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SUPPLEMENTARY INFORMATION

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Supplementary Information for maleimide–thiol adducts stabilized through stretching

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This file includes:

Supplementary Methods

Materials

3-Mercaptopropyltrimethoxysilane (MPTMS), (3-aminopropyl)triethoxysilane (APTES), tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 11-mercaptoundecanoic acid (MUA), *N*-hydroxysuccinimide (NHS), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC), *N*-ethylmaleimide (NEM), mercaptoethanol, 4,4'-bismaleimidodiphenylmethane (bis-maleimide), triethylamine, ethylenediamine, bromothymol blue and PBS tablet were purchased from Sigma-Aldrich. Silane-PEG-Mal (MW 5000), NHS-PEG-Mal (MW 5000), NHS-PEG-NHS (MW5000), mPEG-SH (MW 5000) and mPEG-Mal (MW 5000) were purchased from NanoCS. Anhydrous acetonitrile with molecular sieves was purchased from Energy Chemical, and anhydrous dichloromethane (DCM) was distilled from CaH_2 under argon before use. Monoclonal antibody anti-Her2 trastuzumab was purchased from Efebio (Shanghai).

Preparation of thiol-decorated substrates and maleimide-functionalized AFM cantilevers

To measure the rupture forces of maleimide–thiol adducts by AFM-based single-molecule force spectroscopy (SMFS), we prepared maleimide-functionalized AFM cantilevers, which were brought into contact with the thiol-functionalized glass substrate to form thiosuccinimide in the first two AFM single-molecule experiments.

Silicon nitride (Si_3N_4) cantilevers (MLCT−D, Bruker) were first cleaned with Milli-Q water, and then placed in a chromic mixture (chromic acid) at 90 ℃ for 30 min. After that, the cantilevers were washed with water, then ethanol, and dried under a steam of nitrogen. Then the hydroxylated cantilevers were immersed in 1% (v/v) APTES in toluene for 1 h. After that, they were rinsed with toluene, then ethanol, dried under a nitrogen stream, and incubated at 90 ℃ for 30 min. Next, the cantilevers were immersed in DMSO containing 0.2 mM Mal-PEG-NHS (MW: 5000 Da) for 1 h. After being washed with DMSO, ethanol and dried under a nitrogen stream, the resulting Mal-coated cantilevers were kept dry at -20 ℃ for use in single-molecule experiments.

The glass substrates were cut into 1 × 1 cm^2 slides, soaked in a chromic mixture overnight, thoroughly washed with deionized (DI) water, ethanol and acetone successively, and then dried under a steam of nitrogen to produce surfaces with exposed hydroxyl groups. These substrates were immersed in an anhydrous toluene solution containing 0.5 % (v/v) MPTMS at room temperature (R.T.) for 1 h for sulfhydrylation. Then, they were washed with toluene and ethanol, dried under a nitrogen flow, and stored in a desiccator. The thiolated substrates were affixed to the AFM sampling chamber using an epoxy resin adhesive, and they were treated with 10 mM TCEP for 15 min and rinsed with DI water and dried under a nitrogen stream before single-molecule experiments.

Preparation of maleimide-decorated substrates and thiol-functionalized AFM cantilevers for reverse geometry of maleimide–thiol adduct

Silicon nitride (Si_3N_4) cantilevers (MLCT−D, Bruker) were soaked in a chromic mixture (chromic acid) overnight, thoroughly washed with deionized (DI) water, ethanol and acetone successively, and then dried under a steam of nitrogen to produce surfaces with exposed hydroxyl groups. These cantilevers were immersed in an anhydrous toluene solution containing 0.5 % (v/v) MPTMS at room temperature (R.T.) for 1 h for sulfhydrylation. Then, they were washed with toluene and ethanol, dried under a nitrogen flow, and stored in a desiccator. The thiolated cantilevers were treated with 10 mM TCEP for 15 min and rinsed with DI water and dried under a nitrogen stream before single-molecule experiments.

The glass substrates were cut into 1 × 1 cm^2 slides, first cleaned with DI water, and then placed in a chromic mixture (chromic acid) at 90 ℃ for 30 min. After that, the substrates were washed with water, then ethanol, and dried under a steam of nitrogen. Then the hydroxylated substrates were immersed in 1% (v/v) APTES in toluene for 1 h. After that, they were rinsed with toluene, then ethanol, dried under a nitrogen stream, and incubated at 90 ℃ for 30 min. Next, the substrates were immersed in DMSO containing 0.2 mM Mal-PEG-NHS (MW: 5000 Da) for 1 h. After being washed with DMSO, ethanol and dried under a nitrogen stream, the resulting Mal-coated substrates were affixed to the AFM sampling chamber using an epoxy resin adhesive for use in single-molecule experiments.

Preparation of pre-hydrolyzed SMFS experiments on the maleimide–thiol conjugates

The 1 × 1 cm^2 glass substrates were immersed in a chromic mixture overnight and then thoroughly washed with DI water, ethanol and acetone successively and dried under a steam of nitrogen to produce surfaces with exposed hydroxyl groups. Then the hydroxylated cantilevers were immersed in 1% (v/v) APTES in toluene for 1 h. After that, they were rinsed with toluene, then ethanol, dried under a nitrogen stream, and incubated at 90 ℃ for 30 min. Next, these substrates were rinsed with DMSO containing 0.2 mM NHS-PEG-Mal (MW: 5 kDa) at R.T for 1h. After washing with DMSO, then ethanol and drying under N_2, the substrates were then rinsed in DMSO containing 10 mM ethylenediamine to introduce an amino group to the free end of the polymer. Subsequently, the substrates were washing with DMSO, then ethanol and drying under N_2, before being incubated in basic PBS buffer (pH = 8.1) at 37 ℃ until the five-membered ring was completely hydrolyzed (~ 5 days)[1]. The substrates were stored in neutral PBS. The amino group could be picked up by an NHS-PEG functionalized cantilever in the pre-hydrolyzed experiment.

NHS-PEG cantilevers were modified following a similar procedure. The MLCT cantilevers were first rinsed in a chromic mixture at 90 ℃ for 30 min. After that, the cantilevers were washed with water and then ethanol and dried under a steam of nitrogen. Then, the cantilevers were coated with the amino group in 1% (v/v) APTES in toluene for 1 h. After incubation at 90 ℃ for 30 min, the cantilevers were soaked in a DMSO solution containing 0.2 mM NHS-PEG-NHS (MW: 5 kDa) for 1 h. After being washed with DMSO, then ethanol and dried, the resulting NHS-coated cantilevers were kept dry and used for single-molecule AFM experiments.

SMFS experiments

Three experiments were conducted in different solvents to measure the rupture force of the thiosuccinimide and its hydrolyzate. In the first two experiments, the

maleimide-functionalized cantilever was put into contact with the thiol-substrate with a set-point force of 1nN for 1 s to trigger the formation of the maleimide–thiol conjugate between the cantilever tip and the substrate. Then the cantilever was retracted away from the substrate at a constant pulling speed to obtain the force-extension curves of the thiosuccinimide-containing linkers under a stretching force in different solvents (anhydrous acetonitrile and aqueous PBS solution at pH=6.4, 7.4, and 8.1).

Similarly, the third series of pre-hydrolyzed experiments were carried out in acetonitrile and neutral PBS solution. The NHS-PEG cantilever was put into contact with the glass surface to pick up the amino group at the very end of the hydrolyzate-containing linker at a constant force of 1 nN for 1 s. Then the cantilever was retracted from the substrate at a constant pulling speed to obtain the force-extension curves of the hydrolyzed thiosuccinimide-containing linkers under a stretching force (Supplementary Fig. 1c).

In the quenching experiments, the maleimide-PEG cantilevers were incubated in 10 mM cysteine PBS buffer for 2 h or basic PBS buffer (pH=8.5) for 2 h before use.

In a control experiment using NHS-PEG-NHS (MW: 5 kDa)-functionalized amino-cantilevers, the rupture from the amino-coated substrate showed that the rupture force of the amino-NHS-PEG linker is higher than the rupture force of the thiosuccinimide, ensuring that the single force peak derived from the thiosuccinimide, which is the weakest part of the linker (Supplementary Fig. 4b). The cantilevers were first coated with the amino group in 1% (v/v) APTES in toluene. After incubation at 90 ℃ for 30 min, the cantilevers were soaked in a DMSO solution containing 0.2 mM NHS-PEG-NHS (MW: 5 kDa) for 1 h. After being washed with DMSO, then ethanol and dried, the resulting NHS-coated cantilevers were kept dry and used for control experiments. The amino group-coated substrates were modified following a similar method by immersion in an anhydrous toluene solution containing 0.5 % APTES at RT for 1 h.

Multiple cantilevers and substrates (at least three) were used for each type of experiments.

All the force curves were collected by commercial software from JPK and analyzed using custom-written protocol in Igor 6.35 (Wavemetrics, Inc.). For the loading rate dependence experiments, the pulling speed was changed every 50 events (from 100, 400, 1000, 4000, 8000, to 20000 nm/s) to determine the loading rate-dependence of a single cantilever. In the control experiment at different contact time, the contact time was changed every 50 events (0 s, 0.5 s, 1.0 s, and 2.0 s) while the contact force was fixed at 1.0 nN. In the control experiment at varying contact forces, the contact force was changed every 50 events (0.1 nN, 0.5 nN, 1.0 nN, and 2.0 nN) while the contact time was fixed at 1.0 s. The pulling speed for control experiments was 4000 nm s^{-1}. We converted the pulling speeds to loading rates following a published procedure[2]. We first converted all the force-extension curves to force-time curves. Then, the average slope (k) of the force-time curves immediately before the rupture event was taken to estimate the average loading rate for the non-equilibrium constant-speed pulling. In this way, the contribution of the stiffness of the whole mechanical linkage, including the cantilever, the PEG linker, and the target molecules, was considered in the loading rates.

Fingerprint experiment

The gene encoding polyprotein Cys-$(GB1)_4$-Spycatcher was constructed in plasmid pQE-80L and transformed into *E. coli* (BL21). The polyprotein containing a cysteine at the N-terminus after the histidine tag and a Spycatcher at the C-terminus was expressed and purified using Co^{2+} - resin (TALON). Maleimide-PEG substrates were modified following the Method above. Then the substrates were covered by a PBS buffer containing 0.1 mM of polyprotein and incubated for 1 h. After that, the substrates were completely rinsed by PBS buffer and stored in PBS at 4 ^{o}C. Maleimide-PEG cantilever were modified following the Method above, then incubated in PBS buffer containing 0.1 mM of Spytag and 1 mM of TCEP. After being completely rinsed by PBS buffer, the substrates were stored in PBS at 4 ^{o}C for use.

In the fingerprint experiment, the Spytag modified cantilever was brought into contact with the substrate at low contact force of ~100 pN for 30 s and then retracted. All events containing four peaks with the characteristic unfolding force and contour length increment of GB1 were used for data analysis.

The sequence of GB1 protein:
MDTYKLILNGKTLKGETTTEAVDAATAEKVFKQYANDNGVDGEWTYDDAT
KTFTVTE

Pre-stretching SMFS experiments

The rupture forces at different pre-stretching times were plotted (Figure 2d), and those data were fitted by two-state transition theory to calculate the lifetime, τ, and rate constant of the ring-opening hydrolysis at different pre-stretching forces. For simplicity we considered the ring-opening hydrolysis at a constant pre-stretching force as a first-order reaction. Therefore, the probability of thiosuccinimide in initial state A (P_A) can be described as follows:

$$\frac{dP_A}{dt} = -k(f_p)P_A \qquad (1)$$

where, $k(f_p)$ was the pre-stretching force-dependent rate constant.

$$P_A = e^{-k(f_p)t} \qquad (2)$$

where, t was the pre-stretching time. Therefore, the final rupture force after pre-stretching can be described as the following:

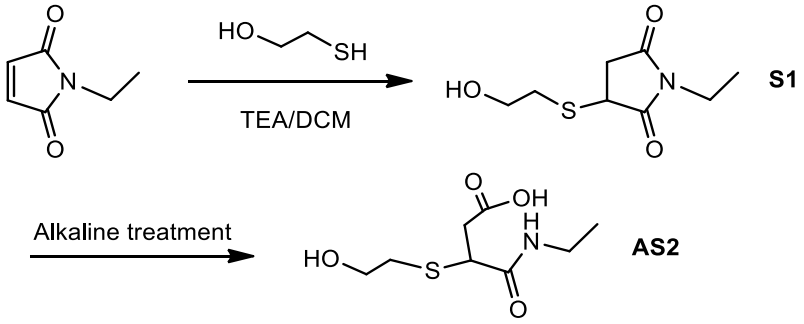
$$F = F_1 e^{-k(f_p)t} + F_2(1 - e^{-k(f_p)t}) \qquad (3)$$

where, F_1 was the rupture force for the retro-Michael reaction, and F_2 was the rupture force of the hydrolyzate of the maleimide–thiol conjugate.

Method of calculating lifetime from biomodal fitting:

Similarly, the lifetime or rate constant at different pre-stretching forces can also be calculated by bimodal fitting. The ratios of un/hydrolyzed maleimide–thiol adducts (R) can be calculated from the areas of two peaks. The probability of unhydrolyzed thiosuccinimide (P_A) equals $R/(1+R)$. Thus, the lifetime or rate constant at each pre-stretching can be calculated using eq. 2.

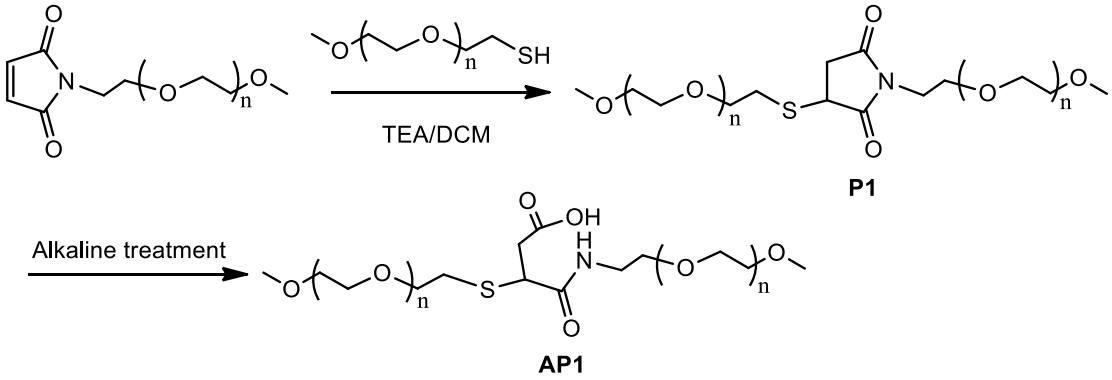
Synthesis



1-Ethyl-3-((2-hydroxyethyl)thio)pyrrolidine-2,5-dione (small-molecule adduct 1, S1)

N-Ethylmaleimide powder (NEM, 625 mg, 5 mmol) was dissolved in 5 mL of dry dichloromethane (DCM), and the solution was stirred at room temperature. After the dropwise addition of mercaptoethanol (365 μL, 5 mmol), trimethylamine (7 μL, 0.01 equiv.) was slowly added to the mixture to start the Michael-addition reaction. The reaction was subsequently maintained at R.T. for 12 h while stirring. After removal of the solvent by rotary evaporation, the resulting powder was subject to silica flash chromatograph using DCM:MeOH (99:1 v/v) as the eluent and provided model compound **1** (yield: 96%) as a blue-black solid.
^{1}H NMR (500 MHz, D_2O, r.t.): δ (*ppm*) 4.02-4.06 (dd, 1H, J=9.0 Hz, 4.0 Hz), 3.78 (t, 2H, J=6.06 Hz), 3.52 (q, 2H, J=7.33 Hz), 3.29 (dd, 1H, J=18.79 Hz, 8.91 Hz), 2.92-2.98 (dt, 1H, J=13.71 Hz, 6.00 Hz), 2.83-2.89 (dt, 1H, J=13.99 Hz, 6.00 Hz), 2.70-2.76 (dd, 1H, J=18.78 Hz, 4 Hz)
ESI-MS (+): calcd for $C_8H_{13}NO_3S$ $[M+Na]^+$ 226.26; found 226.10.

4-(Ethylamino)-3-((2-hydroxyethyl)thio)-4-oxobutanoic acid (alkaline-treated S1, AS1)
Small-molecule adduct **S1** (100 mg) was dissolved in 5 mL basic PBS solution at pH=8.1. The reaction was incubated at 37 °C for 5 days until the ring opening was complete. Additional 1 M NaOH was added every 24 h to keep the pH at 8.1. The transparent solution was collected, and 0.5% formic acid was added to reduce the pH to 1~2. The crude product was purified using a desalination column. Purified fractions were collected and freeze-dried. The hydrolyzed product of model compound **S1** was obtained as blue-black oil (yield: 90%). Note that two isomers of the hydrolyzate are possible depending on the ring-open site of the five-membered ring.
^{1}H NMR (500 MHz, D_2O, r.t.): δ (*ppm*) 4.02-4.06 (dd, 1H, J = 9.0 Hz, 4.0 Hz), 3.78 (t, 2H, J = 6.06 Hz), 3.70-3.75 (m, 4H), 3.66 (t, 1H, J=8.03 Hz), 3.52 (q, 2H, J=7.33 Hz), 3.29 (dd, 1H, J=18.79 Hz, 8.91 Hz), 3.18 (q, 3H, J=7.35 Hz), 2.92-2.98 (dt, 1H, J= 13.71 Hz, 6.00 Hz), 2.68-2.90 (m, 8H), 2.54-2.60 (dt, 1H, J=10.51 Hz, 7.47 Hz)
ESI-MS (+): calcd for $C_8H_{15}NO_4S$ $[M+Na]^+$ 244.26; found 244.10.

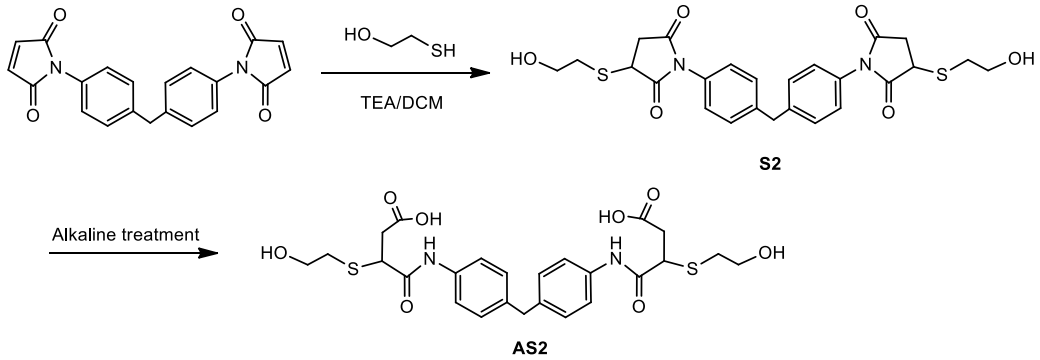


PEG-Mal-thiol-PEG, P1

mPEG-SH (100 mg, 0.02 mmol) and mPEG-Mal (100 mg, 0.02 mmol) were separately dissolved in 1 mL of dry DCM and mixed in flasks. Trimethylamine (0.01 equiv.) was slowly added to 2 mL of the solution to start the Michael-addition reaction. The reaction was subsequently maintained at R.T. for 12 h with stirring. After removal of the solvent by rotary evaporation, the resulting product was collected to give **P1** as a white powder. Based on the ^{1}H NMR spectrum (500 MHz, DMSO-d_6, r.t.), the maleimide was fully reduced (ppm ~6.9).

Alkaline-treated PEG-Mal-thiol-PEG, AP1

P1 (100 mg) was dissolved in 5 mL PBS solution at pH=8.1. The reaction was incubated at 37 ℃ for 5 days until ring opening was complete. Additional 1 M NaOH was added every 24 h to keep the pH at 8.1. The resulting solution was collected, and 0.5% formic acid was added to reduce the pH to 1~2. The crude product was purified using a desalination column. Purified fractions were collected and freeze-dried. **AP1** was obtained as a white solid.



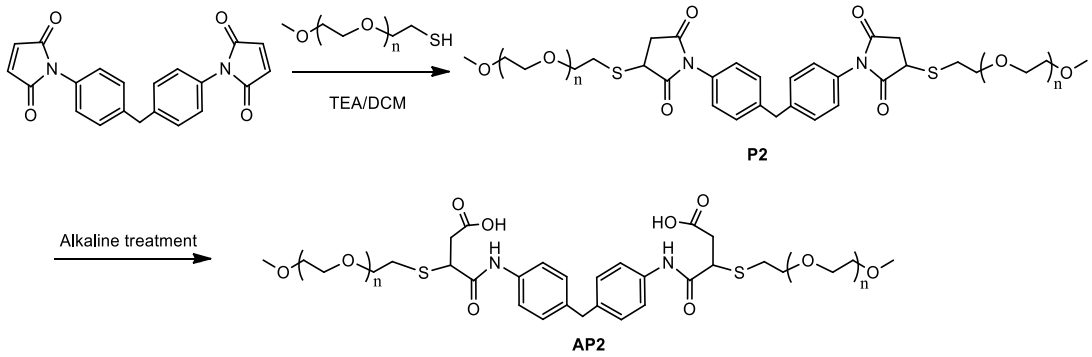
1,1'-(Methylenebis(4,1-phenylene))bis(3-((2-hydroxyethyl)thio)pyrrolidine-2,5-dione) (small-molecule adduct 2, S2)

4,4'-Bismaleimidodiphenylmethane (bis-maleimide, 1.79 g, 5 mmol) was dissolved in 10 mL of dry DCM and stirred at room temperature. After dropwise addition of mercaptoethanol (730 μL, 10 mmol), 0.01 equiv. trimethylamine was slowly added to the mixture to initiate the Michael-addition reaction. The reaction was subsequently maintained at R.T. for 12 h with stirring. After removal of the solvent by rotary evaporation, the resulting powder was subject to flash chromatograph on silica gel using DCM:MeOH (99:1 v/v) as the eluent and provided **S2** as a red solid. The ^{1}H

NMR spectrum (500 MHz, DMSO-d_6, r.t.) showed the maleimide was fully converted (ppm ~7.16).
^{1}H NMR (500 MHz, DMSO-d6, r.t.): δ (*ppm*) 7.37 (d, 4H, J = 8.4 Hz), 7.20 (d, 4H, J= 8.4 Hz), 4.9 (t, 2H, J=5.36 Hz), 4.16 (dd, 2H, J=9.09 Hz, 4.19 Hz), 3.62 (dd, 4H, J=11.5 Hz, 6.17 Hz), 3.28-3.36 (m, 4H), 2.88-2.95 (m, 2H), 2.74-2.81 (m, 2H), 2.66-2.73 (dd, 2H, J= 18.28 Hz, 4.29 Hz)
ESI-MS (+): calcd for $C_{25}H_{26}N_2O_6S_2$ $[M+Na]^+$ 537.61; found 537.20.

4,4'-((Methylenebis(4,1-phenylene))bis(azanediyl))bis(3-((2-hydroxyethyl)thio)-4-oxobutanoic acid) (alkaline-treated S2, AS2)

Model compound **S2** (100 mg) was dissolved in 2 mL of acetonitrile, and 2 mL of PBS solution at pH=8.1 was added. The reaction was incubated at 37 ℃ for 5 days until the ring opening was complete. Additional 1 M NaOH was added every 24 h to keep the pH at 8.1. The transparent solution was collected, and 0.5% formic acid was added to reduce the pH to 1~2. The crude product was purified using a desalination column. Purified fractions were collected and freeze-dried. Note that owing to the symmetry or asymmetry of the ring-opening of the two thiosuccinimides, two isomers of the hydrolyzate are possible.
^{1}H NMR (500 MHz, DMSO-d6, r.t.): δ (*ppm*) 7.58 (dt, 2H, J=8.28 Hz, 1.96 Hz), 7.47 (dd, 2H, J=16.02 Hz, 8.65 Hz), 7.1-7.2 (m, 4H), 4.86 (t, 1H, J=5.36 Hz), 3.83-3.87 (m, 2H), 3.54 (dd, 2H, J=11.83 Hz, 6.55 Hz), 2.65-2.76 (m, 2H)
ESI-MS (+): calcd for $C_{25}H_{30}N_2O_8S_2$ $[M-2CO+H]^+$ 496.64; found 495.20.



PEG-thiol-bis-maleimide–thiol-PEG, P2
mPEG-SH (100 mg, 0.02 mmol) and bis-maleimide (3.58 mg, 0.01 mmol) were dissolved in 2 mL of dry DCM. Trimethylamine (0.01 equiv.) was slowly added to the mixture to initiate the Michael addition. The reaction was incubated at R.T. for 12 h with stirring. After removal of the solvent by rotary evaporation, the resulting product was collected to give **P2** as a canary yellow powder. The ^{1}H NMR spectrum (500 MHz, DMSO-d_6, r.t.) showed the maleimide was fully consumed (ppm ~7.16).

Alkaline-treated P2, AP2
P2 (50 mg) was dissolved in 5 mL basic PBS solution at pH=8.1. The reaction was incubated at 37 ℃ for 5 days until the ring opening was complete. Additional 1 M NaOH was added every 24 h to keep the pH at 8.1. The resulting solution was collected, and 0.5% formic acid was added to reduce the pH to 1~2. The crude product was purified using a desalination column. Purified fractions were collected and freeze-dried.

Pulsed ultrasonication

The ultrasonic intensity was calibrated using a calorimetry method[3]. The probe was submerged approximately half way into a Dewar filled with exactly 250 mL of Milli-Q (250 g) water. The exact height was marked for reproducibility. The temperature was recorded using an infrared thermometer and allowed to stabilize. The amplitude on the processor was set to 50% and sonication was started. The temperature of the water was recorded every 30 s for 5 min, and these values were plotted against time to determine Δtemperature/Δtime from the slope of the line. This procedure was replicated for amplitudes of 20, 40, 50, 60, 80, and 100%. The heat due to cavitation in J/s or in W was determined from the following equation: $Q = \Delta_{temperature} \times m \times C_{water} / \Delta_{time}$, where m is the mass of water (250 g), C_{water} is the heat capacity of water (4.179 J g) and $\Delta_{temperature}/\Delta_{time}$ is the slope of the line. The resulting value, Q, was divided by the surface area of the probe tip (0.2826 cm^2 for a 6-mm tip) to find the ultrasonic intensity, W/cm^2. Finally, the power produced was plotted against the percentage amplitude to determine the average ultrasonic intensity.

Chemical characterization

^{1}H NMR

^{1}H NMR spectra were recorded on a Bruker 500 MHz NMR spectrometer in dimethyl sulfoxide-d_6 (DMSO-d_6), deuterium oxide (D_2O) or chloroform-d ($CDCl_3$), and chemical shifts are reported in δ (ppm) relative to the residual solvent peak (DMSO-d_6 = 2.5 ppm, D_2O=4.79 ppm, and $CDCl_3$=7.26 ppm). Coupling constants (J) are reported in Hertz (Hz). Splitting patterns are designated s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), m (multiplet), and br (broad). For high-molecular weight polymers, samples were dissolved in DMSO-d_6 at a high concentration (100 mg/mL), and the spectroscopy was emerged from more than 128 scanning times to improve the low signal intensity of the target compound (maleimide–thiol). However, the PEG linker exhibited large peaks at 3-4 ppm, which masked the core signal of the mechanical hydrolysis of maleimide–thiol (Supplementary Fig. 18b). In the present work, **P2** was designed and synthesized, and the adjacent aromatic protons in this compound (located at ~ 7 ppm) were used to track the mechanical transition signal (Figure 3 and Supplementary Fig. 19).

FT-IR

Fourier transform infrared (FT-IR) spectroscopy was performed on a Nicolet iS50 FT-IR instrument (Thermo Scientific) with ATR. Samples were dissolved in $CDCl_3$, and ~20 μL drops of the solution were added to the detector after baseline correction. The spectra were recorded from 400 to 4000 cm^{-1}, and the resolution was set at 0.4 cm^{-1}.

UV-vis

UV-vis absorption spectra were recorded on a JASCO V550 spectrometer (Jasco, Japan). A quartz cell with a 1 mm path length was used, and the samples were dissolved in DMSO or water. The concentrations were ~ 20 mg mL^{-1} for small-molecule adducts and ~ 100 mg mL^{-1} for polymers. The spectra was recorded from 190 nm to 400 nm.

pH measurements

A pH meter equipped with an H^+ ion selective electrode was used to record the pH of the ultrasonicated solutions. The electrode was calibrated using standard buffers of

pH 4, 7 and 9. During the ultrasonication of ~10 mL pure water (Milli-Q) containing 2 mg/mL **P2**, the pH was recorded every 5 min for 60 min. The solution was degassed with bubbling N_2 during the ultrasonication experiment, and the pH was measured to prevent CO_2 absorption. The ultrasound was pulsed (1.0 s on and 1.0 s off) at 11.81 W/cm^2. The acid-base indicator bromothymol blue was added to the solution (5% v/v) to confirm the pH change by the color transition. An image was recorded every 15 min. We carried out two control experiments, with a PEG linker under ultrasonication for 60 min and **P2** without ultrasonication, which showed minimal pH changes and no color changes (Supplementary Fig. 23).

Moreover, we roughly estimated the theoretical change in pH based on the concentration of maleimide–thiol-PEG in the solution. In the pH change experiment, 2 mg mL^{-1} of maleimide–thiol-PEG adduct could produce 0.4 mM of hydrolyzate with ring-opening succinamic acid thioether in 100% yield. If the pKa of succinamic acid thioether was around 6.23 (pKa_2 of maleic acid), the ideal pH drop should be ~ 2.0. The observed decrease of pH from 7.7 to 6.8 should be reasonable, as the yield of the ultrasound-treated reaction did not reach 100% under our experimental conditions.

Antibody-PEG conjugate stability

The antibody trastuzumab has four cross-linked disulfide bonds between its two heavy chain and light chain. After being treated with excess TCEP, there are three exposed cysteines on each heavy chain and one exposed cysteine on each light chain (marked red in the following sequence).

The sequence of the antibody trastuzumab is following:
Heavy chain:
EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIY
PTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDG
FYAMDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPE
PVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHK
PSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEV
TCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL
HQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKN
QVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDK
SRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG
Light chain:
DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASF
LYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIK
RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNS
QESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNR
GEC

Alkaline-treated Ab-PEG samples: The Ab-PEG conjugates were put in Tris buffer (pH 9.2), and incubated at 37 ^{o}C for 24 h until the majority of the maleimide–thiol adducts were hydrolyzed[4]. Then, the alkaline-treated sample was transferred into PBS buffer (pH=7.4) containing 1 mM of reduced GSH to test the stability of the alkaline-treated Ab-PEG.

Enzyme-linked immunosorbent assay (ELISA): Each well of the ELISA plates was coated with 0.5 μg mL^{-1} of antigen Her2 protein (Cat: 10004-H08H, Sino Biological,

China), the volume was 50 μL per well and the plates were incubated overnight at 4 ℃. Next day, 200 μL of the washing buffer (PBS, containing 0.05% Tween 20 (TBST)) was used to wash the plates once. Then, 200 μL of the blocking buffer (Blocker™ Casein in PBS, Cat: 37582, ThermoFisher, USA) was added into the plates and incubated for 1 h at RT. Next, the plates were washed by the washing buffer for three times before adding 50 μL of trastuzumab at different dilution gradient. The highest concentration of trastuzumab was 1.0 μg mL^{-1}. After incubation for 1 at RT, the plates were washed for three-times using the washing buffer. Then 50 μL of the secondary antibody (diluted as 1:1000 ratio) (Goat anti-Human IgG antibody-HRP, Cat: A0201, Beyotime, China) was added and incubated for 1 h at RT. The plates were washed six times before adding 50 μL of TMB (3,3′,5,5′-Tetramethylbenzidine Liquid Substrate, Cat: T4444, Sigma-Aldrich, Germany) into each well. After incubating for 6 min, the absorbance at 450 nm and 620 nm was detected by the plate reader (SpectraMax M5/M5e, Molecular Devices, USA) immediately.

The stability of the ultrasound-treated, alkaline-treated and untreated Ab-PEG samples were evaluated *in vitro* at a concentration of 20 μM in a PBS buffer (pH=7.4) containing 1 mM reduced glutathione. GB1 protein (MW 8k) as an internal reference was added into the solutions to monitor the protein degradation. The solutions were filtered using a 0.22-μm filter and incubated at 37 °C. At each time point, a 100-μL aliquot was removed and frozen at −80 °C.

Western Blot

The concentrations of antibody samples were calculated by the A280 absorbance measured using Nanodrop (Thermo Scientific, USA) and then adjusted to 0.3 mg mL^{-1} as the final concentration. After adding 5X loading buffer, the samples were heated at 95 ^{o}C for 10 minutes. Every lane of the commercial ExpressPlus PAGE 4-20% Gradient Gel (Genscript, China) was loaded with 2.5 μg of total proteins. The electrophoresis tank (Bio-rad, New South Wales, Australia) was filled with the Tris-MOPS-SDS Running Buffer (Genscript, China) and the electrophoresis current was 10 mA. The wet transfer system with TG (tris-glycine) buffer and 15% ethanol was used to transfer the protein bands to polyvinylidene difluoride (PVDF) membrane. The transferring process was at 150 mA current at 4℃ for 2.5 h. The PVDF membrane was blocked with 5.0% BSA for 1.0 h at room temperature (RT). Mouse anti-Human IgG_1 Fc secondary antibody-Horse Radish Peroxidase (HRP) (Cat: A-10648, ThermoFisher, USA), anti-Human (κ-chain specific), goat $F(ab')_2$ fragment-HRP antibody (Cat: SAB3701414, Sigma-Aldich, Germany) and his-tag (27E8) mouse monoclonal antibody-HRP conjugate (Cat: 9991, Cell Signaling Technology, USA) were diluted by 1:1000 ratio. Then, the PVDF membrane was incubated in secondary antibody solution overnight at 4 ℃. The TBS (Tris-buffered saline) buffer containing 0.1% Tween 20 was used as the washing buffer to wash PVDF membrane for three times at RT. The ECL™ Prime Western Blotting System (Cat: GERPN2232, GE Health, USA) solution was added and exposed at 0.2 second. The obtained graph was analyzed using Gel-Pro Analyzer Software to obtain the gray values of each band.

The average drug antibody ratio (DAR) values of the light chains and heavy chains from Western Blot were obtained as following:

Average DAR of light chains = $Lc_1/(Lc_0+Lc_1)$

Average DAR of heavy chains = $(Hc_1 \times 1+Hc_2 \times 2+Hc_3 \times 3)/(Hc_0+Hc_1+Hc_{2+}Hc_3)$

where, Lc_0 and Lc_1 are the band intensities of the light chain with zero and one payloads (PEGs), respectively, and Hc_0, Hc_1, Hc_2 and Hc_3 are the band intensities for the heavy chains with zero, one, two and three payloads (PEGs), respectively. The antibody-PEG remaining (%) at each day was calculated and normalized to the average DAR at day 0.

The significance of statistic was performed by unpaired two-sided Student's *t*-test with GraphPad Prism 5.0.
For unpaired *t*-test of heavy chain in Figure 4c: 95% confidence intervals, R squared as represented effect size: 0.7493, degree of freedom: 8, P value: 0.0012, and P value summary: **.
For unpaired *t*-test of light chain: 95% confidence intervals, R squared as represented effect size: 0.5635, degree of freedom: 8, P value: 0.0124, and P value summary: *.

Theoretically prediction of force distributions by Monte Carlo method
For simplicity, the retro-Michael reaction and cleavage of hydrolyzate can be represented by a two-state Markovian model with force-dependent rate constants, respectively. This description gives the probability of observing the breaking of any N_b bonds in the time interval dt:
$P_u = k_u(F)N_b dt$ (4)
where, $k_u(F)$ is the force-dependent unbinding rate constant. The effect of an external force on the unbinding rate constant was calculated as the following[5,6]:
$k_u(F) = k_0 exp(\beta F \Delta x^{\ddagger})$ (5)
where, k_0 is the intrinsic unbinding rate in the absence of force, $\Delta x^{\ddagger}$ is the distance to the transition state, $\beta = 1/k_b T$ is the Boltzmann's constant times the absolute temperature as the scale of energy.
The AFM cantilever extends the linked peptide at a pulling speed v, which leads to an extension dx at each time interval dt:
$dx = vdt$ (6)
After each time step the actual force is calculated according to the WLC model:
$F(x) = \frac{k_v T}{p}\left(\frac{1}{4\left(1-\frac{x}{L}\right)^2} - \frac{1}{4} + \frac{x}{L}\right)$ (7)
where, p is the persistence length of the polymer linker and L is the contour length of the polymer.
The combination of those equations, which describe the extension of the linker containing the thiosuccinimide or hydrolyzate by the AFM cantilever and the rupture rate at each time step, can be realized in continuous simple Monte Carlo simulations[7].

The parameters k_0 and $\Delta x^{\ddagger}$ for the two pathways were taken from the Bell-Evans model fittings of the data according to dynamic force spectroscopy (Supplementary Fig. 12 and Supplementary Table 2). Then ~2000 force-extension curves were generated using the Monte Carlo simulation procedures[7] written in Wavemetrics Igor 6.35. The histograms of the simulated final rupture forces can reproduce the experimentally measured force distributions for the retro-Michael reaction and rupture of the ring-opened hydrolyzate reasonably well (Supplementary Fig. 14).

Monte Carlo simulations of the pre-stretching experiment
We only consider one condition: that if the pre-stretching protocol could artificially shift the force population as long as no chemical reactions occurred during the pre-stretching. Without the hydrolysis reaction pathway (for simplicity) the rupture of

thiosuccinimide can be represented by a two-state Markovian model with force-dependent rate constants.

The parameters k_0 and $\Delta x^{\ddagger}$ were taken from the Bell-Evans model fittings of the retro-Michael reaction according to dynamic force spectroscopy (Supplementary Fig. 12 and Supplementary Table 2). The pre-stretching force was set at 300 pN, the pre-stretching time was 0.1 ms and the pulling speed was 1000 nm s^{-1}. We have determined the simulated force-extension curves with no pre-stretching and with pre-stretching at different relaxing forces (0 pN, 100 pN, 200 pN and 300 pN) 2000 times using Wavemetrics Igor 6.35. Those events that did not reach or survive during the pre-stretching were excluded from data analysis. The histograms of the simulated final rupture force showed no shift in the force distribution from the pre-stretching protocol if the molecule was fully relaxed to zero force after the pre-stretching.

Model-free analysis

We have applied the model-free analysis procedure to the single-molecule force spectroscopy data of the retro-Michael pathway and the rupture of the ring-opened hydrolyzates at 4000 nm s^{-1}. We have counted the total time (T_f) of each force was applied based on all force-extension traces, and the bin width of force was 100 pN. Then the number of rupture events counted at each force (N_f from histogram) was divided by the total time of each force. The force-dependent dissociation rates were calculated as $k_f = N_f/T_f$. The logarithm of the dissociation rates (Supplementary Fig. 13c) was then fitted by a straight line ($lnk_f = lnk_0 - \triangle G_f/k_bT$) to obtain k_0 and ΔG_f.

Supplementary Notes

1. Single-molecule data quality control

As summarized by Thomas *et al.*, practical methods ensuring that single molecule force spectroscopy experiments really measure single bonds include: (1) Poisson statistics to estimate the ratios between single bonds and multiple bonds; (2) Low adhesion probabilities (less than 20%); (3) Filtering out most multiple bonds; (4) Extendable molecular linkage; (5) Controlled molecular spacing[8]. Our experimental design employed many controls (see below) to ensure most data indeed corresponded to single molecule events.

We kept the density of the thiol groups on the surface very low, so that in most of the trails (in average of ~92% of the total traces), even the AFM cantilever tip was in contact with the surface, no bonds were formed. The chance to simultaneously pick up two or more maleimide–thiol adducts was minimized.

Based on the Poisson statistics, 96.1% of the total events we obtained were single molecule events, as the pick-up frequency in our experiments was ~8% of the total traces. Assuming the maleimide–thiol bond formation obeyed Poisson statistics, the probabilities of forming a single, double, and triple maleimide–thiol bonds between the AFM tip and the surface were 96.1%, 3.8%, and 0.1%, respectively[9].

Moreover, the elasticity and contour length of the PEG linkers were used to further filter potential multiple events. The WLC model was used to fit the force-extension curves containing only a single peak (fitting to the region close to the rupture peak). The range of contour length and persistence length we used to filter the data was two-sigma (standard deviation) around the mean value, corresponding to a confidence level of ~ 95%. Specifically, the persistence length was in a range between 0.31 nm and 0.55 nm, and the contour length was in a range between 23 nm and 61 nm. The curves corresponding to simultaneous rupture of multiple interactions showed the elasticity of multiple chains which differed largely from that of a single chain[10].

2. AFM single-molecule control experiments

In the quenching experiments, the maleimide modified cantilever was incubated with cysteines to quench most reactive groups before AFM experiment with the thiol-activated surface. Alternatively, the maleimide modified cantilever was incubated in basic PBS buffer (pH=8.5) for 2 h to hydrolyze most of the maleimide. The numbers of successful single-molecule events and the single-molecule ratios drops a lot comparing to untreated cantilever (Supplementary Fig. 3). Both control experiments confirmed indeed the maleimide–thiol bonds were formed and ruptured in the single molecule experiments.

Notably, in the three AFM single-molecule experiments mentioned in the manuscript (anhydrous, aqueous and alkaline-pretreated), the measured rupture events corresponded to the breakage of the weakest part of the linkage between the cantilever tip and the substrate, which was not necessarily the maleimide–thiol conjugate. To confirm that the measured force peaks indeed corresponded to the breakage of the maleimide–thiol adducts, the following two control experiments were performed.

A negative control experiment using a maleimide-functionalized cantilever tip but without the thiol-coated substrate showed a force curve with no observable force peaks, indicating that the force peaks observed in previous experiments could not have resulted from non-specific binding of maleimide with the substrate (Supplementary Fig. 4a).

A second control experiment using the same cantilever and surface modification chemistry but without the maleimide–thiol conjugates in the polymer linkage exhibited rupture forces much higher than 1 nN. Therefore, the other chemical bonds in the linkage between the cantilever tip and the substrate were stronger than both the unhydrolyzed and hydrolyzed maleimide–thiol adducts (Supplementary Fig. 4b).

Considering these observations together, the observed force peaks with rupture forces between 100 pN and 1.5 nN in all three parallel experiments can be attributed to the rupture of maleimide–thiol adducts through different reaction pathways.

3. AFM single-molecule fingerprint experiments

We have designed a single molecule AFM experiment using GB1 proteins as the fingerprint markers. (Supplementary Fig. 7a) We engineered a polyprotein Cys-$(GB1)_4$-Spycatcher, which contains a cysteine at the N-terminus and a Spycatcher at the C-terminus. The polyprotein was anchored onto the maleimide-PEG-functionalized substrate through its N-terminal cysteine. The C-terminal Spycatcher can form a covalent bond with a specific peptide Spytag[11], which was also linked to the cantilever through a maleimide–thiol-PEG linkage. In the fingerprint experiment, the Spytag modified cantilever was brought into contact with the substrate at low contact force of ~100 pN (to avoid non-specific adhesions) for 30 s, because the Spytag Spycatcher reaction was slow. Once the polyprotein was captured by the cantilever, the GB1 domains would be unfolded one by one giving rise to a clear sawtooth like pattern with a characteristic contour length increment of ~18 nm and rupture forces of ~ 220 pN at the pulling speed of 1000 nm s^{-1}, which were consistent with previous reports about GB1 domain[12]. (Supplementary Fig. 7). All events containing four unfolding peaks were used for data analysis. The molecules being picked up by non-specific interactions would have less GB1 domains or different contour length. The last rupture force peak (*) was assigned as the rupture of the maleimide–thiol adducts because the covalent bond formed by Spytag and Spycatcher was reported to rupture at more than 1.5 nN[11]. The force distributions measured using the polyprotein fingerprint agreed well with that obtained using PEG linkers. (Supplementary Fig. 7d) Note that some events with rupture forces below the unfolding forces of GB1 (in the range of 100-350 pN) may be missed in the final data pool, because the molecules were detached before all four GB1 domains were unfolded (These data were undistinguishable from the curves corresponding to nonspecific interactions).

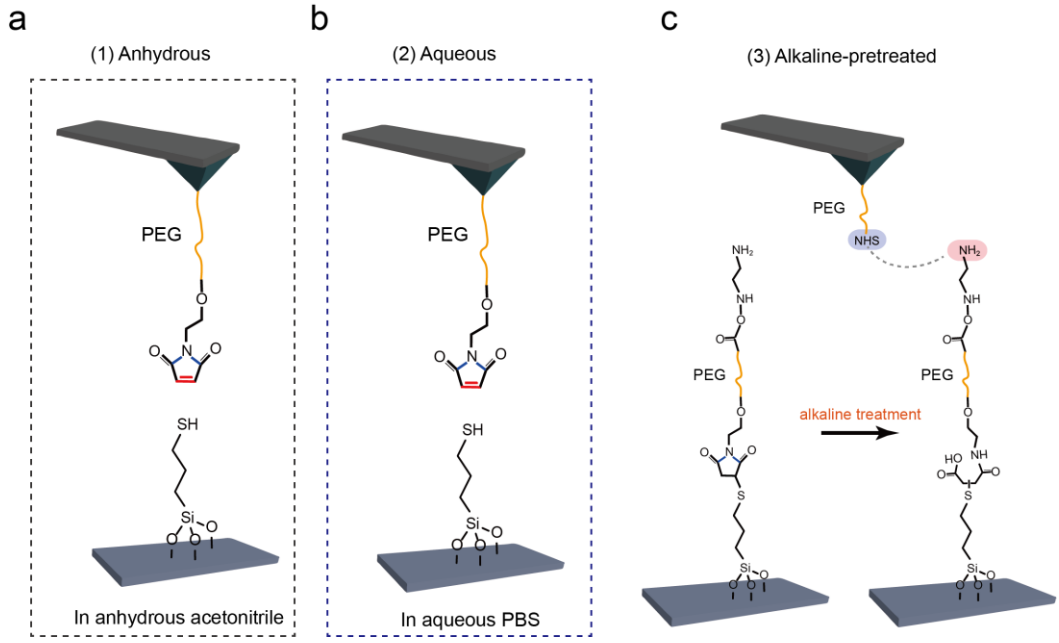
4. The bond being broken in the hydrolyzed product

The bond being broken in the hydrolyzed product should be the C-S bond, which was predicted to be the weakest part in the polymer linkers[13]. (Rupture force: C-S < C-O, C-N and C-C)

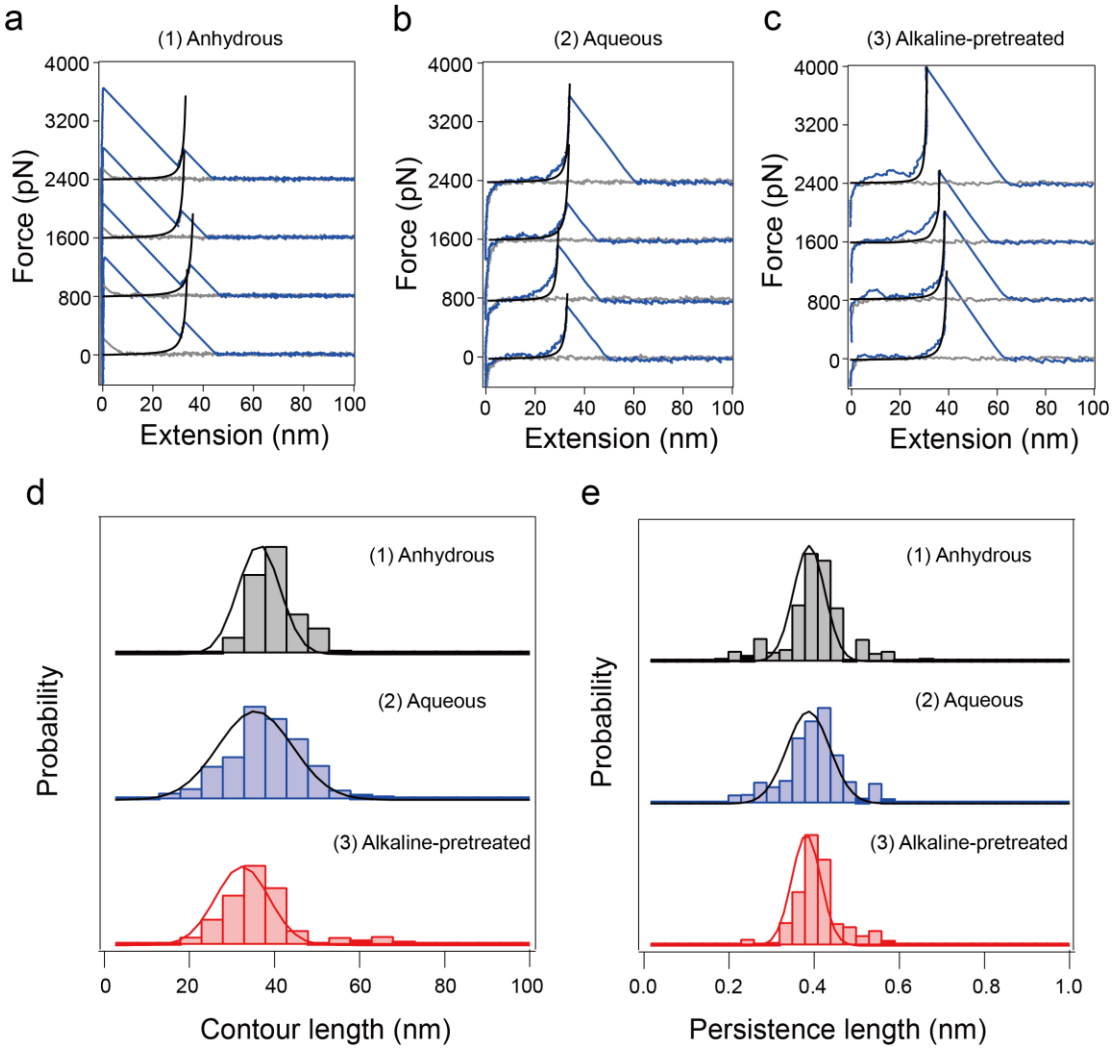
The C-S bonds are typically ruptured to give rise to unstable free radicals, which then quickly quenched in the buffer[13,14]. Therefore, the rupture of the hydrolyzed adducts

is not reversible. Indeed, as shown in Supplementary Fig. 10, the single-molecule pickup ratio became lower when more maleimide–thiol adducts were hydrolyzed by force stretching in the experiments. Because the rupture of the hydrolyzed adducts is irreversible, in our experimental design, we purposely increased the density of molecules on the cantilever and used low density of molecules on the substrate. We were able to change to different spots on the surface automatically in the single molecule experiments to ensure unreacted thiol groups being exposed to the cantilever tip to form new maleimide–thiol adducts. This allowed ~ 500 events can be obtained using each cantilever, while majority of the events (>90%) fulfilled the criteria for single molecule events based on the contour length and the elasticity of the PEG linkers. The single-molecule pickup ratios were ~8% and summarized in Supplementary Table 1, which were controlled by the density of thiol groups on the substrate.

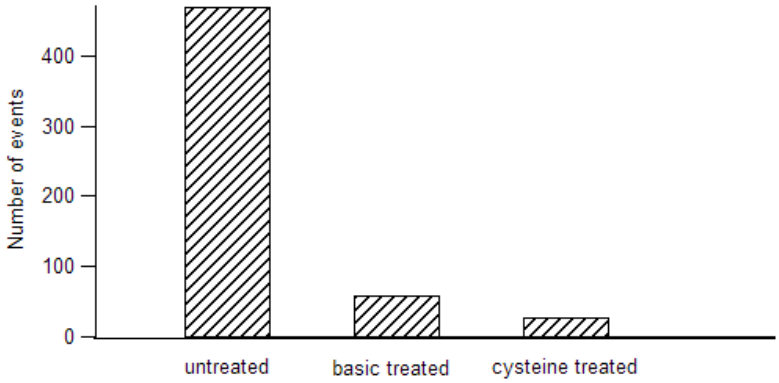
Supplementary Figures



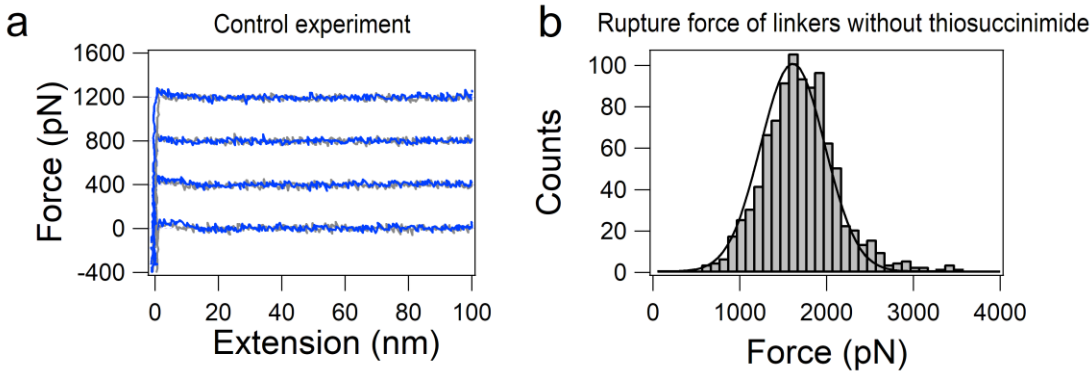
Supplementary Figure 1. Schematic diagram of AFM-based single-molecule force spectroscopy on maleimide–thiol conjugates under various condition (anhydous, aqueous and alkaline-pretreated). In the first two experiment, bifunctionalized polyethylene glycol (PEG, MW: 5 kDa, terminated with a maleimide and an *N*-hydroxysuccinimide (NHS)) was covalently conjugated to an amino-functionalized AFM cantilever tip, which was brought into contact with the thiol-functionalized glass substrate to trigger the formation of a maleimide–thiol conjugate between the cantilever tip and the substrate in anhydrous acetonitrile (a) or in aqueous PBS (b). In the third experiment (c), a bifunctional NHS-PEG-Maleimide (MW: 5 kDa) linker was reacted with surface thiol groups first. Then excessive ethylenediamine was added to introduce an amino group to the free end of the polymer prior to the hydrolysis of the maleimide–thiol conjugate. After incubation at 37 ^{o}C in a basic PBS buffer (pH = 8.1) for 5 d until the five membered ring of the thiosuccinimide was completely converted to succinamic acid thioethers via ring-opening hydrolysis, the terminal amino group was picked up by an NHS-PEG-functionalized cantilever.



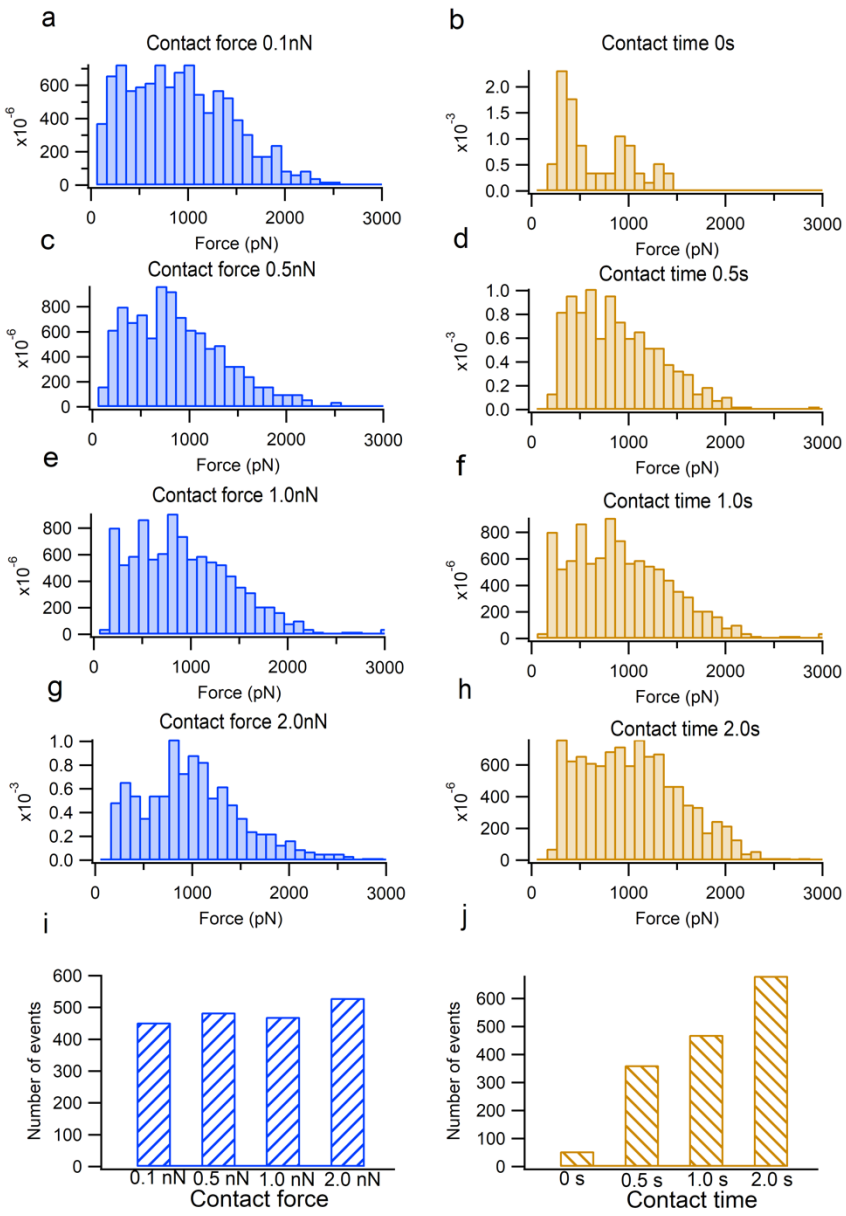
Supplementary Figure 2. Typical force traces for the stretching of the PEG linker containing newly formed thiosuccinimide in anhydrous acetonitrile (pH=7.4) (a) and in aqueous PBS (b). (c) Typical force traces of stretched alkaline-pretreated maleimide–thiol conjugates. Worm-like chain fitting curves are shown in black to highlight the mechanical features. The deviation of the force traces from the WLC model was due to the intrinsic elasticity profile of the PEG linker. Histograms of the (d) contour length (*Lc*) and (e) persistence length (*p*) showed average *Lc* values of 36.36 ± 4.95, 35.47 ± 8.75 and 34.9 ± 6.28 nm, and average *p* value of 0.388 ± 0.037, 0.386 ± 0.050 and 0.381 ± 0.034 nm for thiosuccinimide in acetonitrile, in PBS and the hydrolyzate, respectively (means ± S.D. are shown).



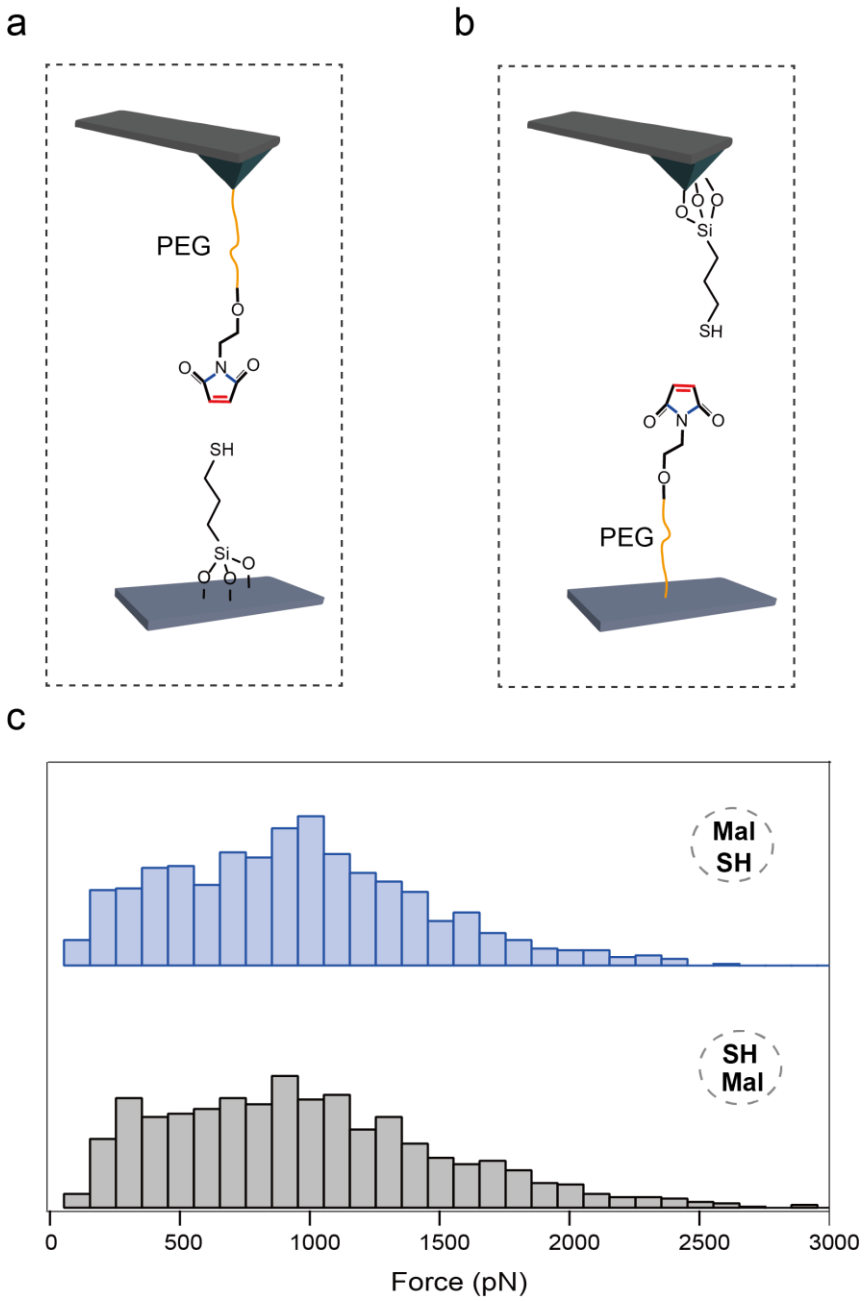
Supplementary Figure 3. The numbers of successful single-molecule events of untreated, basic treated and cysteine treated cantilever. The single-molecule pickup ratios were 7.8%, 0.39% and 0.4%, respectively.



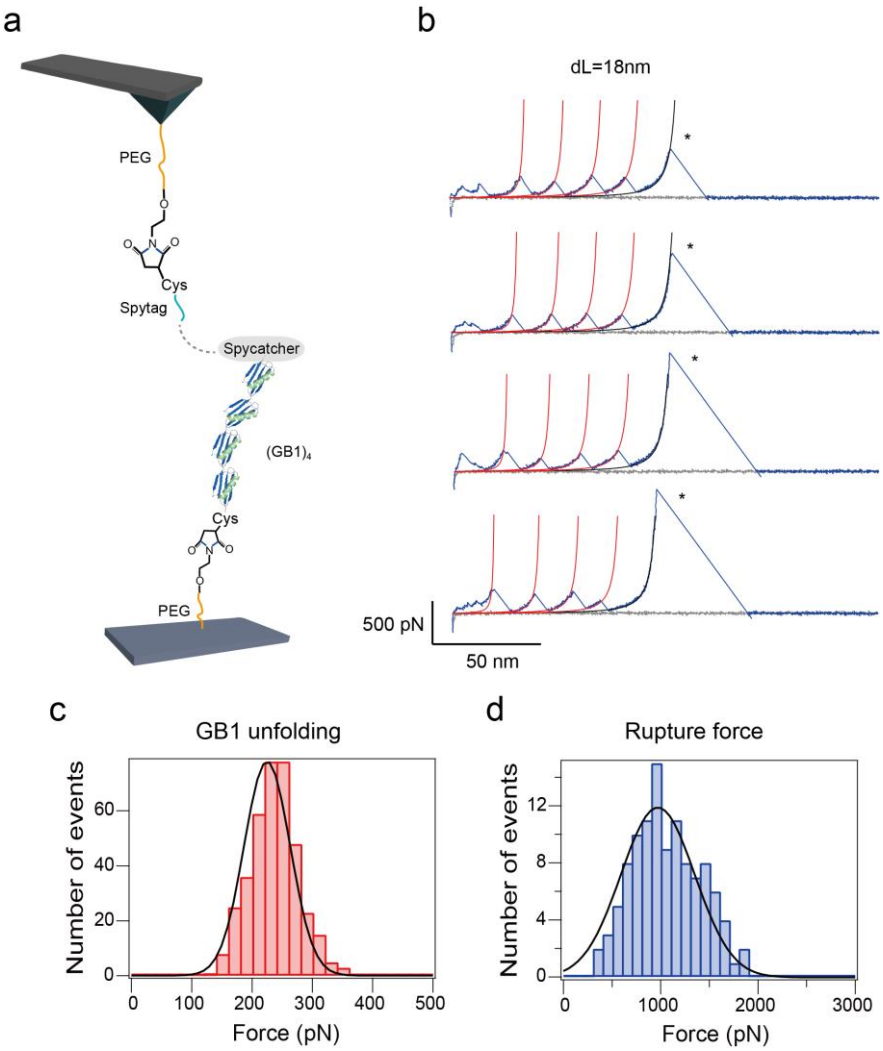
Supplementary Figure 4. (a) Typical force traces of negative control experiments without thiol-coated substrates. (b) Rupture force distribution of NHS-PEG-NHS-coated cantilever rupturing from an amino-functionalized substrate. Gaussian fitting exhibited an average rupture force of 1609±371 pN, ensuring that the single force peaks were derived from the thiosuccinimide, which was the weakest part of the linker (means ± S.D. are shown).



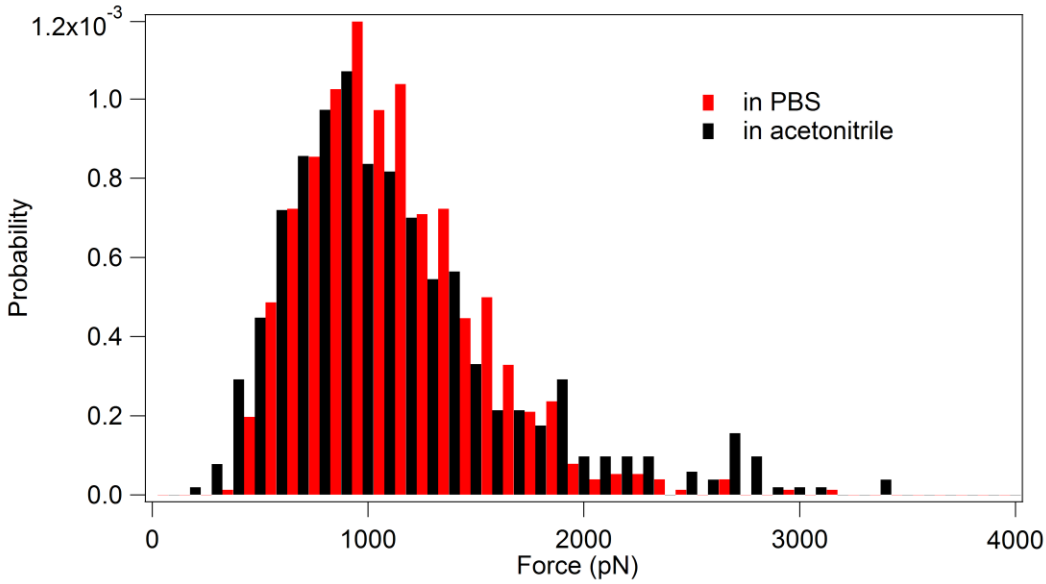
Supplementary Figure 5. Single-molecule experiments at different contact time and contact forces.(a)-(h) Rupture force distributions of the maleimide–thiol adducts in PBS buffer (pH=7.4) with different contact forces: 0.1 nN (a), 0.5 nN (c), 1.0 nN (e) and 2.0 nN (g), the contact time was fixed at 1.0 s. Rupture force distributions of the maleimide–thiol adducts in PBS buffer (pH=7.4) with different contact time: 0 s (b), 0.5 s (d), 1.0 s (f) and 2.0 s (h), the contact force was fixed at 1.0 nN. (i) The number of single-molecule events at different contact forces. The total pulling cycles were 6400 each. (j) The number of single-molecule events at different contact time. The total pulling cycles were 8400 for each experimental condition.



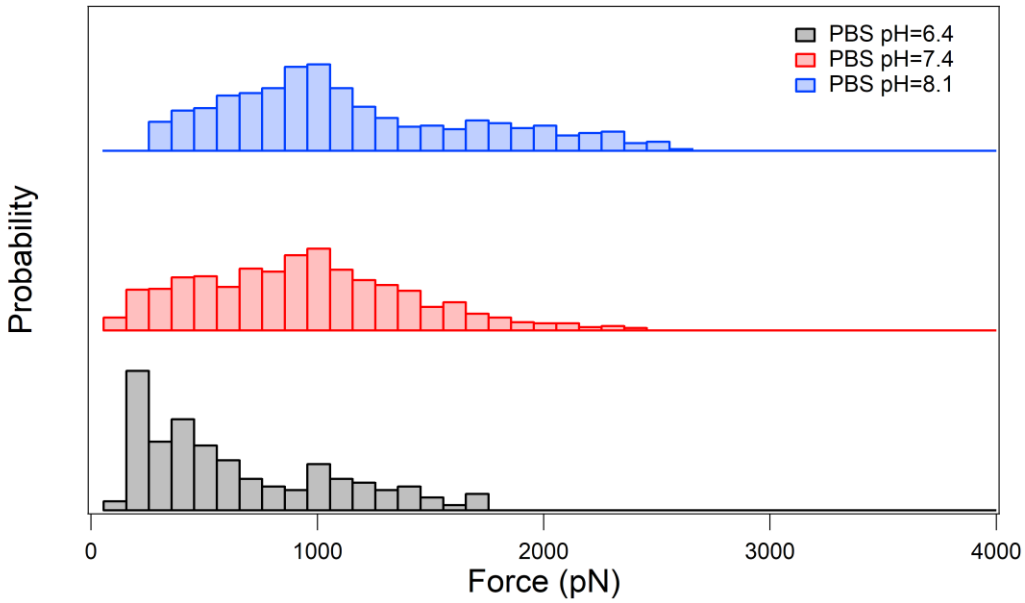
Supplementary Figure 6. Effect of pulling geometry on the rupture forces. (a) Schematic diagram of the AFM experiment of the maleimide–thiol adduct. (b) Schematic diagram of the AFM experiment with reverse geometry (maleimide on the surface and thiol on the cantilever). (c) Rupture force distributions of two AFM experiments with opposite pulling geometries.



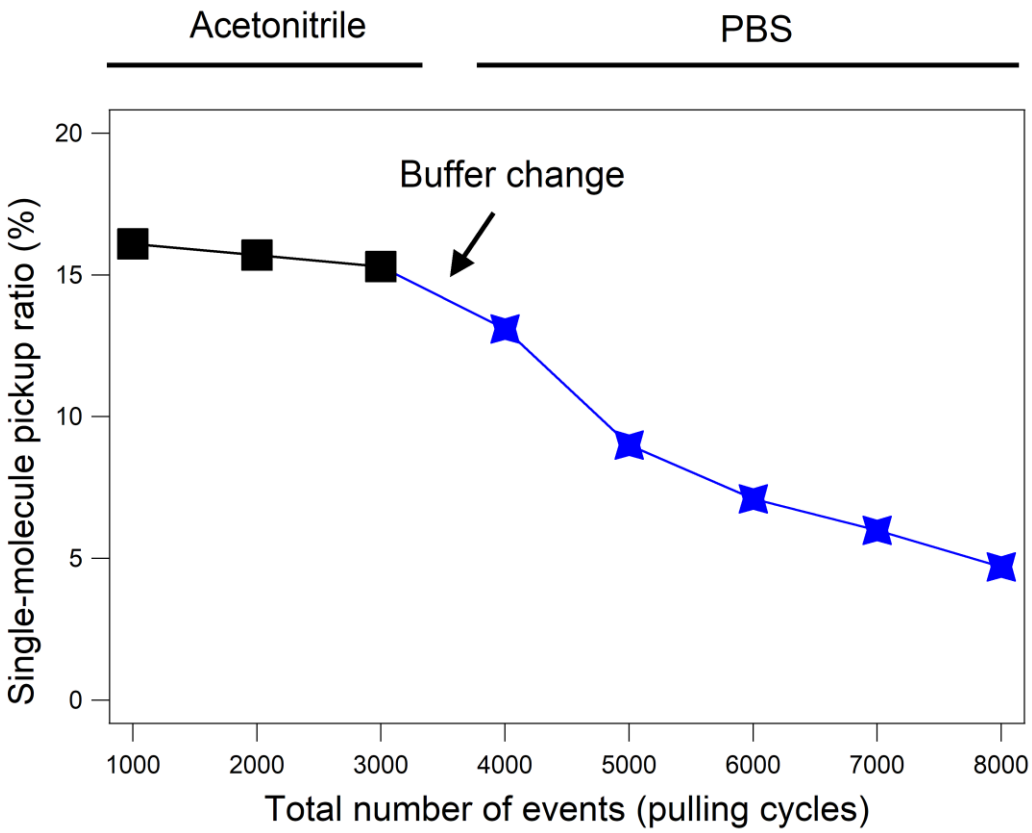
Supplementary Figure 7. Control experiments using $(GB1)_4$ as the fingerprint. (a) Schematic of the fingerprint single molecular force spectroscopy experiment. (b) Typical force-distance traces of pulling GB1-containing polyprotein. Worm-like chain (red) was used to fit the saw-tooth peaks. The last peak marked with asterisk (*) was corresponded to the rupture of the maleimide–thiol adducts. (c) Unfolding force distribution of fingerprint maker GB1 proteins. Gaussian fitting shows the average unfolding force is 225 ± 38 pN. (d) Rupture force distribution of maleimide–thiol adducts in the polyprotein shows an average force centered at 1021 ± 378 pN. The error values represent the mean ± S.D.



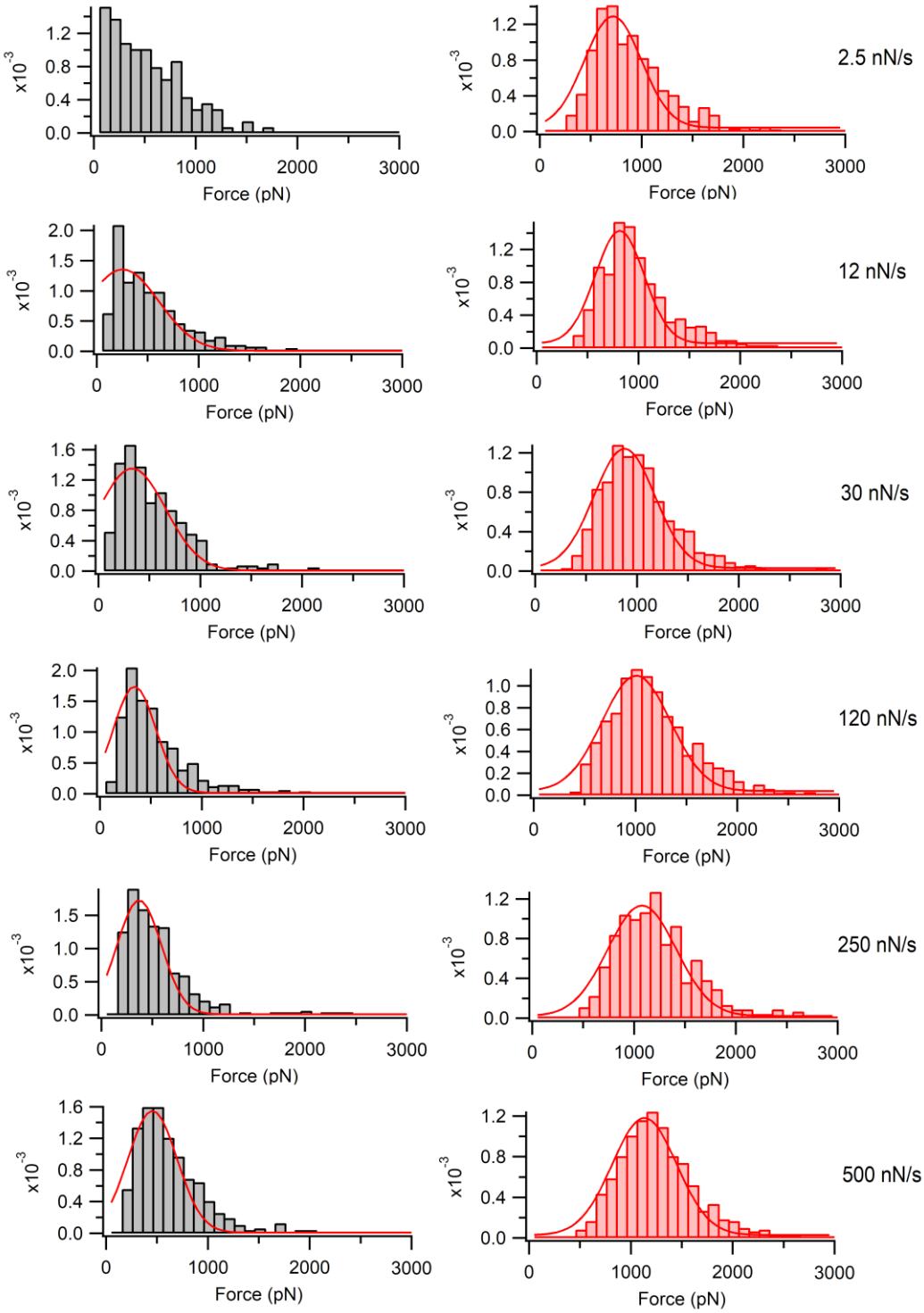
Supplementary Figure 8. Rupture force distribution of ring-open hydrolyzed maleimide–thiol conjugates in acetonitrile (black) and in 1 × PBS (pH=7.4); the data show no solvent-dependence.



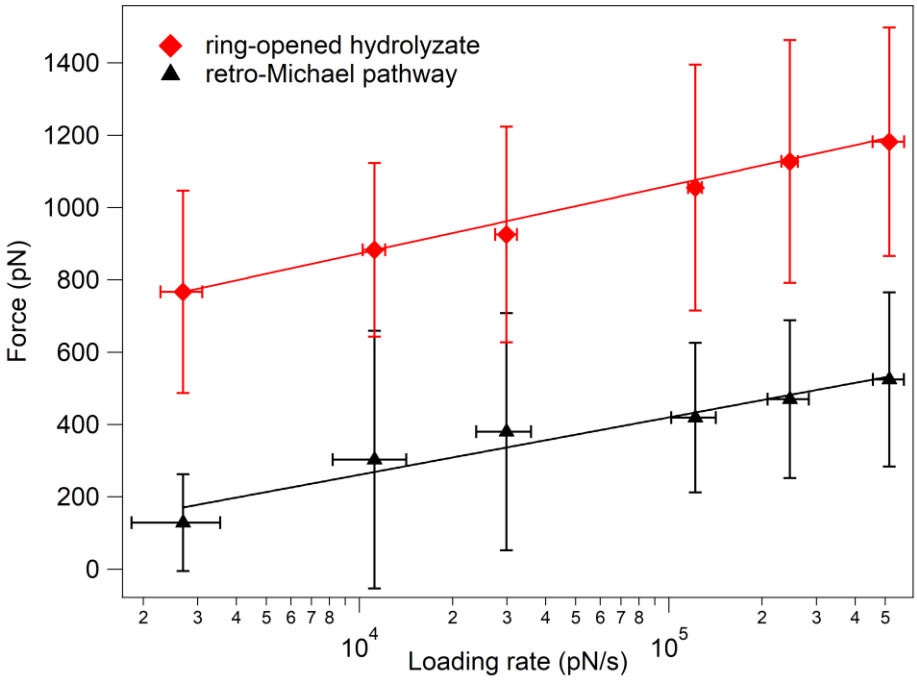
Supplementary Figure 9. Rupture force histograms of newly formed maleimide–thiol conjugates in PBS at different pH values.



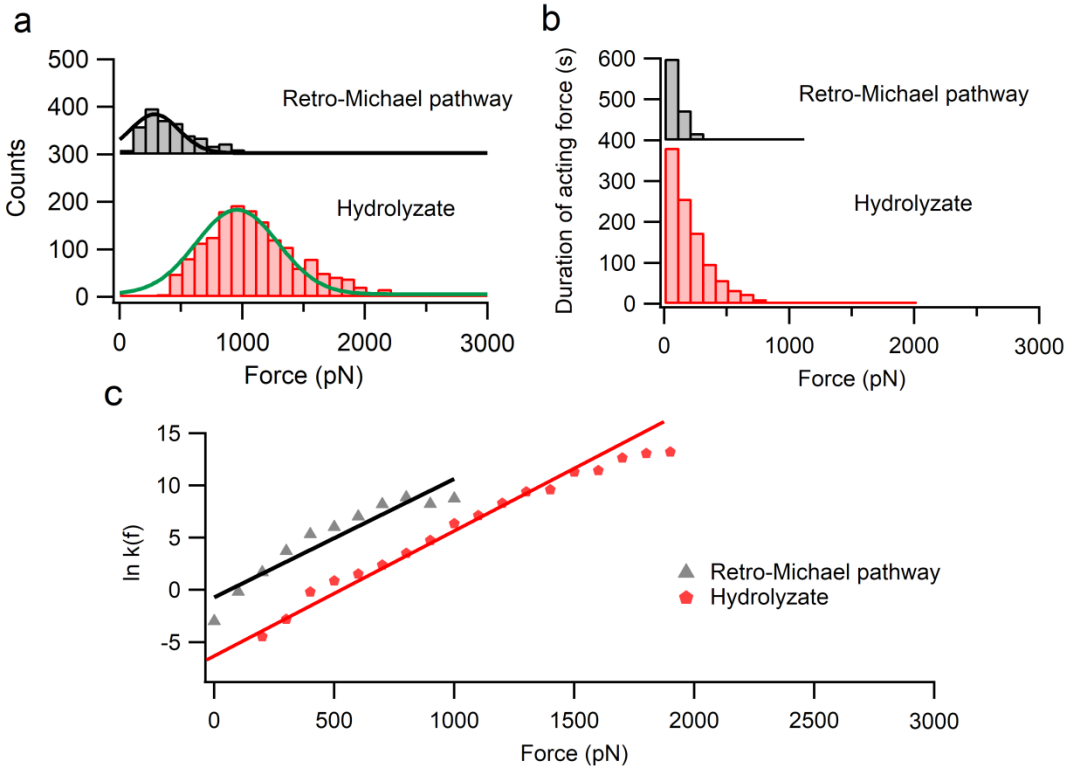
Supplementary Figure 10. Single-molecule pickup ratios in a single-molecule force spectroscopy (SMFS) experiment using one cantilever in anhydrous and aqueous solutions. The buffer was changed from anhydrous acetonitrile to aqueous PBS after ~3000 SMFS events. The pulling speed was 4000 nm s^{-1} and the total time was ~2.8 h.



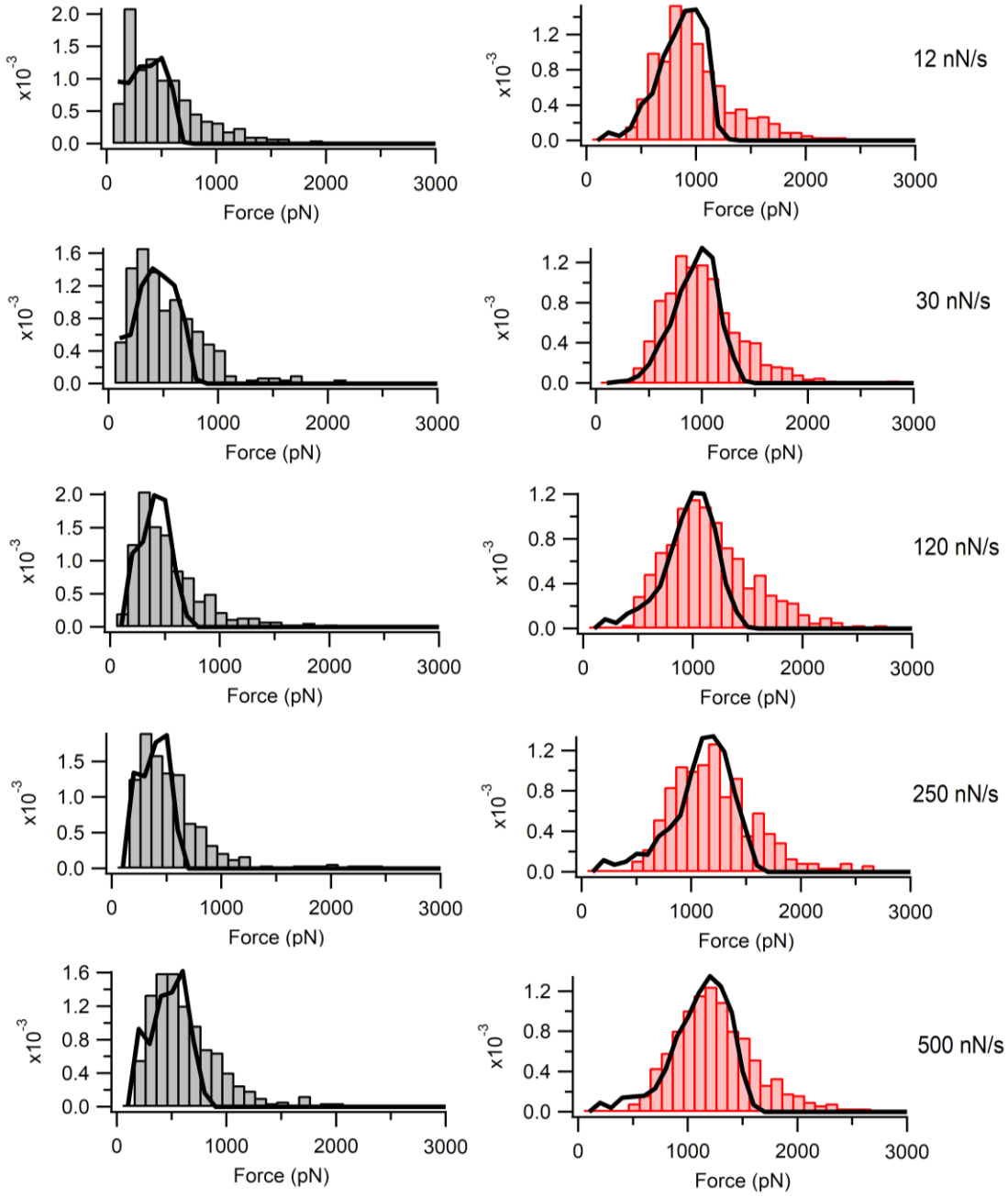
Supplementary Figure 11. Rupture force histograms of the force-induced retro-Michael reaction (left) and rupture force of alkaline-pretreated ring-opened hydrolyzate (right). The loading rates are 2.5, 12, 30, 120, 250 and 500 nN s^{-1} from top to bottom. Y-axis represents the probability of rupture force. Gaussian fittings are shown in red curves.



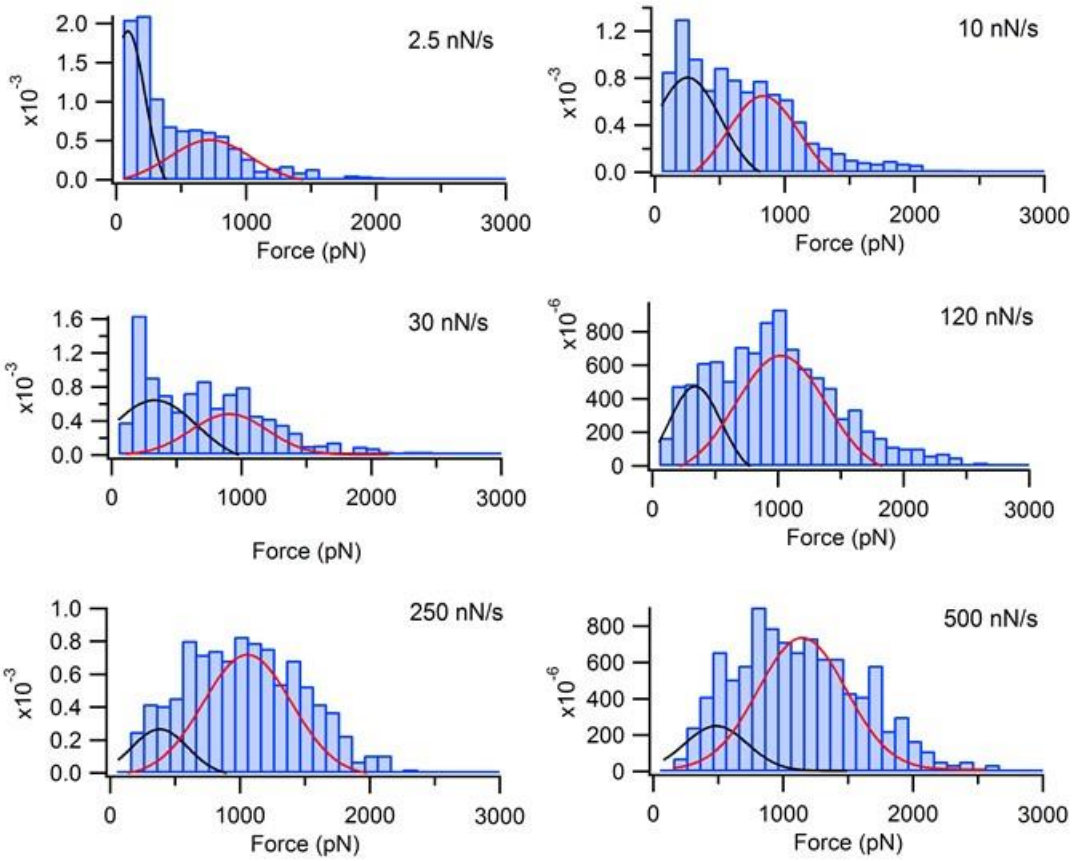
Supplementary Figure 12. Mean rupture forces of the retro-Michael reaction (black) and ring-opened hydrolyzate (red) at different loading rates. Error bars indicate the mean S.D. Parameters from the Bell-Evans fittings were $\Delta x^{\ddagger}$= 0.0559 nm and k_0= 2.1 s^{-1} for the rupture of the retro maleimide–thiol reaction and $\Delta x^{\ddagger}$=0.0505 nm and k_0= 0.00035 s^{-1} for the rupture of the ring-opened hydrolyzate.



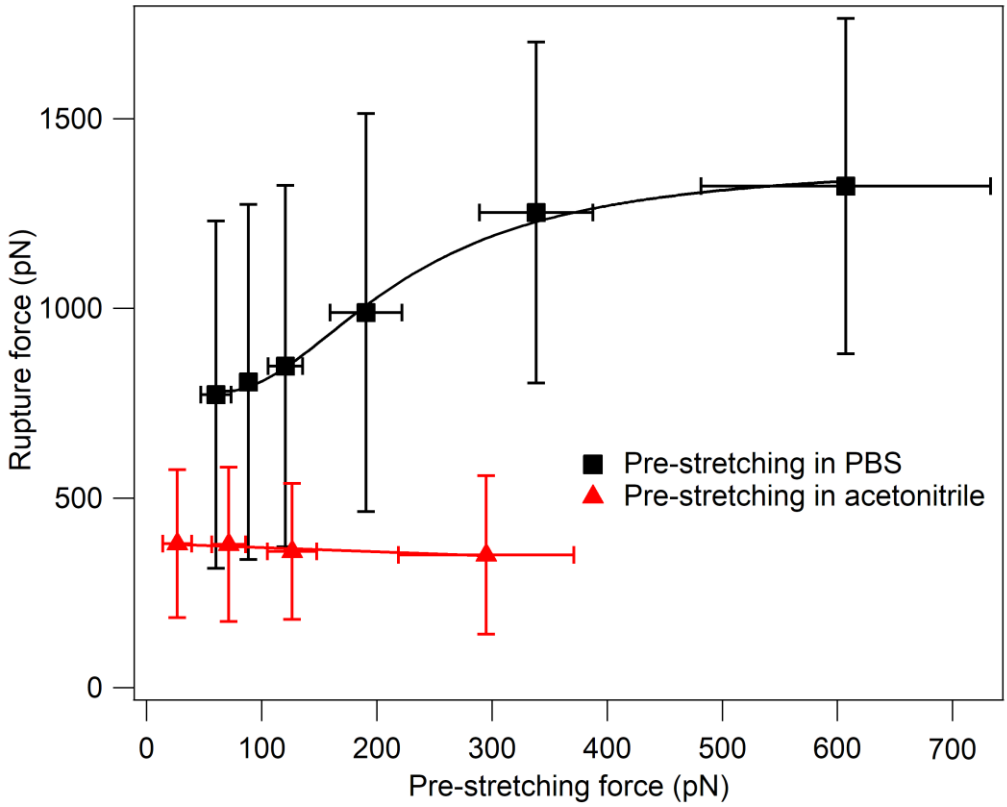
Supplementary Figure 13. Data analysis using the model-free method (a) Rupture force distributions of retro-Michael reaction (top) and hydrolyzate (bottom). (b) The total acting time of each force from all the force-extension traces. (c) The logarithm of the dissociation rates (ln *k(f)*) with a linear fit to directly measure the force-dependent dissociation free energy. The bin size is 100 pN.



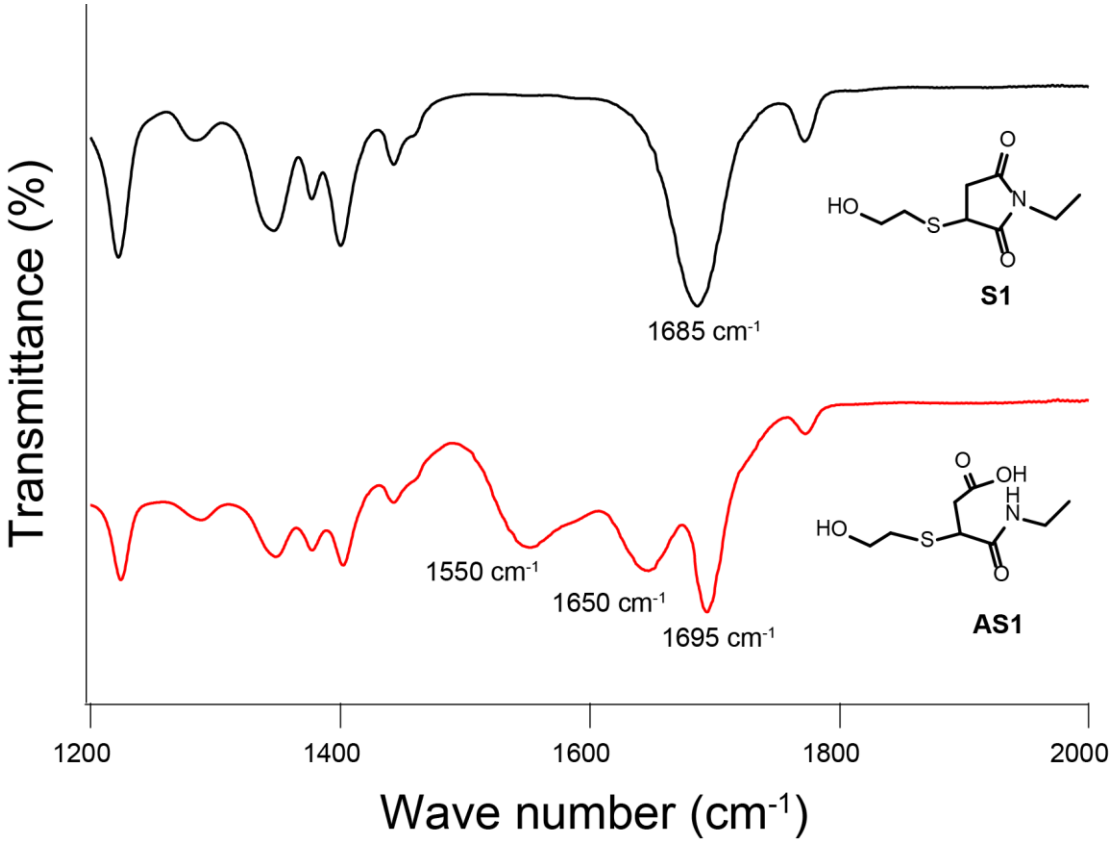
Supplementary Figure 14. Theoretically prediction force distributions by Monte Carlo method using the parameters from the dynamic force spectroscopy.



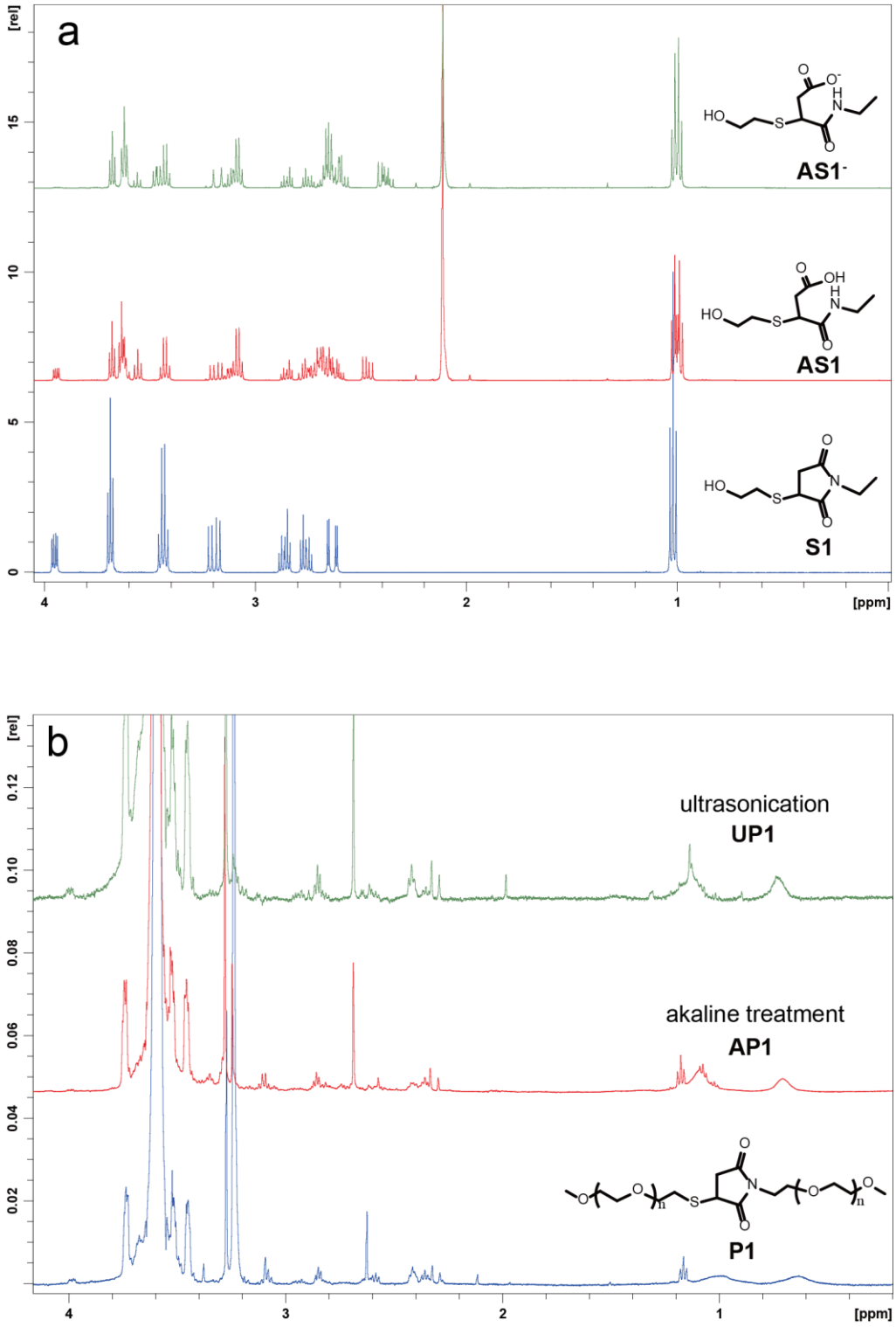
Supplementary Figure 15. Histograms of the maleimide–thiol rupture forces in PBS (pH = 7.4) buffer undergoing both the retro-Michael reaction and ring-opening hydrolysis pathway. The loading rates are 2.5, 12, 30, 120, 250 and 500 nN s^{-1} from top to bottom. Y-axis represents the probability of rupture force. Two-peak Gaussian fittings based on the retro-Michael and ring-opened hydrolyzate distributions are shown in black curves and red curves, respectively.



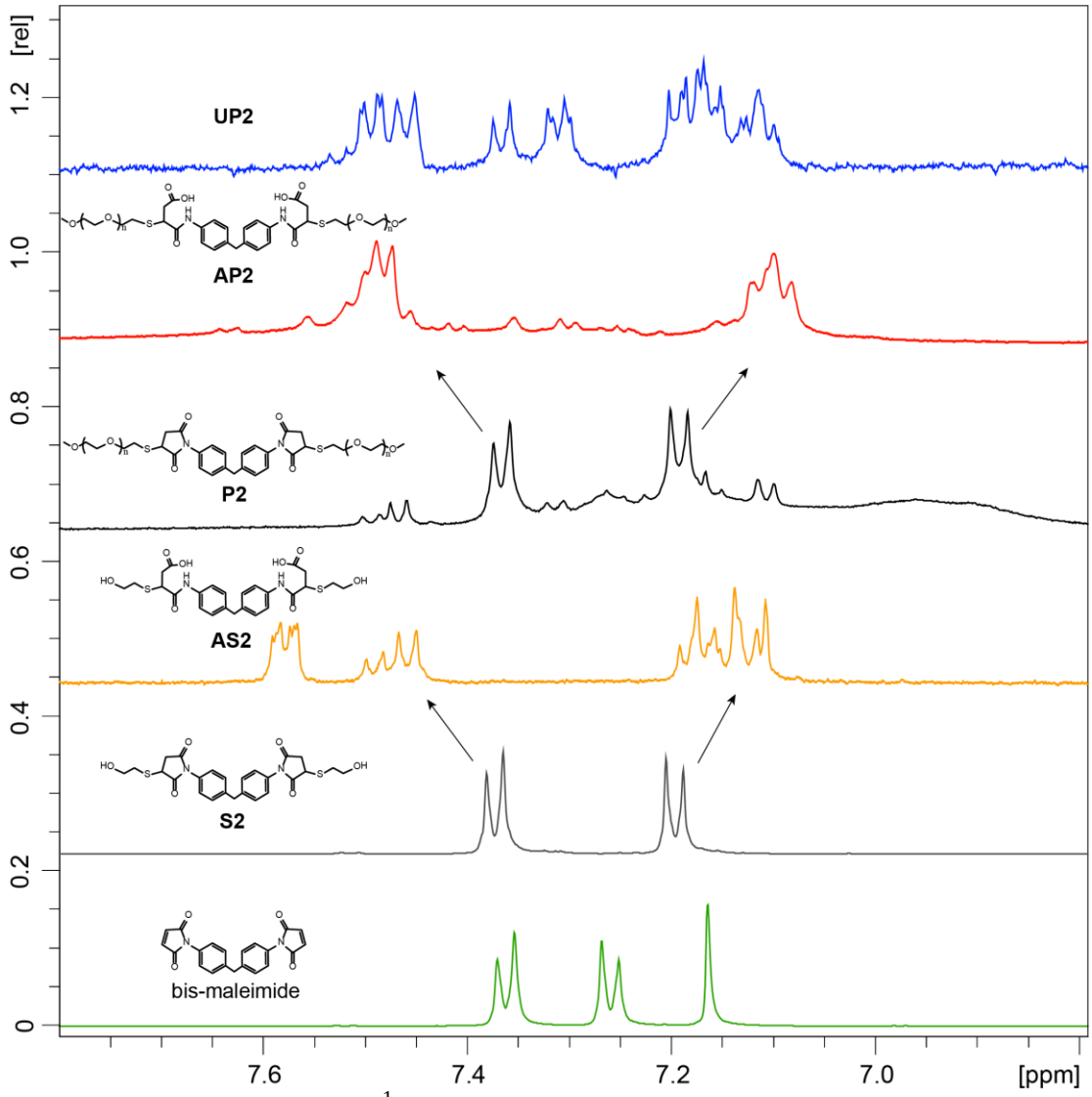
Supplementary Figure 16. Pre-stretching experiments in PBS and in acetonitrile. Error bars indicate the mean S.D.



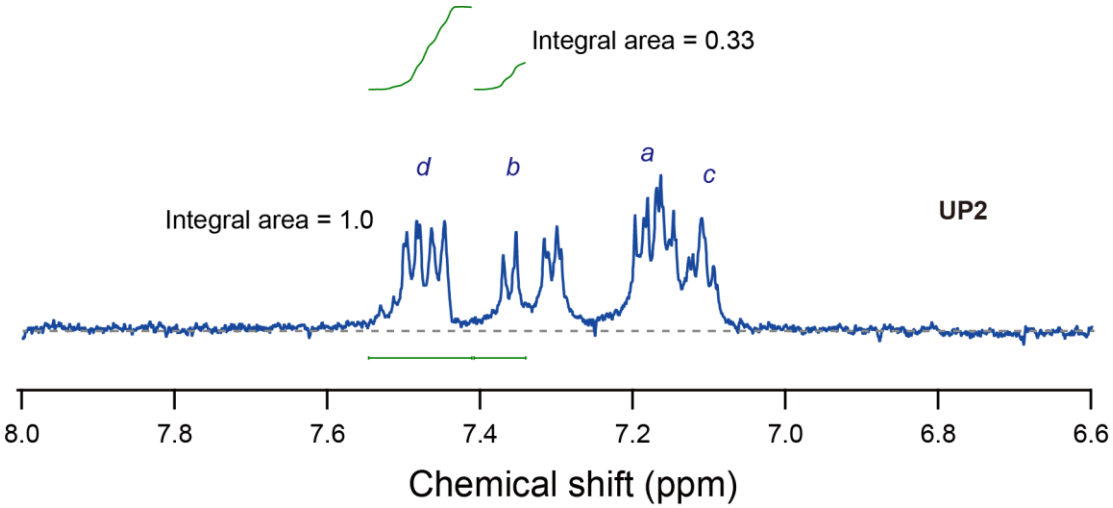
Supplementary Figure 17. FT-IR spectra of model compound **S1** and its ring-opened product, **AS1**. The appearance of new transmittance peaks at 1695 cm^{-1}, 1650 cm^{-1} and 1550 cm^{-1} after alkaline treatment (red curve) are due to the two asymmetric carbonyls and the NH of the CONH moiety formed after ring-open hydrolysis, respectively. The as-synthesized model compound has only one peak at 1685 cm^{-1} in this region, which comes from the two symmetric carbonyls on the maleimide five-membered ring.



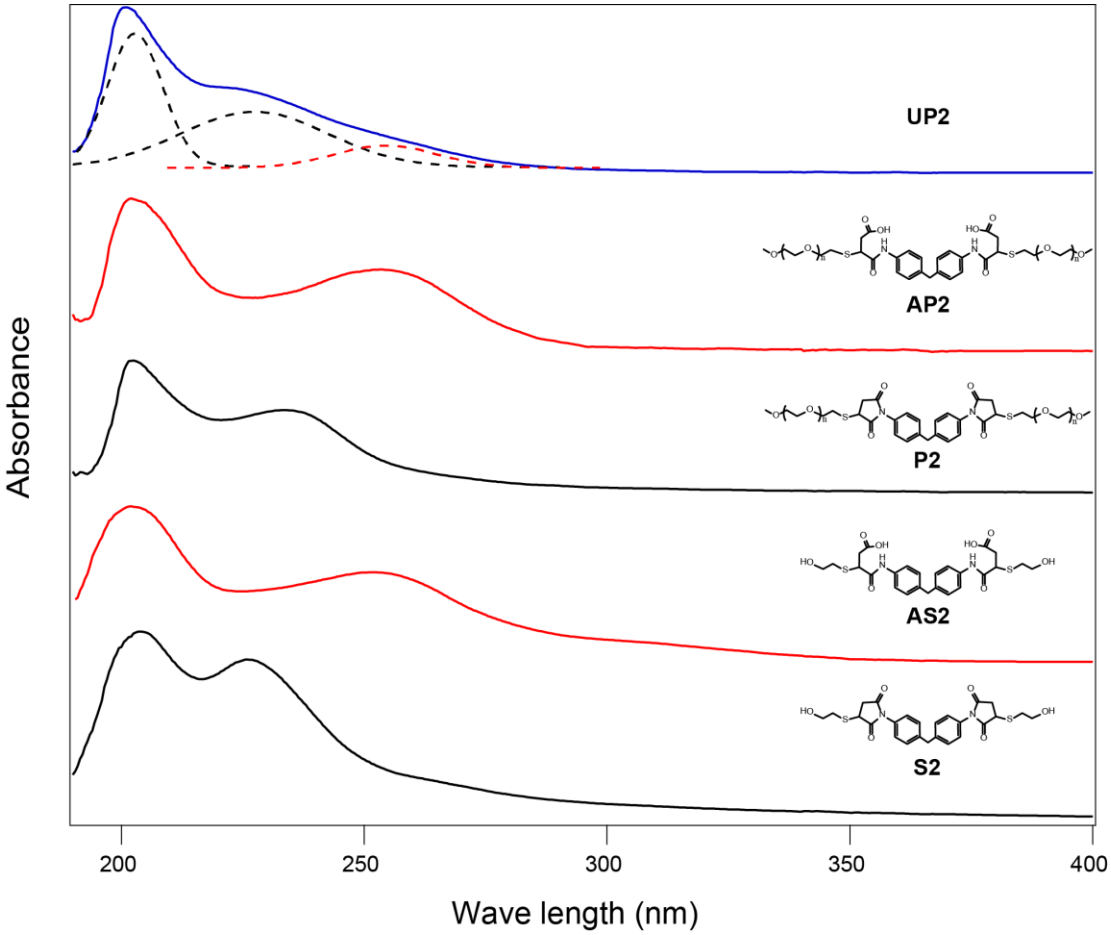
Supplementary Figure 18. (a) ^{1}H NMR spectrum of model compound **S1** and its ring-opened product **AS1** and **AS1**$^{-}$. Chemical shifts between 2 and 4 ppm were observed. (b) ^{1}H NMR spectrums of **P1, AP1** and **UP1**. Chemical shifts from the ring-opening hydrolysis were masked by the PEG signals.



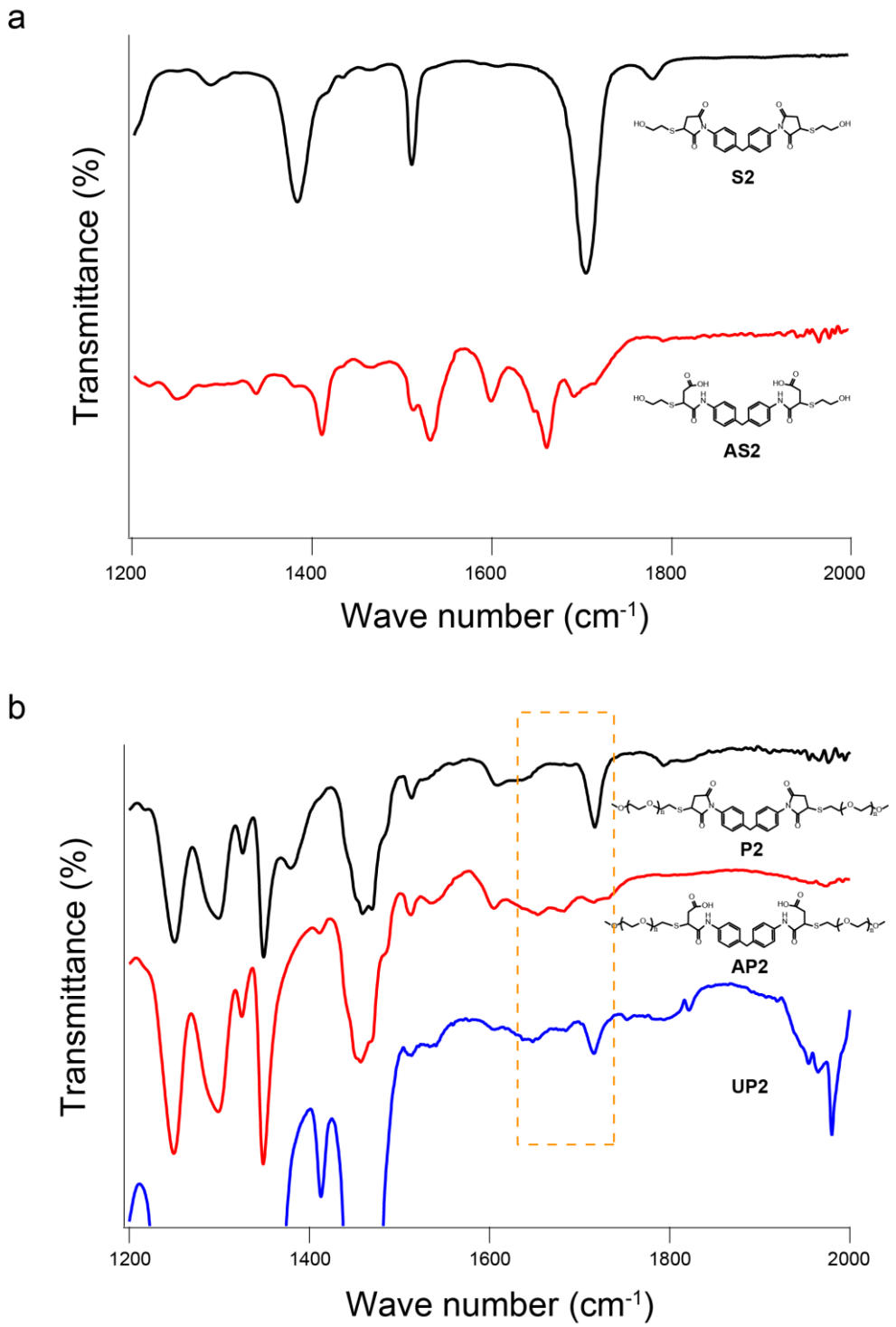
Supplementary Figure 19. ^{1}H NMR spectra of bis-maleimide model polymer P**2** (black spectrum), its alkaline-treated ring-opened product **AP2** (red spectra) and ultrasound-treated product **UP2** (blue spectra), as well as the small-molecule analogs (bottom 3 spectra) The chemical shifts of the bis-maleimide were centered at 7.360 and 7.260 ppm, which result from the symmetric aromatic protons as well as contributions from the carbon-carbon double bond of the maleimide centered at 7.165 ppm (green). Upon the addition of mercaptoethanol and thiol-PEG, the aromatic protons shift downfield and upfield respectively (two peaks centered at 7.370 and 7.190 ppm), and the peak at 7.165 ppm vanished, indicating the production of the conjugate (gray and black). After 5 days of incubation in basic PBS buffer (pH=8.1) at 37 ℃, all the thiosuccinimide was ring-opened, which was confirmed by the shifts in the aromatic proton signals from 7.370 and 7.190 ppm to 7.475 and 7.146 ppm, respectively (orange and red). Similar chemical shifts were observed for the polymers after ultrasonication (blue).



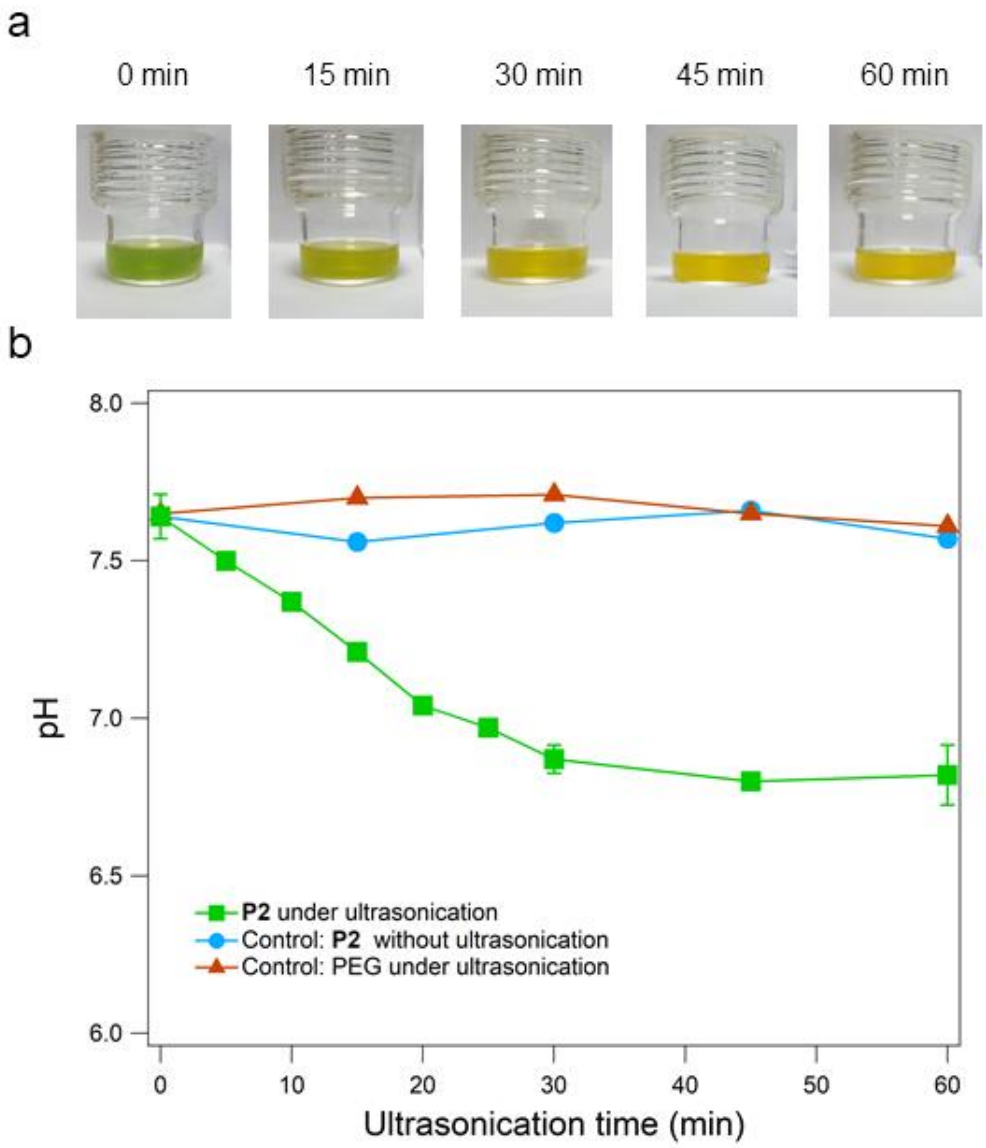
Supplementary Figure 20. Based on the integrals of peaks *b* and *d*, 75% of the thiosuccinimide-containing polymers were mechanically hydrolyzed by the applied force.



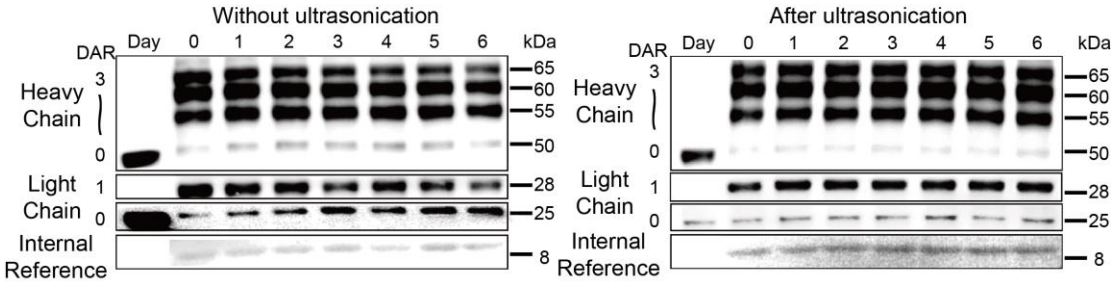
Supplementary Figure 21. UV-vis spectra showing the shift in the absorption peak from approximately 230 to 255 nm after alkaline treatment for small-molecule adduct (**S2** to **AS2**) and polymer (**P2** to **AP2**). It is obvious that the hydrolysis of the thiosuccinimide ring could occur after ultrasonication (**UP2**).



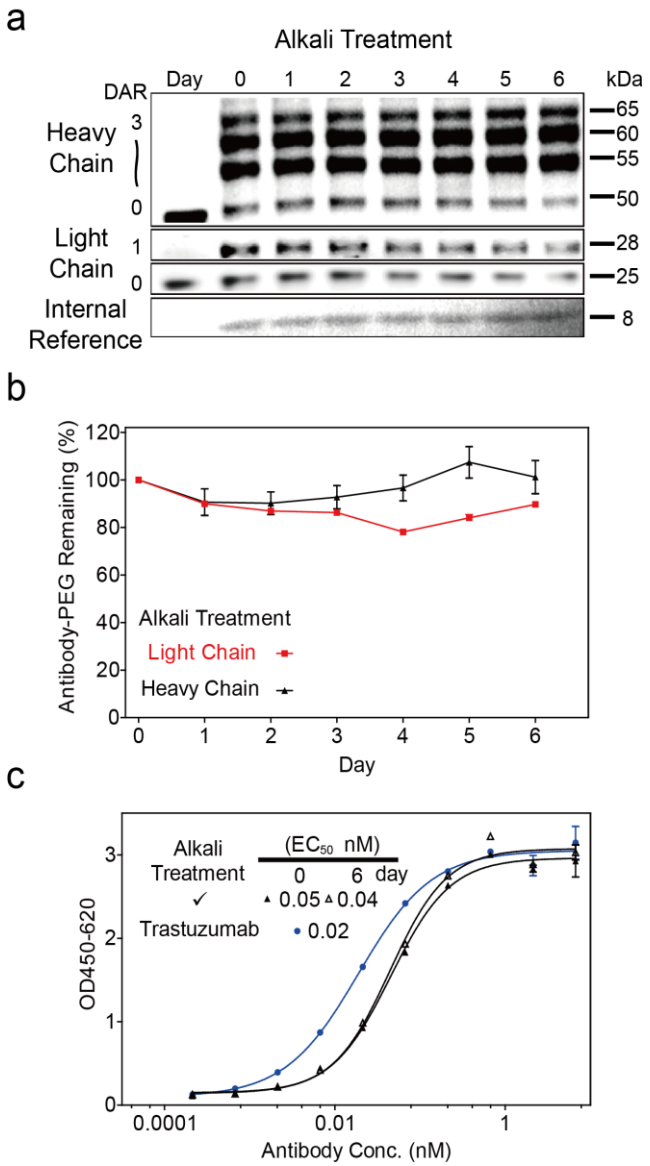
Supplementary Figure 22. FT-IR spectra of bis-maleimide model compounds and polymers. (a) FT-IR transmittance spectra of small-molecule adduct **S2** (black) and its hydrolyzate **AS2** (red) after alkaline treatment showing the appearance of peaks at approximately 1650 cm^{-1} from the ring-opened thiosuccinimide. (b) FT-IR transmittance spectra of **P2** (black), its hydrolyzate **AP2** after alkaline treatment (red) and **UP2** after pulsed ultrasonication (blue) showing similar peaks around 1650 cm^{-1} after both alkaline treatment and ultrasonication.



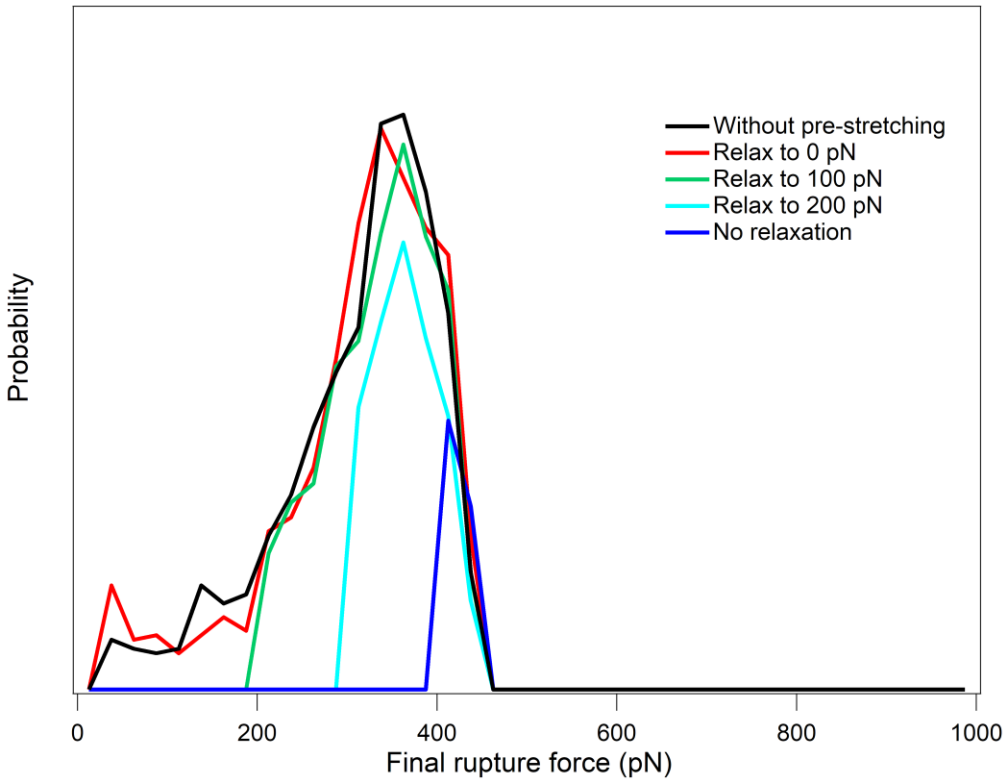
Supplementary Figure 23. pH levels of polymer **P2** during ultrasonication in pure water. (a) 5% (v/v) acid-base indicator bromothymol blue in the solution during ultrasonication changed color from green to deep yellow, indicating a drop in pH. Images were recorded every 15 min. (b) the solution pH recorded by a pH meter showed a pH drop from ~7.5 to 6.8 for polymer **P2** (green). Two control experiments, with the PEG linker under ultrasonication for 60 min (blue) and **P2** without ultrasonication (red), showed minimal pH changes and no color changes.



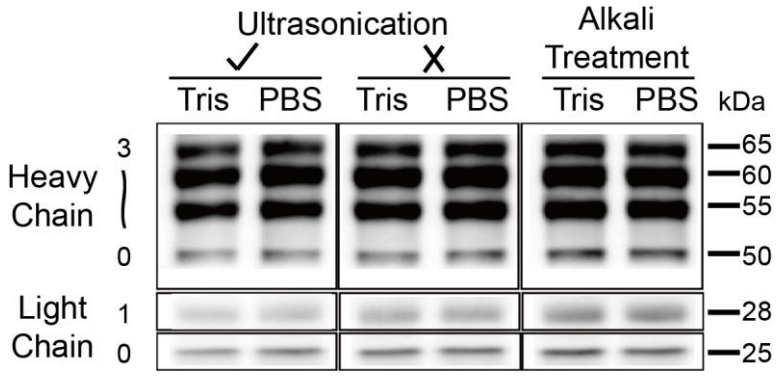
Supplementary Figure 24. Western Blot analysis of antibody-PEG conjugates without (left) or with (right) ultrasonication after incubation in a solution containing a physiological level of free thiol (1 mM glutathione solution) for 6 d. Bands at different drug antibody ratios (DAR) correspond to the light chain and heavy chain conjugates with different payloads (PEG). From top to bottom: heavy chain-3PEG (DAR=3), heavy chain-2PEG (DAR=2), heavy chain-PEG (DAR=1), heavy chain (DAR=0), light chain-PEG (DAR=1), light chain (DAR=0), and internal reference (GB1 protein). Without ultrasound treatment, the band intensity of the light chain-PEG conjugates decreased and the band intensity of free antibody (DAR=0) increased gradually at increased incubation time, suggesting the loss of PEG modification due to the rupture of the maleimide–thiol linkage. On the contrary, for the ultrasound-treated samples, the band intensity of the light chain-PEG conjugates did not change with time. For heavy chain-PEG, the degradation led to gradual decrease of DAR and the change was complicated. The loss of conjugated PEG due to the rupture of the maleimide adduct was systematically evaluated by the method shown in the Western Blot section in Method and the results were shown in Figure 4c. The Western Blot experiments were repeated independently for three times (n = 3) with similar results.



Supplementary Figure 25. Stability of alkaline-treated Ab-PEG conjugates. (a) Western Blot analysis of alkaline-treated Ab-PEG conjugates after incubation in a solution containing a physiological level of free thiol (1 mM glutathione solution) for 6 d. Bands at different drug antibody ratios (DAR) correspond to the light chain and heavy chain conjugates with different payloads (PEG). From top to bottom: heavy chain-3PEG (DAR=3), heavy chain-2PEG (DAR=2), heavy chain-PEG (DAR=1), heavy chain (DAR=0), light chain-PEG (DAR=1), light chain (DAR=0), and internal reference (GB1 protein). The Western Blot experiments were repeated independently for three times with similar results. (b) Quantitation of the remaining antibody-PEG conjugates normalized to the signal at day 0. Each data point represents the mean of three independent experiments (n = 3). Error bars indicate the mean ± S.D. (c) Quantification of the binding activity of alkaline-treated Ab-PEG conjugates (black) to its antigen Her2 protein by an ELISA assay. The unmodified antibody trastuzumab (blue) was used as a control. The representative data shown are mean ± S.D. of n = 2 biological replicate samples, EC_{50} were calculated from four-parameter cure fitting to the data. The ELISA experiments were repeated independently for four times with similar results.



Supplementary Figure 26. Monte-Carlo prediction of the final rupture force histograms with or without pre-stretching. The predicted histograms were generated by Monte Carlo simulation using Bell-Evans models with the parameters and details listed in Supplementary Method. If no chemical reactions occurred during the pre-stretching, and then the molecule was relaxed to zero force, the pre-stretching protocol does not shift the force distribution. However, if the molecule was not fully relaxed before the final stretch, the rupture force was shifted to a higher force.



Supplementary Figure 27. Western Blot analysis of untreated, ultrasound-treated and alkaline-treated antibody-PEG conjugates in PBS buffer and Tris buffer. Bands at different drug antibody ratios (DAR) correspond to the light chain and heavy chain conjugates with different payloads (PEG). From top to bottom: heavy chain-3PEG (DAR=3), heavy chain-2PEG (DAR=2), heavy chain-PEG (DAR=1), heavy chain (DAR=0), light chain-PEG (DAR=1), light chain (DAR=0). The Western Blot experiments in PBS buffer and Tris buffer were repeated independently for three times with similar results.

Supplementary Table 1. Summary of the number of successful pulling cycles over the course of all single-molecule experiments.

Experiment	Pulling speed (nm/s)	Total number	Single-molecule number	Single-molecule ratio (%)
Anhydrous (retro-Michael)	100	3385	138	4.07
	400	3124	362	11.58
	1000	3450	383	11.10
	4000	3314	477	14.39
	8000	3512	451	12.84
	16000	3486	462	13.25
Aqueous	100	10585	982	9.28
	400	11708	905	7.72
	1000	12564	1153	9.18
	4000	12734	1002	7.87
	8000	12596	957	7.60
	16000	12558	630	5.02
Alkaline-pretreated (hydrolyzate) in PBS	100	9824	407	4.14
	400	9874	405	4.10
	1000	9824	521	5.30
	4000	23475	1031	4.39
	8000	9924	458	4.61
	16000	10074	601	5.97
Alkaline-pretreated (hydrolyzate) in acetonitrile	4000	9742	552	5.67
pH=6.4	4000	8064	192	2.38
pH=8.1	4000	8835	720	8.15
Control experiment tip-Mal-glass surface	4000	5603	15	0.27
Control experiment tip-NHS sub-APTES	4000	10889	1023	9.39
Control experiment basic treated	4000	15809	61	0.39
Control experiment cysteine treated	4000	7416	30	0.40
Control experiment reverse	4000	10993	1026	9.33

geometry				
Fingerprint experiment	1000	5425	109	2.01
Control experiment contact force change	**Contact force (nN)**	**Total number**	**Single-molecule number**	**Single-molecule ratio (%)**
	0.1	6400	453	7.08
	0.5	6400	485	7.58
	1.0	6400	471	7.36
	2.0	6400	530	8.28
Control experiment contact time change	**Contact time (s)**	**Total number**	**Single-molecule number**	**Single-molecule ratio (%)**
	0	8400	55	0.86
	0.5	8400	363	5.67
	1.0	8400	471	7.36
	2.0	8400	682	10.66
Pre-stretching experiment in PBS	**Pulling speed (nm/s)**	**Total number**	**Single-molecule number**	**Single-molecule ratio (%)**
	4000	71470	3938	5.51
Pre-stretching experiment in acetonitrile	4000	28798	1797	6.24
Time-dependent pre-stretching experiment	**Pre-stretching time (ms)**	**Total number**	**Single-molecule number**	**Single-molecule ratio (%)**
	50	10,329	610	5.90
	200	8047	439	5.45
	400	10301	442	4.29
	800	36515	1281	3.51
	1600	31391	750	2.39

Supplementary Table 2. Comparison of the positions of the transition state and the rate constants at zero force for the ring-opening pathway and the retro-Michael pathway calculated from Bell-Evans model and model-free analysis. The error values represent the mean S.D.

	Bell-Evans model		**Model-free**	
	$\Delta x^{\ddagger}$**/nm**	k_0**/s^{-1}**	$\Delta x^{\ddagger}$**/nm**	k_0**/s^{-1}**
Ring-opening pathway	0.0505 ±0.0073	3.5 ±0.6 ×10^{-4}	0.0413 ±0.0014	6.39 ± 4.10 ×10^{-4}
Retro-Michael pathway	0.0559 ±0.0034	2.1 ±0.79	0.0465 ±0.0049	1.44 ±0.74

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