sigma-Aldrich)/0.02% acetic acid.
   - 100 mM DTT.
   - 3 mg/ml maleimide-C3-NTA (Dojindo Laboratories) dissolved in 10 mM MOPS [pH 7.0].
   - 10 mM $NiCl_2$.

2.3. Data Acquisition in SMFS

All experiments with IDPs using the pFS-2 vector were performed in the length-clamp mode of the AFM (see below; (6)). Force-clamp mode and "refolding" protocols to analyze structure formation can also be used (28). A basic protocol for length-clamp nanomechanical analysis of IDPs is presented here focusing on the "unfolding" process to analyze the breakage of structures.

1. 10–20 μl of the pFS-2 polyprotein carrying the IDP (see Subheading 2.1) at a concentration of 2–3 μM.
2. AFM substrates, as described in Subheading 2.2.
3. The AFM apparatus:
   - Multimode AFM head: TVOH-MMAFMLN (Veeco).
   - Laser System (Schäfter + Kirchhoff): power supply SK9732C, laser diode collimator 50BM, laser-beam coupler 60SMS-1-4-A8-07, single mode fiber cable SMC630-5-NA010-3APC-0-50, collimator lens 60 FC-4-M12.
   - Fluid cell (Veeco).
   - Multiaxis closed-loop PicoCube P-363.3CD piezoelectric positioner (Physik Instrumente). This piezoelectric positioner is equipped with a capacitive sensor (PZT-Servocontroller E-509.C3A, Physik Instrumente) that allows subnanometer resolution in its displacement.

- AFM controller (JRC Instruments).
- Dulcinea high voltage unit (Nanotec Electrónica S.L.).
- Data acquisition boards (PCI-6052E and PCI-6703, National Instruments) mounted into a personal computer.
- Commanding software: Igor Pro (Wavemetrics) and WSxM (Nanotec Electrónica S.L.).

4. Si_3N_4 AFM cantilevers: Biolever (Olympus) or MLCT-AUNM (Veeco).
5. UV/Ozone ProCleaner™ Plus lamp (Bioforce Nanosciences Inc.).
6. Experimental buffer (0.22 μm filtered).
7. Vibration isolation table (Nano-K 25BM-4; Minus K Technology).

2.4. SMFS Data Analysis

All SMFS data were collected and analyzed in Igor Pro (Wavemetrics) using home-made protocols. The analysis is based on the fitting of the recordings to the WLC, which models the elasticity of polymers (7). The following parameters are typically analyzed: p (persistence length), F_u (unfolding force), ΔL_c (increase in contour length), total length of the molecule, and extension at which the force peak appears.

3. Methods

3.1. Cloning, Expression, and Purification of IDPs in the pFS-2

As an example of the use of the pFS-2 vector to analyze IDPs, we focus on the nanomechanical analysis of the Sup35NM prion from *Saccharomyces cerevisiae* (residues 1–253, UniProtKB/Swiss-Prot code P05453; (29)), which was cloned into the pFS-2 using the carrier-guest strategy (16).

Cloning

1. The codifying sequence for Sup35NM was PCR-cloned using the pJCSUP35 plasmid (Addgene) as the template. As the sites chosen for the cloning of this sequence into the multi-cloning site inside the I27 module were AgeI and SmaI, the synthesized oligonucleotides primers should contain these sites immediately flanking the Sup35NM sequence of the oligonucleotide. The sequences of the primers were (in 5′→3′):

   Forward oligonucleotide: *ACCGGT*ATGTCGGATTCAAACCAAGGC

   Reverse oligonucleotide: *CCCGGG*ATCGTTAACAACTTCGTCATCC

   The chosen restriction sites are shown in italics (*ACCGGT* for AgeI and *CCCGGG* for SmaI).

2. The AgeI-Sup35NM-SmaI sequence is amplified by conventional PCR using Taq DNA polymerase (New England Biolabs) and the PCR product is then purified by electrophoresis in a 1.5–2% agarose gel to clone it into a convenient vector to verify its sequence before subcloning it into the expression vector.
3. The gel-purified PCR product (AgeI-Sup35NM-SmaI) is ligated into the pCR2.1 vector (Invitrogen) using T4 DNA ligase (Fermentas).
4. Transform competent ("Z-competent", Zymo Research) XL1-blue cells with the ligation mix, plate them on LB + agar plates containing antibiotic (carbenicillin), and incubate overnight at 37°C.
5. Using a toothpick, isolate single colonies and grow them overnight in 5 ml of LB + carbenicillin at 37°C with agitation (280 rpm) to screen the colonies for the correct insert sequence. The plasmid DNA is then obtained from the colonies using the conventional mini-prep method (30), and it is digested with appropriate enzymes to confirm the presence of the insert and size by gel electrophoresis. Both strands of the insert are sequenced to verify it has the correct sequence.
6. Once the sequence of the insert is confirmed, repeat steps 2–5 in order to subclone AgeI-Sup35NM-SmaI (already cloned into pCR2.1) into dephosphorylated AgeI-pFS-2-SmaI. It is now not essential to confirm the correct sequence of the insert as the probability of introducing errors in the sequence with this additional cloning step is extremely low. The resulting expression plasmid will be pFS-2 + Sup35NM.

Expression

1. Transform Z-competent C41(DE3) cells (23) with pFS-2 + Sup35NM, plate the bacteria on LB + agar Petri dishes containing antibiotic (carbenicillin) and incubate the inverted dishes overnight at 37°C.
2. The following day, inoculate three colonies in separate tubes containing 5 ml of LB medium + carbenicillin and incubate them overnight at 37°C with agitation.
3. The following morning, inoculate fresh media with a small volume of the overnight cultures and incubate them until an OD_{595} of 0.6–0.8 is reached. Induce protein expression by adding 1 mM IPTG and incubating for 3–4 additional hours at 37°C with agitation.
4. Harvest cells by pelleting at 4,000 × *g* for 10 min and lyse them for 4 min at 98°C in Laemmli Sample Buffer (5× LSB: 156.25 mM Tris–HCl [pH 6.8], 5% (w/v) SDS, 25% (v/v) glycerol, and 0.1% (w/v) bromophenol blue). Run an aliquot of the lysed cultures in 8% SDS-PAGE gels and determine which colony yields the stronger over-expression of the full-length

pFS-2 + Sup35NM recombinant polyprotein with the least degradation, by Coomassie Blue staining or Western blotting. Then, take 500 μl of this clone (as the expression clone stock), pellet the cells, add fresh medium with 10% glycerol, and freeze at −80°C for storage.

5. Inoculate 5 ml of LB + carbenicillin with this clone and incubate overnight at 37°C with agitation. The following day, inoculate 500 ml of LB + carbenicillin with the overnight culture and repeat step 3.

Purification

1. Harvest the cells by centrifugation (6,000 × *g* for 10 min) and then resuspend them in 20 ml of Ni^{2+}-affinity binding buffer (see Subheading 2.1). Add a 1:1,000 dilution of protease inhibitor cocktail (Calbiochem) and then snap freeze (liquid N_2) and snap thaw (42°C) the suspension to facilitate cell lysis.
2. Add lysozyme (Calbiochem) to a final concentration of 1 mg/ml and incubate the cells for 30 min at 4°C. Add Triton X-100 to a final concentration of 1%, and DNase I and RNase A (Sigma-Aldrich) to a final concentration of 5 μg/ml each.
3. Incubate for 30 min in a rocking platform at 4°C.
4. Remove the insoluble debris by centrifugation at 18,100 × *g* for 20 min and filter the supernatant through a 0.45 μm filter to prevent clogging of the resin. Store the pellet at −80°C as this may contain the recombinant protein insoluble in the form of inclusion bodies.
5. Perform FPLC Ni^{2+}-affinity chromatography purification using Histrap HP columns (see the manufacturer's instructions for the column specifications; GE Healthcare).
6. Separate the eluted fractions by 8% SDS-PAGE to verify the purification procedure and monitor the proteins in the fractions using Coomassie Blue staining or Western blotting (using antibodies against the N-terminal His-tag or the C-terminal Strep-tag).
7. Pool the correct elution fractions and perform ultrafiltration using Amicon 10K filters (Millipore). Repeat the ultrafiltration step several times to obtain the sample in a convenient buffer and volume for the next step.
8. Repurify the sample by size exclusion chromatography using the buffer and column specified in Subheading 2.1. Select the appropriate flow rate based on the molecular weight of the recombinant protein of interest.
9. Extensively dialyze the fractions containing the protein of interest against the final SMFS buffer: Tris–HCl 10 mM [pH 7.5]. Repeat steps 6–7, and leave the sample in the experimental buffer.

10. Determine the protein concentration by absorbance at 280 nm using its theorethical molar extinction coefficient.
11. Add 5 mM DTT to avoid disulphide bonding through the C-terminal cysteine residues present in the pFS-2, and divide the sample into small aliquots (~100 µl). Snap freeze (if the recombinant protein of interest tolerates freezing/thawing) in liquid N_2 and store at −80°C. Aliquots should be snap thawed (at 42°C) before use.

3.2. Preparation of NTA-Ni^{2+} Functionalized Glass Coverslips

As mentioned above, two different substrates can be used with the pFS polyproteins. The following is the protocol used to prepare NTA-Ni^{2+} functionalized glass coverslips (27), as gold substrates are commercially available (Arrandee).

1. Immerse the coverslips overnight in a 20N KOH solution.
2. Place the coverslips under a MilliQ water flow for 1 h and then transfer them to a solution of 2% 3MPTS/0.02% acetic acid for 1 h at 90°C.
3. Wash the coverslips in a MilliQ water flow for 1 h and then cure them for 15 min in an oven at 120°C. Then, cool them at room temperature for about 10 min.
4. Next, transfer the coverslips to a 100 mM DTT solution for 15 min and wash under a MilliQ water flow for 1 h.
5. To each coverslip, add a ~50 µl drop of a solution containing 3 mg/ml maleimide-C3-NTA dissolved in 10 mM MOPS [pH 7.0]. Incubate the coverslips for 30 min. Keep the orientation of the coverslips from now on.
6. Wash quickly in MilliQ water each coverslip while holding it. To each, add a drop (60 µl) of 10 mM $NiCl_2$ and incubate them for 10 min.
7. Wash coverslips briefly in MilliQ water (as in step 6) prior to storage.

3.3. SMFS of pFS-2 + Sup35NM (see Notes 3 and 4)

1. Glue one NTA-Ni^{2+} functionalized coverslip (see Subheading 3.2) on a metal support disc using double-sided tape.
2. Wash the coverslip briefly with 20 µl of experimental buffer and repeat a few times (previously filtered through a 0.22 µm filter).
3. Mount the disc onto the AFM head and place a drop of the buffer (20 microlitersl) and ensure that the laser spot is focused directly on the center of the drop. Be careful when mounting the disc as the magnets on top of the piezoelectric device can displace the disc and break the coverslip.
4. Switch off the laser and then add 15–20 µl of the sample (protein concentration 2–3 µM) to the drop of buffer. Incubate for 15–30 min at room temperature.

5. In the meantime, switch on the JRC controller and the oscilloscope.
6. Introduce a cantilever (we typically use Biolever cantilevers, Olympus) into the UV cleaner and switch on the UV for 15–30 s. Leave the cleaner closed as the ozone formed will further clean the cantilever.
7. Meanwhile, fill two 1 ml syringes with filtered buffer (0.22 μm filter) and insert them into the ports of the fluid cell. Once ready, quickly place the clean correctly positioned cantilever into the fluid cell. Keep the cantilever hydrated with a drop of buffer.
8. Recover the unbound sample from the substrate. Wash gently three times with buffer, trying to leave the least amount of liquid possible on the coverslip.
9. Mount the fluid cell. Remove the buffer from the chamber to avoid overflow of fluid from the o-ring when pressing the fluid cell onto the coverslip. Once firmly adjusted, gently apply pressure to the syringes to fill the chamber up.
10. Switch on the laser and reposition the mirrors in order to focus the laser directly onto the tip of the selected cantilever and to obtain the most intense signal in the photodiode.
11. Open Igor Pro and compile the appropriate data acquisition and analysis procedures. In our case, these are custom-made.
12. A spectrum of thermal fluctuations must be acquired. We select here the first resonance peak of the cantilever (for Biolever cantilevers the nominal value is 37 kHz in air and a lower value for our experiments in liquids) in order to calibrate its elastic constant by the equipartition theorem (31).
13. Switch on the Dulcinea and Physik Instrumente units and open the WSxM program (19). Activate the external controllers, which will send a voltage signal to the Dulcinea controller. The latter will amplify both voltage signals (the signal from the external controllers and that generated by the Dulcinea itself) before sending them to the piezoelectric device.
14. Switch on the motor and approach the sample until the piezoelectric positioner enters in range. Then, the position detected by WSxM will be taken as 0 and the SMFS experiments can start.
15. Calibrate the cantilever. After measuring the thermal spectrum (step 12) of the cantilever, its sensitivity must be measured (Sens [nm/V]) by recording an F–z curve. This should be performed in a clean zone of the substrate choosing a region of the recording where the tip is pressing the surface and the forward and backward traces of the piezoelectric movement overlap. The elastic constant of the cantilever detected by Igor must be close to the nominal value specified by the manufacturer (close to 30 pN/nm

for Biolever cantilevers). Otherwise, the cantilever may be defective and should be replaced.

16. Once calibrated, choose the SMFS mode (*length-clamp*, *force-clamp* or its variant *force-ramp*). For length-clamp set the following parameters: pulling speed, extension of pulling, and the fraction of contact between tip and substrate (see Note 3).
17. Thermal drift must be controlled, particularly at the beginning of the experiment, as the temperature inside the fluid cell will steadily increase by the action of the laser heating until it stabilizes. Withdraw and approach with the motor (or retract the piezoelectric positioner) each time the position in z is corrected, to avoid breaking the cantilever.
18. On completing the experiment, retract the piezoelectric positioner and withdraw it with the motor. Restore the force and position offsets (button "Zero") before switching off the controlling programs.
19. Switch off all the electronics, the laser, and the oscilloscope.

3.4. Data Analysis

1. The data must be analyzed peak by peak, setting the *zero* position as close as possible to the *zero* measured by Igor.
2. The adjustable parameters that can be modified to best fit the curve to the WLC are the total length, p, and the ΔL_c.
3. Create a table and record in the analysis procedure all the following parameters for every force peak: p, F_u, ΔL_c, total length of the molecule, and extension at which the force peak appears.
4. Once the table is complete, a histogram for any variable can be constructed, and it can be normalized and fitted to any function of interest (Gaussian, Log-Normal, etc.).

   The protocol above is suitable for the SMFS analysis of proteins with defined folds, the mechanical properties of which are usually fixed (6). However, analyzing IDPs is not that simple particularly when they display conformational polymorphism (e.g., amyloidogenic IDPs like neurotoxic proteins), as these proteins do not exhibit a single and reproducible mechanical feature ("signature" or "fingerprint") in force-extension recordings but rather, multiple mechanical events. In order to characterize and unambiguously quantify such polymorphisms, we designed the carrier-guest strategy (15, 16).
5. Select good single-molecule IDP recordings (see Note 4). The following criteria must be applied in order to select good single-molecule IDP recordings when using this strategy for the nanomechanical analysis of IDPs with conformational polymorphism:
   (a) The spacer present in the pFS-2 (N2B fragment) unfolds without detectable mechanical resistance and will appear in the proximal region of the force-extension recordings

(~70 nm if the polyprotein is being pulled from its termini). This allows us to avoid the noisy proximal region of the force-extension spectra. As such, any recording where the putative IDP force peak appears in this region is discarded (Fig. 1d).

(b) The recording should show several (less than 6) equally spaced force peaks as markers, attributable to the unfolding of the ubiquitin repeats present in the pFS-2 vector, the F_u and ΔL_c of which are characteristic and well described in the literature: $F \approx 200$ pN and $\Delta L_c \approx 23$ nm (22).

(c) The polyprotein should not exhibit more force peaks than the number expected based on the construction of the pFS-2 protein (excluding those derived from the unfolding of the IDP due to its mechanical plasticity).

(d) The total length of the unfolded molecule should not be greater than that of the extended polypeptide (considering a gain in length of 0.4 nm per stretched amino acid; (12)).

(e) In the carrier-guest strategy, the guest IDP is "force hidden" inside the carrier module (Fig. 1b) and therefore, the force peak originated from the unfolding of the carrier module should always precede (although not necessarily immediately) the force peaks corresponding to the unfolding of the grafted IDP (Fig. 1d).

(f) Any force peak that appears at an extension shorter than that corresponding to the complete unfolding of the carrier module (29.5 nm for the carrier I27 and 25.5 nm for the carrier ubiquitin) is excluded from our analyses as, in principle, it may originate from spurious interactions between the IDP and the carrier module.

(g) Only the force data with an ΔL_c value that, when summed, coincides exactly with the ΔL_c of the carrier-guest construction are included in our analyses. In the specific case of I27 + Sup35NM, the latter value is 29.5 nm from the unfolding of the carrier I27 ("a" in Fig. 1d) and 101 nm from the stretching of the grafted Sup35NM ("b" and "c" in Fig. 1d). This ensures an exact measurement of the expected ΔL_c to detect the force events, and allows these events to be observed far from the problematic proximal region of the force-extension recordings. Alternatively, if a classical hetero-polyprotein strategy (10) were used, in which the protein of interest is placed in series with the repeats of the marker, many of the force events of the protein of interest could be hidden by nonspecific interactions between the tip and the sample (particularly those lower than the mechanical stability of the markers), introducing false positive data (contamination) into the analyses.

4. Notes

1. As the carrier-guest strategy described here for unequivocal SMFS of neurotoxic proteins implies the insertion of these proteins into a carrier, several structural controls for the carrier-guest protein should be performed in order to rule out the possibility that artifactual effects may occur in and between both proteins (e.g., ^{1}H monodimensional nuclear magnetic resonance or circular dichroism), which could induce structural changes in the grafted neurotoxic protein. Furthermore, controls must be included to ensure that the neurotoxic protein maintains its amyloidogenic properties when hosted in the carrier (16), such as amyloid aggregation (turbidometry, congo Red or thioflavin binding assays) and fibrillogenesis (imaging AFM or transmission electron microscopy).
2. The specific configuration of our custom-made AFM is protected by an international patent (PCT/ES2008/070130) licensed to Nanotec Electrónica S.L. while the use of pFS vectors is protected by another international patent (PCT/ES2011/070867). The procedures for data acquisition and data analysis were modified (for connecting with Dulcinea control unit) from those originally developed by Prof. Julio M. Fernández (http://fernandezlab.biology.columbia.edu).
3. The protocol described in this section is specific for the experiments performed using our AFM setup. As mentioned above, our AFM combines three electronics: the AFM controller, the piezoelectric sensors and the high-voltage unit, which itself can be used also to control the AFM. To that end, several command lines were added to the original custom-made software written in Igor to allow crosstalk with WSxM (18, 19).
4. The procedure for refolding (used as a control for unwanted interactions) includes the following steps: unfolding without detachment, a series of cycles of limited approach/extension (to relax and unfold the same molecule away from the substrate several times), and the final complete stretching. We perform it using a home-made procedure.

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