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TITLE:

Covalent Attachment of Single Molecules for AFM-based Force Spectroscopy

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SUMMARY:

Covalent attachment of probe molecules to atomic force microscopy (AFM) cantilever tips is an essential technique for the investigation of their physical properties. This allows us to determine the stretching force, desorption force and length of polymers via AFM-based single molecule force spectroscopy with high reproducibility.

ABSTRACT:

Atomic force microscopy (AFM)-based single molecule force spectroscopy is an ideal tool for investigating the interactions between a single polymer and surfaces. For a true single molecule experiment, covalent attachment of the probe molecule is essential because only then can hundreds of force-extension traces with one and the same single molecule be obtained. Many traces are in turn necessary to prove that a single molecule alone is probed. Additionally, passivation is crucial for preventing unwanted interactions between the single probe molecule and the AFM cantilever tip as well as between the AFM cantilever tip and the underlying surface. The functionalization protocol presented here is reliable and can easily be applied to a variety of polymers. Characteristic single molecule events (i.e., stretches and plateaus) are detected in the force-extension traces. From these events, physical parameters such as stretching force, desorption force and desorption length can be obtained. This is particularly important for the precise investigation of stimuli-responsive systems at the single molecule level. As exemplary

systems poly(ethylene glycol) (PEG), poly(*N*-isopropylacrylamide) (PNiPAM) and polystyrene (PS) are stretched and desorbed from SiO_x (for PEG and PNiPAM) and from hydrophobic self-assembled monolayer surfaces (for PS) in aqueous environment.

INTRODUCTION:

Since its invention in the 1980s[1], the atomic force microscope (AFM) has become one of the most important imaging techniques in natural science featuring sub-nanometer spatial resolution, sub-piconewton force resolution and the possibility of measuring in various solvent and temperature conditions[2-7].

Apart from imaging[8,9], AFM is used to perform single molecule force spectroscopy (SMFS) giving insight into adhesive interactions between a single polymer and surfaces, physical properties of single polymers and unfolding mechanisms of proteins[7,10-16]. In a regular SMFS experiment, the functionalized cantilever tip is brought into contact with surfaces so that the polymer at the cantilever tip physisorbs to this surface. By retracting the AFM cantilever tip from the surface, a change in the deflection of the AFM cantilever is converted into a force leading to a force-extension curve[4]. Physical parameters such as stretching force, desorption force and desorption length can be determined as dependent on different parameters such as pulling velocity, dwell time on surface, indentation depth into the surface, temperature, solvent[17,18] and different surfaces like solid substrates, polymer films or supported lipid bilayers[19-22]Klicken oder tippen Sie hier, um Text einzugeben.. Furthermore, a polymer can be probed in different spatial directions thus investigating the frictional properties of the polymer[23-26].

A covalent attachment of the investigated polymer to an AFM cantilever tip is essential for such studies. Thus, a high yield of single molecule events with one and the same polymer bound to an AFM cantilever tip prevents any bias of the results due to calibration of the spring constant of the AFM cantilever[27,28], varying attachment points[29] or varying polymers (with different contour lengths) such as in the case of nanofishing experiments[30–32]. Also, interactions with other polymers as well as averaging effects can be widely prevented[18,28]. For the covalent attachment of a polymer to the AFM cantilever tip, different types of chemical modifications can be applied, many of which are summarized in the book by Hermanson[33]. Amine and thiol-based linking reactions as well as click chemistry represent the most commonly used methods in AFM cantilever tip functionalization[34–42]. Becke et al.[40] show how to use 1-ethyl-3-(3-dimethylaminopropyl)carbodiimid (EDC)/NHS chemistry to attach a protein to an AFM cantilever tip. However, the said functional groups tend to crosslink, thus leading to a loss of functionality[43,44]. Also, carbodiimides show a tendency to fast hydrolysis in solution[43]. Maleimide and thiol groups are generally more stable and do not show crosslinking reactions. The presented protocol is an optimization of the previously published protocols given in references [35,39].

Here, a reliable functionalization protocol is presented that can be easily adjusted to a large number of different polymers, independently of properties such as contour length or hydrophobicity. Three different polymers were chosen by way of example: hydrophilic polyethylene glycol (PEG) and poly(*N*-isopropylacrylamide) (PNiPAM) as well as high molar mass hydrophobic polystyrene (PS). In order to provide for a covalent binding capability with the

appropriate linker molecule the three polymers were selected for featuring a telechelic thiol moiety as functional end group. The linker molecule itself is typically a short PEG polymer with two active sites, a silane group at one end and a maleimide group at the other end. The former enables a covalent attachment to the AFM cantilever tip and the latter a binding reaction with the thiol group of the functionalized high molar mass polymer. Furthermore, inactive PEG linker molecules serve as a passivation layer to prevent unwanted interactions between the probe polymer and the AFM cantilever tip as well as between the AFM cantilever tip and the underlying surface.

PROTOCOL:

NOTE: See **Figure 1** for a schematic overview.

1. Reagent setup

NOTE: The polymers used for this protocol are: maleimide-polyethylene glycol-triethoxysilane (silane-PEG-mal, 5 kDa), thiol-polyethylene glycol-thiol (HS-PEG-SH, 35 kDa), thiol terminated poly(*N*-isopropylacrylamide) (PNiPAM-SH, 637 kDa) and thiol terminated polystyrene (PS-SH, 1.3 mDa).

1.1. Prepare the well-defined and high molar mass PNiPAM-SH via atom transfer radical polymerization, followed by conversion and reduction of the functional end group for the introduction of a thiol moiety, as described in the literature[18]. Please see **Figure 1** for the detailed structures.

1.2. For storing of the chemicals, prepare smaller aliquots within a dry glovebox system with nitrogen atmosphere to avoid exposure to atmospheric oxygen and moisture. PEG and PNiPAM are hygroscopic[45,46] and the functional end groups of PEG, PNiPAM and PS are known to become easily oxidized when stored at ambient conditions[33,47,48]. All chemicals have to be stored at -20 °C.

1.3. Use analytical grade solvents or higher. Moreover, use ultrapure water to rinse AFM cantilevers and glassware because single molecule experiments are very sensitive to all contamination.

2. Equipment Setup

NOTE: Use tweezers and beakers made of stainless steel or glass. Use inverted tweezers for a safe grip (e.g., model R3 SA having a low spring constant).

2.1. Prepare RCA (ultrapure water, hydrogen peroxide and ammonia (5:1:1)) solution to clean glassware and tweezers.

2.2. Put the vessels in a beaker and fill it with RCA until glassware or tweezers are fully covered.

2.3. Heat the beaker from step 2.2 for 1 h at 80 °C.

2.4. Rinse the vessels subsequently with ultrapure water until no pungent smell is ascertainable anymore (at least three times).

2.5. Dry glassware and tweezers in an oven (120 °C).

3. Tip functionalization

NOTE: Perform all steps in a hood to avoid inhalation of organic vapors. Additionally, gloves, lab coat and eye protection are required. Use nitrile or latex gloves for every step to avoid contamination. Wear solvent resistant gloves when using toluene. All steps, unless specified otherwise, are done at RT. Use fresh equipment and gloves for every step to avoid possible cross-contamination.

3.1. Perform surface activation by applying oxygen plasma to the AFM cantilever chip MLCT-Bio-DC.

NOTE: The efficiency of the plasma treatment for further functionalization steps scales with the content of oxygen in the plasma chamber.

3.1.1 Use freshly cleaned tweezers to place AFM cantilever chips in a plasma chamber (40 kHz, 600 W).

3.1.2. Use custom-modified activation program: evacuation (0.1 mbar) – flooding with oxygen to a pressure of: 0.2 mbar (4 min) – plasma process (power: 40%, duration: 2 min, process pressure: 0.2 mbar).

3.1.3. Ventilate chamber and carry on with step 3.2.2 immediately in order to prevent any adsorption of contaminants to AFM cantilever chips from air.

3.2. Silanization and PEGylation

NOTE: Timing is a critical parameter between the steps. Prepare solutions as fresh as possible during the waiting times. Maleimide groups are subject to hydrolysis in aqueous media and thiols easily become oxidized to disulfides in solution[33,47] impeding AFM tip functionalization reactions.

3.2.1. Prepare 6 mL of silane-PEG-mal solution in toluene (1.25 mg/mL) in two flat Petri dishes, 3 mL each.

NOTE: If binding of multiple probe polymers is observed in the SMFS experiment, mixing silane-PEG-mal with non-functional silane-PEG can reduce the number of anchoring points. For the adjustment of the passivation layer PEG with different masses (i.e., contour lengths) can be used[27].

3.2.2. Incubate the AFM cantilever chips immediately after step 3.1.3 in the silane-PEG-mal solution (5 chips per Petri dish) for 3 h at 60 °C[35].

3.2.3. Take Petri dishes out of the oven and let the solution cool down for at least 10 min.

3.2.4. Rinse each AFM cantilever chip carefully.

3.2.4.1. For PEG and PS polymers, rinse three times with toluene.

3.2.4.2. For PNiPAM polymer, rinse once with toluene and twice with ethanol.

3.2.5. Choose at least two cantilever chips as control cantilever chips, skipping step 3.3 and rinse them as follows to increase the polarity of the solvent:

3.2.5.1. For PEG and PS polymers, rinse twice with ethanol and once with water.

3.2.5.2. For PNiPAM polymer, rinse twice with water.

NOTE: Control AFM cantilever chips have gone through all functionalization steps except the polymer attachment (step 3.3). They serve to prove the cleanliness of the functionalization process, the AFM cantilever chip holder system, the surfaces and the solvents used for the SMFS experiment.

3.3. Covalent polymer attachment

NOTE: Even though the AFM cantilever is expected to be completely covered with maleimide groups, there are just a few binding sites for the single probe polymer, because maleimide undergoes hydrolysis in water leading to inactive PEGs[47]. These inactive PEGs act as a passivation layer, as described above.

3.3.1. Incubate AFM cantilever directly after step 3.2.5 in one of the following polymer solutions in 3 mL Petri dishes. If the respective polymer is not dissolved properly, use a 40 °C water bath and stir the solution well.

NOTE: As the use of thiol terminated polymers might lead to the formation of disulfide bonds hampering the reaction with the maleimide groups of silane-PEG-mal, a reducing agent is recommended, in particular if step 3.3 is applied in aqueous buffers for water soluble polymers[33].

3.3.1.1. For PEG and PS polymers, use a concentration of 1.25 mg/mL in toluene for 1 h at 60 °C.

3.3.1.2. For PNiPAM polymer, use a concentration of 1.25 mg/mL in ethanol for 3 h at RT.

NOTE: If binding of multiple probe polymers is observed in the SMFS experiment, the concentration of the polymer should be reduced.

3.3.2. Carefully rinse each AFM cantilever chip.

3.3.2.1. For PEG and PS polymers, rinse twice with toluene, twice with ethanol and once with ultrapure water after a 10 min cooldown.

3.3.2.2. For PNiPAM polymer, rinse twice with ethanol and twice with ultrapure water.

3.3.3. Store each AFM cantilever chip separately in a small (1 mL) Petri dish filled with ultrapure water at 4 °C until use in experiment.

4. Surface preparation

4.1. Silicon oxide wafer

NOTE: This surface was used for SMFS with PEG and PNiPAM.

4.1.1. Cut a silicon oxide wafer in small pieces using a diamond knife.

4.1.2. Put the silicon oxide pieces separately in microcentrifuge tubes and fill these tubes with ethanol.

4.1.3. Sonicate the silicon oxide pieces for 10 min.

4.1.4. Rinse the silicon oxide pieces with ethanol twice and dry them under a nitrogen flow carefully. Use the silicon oxide pieces immediately.

4.2. Self-assembled monolayer of hydrophobic alkane thiol on gold (SAM)

NOTE: This surface was used for SMFS with PS. See literature[39,49] for more information about SAMs.

4.2.1. Use gold-coated silicon wafer (A [100], 5 nm titanium, 100 nm gold) to perform steps 4.1.1 – 4.1.4.

4.2.2. Incubate the surface pieces in a 1-dodecanthiol solution (2 mM) for 18 h.

4.2.3. Rinse the freshly prepared SAMs in ethanol twice.

4.2.4. Dry SAMs with nitrogen flow for direct use or store them in ethanol for up to 4 days.

5. Data acquisition

NOTE: All measurements shown here were performed in ultrapure water with a Cypher ES AFM using a heating and cooling sample stage for temperature variation. Generally, all AFMs providing the capability to measure in liquids can be used.

5.1. Insert the AFM cantilever chip into the AFM.

5.2. Glue the prepared surface into a sample holder that is suitable for measuring in liquids (e.g., High Resolution Replicating Compound 101RF).

NOTE: This bonding agent is highly inert and resistant to a large number of polar solvents. Nonpolar solvents (e.g., toluene or hexane) and high temperatures should be avoided. In these cases, an UV curable adhesive can be used.

5.3. Immerse the AFM cantilever and the probe sample in the liquid, here: ultrapure water.

NOTE: A solvent drop (about 100 µL) can be deposited on the AFM cantilever chip holder. Covering the AFM cantilever chip with solvent reduces capillary forces, which would otherwise act on the AFM cantilever when approaching the sample surface passing through the air-solvent interface.

5.4. If required, adjust the temperature and let the system equilibrate.

NOTE: Temperature changes may result in a deflection of the AFM cantilever due to a bimetallic effect for AFM cantilevers with a reflective coating like aluminum or gold. Equilibration should be performed away from the surface (several µm) until no further change of the deflection signal is observed (up to 15 min for MLCT-Bio-DC).

5.5. Vary the temperature randomly to exclude any effects of ageing of the functionalization. Make sure that the temperatures applied do not lead to an irreversible bending of the AFM cantilever.

NOTE: Any temperature effects on solvent properties (such as evaporation or changes in viscosity) might hamper your experiments. In the presented examples, the temperature was varied over a range of up to 40 K in steps of 10 K taking water as a solvent (e.g., from 278 K to 318 K).

5.6. Approach the surface and determine the InvOLS (inverse optical lever sensitivity) by taking force-extension curves on a hard surface (such as silicon oxide). For this, take the deflection signal of the photodetector (in V) vs piezo distance and determine the slope of the part representing the indentation of the AFM cantilever tip into the underlying surface (repulsive regime) using a linear function. In order to reduce errors, take the average of at least five values as final InvOLS value. For further details, see the literature [4,39].

NOTE: The InvOLS can only be reliably determined on hard surfaces. In the case of experiments on soft surfaces or interfaces make sure you place a hard surface close to your soft surfaces. Then, the InvOLS calibration can be done before or after your soft surface experiments without the need of disassembling the AFM setup.

5.7. For spring constant determination, move the AFM cantilever to a height with neither attractive nor repulsive interactions between AFM cantilever tip and surface (several µm). Then, take a thermal noise spectrum where the power spectral density (PSD) vs frequency is plotted. The following steps are usually performed by automated built-in functions in commercial AFM software: first, the acquired thermal noise spectrum is analyzed by fitting a function to the PSD, e.g., a simple harmonic oscillator (SHO). The fit is done up to the minimum between the first and second resonance. Second, the area under the fitted part of the PSD vs frequency plot is determined representing the mean square displacement of the AFM cantilever in vertical direction. Finally, the equipartition theorem is used to obtain the AFM cantilever force constant[28,50].

NOTE: An appropriate frequency range should be used comprising the first resonance peak of the AFM cantilever. To get a satisfactory signal-to-noise ratio, at least 10 PSDs should be accumulated with the highest possible frequency resolution.

5.8. Start the experiment. Record force maps by scanning the surface in grid-like fashion (e.g., 10 x 10 points for an area of 20 x 20 μm^2) to avoid any surface spot specific effects (e.g., impurities, dislocations) and to average different surface areas.

NOTE: Typical parameters are a velocity of 1 µm/s and a sampling rate of 5 kHz to ensure sufficient resolution. The sampling rate should be adapted when the velocity is varied. The force distance should be adapted to the contour or desorption length of the measured polymer (approx. twice the expected length).

5.9. Use and vary the dwell time towards the surface to allow the single polymer to adhere to the surface (typically 0 – 5 s).

5.10. Repeat the determination of the cantilevers InvOLS and spring constant to ensure the calibration values at the end of the experiment.

NOTE: For strong adhesion between polymer and surface, the calibration can be done after the actual experiment to preserve the functionalization.

6. Data evaluation

NOTE: For data evaluation, a custom-written software based on Igor Pro was used for performing the following steps.

6.1. Convert the raw deflection signal (in Volts) into force values (in Newtons) by multiplication with the recorded InvOLS and the determined spring constant.

6.2. Subtract the deflection of the AFM cantilever (after multiplication of the raw deflection signal with the InvOLS) from the distance driven by the piezo elements in vertical direction in order to obtain the true extension (tip-surface distance)[4].

6.3. Correct the force-distance curves obtained for drift by fitting a linear function to the baseline after the last event and subtracting the same from the force-distance curve. The fitted part should represent a sufficient distance from the surface where neither attractive nor repulsive interactions are observed between AFM cantilever tip and underlying surface. Then, the baseline is set to the zero-axis.

NOTE: In the case of measurements on highly reflective surfaces like gold, interferences might appear. These result from partial reflection of the laser beam from the surface and from the backside of the AFM cantilever. So, the obtained force-extension curves might show a sinusoidal force signal artifact along the vertical extension. This is an artifact that hampers the final force values. In order to still take these force-extension curves into account, a correction is possible (**Figure 2**).

6.4. If the interferences appear in the force-extension curves, select a representative force-extension curve (retraction curve) showing no other events than a peak of unspecific adhesion and the same sinusoidal artifact (i.e., amplitude and phase) (**Figure 2A**).

NOTE: Smooth the representative force-extension curve in order to obtain the low frequency pattern of the interference.

6.5. Select a force-extension curve that is to be corrected (**Figure 2B**).

6.6. Overlay both force-extension curves from steps 6.4. and 6.5. to make sure that both show the same sinusoidal artifact (i.e., amplitude and phase) (**Figure 2C**).

6.7. Subtract the (smoothed) representative force-extension curve from the force-extension curve to be corrected leading to a straight rather than a sinusoidal baseline (**Figure 2D**).

NOTE: Take care that the unspecific adhesion peak of the representative curve is distinct from any single molecule events appearing in the force-extension curves to be corrected. In fact, the selection of the representative curve is crucial for a proper correction.

REPRESENTATIVE RESULTS:

The following examples show results of single molecule stretching and desorption of the polymers PEG, PNiPAM and PS. All AFM cantilever tips were functionalized with the protocol given above. PEG and PNiPAM were measured on SiO_x with temperature variation. For a detailed discussion of the resulting temperature-dependent stretching curves for PEG and PNiPAM, see Kolberg et al.[18]

A different force-extension motif is a plateau of constant force (e.g., when desorbing PS from self-assembled monolayers of methyl terminated alkane thiols on gold (SAM) in water[4,27,39,51]).

Example 1: Stretching of PEG and PNiPAM in water

The temperature-dependent stretching behavior in water was measured using single PNiPAM and PEG polymers covalently bound to an AFM cantilever tip at one end and physisorbed on a SiO_x surface at the other end. After the calibration and clean control experiments (less than 2% of the force-extension curves show single molecule events), at least two force maps were recorded for each AFM cantilever. The temperature-dependent experiment was performed by recording at least one force map at each temperature. When only few stretching events appeared, the respective AFM cantilever was discarded and the next AFM cantilever of the chip was taken (usually in the order C, B, D and E of MLCT-Bio-DC). For the exemplary data of PEG, a single stretching event was observed in 95 out of 500 measured force-extension curves (19%). For PNiPAM, 252 out of 600 force-extension curves showed a stretching pattern (42%). For a better comparison of the force-extension curves, a single master curve for every temperature was generated. For this purpose, only those curves with a stretching event to at least 500 pN, where conformational fluctuations and solvent effects are negligible, were chosen[52]. The final number of stretches taken into account was 3 at 278 K, 7 at 298 K and 4 at 318 K for PEG and 4 at 278 K, 3 at 298 K and 3 at 318 K for PNiPAM[18].

The procedure for generating master curves is given in **Figure 3.** The force-extension curves chosen (**Figure 3A**) are rescaled to a length L_0 (extension at a force of 500 pN), see **Figure 3B**. The adhesion peak shows a large variation of unspecific adhesion between the surface and the AFM cantilever tip, but does not influence the polymer stretching behavior. After merging the rescaled force-extension curves they are averaged by a binominal smoothing as presented in **Figure 3C**. For this, a Gaussian filter convolves the data with normalized coefficients derived from Pascal's triangle at a level equal to the smoothing parameter 20[53]. Finally, a master curve was obtained for every temperature as given in **Figure 3D**. The zoom-in shows the range where the temperature effect on the force-extension behavior is most pronounced.

A comparison of the temperature behavior of PEG (**A**) and PNiPAM (**B**) can be found in **Figure 4**. For PEG a decrease of the stretching force with increasing temperature is observed. An increase of approximately 5% of rescaled extension at 100 pN is observed when increasing the temperature from 278 to 318 K. For PNiPAM, an opposite temperature-dependent shift can be revealed. A decrease of approximately 1% of rescaled extension at 100 pN is observed when the temperature is increased from 278 to 328 K. Additionally, the stretching free energy can be obtained from the force-extension master curves by determining the area under the curve for any given force value. This can be used for extracting energetic and entropic contributions of the stretching free energy with the help of molecular dynamics (MD) simulations[18].

Example 2: Desorption of PS from a SAM surface in water

The desorption of PS from a SAM surface in water can be used to determine the desorption force and length and thereby quantify the hydrophobic interaction. After calibration, at least two force maps were recorded at two different spots of the surface. When the polymer attachment is

successful, the force-extension curves show plateaus of constant force, as characteristic feature, see **Figure 5A** and **Figure 5C**. Plateau-like desorption is observed when the dynamics of the probed bonds are much faster than the pulling rate of the AFM cantilever tip (quasi-equilibrium). Desorption forces of plateau-like force-extension curves directly provide adhesion free energies by integrating the force-extension trace[54]. They have been used to determine electrostatic, dispersive and hydrophobic interactions as well as friction properties of single polymers on surfaces in liquid environment[2,4,23,51,54,55].

Each plateau of constant force was fitted with a sigmoidal curve to determine the desorption force and desorption length, which were then plotted in histograms. The histograms were fitted with a Gaussian to extract the maximum value and standard deviation. For a better overview, the desorption force and length values were displayed together in a scatter plot, as given in **Figure 5B** and **Figure 5D**.

For polystyrene on SAM in water, the determined desorption forces correspond to previously obtained values[19,23]. As the desorption length correlates with the polymer contour length[51], the desorption length distribution can be used as a proof of the covalent binding of the respective polymer to the AFM cantilever tip. Thus, the desorption length serves as a fingerprint.

For more than one polymer attached to the AFM cantilever tip, cascades of plateaus (discrete steps) are observed in the force-extension curves[56]. Each plateau represents the desorption of a polymer at a different extension. The experiment given in **Figure 5C** and **Figure 5D** shows a typical case of two polymers attached to the cantilever tip at the same time. By fitting the final rupture, a bimodal distribution can be found for the desorption length, while the desorption force shows a narrow distribution. In this case, the smaller desorption length could be found in 90% of the force-extension curves, either as a single plateau or as an additional plateau on the longer plateau, as shown in **Figure 5C**. The higher desorption length was found in 37% of the obtained force-extension curves. Thus, the desorption length distribution can be used to determine the number of different polymers attached to the AFM cantilever tip. Furthermore, a narrow distribution of the desorption length values is a good indication that one and the same single polymer was probed in the obtained force-extension curves. At the same time, a superposition of the respective forces-extension can be used to decide whether one and the same single polymer was measured.

After this experiment, further experiments with the PS polymer can be performed varying substrate (solid surface as well as polymer films), solvent conditions, temperature, velocity or dwell time.

FIGURES AND TABLE LEGENDS:

Figure 1: Schematic overview of the tip functionalization process. Includes the chemical modification of the AFM cantilever tip after (**1**) plasma activation (**2**) silanization/PEGylation and (**3**) polymer attachment. Additionally, the detailed chemical structures of the polymers used, namely PEG, PNiPAM and PS are shown.

Figure 2: Elimination of interferences in force-extension curves. (**A**) Find a force-extension curve showing a sinusoidal force signal artifact along the extension but having no single molecule stretching event. (**B**) Choose a force-extension curve with a single molecule event, which is to be corrected from the sinusoidal artifact. (**C**) Superimpose the curves to control if the sinusoidal artifacts of the curves really match. (**D**) By subtracting the force-extension curve (**A**) from (**B**) a force-extension curve with a straight baseline is obtained. Although the adhesion peak cannot be used for further analysis, the force-extension curve is now corrected for the artifact leading to much more accurate force values in the region of the single molecule event (here: > 0.2 µm of extension).

Figure 3: Determination of master curves from force-extension curves of PEG at 298 K. (**A**) Experimental data at 298 K, using 7 force-extension curves. After rescaling to a length L_0 at a force of 500 pN (**B**), the force-extension curves can be merged and averaged by binominal smoothing obtaining a master curve (**C**). The rescaled curves are given as dots while the master curve is shown as a solid line. Finally, the obtained master curves for different temperatures can be compared (**D**). The zoom-in indicates the range where the temperature effect on the force-extension behavior is most pronounced.

Figure 4: Comparison of the temperature-dependent master curves of PNiPAM and PEG. For PEG an increase of rescaled extension at 100 pN (mid-force range) is observed when increasing the temperature (**A**), while for PNiPAM an opposite temperature-dependent shift is revealed (**B**).

Figure 5: Analysis of force-extension curves of PS on SAM in water. (**A**) Exemplary force-extension curve (blue) with a sigmoidal fit of the plateau (purple). Additionally, the arrows mark the determined force (red) and length (green) of the plateau. The desorption force and desorption length values obtained by sigmoidal fits are displayed in a scatter plot and the resulting histograms are fitted with a Gaussian. (**B**) The determined average desorption force and desorption length values are (112 ± 6) pN and (659 ± 7) nm, wherein 93% of the force-extension curves show such single plateau events. (**C**) Exemplary force-extension curve (blue) for two polymers attached to the cantilever tip at the same time. Here, the desorption force shows a unimodal distribution with an average force value of (117 ± 5) pN, while a bimodal distribution can be found for the desorption length values leading to average length values of (656 ± 9) nm and (1050 ± 16) nm. (**D**) 90% of the sampled force-extension curves show only single plateau events.

DISCUSSION:

AFM-based single molecule force spectroscopy is one of the major tools for investigating single molecule interactions in polymer physics. For a true single molecule experiment, covalent attachment of the probe polymer to an AFM cantilever tip is essential.

Many previous works are based on nanofishing experiments, in particular for PNiPAM, where polymers are adsorbed onto a surface and then stretched by randomly picking them from the substrate using an AFM cantilever tip[30,31]. This might alter the results and lead to misinterpretation of the single molecule behavior. There, cooperative effects might dominate the results because interactions with neighboring polymers cannot be excluded. This has a large impact on the results, especially for polymers that show significantly different behavior in bulk compared to single isolated molecules[57,58].

The functionalization protocol presented here is reliable and can be easily applied to different polymers, independently of their contour length, hydrophobicity or the steric hindrance of the monomers. Additionally, a passivation is provided to prevent unwanted interactions between the single probe polymer and the AFM cantilever tip as well as between the AFM cantilever tip and the underlying surface. Furthermore, the evaluation of force-extension curves showing stretching events is shown. There, a procedure is proposed for the determination of master force-extension curves. This offers a better means of revealing (e.g., temperature-related effects on the force-extension behavior). Furthermore, the analysis of single molecule desorption events featuring constant force plateaus is provided. Also, a simple way of correcting sinusoidal force signal artifacts in force-extension curves is given which might otherwise impair the results of the experiment.

Compared to Stetter et al.[39], the functionalization procedure presented here is reduced to three steps instead of four and the robustness of the procedure is improved. The major benefit of performing PEGylation and silanization in one step is to have a better-controlled reaction and to increase the yield. Furthermore, fewer solutions need to be prepared and fewer rinsing steps are required. This reduces the effort and time for preparation and increases the reproducibility. Furthermore, moving AFM cantilevers is always a critical part of the functionalization process. A transfer from one solution to the other always runs the risk of strongly influencing the functionalization quality due to transfers through the air-water interface or of losing AFM cantilevers by improper use of tweezers.

In order to prove proper covalent attachment of a single polymer to an AFM cantilever tip different conditions have to be met. First, control AFM cantilevers are of significant importance and should be prepared for every functionalization. The functionalization process and the fluid cell for performing the experiments are only considered to be clean, if a small number of force-extension curves show stretches or plateaus in the control experiment (in the presented examples less than 2%).

A clear stretching pattern with no further drops or maxima is essential for having proper single molecule stretching events. Additionally, the dependence of rupture force on the force loading

rate at rupture or the complete elastic response of the stretching curve should be analyzed in order to exclude simultaneous desorption of multiple polymers[59,60]. For PEG and PNiPAM, 19% and 42% of the force-extension curves taken at different positions of the surface show such a stretching pattern, respectively. In order to obtain stretching events, the physisorption of the polymer to the respective underlying surface must be strong. Otherwise a plateau-like desorption event is observed. This is even more decisive for the detection of stretching events at high forces (up to 500 pN or more). As this strong physisorption is not met for every force-extension curve, the yield of such events is less than for pure plateau-like desorption events. As an alternative, strongly adhering groups such as catechols or chemisorption between polymer and underlying surface can be used. However, this requires the introduction of further functional groups or coupling sites at the polymer[61,62].

In fact, the mass (i.e., contour length) of the polymer provides a valuable fingerprint. Although the mass cannot directly be translated into the measured contour length for the following reasons, the length distribution is very valuable to define single-molecule events. In the case of a PNiPAM polymer with a low polydispersity ($Đ$ = 1.28), we found significant differences in the extension values for the obtained stretching events (and thus in the polymer length) in the experiments. One reason for this could be the determination of the polymer length and its distribution. In size-exclusion chromatography (SEC), a relative weight of the target polymer is determined in comparison with standards like PS or poly (methyl methacrylate) (PMMA)[63]. The presumed relative weight is expected to deviate from the absolute molecular weight because the hydrodynamic radius of the target polymer and the standard can significantly differ. Additionally, the silane layer might be oligomerized by spurious water in toluene during the functionalization process. The attachment of such oligomers to the AFM cantilever tip leads to a more flexible layer with fewer anchor points[64]. Also, the attachment point of the polymer to the silicon layer might not necessarily be at the apex leading to a shift of the detected length values[29]. While a polymer model such as the wormlike chain (WLC) or the freely jointed chain (FJC) model cannot reproduce the respective force-extension behavior for PEG or PNiPAM properly over the entire extension range[18,29,41,65,66], such a polymer model might be valuable for other polymeric and protein systems [10,15,67,68].

The covalent attachment of a single PS polymer (with a contour length of more than 1 µm) is only considered to be successful, when a considerable number of force-extension curves show a long enough plateau of constant force (**Figure 5**). A plateau resulting from desorbing a single polymer is defined by a single sharp drop of a constant force to the baseline at a certain extension, as given in **Figure 5A**. If more polymers are attached to the AFM cantilever tip, a cascade of plateaus is observed[56] (**Figure 5C**). The plateau length (desorption length), correlating with the polymer contour length[51], has to be significantly longer than any adhesion peak due to unspecific adhesion of the AFM cantilever tip to the underlying surface (here around 200 nm). Features appearing solely in a single force-extension curve, should not be interpreted. In the presented experiments, at least 80 out of 100 curves showed a plateau longer than 200 nm in at least two force maps at two different spots on the surface. Furthermore, the distribution of desorption lengths, using scatter plots such as given in **Figure 5B** and **5D**, reveal if and how many polymers are bound to the AFM cantilever tip. In the case of PS, a narrow distribution of desorption force and length

taken from plateaus of the force-extension curves serves as an evidence of a successful covalent attachment. This finally proves the success of the functionalization protocol. Thus, we strongly recommend to present such force and length distributions in publications.

Evaluating force-extension curves using built-in algorithms that comprise many pre-set parameters should be done with care. Reasons are for example that a fixed sampling rate is not appropriate for every applied pulling velocity or that an automated smoothing of the force-extension curves might average out important details. Usually a proper understanding of the respective evaluation procedure can prevent errors in the evaluation procedure, which can in turn strongly influence the final findings of an AFM-based force spectroscopy experiment.

In summary, we present a functionalization protocol that is reliable and can be easily applied to a variety of polymers. Furthermore, proper evaluation of single molecule force-extension curves is presented, allowing the determination of physical parameters such as stretching force, desorption force and desorption length. The presented protocols and procedures are valuable for the investigation of stimuli-responsive systems at the single molecule level.

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DISCLOSURES:

The authors declare that they have no competing financial interest.

REFERENCES:

(1) Binnig, G., Quate, C. F. Gerber, C. Atomic Force Microscope. *Physical Review Letters*. **56** (9), 930–933 (1986).

(2) Hugel, T. Seitz, M. The Study of Molecular Interactions by AFM Force Spectroscopy. *Macromolecular Rapid Communications*. **22** (13), 989–1016 (2001).

(3) Butt, H.-J., Cappella, B. Kappl, M. Force measurements with the atomic force microscope: Technique, interpretation and applications. *Surface Science Reports*. **59** (1-6), 1–152 (2005).

(4) Balzer, B. N. Hugel, T. Single-Molecule Detection and Manipulation. In *Reference Module in Materials Science and Materials Engineering*; Hashmi, S., Ed.; Elsevier: Amsterdam (2016).

(5) Krieg, M. et al. Atomic force microscopy-based mechanobiology. *Nature Reviews Physics*. **1** (1), 41–57 (2019).

(6) Edwards, D. T., Faulk, J. K., LeBlanc, M.-A. Perkins, T. T. Force Spectroscopy with 9-μs Resolution and Sub-pN Stability by Tailoring AFM Cantilever Geometry. *Biophysical journal*. **113** (12), 2595–2600 (2017).

(7) Alsteens, D. et al. Nanomechanical mapping of first binding steps of a virus to animal cells. *Nature Nanotechnology*. **12** (2), 177–183 (2017).

(8) Kodera, N., Yamamoto, D., Ishikawa, R. Ando, T. Video imaging of walking myosin V by high-speed atomic force microscopy. *Nature*. **468**, 72–76 (2010).

(9) Shibata, M. et al. Real-space and real-time dynamics of CRISPR-Cas9 visualized by high-speed atomic force microscopy. *Nature Communications*. **8** (1), 1–9 (2017).

(10) Rief, M., Gautel, M., Oesterhelt, F., Fernandez, J. M. Gaub, H. E. Reversible unfolding of individual titin immunoglobulin domains by AFM. *Science*. **276** (5315), 1109–1112 (1997).

(11) Oesterhelt, F., Oesterhelt, D., Pfeiffer, M., Engel, A., Gaub, H. E. Müller, D. J. Unfolding pathways of individual bacteriorhodopsins. *Science (New York, N.Y.)*. **288** (5463), 143–146 (2000).

(12) Hugel, T., Holland, N. B., Cattani, A., Moroder, L., Seitz, M. Gaub, H. E. Single-molecule optomechanical cycle. *Science (New York, N.Y.)*. **296** (5570), 1103–1106 (2002).

(13) Yu, H., Siewny, M. G. W., Edwards, D. T., Sanders, A. W. Perkins, T. T. Hidden dynamics in the unfolding of individual bacteriorhodopsin proteins. *Science (New York, N.Y.)*. **355** (6328), 945–950 (2017).

(14) Erlich, K. R., Sedlak, S. M., Jobst, M. A., Milles, L. F. Gaub, H. E. DNA-free directed assembly in single-molecule cut-and-paste. *Nanoscale*. **11** (2), 407–411 (2019).

(15) Rico, F., Russek, A., González, L., Grubmüller, H. Scheuring, S. Heterogeneous and rate-dependent streptavidin-biotin unbinding revealed by high-speed force spectroscopy and atomistic simulations. *Proceedings of the National Academy of Sciences of the United States of America*. **116** (14), 6594–6601 (2019).

(16) Löf, A. et al. Multiplexed protein force spectroscopy reveals equilibrium protein folding dynamics and the low-force response of von Willebrand factor. *Proceedings of the National Academy of Sciences of the United States of America*. **116** (38), 18798–18807 (2019).

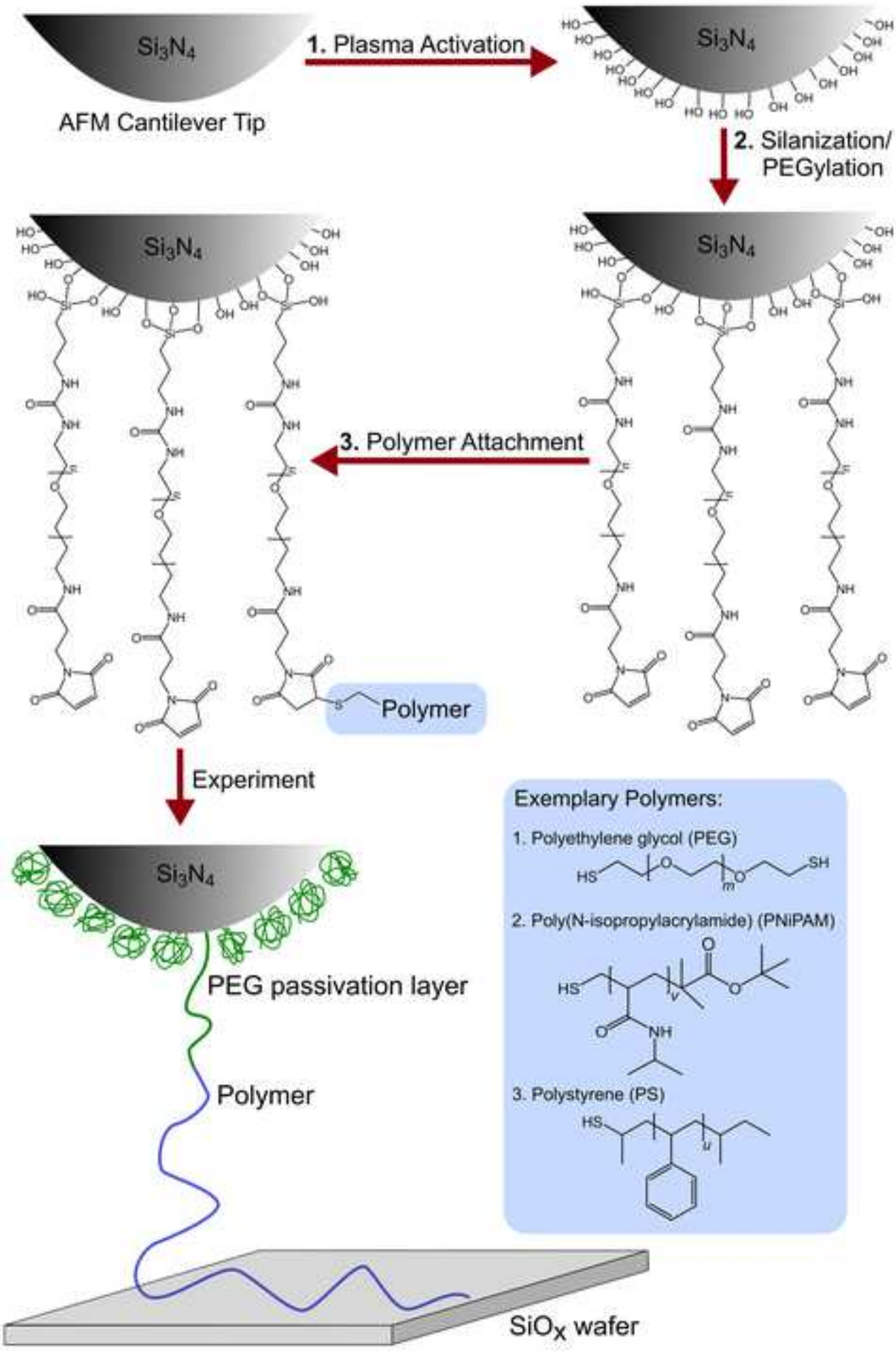
(17) Kienle, S., Liese, S., Schwierz, N., Netz, R. R. Hugel, T. The effect of temperature on single-polypeptide adsorption. *Chemphyschem : a European journal of chemical physics and physical chemistry*. **13** (4), 982–989 (2012).

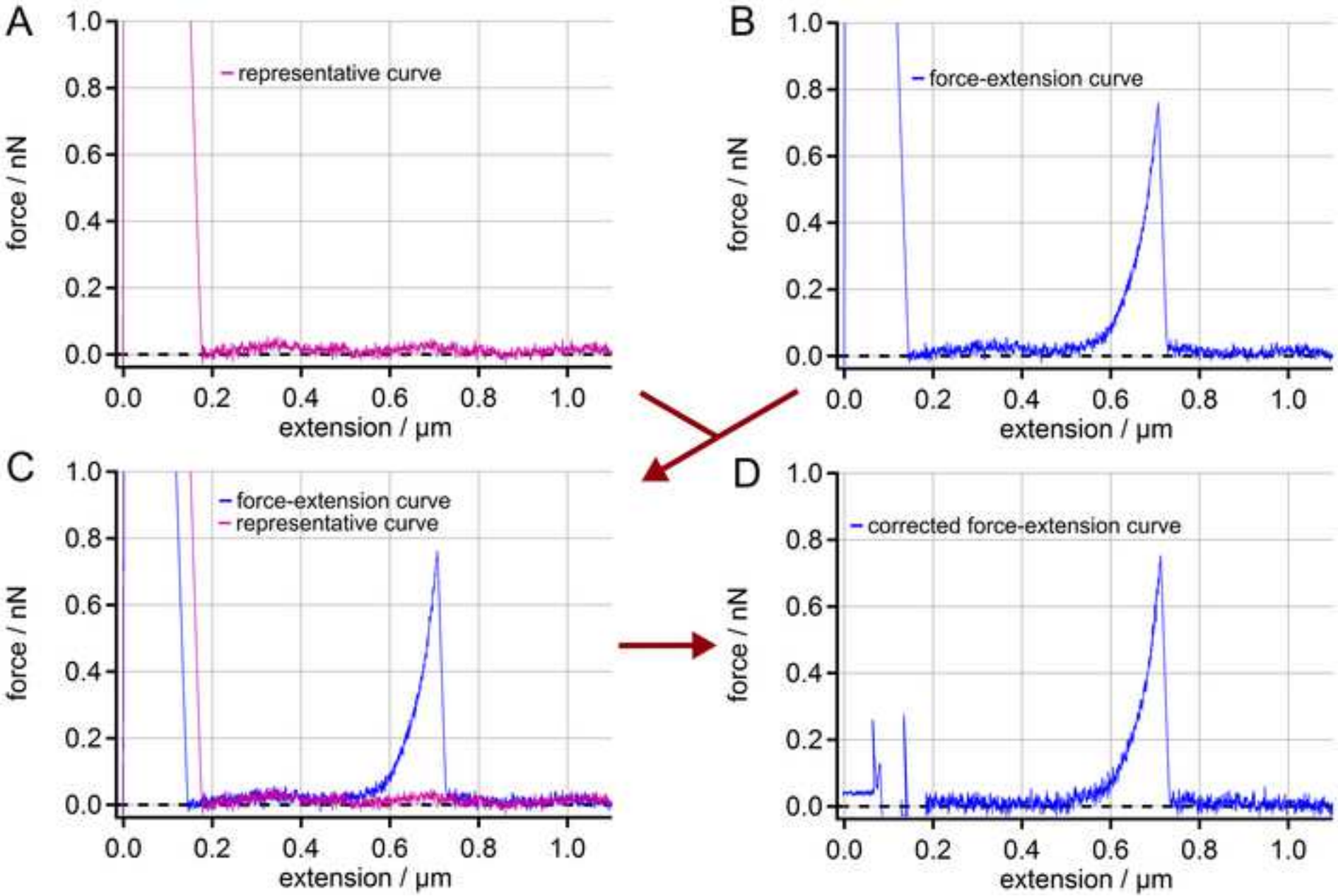
(18) Kolberg, A. et al. Opposing Temperature Dependence of the Stretching Response of Single PEG and PNiPAM Polymers. *Journal of the American Chemical Society*. **141** (29), 11603–11613 (2019).

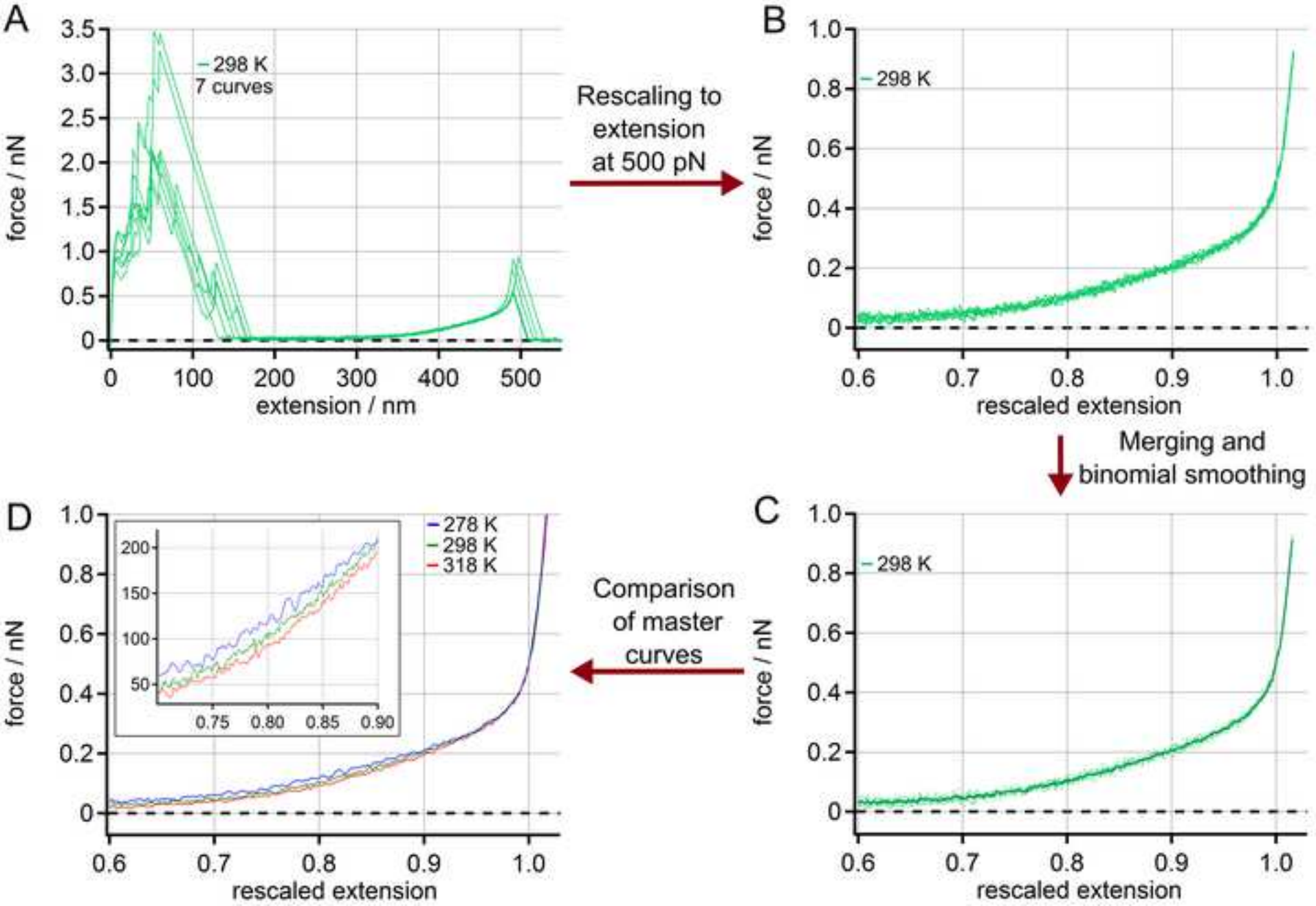
(19) Balzer, B. N. et al. Cohesion Mechanisms of Polystyrene-Based Thin Polymer Films. *Macromolecules*. **46** (18), 7406–7414 (2013).
(20) Balzer, B. N. et al. Adhesion property profiles of supported thin polymer films. *ACS Applied Materials & Interfaces*. **5** (13), 6300–6306 (2013).
(21) Stetter, F. W.S., Cwiklik, L., Jungwirth, P. Hugel, T. Single Lipid Extraction: The Anchoring Strength of Cholesterol in Liquid-Ordered and Liquid-Disordered Phases. *Biophysical /journal*. **107** (5), 1167–1175 (2014).
(22) Schwierz, N., Krysiak, S., Hugel, T. Zacharias, M. Mechanism of Reversible Peptide-Bilayer Attachment: Combined Simulation and Experimental Single-Molecule Study. *Langmuir*. **32** (3), 810–821 (2016).
(23) Balzer, B. N. et al. Nanoscale Friction Mechanisms at Solid–Liquid Interfaces. *Angewandte Chemie International Edition*. **52** (25), 6541–6544 (2013).
(24) Balzer, B. N., Kienle, S., Gallei, M., Klitzing, R. von, Rehahn, M. Hugel, T. Stick-Slip Mechanisms at the Nanoscale. *Soft Materials*. **12**, 106-114 (2014).
(25) Kühner, F., Erdmann, M., Sonnenberg, L., Serr, A., Morfill, J. Gaub, H. E. Friction of single polymers at surfaces. *Langmuir*. **22** (26), 11180–11186 (2006).
(26) Grebíková, L., Gojzewski, H., Kieviet, B. D., Klein Gunnewiek, M. Vancso, G. J. Pulling angle-dependent force microscopy. *The Review of Scientific Instruments*. **88** (3), 33705 (2017).
(27) Geisler, M. et al. Hydrophobic and Hofmeister effects on the adhesion of spider silk proteins onto solid substrates: an AFM-based single-molecule study. *Langmuir*. **24** (4), 1350–1355 (2008).
(28) Pirzer, T. Hugel, T. Atomic force microscopy spring constant determination in viscous liquids. *Review of Scientific Instruments*. **80** (3), 35110 (2009).
(29) Liese, S. et al. Hydration Effects Turn a Highly Stretched Polymer from an Entropic into an Energetic Spring. *ACS Nano*. **11** (1), 702–712 (2017).
(30) Cui, S., Pang, X., Zhang, S., Yu, Y., Ma, H. Zhang, X. Unexpected Temperature-Dependent Single Chain Mechanics of Poly(N-isopropyl-acrylamide) in Water. *Langmuir*. **28** (11), 5151–5157 (2012).
(31) Liang, X. Nakajima, K. Nanofishing of a Single Polymer Chain: Temperature-Induced Coil-Globule Transition of Poly(N -isopropylacrylamide) Chain in Water. *Macromolecular Chemistry and Physics*. **219** (3), 1700394 (2018).
(32) Zhang, W., Zou, S., Wang, C. Zhang, X. Single Polymer Chain Elongation of Poly(N -isopropylacrylamide) and Poly(acrylamide) by Atomic Force Microscopy. *The Journal of Physical Chemistry B*. **104** (44), 10258–10264 (2000).
(33) Hermanson, G. T. *Bioconjugate techniques - 3rd Edition.* Academic Press: San Diego (2013).
(34) Leitner, M. et al. Single-molecule AFM characterization of individual chemically tagged DNA tetrahedra. *ACS Nano*. **5** (9), 7048–7054 (2011).
(35) Walder, R. et al. Rapid Characterization of a Mechanically Labile α-Helical Protein Enabled by Efficient Site-Specific Bioconjugation. *Journal of the American Chemical Society*. **139** (29), 9867–9875 (2017).
(36) Tang, J. et al. High-affinity tags fused to s-layer proteins probed by atomic force microscopy. *Langmuir*. **24** (4), 1324–1329 (2008).
(37) Wildling, L. et al. Linking of sensor molecules with amino groups to amino-functionalized AFM tips. *Bioconjugate Chemistry*. **22** (6), 1239–1248 (2011).

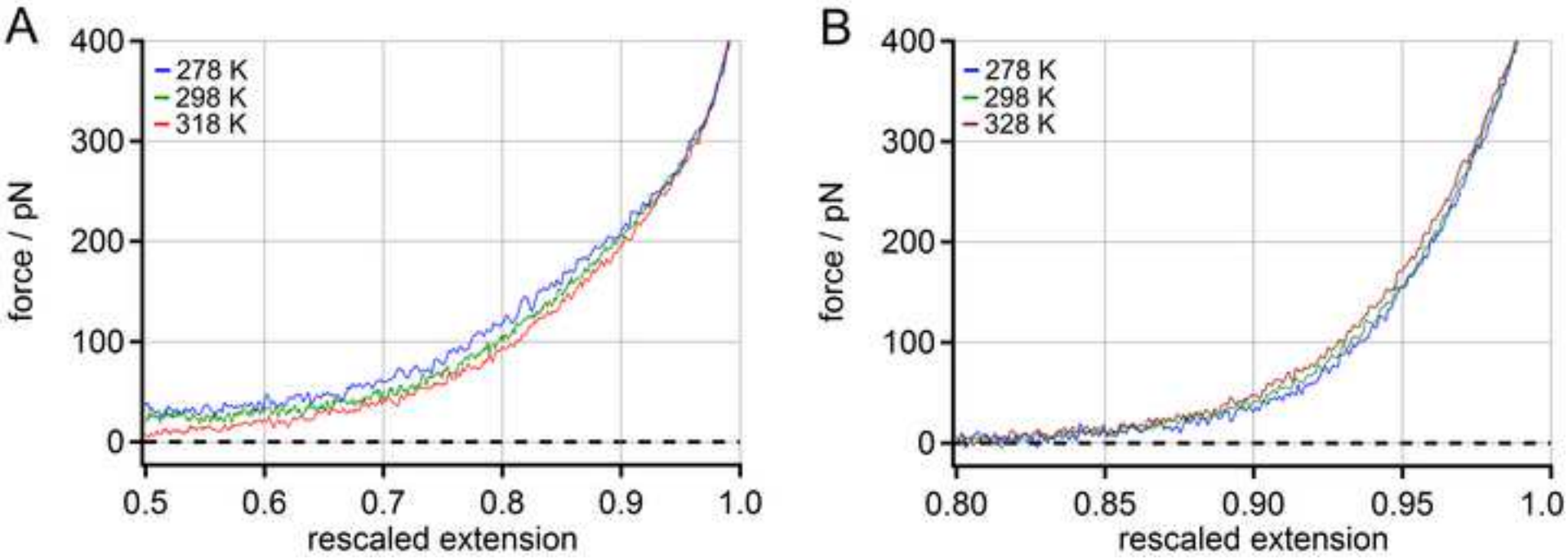
(38) Maity, S., Viazovkina, E., Gall, A. Lyubchenko, Y. A Metal-free Click Chemistry Approach for the Assembly and Probing of Biomolecules. *Journal of Nature and Science*. **2** (4), e187 (2016).
(39) Stetter, F. W. S., Kienle, S., Krysiak, S. Hugel, T. Investigating Single Molecule Adhesion by Atomic Force Spectroscopy. *Journal of Visualized Experiments* (96), e52456 (2015).
(40) Becke, T. D. et al. Covalent Immobilization of Proteins for the Single Molecule Force Spectroscopy. *Journal of Visualized Experiments* (138), e58167 (2018).
(41) Ott, W. et al. Elastin-like Polypeptide Linkers for Single-Molecule Force Spectroscopy. *ACS Nano*. **11** (6), 6346–6354 (2017).
(42) Newton, R. et al. Combining confocal and atomic force microscopy to quantify single-virus binding to mammalian cell surfaces. *Nature Protocols*. **12** (11), 2275–2292 (2017).
(43) Staros, J. V., Wright, R. W. Swingle, D. M. Enhancement by N-hydroxysulfosuccinimide of water-soluble carbodiimide-mediated coupling reactions. *Analytical Biochemistry*. **156** (1), 220–222 (1986).
(44) Grabarek, Z. Gergely, J. Zero-length crosslinking procedure with the use of active esters. *Analytical Biochemistry*. **185** (1), 131–135 (1990).
(45) Baird, J. A., Olayo-Valles, R., Rinaldi, C. Taylor, L. S. Effect of Molecular Weight, Temperature, and Additives on the Moisture Sorption Properties of Polyethylene Glycol. *Journal of Pharmaceutical Sciences*. **99** (1), 154–168 (2010).
(46) Halperin, A., Kröger, M. Winnik, F. M. Poly(N-isopropylacrylamide) Phase Diagrams: Fifty Years of Research. *Angewandte Chemie International Edition*. **54** (51), 15342–15367 (2015).
(47) Barradas, R. G., Fletcher, S. Porter, J. D. The hydrolysis of maleimide in alkaline solution. *Canadian Journal of Chemistry*. **54** (9), 1400–1404 (1976).
(48) Kharasch, N., Tarbell, D. S. Chapter 10 - The Mechanism of Oxidation of Thiols to Disulfides. In *Organic Sulfur Compounds*, 97–102 (1961).
(49) Folkers, J. P., Laibinis, P. E. Whitesides, G. M. Self-assembled monolayers of alkanethiols on gold: comparisons of monolayers containing mixtures of short- and long-chain constituents with methyl and hydroxymethyl terminal groups. *Langmuir*. **8** (5), 1330–1341 (1992).
(50) Hutter, J. L. Bechhoefer, J. Calibration of atomic-force microscope tips. *Review of Scientific Instruments*. **64** (7), 1868–1873 (1998).
(51) Krysiak, S., Liese, S., Netz, R. R. Hugel, T. Peptide desorption kinetics from single molecule force spectroscopy studies. *Journal of the American Chemical Society*. **136** (2), 688–697 (2014).
(52) Hugel, T., Rief, M., Seitz, M., Gaub, H. E. Netz, R. R. Highly Stretched Single Polymers: Atomic-Force-Microscope Experiments Versus *Ab-Initio* Theory. *Physical Review Letters*. **94** (4), 48301 (2005).
(53) Marchand, P. Marmet, L. Binomial smoothing filter: A way to avoid some pitfalls of least-squares polynomial smoothing. *Review of Scientific Instruments*. **54** (8), 1034–1041 (1983).
(54) Horinek, D. et al. Peptide adsorption on a hydrophobic surface results from an interplay of solvation, surface, and intrapeptide forces. *Proceedings of the National Academy of Sciences of the United States of America*. **105** (8), 2842–2847 (2008).
(55) Friedsam, C., Gaub, H. E. Netz, R. R. Adsorption energies of single charged polymers. *EPL (Europhysics Letters)*. **72** (5), 844–850 (2005).
(56) Scherer, A., Zhou, C., Michaelis, J., Brauchle, C. Zumbusch, A. Intermolecular Interactions of Polymer Molecules Determined by Single-Molecule Force Spectroscopy. *Macromolecules*. **38** (23), 9821–9825 (2005).

(57) Abbott, L. J., Tucker, A. K. Stevens, M. J. Single Chain Structure of a Poly(N-isopropylacrylamide) Surfactant in Water. *The Journal of Physical Chemistry B*. **119** (9), 3837–3845 (2015).
(58) Okano, T., Bae, Y. H., Jacobs, H. Kim, S. W. Thermally on-off switching polymers for drug permeation and release. *Journal of Controlled Release*. **11** (1), 255–265 (1990).
(59) Sulchek, T., Friddle, R. W. Noy, A. Strength of multiple parallel biological bonds. *Biophysical Journal*. **90** (12), 4686–4691 (2006).
(60) Sulchek, T. A. et al. Dynamic force spectroscopy of parallel individual Mucin1-antibody bonds. *Proceedings of the National Academy of Sciences of the United States of America*. **102** (46), 16638–16643 (2005).
(61) Krysiak, S., Wei, Q., Rischka, K., Hartwig, A., Haag, R. Hugel, T. Adsorption mechanism and valency of catechol-functionalized hyperbranched polyglycerols. *Beilstein Journal of Organic Chemistry*. **11**, 828–836 (2015).
(62) Jobst, M. A., Schoeler, C., Malinowska, K. Nash, M. A. Investigating receptor-ligand systems of the cellulosome with AFM-based single-molecule force spectroscopy. *Journal of Visualized Experiments* (82), e50950 (2013).
(63) Trathnigg, B. Determination of MWD and chemical composition of polymers by chromatographic techniques. *Progress in Polymer Science*. **20** (4), 615–650 (1995).
(64) Blass, J., Albrecht, M., Wenz, G., Zang, Y. N. Bennewitz, R. Single-molecule force spectroscopy of fast reversible bonds. *Physical Chemistry Chemical Physics.* **19** (7), 5239–5245 (2017).
(65) Oesterhelt, F., Rief, M. Gaub, H. E. Single molecule force spectroscopy by AFM indicates helical structure of poly(ethylene-glycol) in water. *New Journal of Physics*. **1**, 6.1 - 6.11 (1999).
(66) Xue, Y., Li, X., Li, H. Zhang, W. Quantifying thiol-gold interactions towards the efficient strength control. *Nature Communications*. **5**, 4348 (2014).
(67) Lyu, X., Song, Y., Feng, W. Zhang, W. Direct Observation of Single-Molecule Stick–Slip Motion in Polyamide Single Crystals. *ACS Macro Letters*. **7** (6), 762–766 (2018).
(68) Hugel, T. et al. Elasticity of Single Polyelectrolyte Chains and Their Desorption from Solid Supports Studied by AFM Based Single Molecule Force Spectroscopy. *Macromolecules*. **34** (4), 1039–1047 (2001).









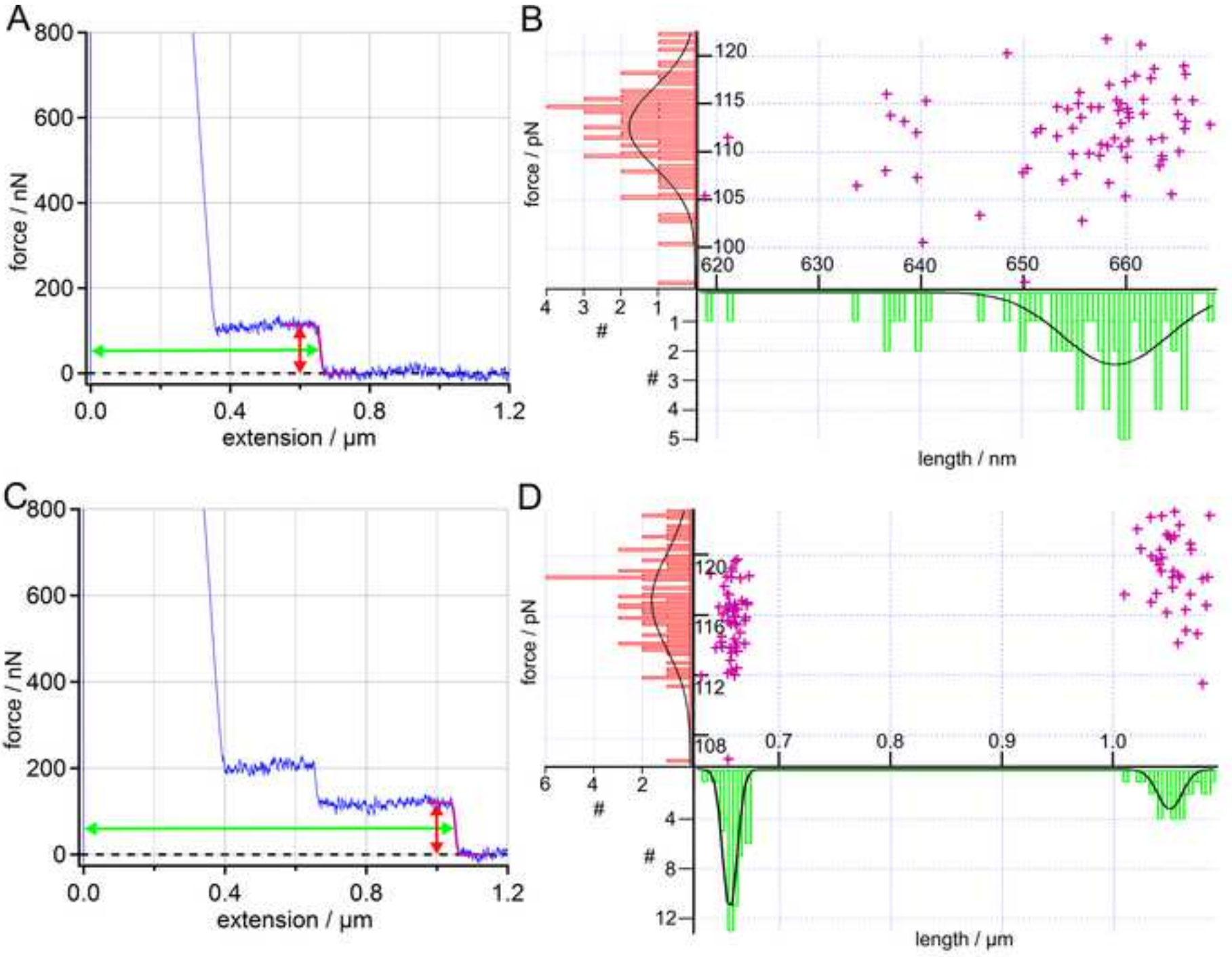


Figure 1


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Figure 2

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Figure 3

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Figure 4

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Figure 5 revised

Table of Materials

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Name of Material/ Equipment
1-Dodecanethiol (≥98%)
Ammonia solution (30%)
Cypher ES
Ethanol (≥99.9%)
Gold coated silicon wafer
High Resolution Replicating Compound
Hydrogen peroxide solution
Igor Pro
Tetra-30-LF-PC
Maleimide-polyethylene glycol-triethoxysilane
MLCT-Bio-DC
Prime CZ-Si wafer, n-type (Phosphor) TTV < 10 µm
Purelab Chorus 1, 18.2 MΩ cm
R3 SA
Thiol terminated poly(*N* -isopropylacrylamide)
Thiol terminated polystyrene
Thiol-polyethylene glycol-thiol
Toluene (99.99%)

Company	Catalog Number
Sigma-Aldrich, USA	417364-500ML
Roth, Germany	CP17.2
Asylum Research, an Oxford Instruments company, USA	-
Roth, Germany	PO76.1
Fraunhofer Institute for Applied Solid State Physics IAF, Germa	-
Microset Products Ltd, UK	101RF
Sigma-Aldrich, USA	H1009
Wavemetrics, USA	-
Diener Electronic, Germany	-
Creative PEG works, USA	PHB-1923
Bruker, USA	MLCT-Bio-DC
MicroChemicals, Germany	WSA40600250 P1314SNN1
Elga LabWater, Germany	10034-540
Vomm GmbH, Germany	5803 Blank
Gallei Group, Saarland University, Germany	-
Polymer Source, Canada	P40722-SSH
Creative PEGWorks, USA	PSB-615
Fisher Chemicals	T324-500

Comments/Description
Used for SAM
Used for cleaning
AFM
Solvent
Used for SAM
Bonding agent
Used for cleaning
Software environment
Plasma chamber
Linker polymer
AFM cantilever
Silicon wafer
Ultrapure water source
Tweezers
PNiPAM probe polymer
PS probe polymer
PEG probe polymer
Solvent

Dear Dr. Nguyen,

thank you for the helpful comments on our manuscript "Covalent Attachment of Single Molecules for AFM based Force Spectroscopy". We have rewritten, clarified and streamlined several parts of the manuscript in response to the editorial and reviewers' comments. In addition, we have optimized some representation of the data for better visualization, in particular Figure 5. We believe that we could address every single concern as detailed in the point by point response (see below).

None of the figures has been previously used for publication. The manuscript has been proofread by a native speaker.

We hope that this revised manuscript is acceptable for publication in JoVE and we are looking forward to your response.

Kind regards,

B.N. Balzer

Point by point response to **reviewer comments**

Reviewer #1

The manuscript by Kolberg et al presents a progress in sample preparing for single-molecule AFM. They use a short PEG linker for further attachment of long polymers. The manuscript can be accepted after revision.

We thank the reviewer for acknowledging our manuscript.

1. What is the exact structure of the silane end group for maleimide-polyethylene glycol-silane?

The term "silane" has been changed to "triethoxysilane" in the manuscript and in the material list for a better description of the end group. It reads now:
"The polymers used for this protocol are: maleimide-polyethylene glycol-triethoxysilane (silane-PEG-mal, 5 kDa), […]" (cf. lines 104ff.).
The exact chemical structure of the silane-based linkers to a hydroxy-activated substrate could be represented as mixture of different alkoxy- and siloxane moieties, as previously investigated by using different spectroscopic methods (see the literature: Gutmann et al., Dynamic Nuclear Polarization Signal Amplification as a Sensitive Probe for Specific Functionalization of Complex Paper Substrates, The Journal of Physical Chemistry C, 121, 3896-3903, 2017). In brief, both anchoring to the substrate via siloxane-chemistry and the presence of intermolecular –O-Si-O- bonds between the linkers are possible. Accordingly, the detailed structure of maleimide-polyethylene glycol-triethoxysilane is given in ***Figure 1****.*

2. The authors used oxygen for the plasma treatment. It will be better if the performance of plasma treatment using air is provided.

Oxygen plasma treatment is used to introduce hydroxyl groups to the AFM cantilever tips surface, which consists of Si_3N_4, with a high degree of coverage to increase the efficiency of further chemical reactions. The oxidation of silicon nitride surfaces in this way is described in the literature as a reliable procedure (cf. C. Jimenez et al. Surface and Coatings Technology ***25****, 147-154, 1991). Furthermore, our experience in the past 15 years has shown that a high oxygen content for the plasma process leads to a more successful functionalization process. Thus, we added the following to note to step 3.1.:*
"The efficiency of the plasma treatment for further functionalization steps scales with the content of oxygen in the plasma chamber." (cf. lines 150f.).

3. From Fig 3A, one can see that many force signals appear before 150 nm, which can be assigned to multiple chains. Thus, it is important to control the surface density of the attached

polymers. What can be done for this? This is very important since the authors claim that in other studies "cooperative effects might dominate the results because interactions to neighboring polymers cannot be excluded".

The surface density of the attached polymers can be controlled by mixing functional and non-functional linker molecules. Additionally, the concentration of the target polymer (e.g., PS, PEG or PNiPAM) can be varied. The following notes to step 3.2.1. and step 3.3.1.2. account for these aspects:
"If binding of multiple probe polymers is observed in the SMFS experiment, mixing silane-PEG-mal with non-functional silane-PEG can reduce the number of anchoring points." (cf. lines 173ff.) and "If binding of multiple probe polymers is observed in the SMFS experiment, the concentration of the polymer should be reduced" (cf. lines 223f.).

Reviewer #2:

Manuscript Summary:

In their MS entitled "Covalent Attachment of Single Molecules for AFM based Force Spectroscopy", Adrianna Kolberg and coauthors describe a profound and detailed chemistry for specific surface functionalization for single molecule force AFM experiments on polymers. Additionally they give convincing examples of their results obtained from those substrates and strategies to intrinsically monitor the quality of the experiment and the functionalization.

We thank the reviewer for acknowledging our manuscript.

Major Concerns: None

Minor Concerns:

1. The MS is basically written well, but please check for miss-spellings:

P6 L171 "question" -> acquisition?, P8 L248 "you" -> your? L285 "are" -> a and carefully check the chosen words: P13 L432ff "This reduces ... reproducibility" ???

The mentioned miss-spellings have been corrected and the manuscript was proofread by a native speaker.

A few points might be worth to be revised as well:

2. P8 §6: In case of tidy polymers the calibration of the cantilever possibly might be better after the experiment in order to keep the functionalization of the apex in good shape?

We thank the reviewer for the very valuable comment and added the following note to step 5.10.: "For strong adhesion between polymer and surface, the calibration can be done after the actual experiment to preserve the functionalization." (cf. lines 348f.)

3. P13 L429 to what refers the term "the procedure" to?

For clarification, the term "the procedure" has been changed to "functionalization procedure".

4. L438 what exactly are "control AFM cantilevers"? can you by those? are they treated in a specific way...?

The term "control AFM cantilevers" refers to cantilevers that went through all steps of functionalization (including the linker system) except the binding of the respective target polymer (e.g., PS, PEG, PNiPAM). This has been clarified by adding a note to step 3.2.5.: "Control AFM cantilever chips have gone through all functionalization steps except the polymer attachment (step 3.3). They serve to prove the cleanliness of the functionalization process, the

AFM cantilever chip holder system, the surfaces and the solvents used for the SMFS experiment." (cf. lines 196ff.)

5. L439 ff This sentence is rather misleading: do the authors evaluate only from a total of 2% of force curves all their experiments and discard from those even further the nonspecific ones? Or should this describe a control experiment without any functionalization of the cantilever? Please clarify.

This sentence refers to control experiments. All force-extension curves of the respective experiment with the target polymer have been evaluated. The criterion for cleanliness is the following: if less than 2% of the evaluated curves from the control cantilever show events (such as plateaus or stretching events) the control AFM cantilevers are considered as clean. For clarification "in the control experiment" was added:
"The functionalization process and the fluid cell for performing the experiments are only considered to be clean, if a small number of force-extension curves show stretches or plateaus in the control experiment (in the presented examples less than 2%)." (cf. lines 549ff.).

6. P14 L447 please specify or quantify "simple"

The word "simple" has been deleted for clarification.

7. Fig. 3D and 4A and P13 427 fff: how could a master curve as the blue one show such a pronounced undulation on top of the vertical displacement due to the temperature? This must be either a highly reproducible and interesting feature of the polymer itself -since it results from overlaying several curves- or is this just an artifact of the substraction of a "representative" goldlayer-interference pattern curve?

In the latter case I strongly suggest to either substract "master" goldlayer-interference pattern curves or strongly smoothed or a fitted pattern that represents only the low frequency pattern of the interference rather than the full noise and speckle patterns of a single "representative" force curve. Please re-think the deconvolution of the gold surface in particular for experiments with "weak" force patterns or steps close to the noise level...

*We did not apply the interference correction for the curves given in **Figures 3** and **4**. Thus, the undulation on top of the blue master curve does not result from any type of interference pattern or the respective correction algorithm. Nevertheless, thank you very much for your suggestion, because at the same time, introducing a smoothing step for the representative force-extension curve has proven to be very valuable. Thus, we added a note to step 6.4. revised step 6.7.:*
"Smooth the representative force-extension curve in order to obtain the low frequency pattern of the interference." (cf. lines 380f.). and "Subtract the (smoothed) representative force-extension […]" (cf. lines 388f.).

Reviewer #3:

Manuscript Summary:

The authors describe a methodology to link polymers to AFM tips and describe their approach to executing single molecule pulling experiments and data analysis. Their linking chemistry is based on common moieties used for decades now, albeit they emphasize that their approach saves a step. I don't see any issue with the chemistry method as is. However, there are a number of issues that arise when reading the single-molecule pulling portions of the paper. I suggest major revisions.

We thank the reviewer for the comments and rewrote parts of the manuscript according to the reviewers' suggestions. In addition, we like to emphasize that our approach does not only save a step, but is also more robust than previously published approaches. Although some changes might look very minor, altogether these improvements help a lot for stably linking polymers to AFM tips.

Major Concerns:

1. Page 7-8: The steps used to perform a single molecule AFM experiment have been described in far greater detail in several methods/review papers in the literature. Someone reading this manuscript would not be able to carry out the experiments unless, for example, they were also using a machine that automatically calculates the invOLS and spring constant (many systems do not, particularly older models). The authors should either elaborate on these topics, or clearly point to some of the more complete SMFS methodology papers which provide the reader with deeper context and step-by-step explanations accompanied with figures.

We have revised the description for the determination of the InvOLS and spring constant. It does now give a deeper theoretical justification and cites literature that describes both processes in full detail. Steps 5.6. and 5.7. read now:

"Approach the surface and determine the InvOLS (inverse optical lever sensitivity) by taking force-extension curves on a hard surface (such as silicon oxide). For this, take the deflection signal of the photodetector (in V) vs piezo distance and determine the slope of the part representing the indentation of the AFM cantilever tip into the underlying surface (repulsive regime) using a linear function. In order to reduce errors, take the average of at least five values as final InvOLS value. For further details, see the literature [4,39].

NOTE: The InvOLS can only be reliably determined on hard surfaces. In the case of experiments on soft surfaces or interfaces make sure you place a hard surface close to your soft surfaces. Then, the InvOLS calibration can be done before or after your soft surface experiments without the need of disassembling the AFM setup." (cf. lines 306ff.)

and

"For spring constant determination, move the AFM cantilever to a height with neither attractive nor repulsive interactions between AFM cantilever tip and surface (several µm). Then, take a thermal noise spectrum where the power spectral density (PSD) vs frequency is plotted. The following steps are usually performed by automated built-in functions in

commercial AFM software: first, the acquired thermal noise spectrum is analyzed by fitting a function to the PSD, e.g., a simple harmonic oscillator (SHO). The fit is done up to the minimum between the first and second resonance. Second, the area under the fitted part of the PSD vs frequency plot is determined representing the mean square displacement of the AFM cantilever in vertical direction. Finally, the equipartition theorem is used to obtain the AFM cantilever force constant[28,50].
NOTE: An appropriate frequency range should be used comprising the first resonance peak of the AFM cantilever. To get a satisfactory signal-to-noise ratio, at least 10 PSDs should be accumulated with the highest possible frequency resolution." (cf. lines 318ff.)

2. Page 9, line 296-298: Again, a reader new to SMFS would not understand how to perform the tip-surface transformation of the data from this brief mention of a vital part of the processing. To make this method paper valuable to a novice reader, it should be stated more clearly how to find information on performing this operation.

For clarification, we have changed the order of the steps and rewritten step 6.2. (including a reference for further details) which now reads:
"Subtract the deflection of the AFM cantilever (after multiplication of the raw deflection signal with the InvOLS) from the distance driven by the piezo elements in vertical direction in order to obtain the true extension (tip-surface distance)[4]." (cf. lines 358ff.).

3. Page 9-10: The authors claim that seeing a single rupture event is evidence of a single-polymer. However, it very likely that when two polymers of similar length share a load, the rupture of the first bond can lead to a catastrophic failure of the remaining bond. This is because the second suddenly see a spike in their load force upon the breakage of the first. Given that a second bond could fail instantaneously with the first bond, it is not enough to simply state that seeing one rupture event indicates a single-polymer. Rather, a true signature of a single polymer is found from the stiffness of the force-extension trace [See Sulchek et al, Biophys J. 2006]. I suggest the authors clarify their definition of what constitutes a single bond in light of the past work.

Thank you for pointing out that we did not discuss the possibility of two polymers rupturing at the same length. This is, because we consider this scenario as very unlikely and the likelihood is further decreased with the very high resolution in extension (time) current AFMs provide. Nevertheless, we now discuss this possibility in our revised manuscript and therefore complete our definition of a true signature of a single polymer:
"A clear stretching pattern with no further drops or maxima is essential for having proper single molecule stretching events. Additionally, the dependence of rupture force on the force loading rate at rupture or the complete elastic response of the stretching curve should be analyzed in order to exclude simultaneous desorption of multiple polymer[59,60]." (cf. lines 553ff.)

4. Page 10, line 332: A note on nomenclature here. The "contour length" of a polymer defines the maximum length of the polymer at its maximum extension. This is not the same as the extension at rupture. For example, a polymer with a contour length of Lc = 500nm will rupture at a distribution of extensions before 500nm is reached. This should be revised in the text.

Thank you for pointing this out. We try to clarify what we take as the contour length in our revised manuscript. The principal problem is that usually a certain model is used to determine the contour length, i.e., the contour length values depend on the model that is used. One would obtain different contour length when using, e.g., the FJC, WLC or FRJ model to fit the force-extension curves. This becomes even more evident, once extensible versions of these models are used. Therefore, we here decided to be "model free" and therefore use the extension at 500 pN as reference length value for rescaling. In our opinion, this is the best way to compare different measurements in the presented cases. 500 pN are chosen, because then conformational fluctuations and solvent effects are negligible, cf. Hugel et al., Highly Stretched Single Polymers: Atomic-Force-Microscope Experiments versus Ab-Initio Theory. Phys. Rev. Lett.,94, 048301, 2005), see also response to point 5. To avoid confusion, we omitted the sentence:
"[…] of a polymer with a contour length of about 350 nm." (cf. line 332 of the original manuscript).

5. Page 10, line 339-347: The rescaling here is problematic. Naturally, a polymer's extension is scaled by its contour length, Lc, as defined above. All theories of polymer extension follow a dependence on the ratio x/Lc where x is the extension. Hence it is common in the literature to find that authors rescale polymer force-extension profiles to Lc, not an arbitrary force-extension value. Why do the authors not use any of the myriad of polymer models on their data to get the true Lc? They state that the WLC and FJC do not fit the data, but for decades it has been known that these two simple models only fit the low-force entropic regime. The extensible versions of these models fit many polymers very well. In particular, the PEG force-extension curve is well-described by the two-state model of Oesterhelt, Rief and Gaub (New J. Phys, 1999, Equation 2). This equation has also been used by Sulchek et al (PNAS 2005, Biophys J. 2006) to characterize bond number using PEG linkers.

We appreciate the work of Oesterhelt et al. and Sulchek et al. . However, as explained in response to point 4, there is no "true" contour length. In addition, the models mentioned by the referee assume a completely entropic chain (extended with diverse features). Such a description fails for several polymers in solvents, especially for PEG (see literature: Liese et al., Hydration Effects Turn a Highly Stretched Polymer from an Entropic into an Energetic Spring. ACS Nano, 11(1), 702–712, 2017). This can for example be seen if the FJC model is investigated in detail: The Kuhn length one obtains for most (if not all) polymers in aqueous environment has no physical meaning, because it does not match the actual chemical bond length, i.e., the basis of the model. Taking a well-defined extension value corresponding to a certain force

value seems more reliable than taking a polymer model-based contour length Lc. Therefore, we decided to use the respective extension values at a force of 500 pN for rescaling, see response to point 4. We have described the above-mentioned discrepancies by the following paragraph:
"While a polymer model such as the wormlike chain (WLC) or the freely jointed chain (FJC) model cannot reproduce the respective force-extension behavior for PEG or PNiPAM properly over the entire extension range[18,29,41,65,66]*, such a polymer model might be valuable for other polymeric and protein systems* [10,15,67,68]*." (cf. lines 579ff.)*

6. Page 10, line 362-363: It is not clear why the plateau force and extension are important. What is learned from this analysis? In particular, does the force and extension of the plateau relate to the intermolecular interactions between the polymer and surface?

Plateau-like force-extension curves are observed when the polymer is desorbed from a surface in liquid environment in equilibrium on the timescale of the experiment (see literature: Hugel, T., & Seitz, M. The study of molecular interactions by AFM force spectroscopy. Macromolecular Rapid Communications, 22(13), 989–1016, 2001; Horinek et al. PNAS, 105 (8), 2842, 2008; Krysiak et al., Peptide Desorption Kinetics from Single Molecule Force Spectroscopy Studies. Journal of the American Chemical Society, 136(2), 688–697, 2014). These force-extension curves yield free energies of adsorption, equilibrium adsorbed polymer lengths and some information on friction properties. Yes, plateau forces are closely related to the intermolecular interactions between the polymer and the surface (via the adsorption free energy).
Plateau curves have been the topic of many AFM based single molecule publications. In order to give a short introduction instead of an in-depth review we added the following paragraph to the Example 2 section including literature for further understanding of plateau-like force-extension curves:
"Plateau-like desorption is observed when the dynamics of the probed bonds are much faster than the pulling rate of the AFM cantilever tip (quasi-equilibrium). Desorption forces of plateau-like force-extension curves directly provide adhesion free energies by integrating the force-extension trace[54]*. They have been used to determine electrostatic, dispersive and hydrophobic interactions as well as friction properties of single polymers on surfaces in liquid environment*[2,4,23,51,54,55]*." (cf. lines 443ff.)*

7. Page 11, line 366-367: The authors also state that this plateau can serve as a fingerprint. While this may seem likely, do the authors have any data from other polymer/substrate systems that support the idea of using this plateau as a polymer fingerprint?

This is indeed a crucial point, which we tried to explain with Example 2 and Figure 5. We rewrote this part and modified Figure 5 to further clarify that both, the plateau height and the plateau length are single polymer fingerprints. For a single plateau this will be difficult, but if several plateaus are measured, then the force distribution will show distinct values (to first approximation multiples of the lowest force plateau). The lowest force plateau corresponds

then to a single polymer event. See for example the revised Figure 5D or the following literature: Scherer et al., Intermolecular Interactions of Polymer Molecules Determined by Single-Molecule Force Spectroscopy. Macromolecules, 38(23), 9821–9825, 2005; Friedsam et al., Adsorption energies of single charged polymers, Europhys. Lett., 72(5), 844, 2005. In general, the distribution of force values can pinpoint the number of polymers desorbed at the same time. Corresponding force-extension curves show step-like force-extension curves.

The length of the plateau is another very good fingerprint because it correlates with the contour length of the polymer. This has been thoroughly discussed by the following literature: Krysiak et al., Peptide Desorption Kinetics from Single Molecule Force Spectroscopy Studies. JACS, 136(2), 688–697, 2014. For clarification, we rewrote the Example 2 section and revised ***Figure 5*** *taking comparing data sets with one (****Figure 5(A)*** *and* ***(B)****) and two different polymers bound to the AFM cantilever tip (****Figure 5(C)*** *and* ***(D****)):*

"Example 2: Desorption of PS from a SAM surface in water.

The desorption of PS from a SAM surface in water can be used to determine the desorption force and length and thereby quantify the hydrophobic interaction. After calibration, at least two force maps were recorded at two different spots of the surface. When the polymer attachment is successful, the force-extension curves show plateaus of constant force, as characteristic feature, see ***Figures 5****(****A****) and* ***5(C)****. Plateau-like desorption is observed when the dynamics of the probed bonds are much faster than the pulling rate of the AFM cantilever tip (quasi-equilibrium). Desorption forces of plateau-like force-extension curves directly provide adhesion free energies by integrating the force-extension trace[54]. They have been used to determine electrostatic, dispersive and hydrophobic interactions as well as friction properties of single polymers on surfaces in liquid environment[2,4,23,51,54,55].*

Each plateau of constant force was fitted with a sigmoidal curve to determine the desorption force and desorption length, which were then plotted in histograms. The histograms were fitted with a Gaussian to extract the maximum value and standard deviation. For a better overview, the desorption force and length values were displayed together in a scatter plot, as given in ***Figures 5****(****B****) and (****D****).*

For polystyrene on SAM in water, the determined desorption forces correspond to previously obtained values[19,23]. As the desorption length correlates with the polymer contour length[51], the desorption length distribution can be used as a proof of the covalent binding of the respective polymer to the AFM cantilever tip. Thus, the desorption length serves as a fingerprint.

For more than one polymer attached to the AFM cantilever tip, cascades of plateaus (discrete steps) are observed in the force-extension curves[56]. Each plateau represents the desorption of a polymer at a different extension. The experiment given in ***Figures 5****(****C****) and (****D****) shows a typical case of two polymers attached to the cantilever tip at the same time. By fitting the final rupture, a bimodal distribution can be found for the desorption length, while the desorption force shows a narrow distribution. In this case, the smaller desorption length could be found in 90% of the force-extension curves, either as a single plateau or as an additional plateau on the longer plateau, as shown in* ***Figure 5****(****C****). The higher desorption length was found in 37% of the obtained force-extension curves. So, the desorption length distribution can be used to determine the number of different polymers attached to the AFM cantilever tip. Furthermore,*

a narrow distribution of the desorption length values is a good indication that one and the same single polymer was probed in the obtained force-extension curves. At the same, time a superposition of the respective forces-extension can be used to decide whether one and the same single polymer was measured.
After this experiment, further experiments with the PS polymer can be performed varying, e.g., substrate (solid surface as well as polymer films), solvent conditions, temperature, velocity or dwell time." (cf. lines 438ff.).

8. Page 11, line 367-368: The authors suggest the plateau can be used as a diagnostic to prove that a single polymer is formed. This isn't clear and should be explained.

See answer to the previous comment.

Minor Concerns:

9. Figure 4 caption: The stretching "free energy" is mentioned here and in many places within the paper. However, it is not actually measured or shown anywhere. Perhaps the use of the term should be reduced, or some demonstration/discussion of measuring free energy should be performed.

Thank you for pointing this out. We have revised the manuscript by underlining the role of free energy for further analysis:
"Additionally, the stretching free energy can be obtained from the force-extension master curves by determining the area under the curve for any given force value. This can be used for extracting energetic and entropic contributions of the stretching free energy with the help of molecular dynamics (MD) simulations[18]." (cf. lines 433ff.) and "Desorption forces of plateau-like force-extension curves directly provide adhesion free energies by integrating the force-extension trace[54]." (cf. lines 445f.)