Supporting information

Tension Sensing Nanoparticles for Mechano-Imaging at the Living/Nonliving Interface

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Materials

(3-Aminopropyl) trimethoxysilane (97%, APTMS), O-(2-carboxyethyl)-O′-(2-mercaptoethyl)heptaethylene glycol (95%, COOH-EG_8-SH), 2,5-dihydroxybenzoic acid (99%, DHB), triethylamine (99%, TEA), dithiothreitol (99.0%, DTT), triethylammonium acetate (TEAA), gold (III) chloride hydrate (99.0%), sodium citrate (99%), paraformaldehyde (95%, PFA), Hank's balanced salts (#H1387) and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. The heterobifunctional linker azide-NHS (#88902) was purchased from Thermo Fisher Scientific (Rockford, IL). The fluorescent dyes Alexa488-maleimide and Alexa488-NHS, Alexa647 labeled IgG_1 secondary antibody as well as the OliGreen assay were purchased from Life Technologies (Carlsbad, CA). Number two glass coverslips, ascorbic acid (>99.0%), and 96-well plates were purchased from Fisher Chemical & Scientific (Pittsburg, PA). DMF (>99.5%), DMSO (99.5%) and sodium bicarbonate (99.0%) were purchased from EMD chemicals (Philadelphia, PA). Cyclic(RGDfK)C (Fig. S1) was custom synthesized by Peptides International (Louisville, KY). All DNA strands used were custom synthesized and desalted by Integrated DNA Technologies (Coralville, Iowa). Alkyne-PEG-SH (MW 3400), mPEG-NHS (MW 2000), and lipoic acid-PEG-NHS (MW 3400) were purchased from Nanocs (New York, NY). Amine-PEG-SH (MW 3400) was purchased from Creative PEGworks (Winston Salem, NC). $CuSO_4.5H_4O$ was purchased from Mallinckrodt (St. Louis, MO) and P4 gel size exclusion beads were acquired from BioRad (Hercules, CA). IgG1 paxillin-antibody was obtained from EMD Millipore (Billerica, MA). All DI water was obtained from a Nanopure water purification system with a UV sterilization unit and showed a resistivity of 18.2 MΩ.

Methods

Synthesis of gold nanoparticles.[1,2] Citrate-stabilized gold nanoparticles (AuNPs, 14.8 ± 0.8 nm) were prepared using published procedures. Briefly, a 500 mL solution of 1 mM hydrogen tetrachloroaurate (III) trihydrate was brought to a vigorous boil, and once boiling, 50 mL of a 38.8 mM sodium citrate tribasic dihydrate solution was added and allowed to reflux for 15 min. The reaction mixture was filtered using a 0.45 μm acetate filter, producing monodisperse AuNPs. The extinction spectrum of the AuNPs was determined using UV-vis spectrometry, and particle size was verified using transmission electron microscopy (TEM).

Negative staining TEM. The polyethylene glycol (PEG) modification of AuNPs was visualized by negative staining TEM. PEG molecules have poor contrast in conventional TEM due to their low electron density. Therefore, negative staining was used to enhance the contrast and identify the PEG shell surrounding the gold core. Specimens were prepared in DI water. The samples were deposited onto glow discharged 300 mesh carbon coated copper grids (Electron Microscopy Sciences). After a 30 seconds incubation period, excess liquid was wicked away and the specimens were stained with 1% methylamine tungstate (Ted Pella, Inc). Excess stain was wicked away after incubating it on the grid for 1 minute. The sample grids were subsequently dried under vacuum. TEM measurements were acquired on a Hitachi H-7500 transmission electron microscope at an accelerating voltage of 75 kV. The micrographs were recorded at magnifications of 200,000×.

Ensemble fluorescence measurement. The ensemble fluorescence emission intensity of Alexa488 dye from all AuNP conjugates was measured by using a Biotek Synergy HT plate reader at λ = 485 nm/528 nm excitation/emission. Each well in the 96-well plate (Fisher scientific) was filled to a volume of 100 μL. All measurements were performed in triplicate, and the reported error bars represent the standard deviation of these measurements.

HPLC. All PEG conjugated products were purified by using a C18 column (diameter: 4.6 mm; length: 250 mm) in a reverse phase binary pump HPLC that was coupled to a diode array detector (Agilent 1100).

MALDI-Mass Spectroscopy. A 10 mM solution of 2,5-dihydroxybenzoic acid (DHB) was prepared in tetrahydrofuran (THF) as the MALDI matrix and 0.1 M sodium chloride was added as cationization agent.[3] All products were also pre-dissolved in THF and then mixed with an equal volume of DHB matrix. 2 μL of this mixture was added to each well on the MALDI plate. After allowing the solution to dry for 20 min, the sample was analyzed by a high performance MALDI time-of-flight mass spectrometer (Voyager STR).

Optical Microscopy. Living cells were imaged in standard cell imaging buffer (Hank's balanced salt, pH 7.4, 10 mM HEPES without phenol red) at 37 °C, and fixed cells were imaged in 1% BSA in 1× PBS at room temperature. During imaging, physiological temperature was maintained with a warming apparatus consisting of a sample warmer and an objective warmer (Warner Instruments 641674D and 640375). The microscope was Nikon Eclipse Ti driven by the Elements software package. The microscope features an Evolve electron multiplying charge

coupled device (EMCCD; Photometrics), an Intensilight epifluorescence source (Nikon), a CFI Apo 100× (numerical aperture (NA) 1.49) objective (Nikon) and a TIRF launcher with two laser lines: 488 nm (10 mW) and 638 nm (20 mW). This microscope also includes the Nikon Perfect Focus System, an interferometry-based focus lock that allowed the capture of multipoint and time-lapse images without loss of focus. The microscope was equipped with the following Chroma filter cubes: TIRF 488, TIRF 640, FITC and reflection interference contrast microscopy (RICM).

AFM imaging. The density of AuNP tension sensor on the functionalized glass coverslip was measured by using an atomic force microscope mounted on an anti-vibration stage (Asylum Research, CA). Silicon AFM tips (MikroMasch) with a force constant (5.4-16 N/m) were used to image the sample in tapping mode at a scan rate of 1 Hz. All images were processed and rendered using IgorPro.

Cell culture. HCC1143 cells were cultured in RPMI 1640 medium (Mediatech) supplemented with 10% FBS (Mediatech), HEPES (9.9 mM, Sigma), sodium pyruvate (1 mM, Sigma), L-glutamine (2.1 mM, Mediatech), penicillin G (100 IU ml^{-1}, Mediatech) and streptomycin (100 µg ml^{-1}, Mediatech) and were incubated at 37 °C with 5% CO_2. Cells were passaged at 90–100% confluency and plated at a density of 50% using standard cell culture procedures. All experiments were conducted with HCC1143 cells that had been serum-starved for ~12 h.

Paxillin-immunostaining. Paxillin-immunostaining was used to image the location of mature focal adhesion sites at the cell membrane. After allowing HCC1143 cells to engage the surface, cells were fixed by incubation in 1 ml of 4% w/v paraformaldehyde [4] for 10 min. The PFA was subsequently rinsed off using 25 ml of 1 X PBS, and then cells were permeated by incubation with 0.1% (v/v) Triton X-100 for 5 min in PBS. The cells were then rinsed with 25 ml 1 X PBS, and then blocked for 1 h using 1% w/v BSA. Note that the force signal gradually weakens as a function of time after fixation, and therefore, overnight blocking is not recommended. 10 µg/mL of primary IgG_1 anti-paxillin antibody was incubated for 1 h and then rinsed using 25 ml of 1 X PBS. The cells were then incubated using an Alexa647 labeled secondary antibody (Life Technologies) for 1 h. After thorough rinsing, TIRF microscopy was then immediately used to image paxillin.

Synthesis of tension sensor ligand

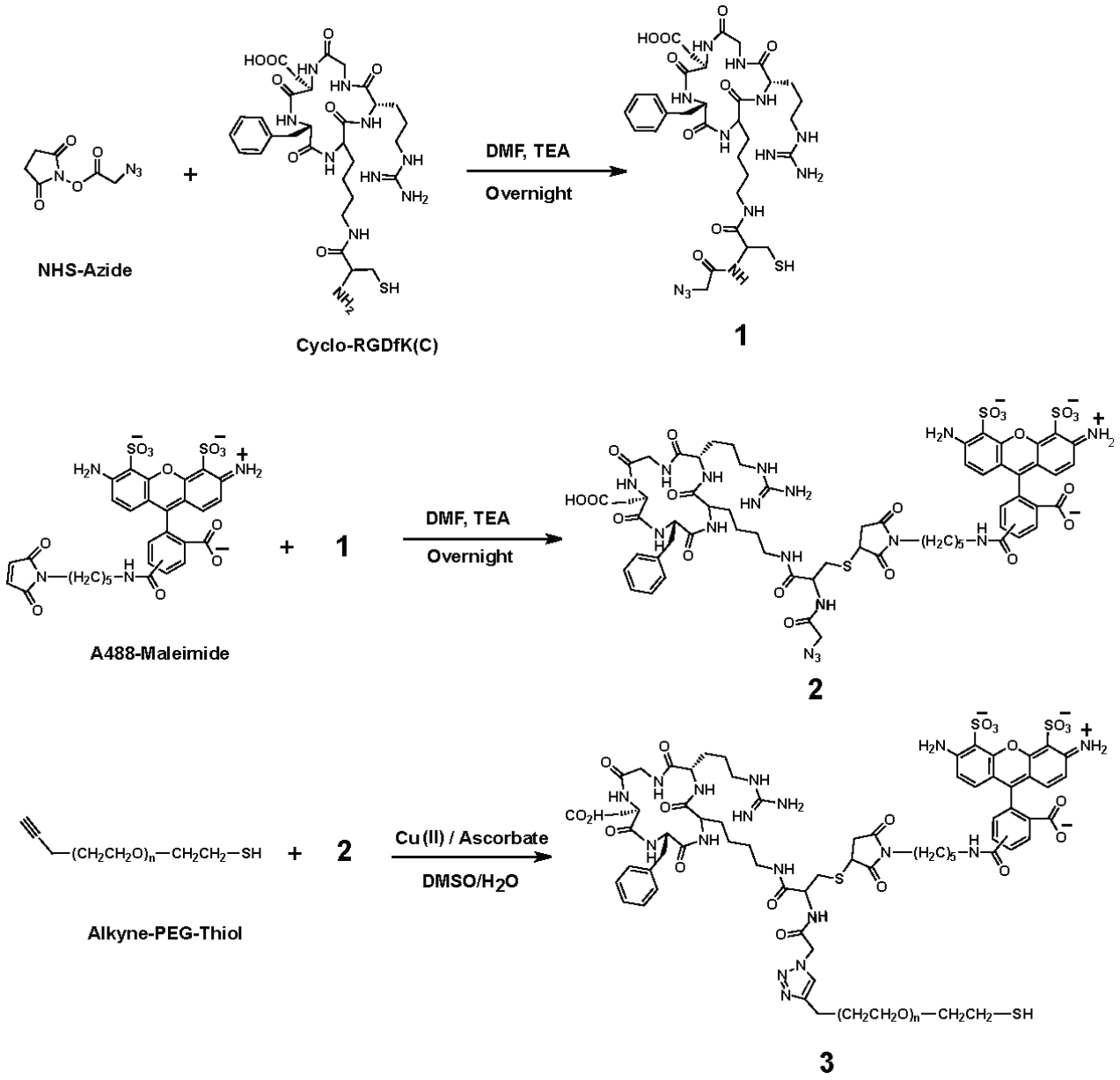

Figure S1. Synthesis of tension sensor ligand

Synthesis of **1**. 200 µg of cRGDfK(C) peptide (MW: 706.81) was reacted with 0.1 mg azide-NHS linker (MW: 198.14) in 15 µL DMF. To this reaction mixture, 0.1 µL of neat triethylamine was added as an organic base and the reaction was allowed to proceed for 12 h. The product, **1**, of this reaction was purified by reverse phase HPLC (flow rate 1 ml/min; solvent A: 99.5% DI water, 0.5% TFA; solvent B: 99.5% acetonitrile 0.5% TFA; initial condition was 10% B with a gradient of 1% per min). The yield of the reaction was determined to be 49% by integrating the HPLC peaks. Note that the yield was low due to the formation of dimer products formed by oxidation of the thiol. Although 0.1 M dithiothreitol (DTT) treatment of the oxidized product **1** minimized dimer formation, DTT was found to inadvertently reduce the azide group to an amine group.[5,6] Thus, the (cRGDfK)C starting peptide was typically pre-treated with 0.1 M DTT before use in order to minimize the dimer starting material.

Synthesis of **2**. A 10-fold molar excess of maleimide-Alexa488 dye in 10 µL DMF was mixed with purified **1** (~1-10 mM) and 0.1 µL of neat TEA overnight. The product of this reaction, **2**, was subsequently purified by HPLC using the same method that was described for purification of **1**. The molecular weight of the product was measured

using MALDI-TOF as 1493 m/z, which was consistent with a calculated m/z of 1501. The concentration of purified product **2** was determined by UV-vis absorbance of Alexa488 dye and the sample was then dried in a Speedvac for 6 h.

Synthesis of **3**. A 0.7 molar ratio of alkyne-PEG-SH (~1 mM) to **2** was dissolved in 10 µL DMSO and 5 µL DI water. 2 µL of 2 M TEAA buffer and 2 µL of 5 mM ascorbic acid was then added to this solution. Finally, 1 µL of $Cu(II)SO_4$ was added to the solution and vortexed. The reaction was incubated overnight at room temperature with foil to protect from ambient light. The reaction mixture was then treated with 0.1 M DTT in phosphate buffer for 1 h to reduce disulfides that formed overnight. Afterwards, size exclusion chromatography was performed with a spin column (cutoff: 4000 MW) to remove all other by-products except the final product **3.** HPLC (method was the same as the one used for **1** and **2**), and MALDI-TOF MS were used to confirm the synthesis of **3** with a near 99% purity, which was based on HPLC peak integration.

HPLC and MALDI characterization

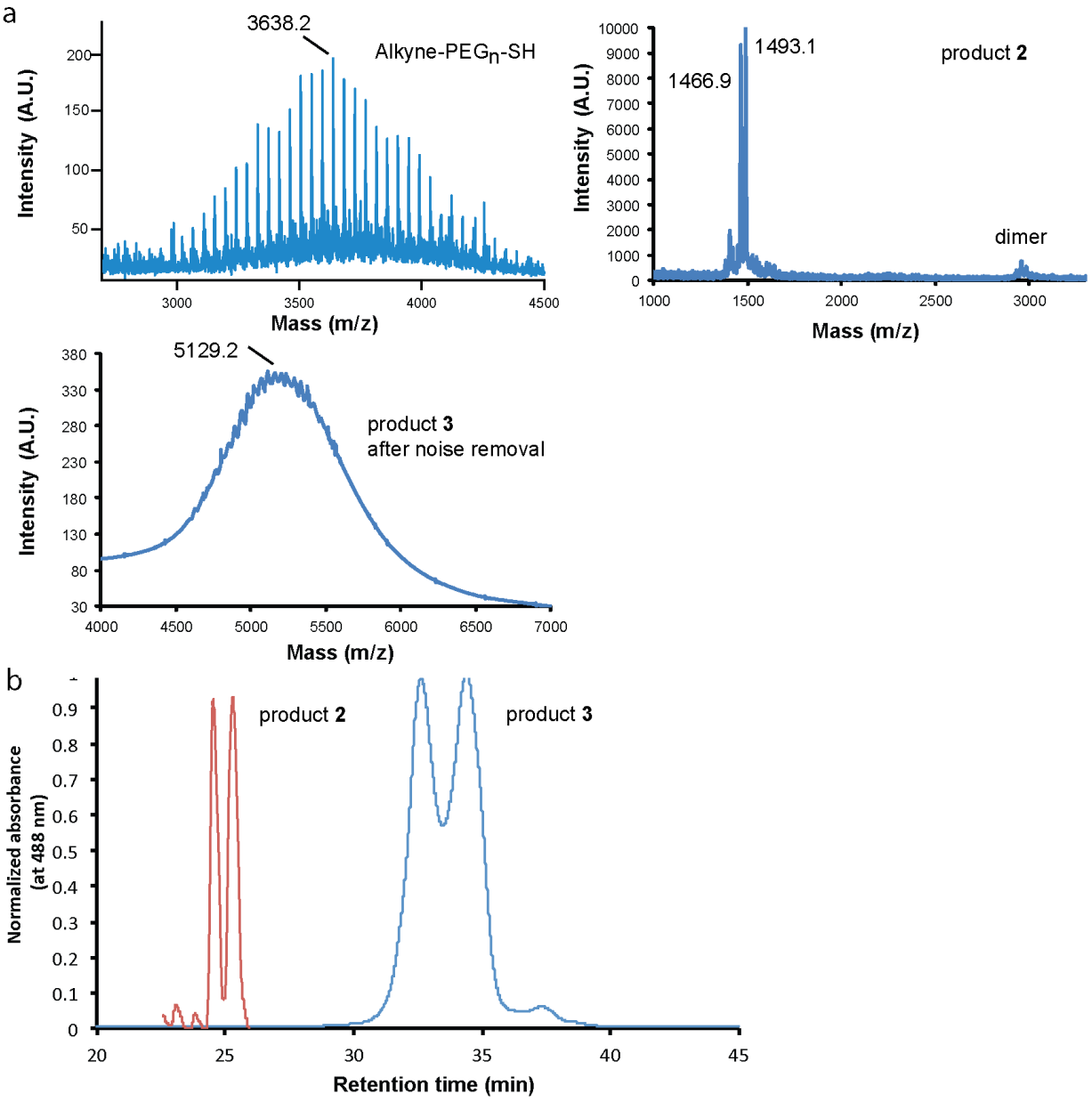

Figure S2. (a) MALDI-TOF mass spectra for alkyne-PEG-SH and Alexa488-RGD-azide **2** and final sensor ligand **3**. (b) HPLC purification of **2** and **3**.

The mass of the alkyne-PEG_n-SH reagent was obtained using MALDI-TOF, and found to be poly-disperse with a maximum intensity at 3638.2 m/z (Figure S2a). In the mass spectrum of the PEG polymer, we observed peak to peak spacings of 44 m/z, corresponding to the mass of one ethylene glycol monomer. Using this value, we determined the average number of the monomers in this PEG molecule to be 80, after subtracting the mass of the alkyne and thiol functional groups along with one sodium ion.[3] The FWHM of the mass peak was determined to be 14, which indicates a standard deviation in the number of monomers corresponding to 80±7 ethylene glycol units per polymer. The mass of **3** was measured at 5129.2 m/z. The difference between the mass of **2** and **3** is 3636.1 m/z, which agrees with the average mass of the alkyne-PEG_{80}-SH starting material (Figure S2a). In the mass spectrum of product **2**, the peak at 1493.1 m/z is the product peak, while the peak at 1466.9 m/z is equal to

the molecular ion peak minus the mass of N_2. The loss of N_2 from the azide is likely due to the MALDI laser excitation.

Importantly, the HPLC chromatograph of **2** and **3** shows a significant shift in their retention times. This is due to the conjugation with alkyne-PEG_{80}-SH, which markedly increase the molecular weight of the product. Moreover, the doublet in the chromatograph of **2** and **3** is likely due to the isomers of the maleimide group in Alexa488 dye molecule.

Synthesis of AuNP sensors

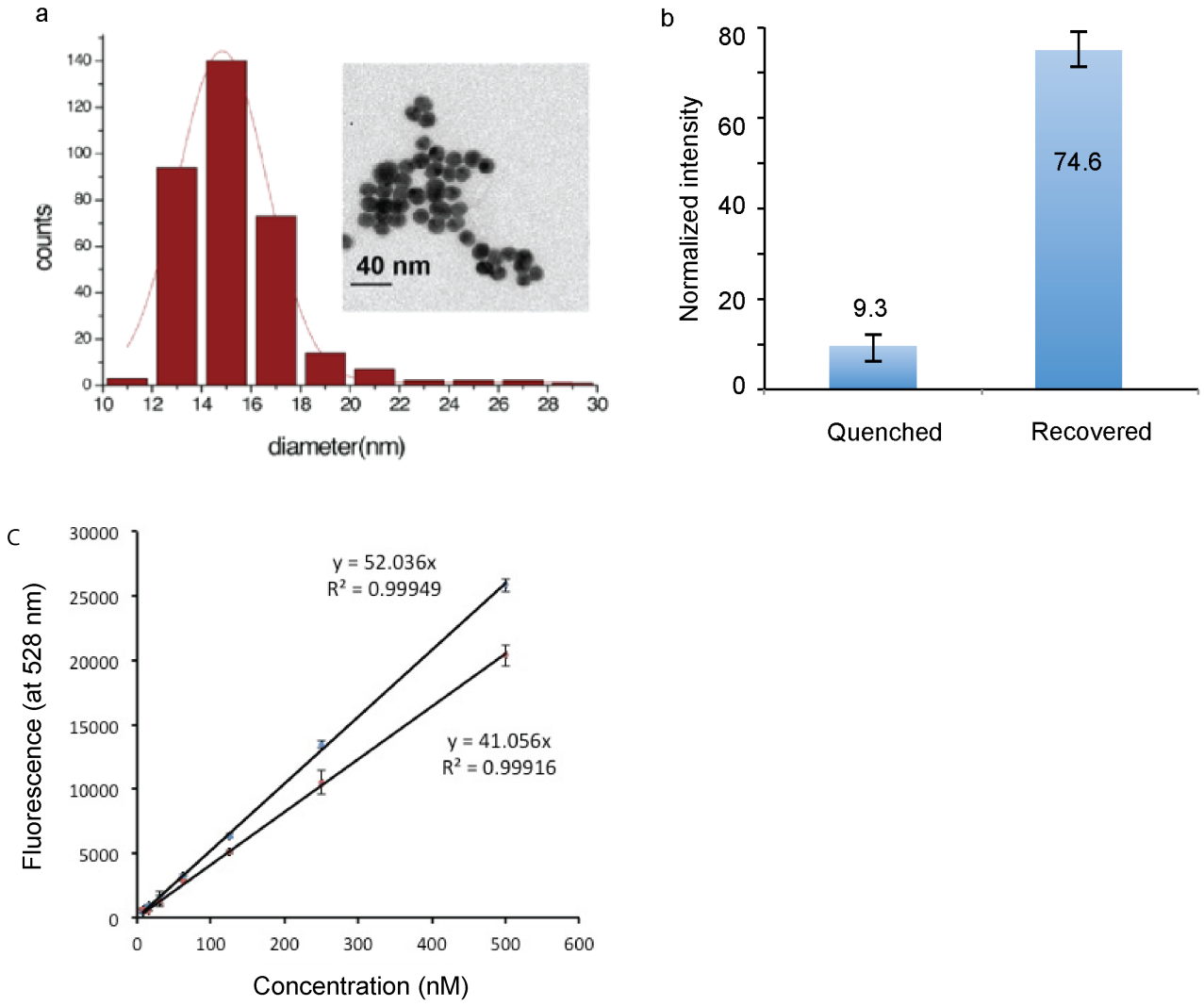

Figure S3. Characterization of gold nanoparticles. (a) Histogram showing the diameter distribution of AuNPs as measured by TEM. (b) The normalized fluorescence intensity of AuNP tension sensors before and after 50 mM KCN treatment. (c) Plot showing a fluorescence calibration curve of Alexa 488 in the presence of 50 mM KCN (blue) and in the presence of 2 nM AuNPs (red).

A 4.1 nM solution of citrate-stabilized AuNPs (15 nm) was incubated with 39.6 μM of COOH-EG_8-SH and 0.4 μM of tension sensor ligand (**3**) to form AuNP-sensor. After 12 h of incubation at room temperature, the AuNP-sensors were purified from excess of unbound ligands by using six rounds of centrifugation (13500 rpm, 25 min each) and washing with DI water.

To determine the quenching efficiency of Alexa488 in **3** by the AuNP, we measured the fluorescence intensity from these nanoparticle conjugates before and after treatment with 50 mM KCN.

Two corrections were applied to ensure the validity of this assay. The first was to account for KCN-induced quenching of Alexa488. The first calibration curve was generated by addition of 50 mM KCN to a range of Alexa488 solutions. This calibration plot is shown in Figure S3(c). The second type of correction accounted for the AuNP filter effect, since AuNPs will block some of the fluorescence excitation source at λ = 490 nm. To measure the filter effect, we doped 2 nM of COOH-EG_8-SH modified AuNP to a series of Alexa488 solutions. By comparing the slope shift, we calculated the filter effect to be 1.3.

Based on this analysis, we determined that the experimental quenching efficiency to be 87.5% for Alexa 488.

AuNP surface loading of PEG molecules

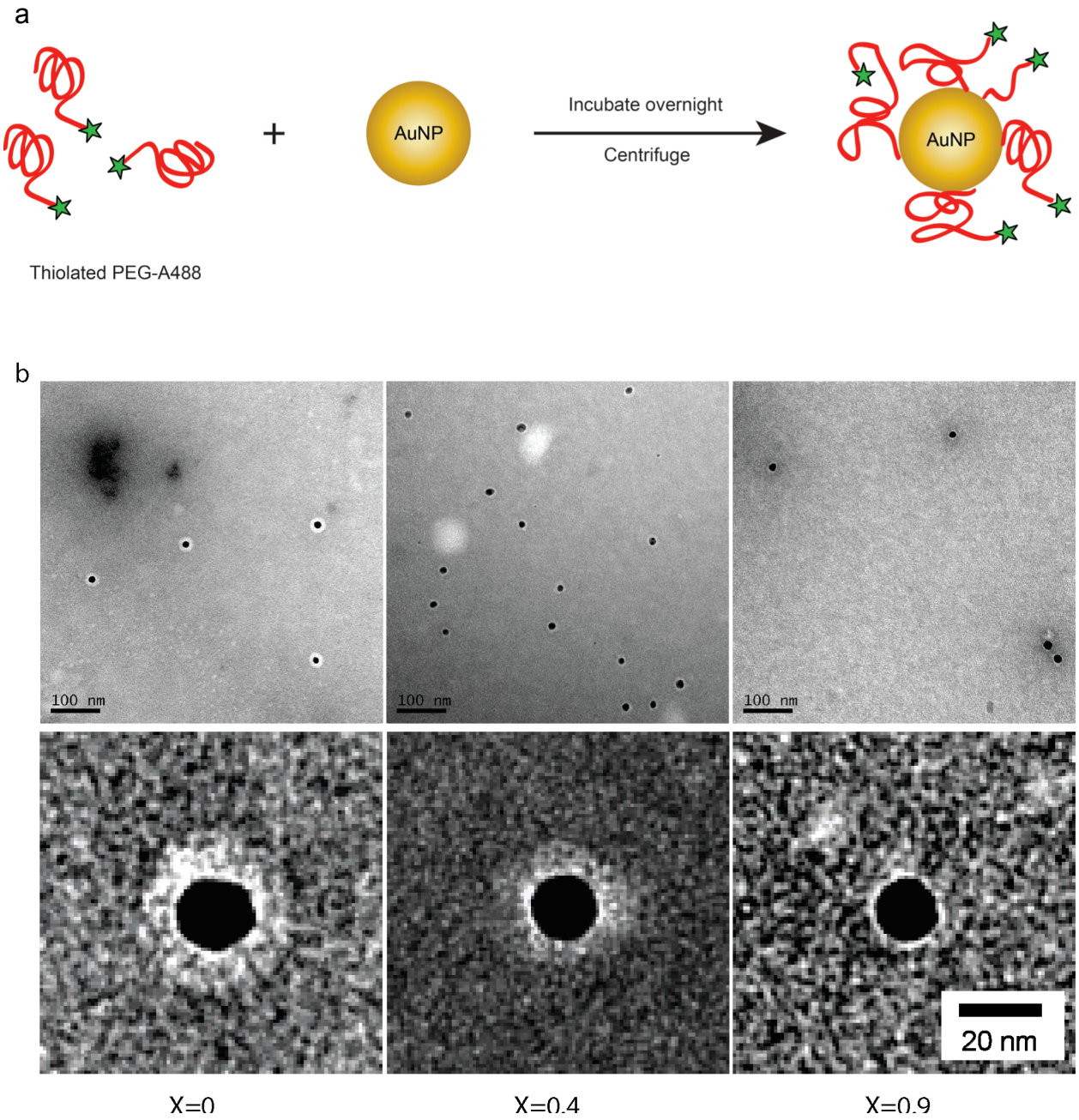

Figure S4. Synthesis of AuNP-PEG-Alexa488 for PEG conformation study. (a) Scheme showing the steps in AuNP conjugate synthesis. (b) Representative negative staining TEM micrographs of particles with X from 0 to 0.9, indicating the effect of polymer density on PEG conformation (Upper, raw image; lower, zoom in image).

10 mM of amine-PEG_{82}-thiol was mixed with 30 mM Alexa488-NHS ester in DMF with 0.1 µL TEA. The reaction was carried out for 12 h and followed by size exclusion chromatography with a 4000 MW cutoff (P4 gel). The purity of the final product was confirmed by HPLC to be 98%. A series of SH-PEG-A488 solutions ranging in concentration from 1 to 80 µM were then mixed with a 4.1 nM solution of citrate-capped AuNPs for 12 h (see main Figure 2). After purification by six rounds of centrifugation and washing, we performed negative staining TEM to analyze each of these samples.

To generate the plot in main Figure 2B, we used a binary PEG system to modify the particles. In these experiments, the total PEG concentration was maintained at 40 μM and the ratio between the COOH-EG_8-SH and A488-PEG_{82}-SH was varied from 0 to 0.99. Also, negative stain TEM was performed to verify the molecular conformation of the PEG_{82} as it transitions from mushroom to brush (see Figure 2b).

Substrate modification with densely packed AuNP sensors

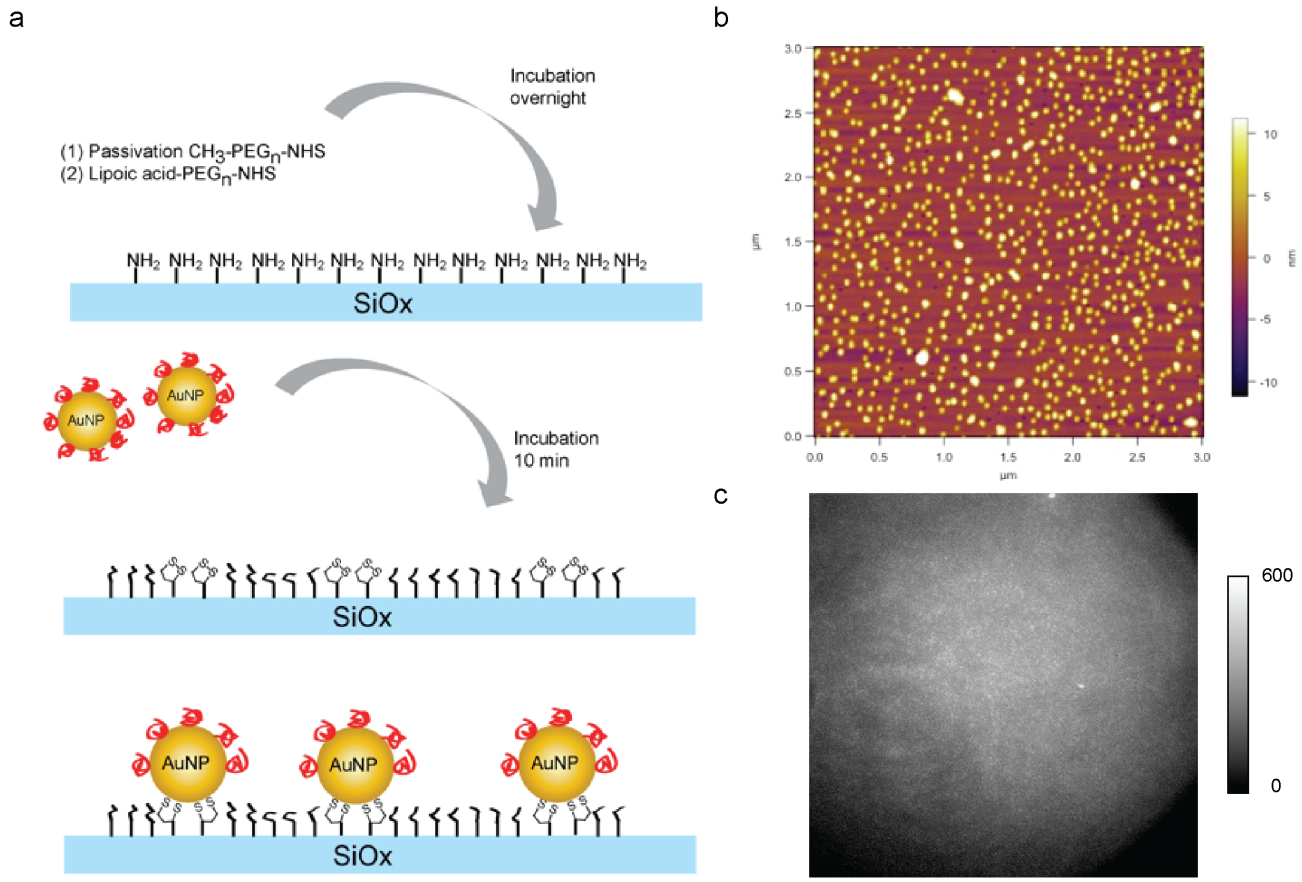

Figure S5. AuNP-sensor immobilization. (a) Scheme showing the reaction scheme used in the modification of the substrate. (b) Representative AFM showing AuNP-sensor distribution on substrate. (c) An average image of 10 quenched fluorescence images of AuNP-sensor modified surface without cells (500 ms acquisition time with instrument background subtraction).

To functionalize the slides with the amine group, No.2 glass coverslips were cleaned with DI water twice, sonicated in DI water, and then sonicated with acetone for 15 min. The cleaned slides were then dried under a stream of high purity N_2. Fresh piranha solution (7:3 v/v = H_2SO_4: H_2O_2) was mixed and then used to clean the substrates for 15 min. Afterwards, the substrates were rinsed with copious amount of DI water. The substrates were then sonicated in acetone to remove excess water and to further clean the substrate. Subsequently, 1% v/v APTMS solution in acetone was added to the slides and incubated for 2 h.

The slides were then thermally annealed for 4 h at 80 °C. The surface was then passivated with 5% w/v mPEG-NHS (MW 2000) and 0.5% w/v lipoic acid-PEG (MW 3400) in 200 μL of 0.1 M fresh sodium bicarbonate solution. After overnight incubation at 4 °C, the excess unreacted PEG molecules were rinsed with DI water. This strategy affords a glass surface with sufficient lipoic acid groups to irreversible anchor AuNP MTFM sensors. Finally, 2 nM of AuNP-sensor solution was incubated for 10 min and then rinsed with DI water to remove nonspecifically bound particles.

Synthesis and quantification of dsDNA-AuNP for NSET calibration

5' Disulfide-modified oligonucleotides were custom synthesized by Integrated DNA Technologies (IDT). The disulfide group was reduced by incubation with 5 nmol of lyophilized oligonucleotide with 100 µL of disulfide cleavage buffer (0.1 M dithiothreitol (DTT), 170 mM phosphate buffer at pH 8.0) for 3 h at room temperature. The reduced oligonucleotides were then purified using a NAP-5 column (GE Healthcare, Piscataway, NJ) using DI water as the eluent.

5' Amine-modified oligonucleotides were custom synthesized by IDT and used without further purification. 10 nmol of the oligonucleotide was reacted with 100 nmol of Alexa488-NHS ester dye in 1X PBS (pH 7.4) for 12 h at room temperature. The product was subsequently purified by reverse phase HPLC (flow rate 1 ml/min, solvent A: 0.1M DI TEAA, solvent B: 100% acetonitrile, initial condition was 10% B with a gradient of 1% per min). The final concentration was determined by using a Nanodrop spectrophotometer.

Equal amounts of 5'SH-DNA and 5' Alexa488-DNA were mixed together at ~25 µM concentrations in 1XPBS and hybridized by heating to 95ºC for 5 min, then cooling at a rate of 3.3 °C/min over a period of 20 min. Afterwards, 8.7 µM of dsDNA was then added to 3.0 mL of 15 nm diameter gold nanoparticles (8.4 nM), bringing the concentration of oligonucleotide and gold nanoparticles to ~2.7 µM, and ~5.8 nM, respectively. The pH of the solution was adjusted to pH 7.4 by adding 296.3 µL of 100 mM phosphate buffer, thus bringing the phosphate buffer concentration to 9 mM. The particles were then stabilized by adding sodium dodecyl sulfate (SDS) to the solution and bringing its final concentration to 0.1% (g/mL) by using a stock solution of 10% SDS. The particles were successively salted with eight NaCl additions that were spaced 20 min apart using a stock solution of 2.0 M NaCl. The final NaCl concentration of the DNA-AuNP solution was increased to 0.3 M. The first two NaCl addition increased the concentration by 0.05 M, while the remaining two NaCl additions increased the NaCl concentration by 0.1 M increments. The fully salted particles were then incubated overnight, in the dark and at room temperature. The following day, the particles were centrifuged five times and reconstituted in DI water for each wash.

The number of dsDNA loaded per AuNP was subsequently quantified using two independent assays, the commercial OliGreen assay kit, and by conventional fluorescence measurement using a calibration curve following KCN dissolution of the Au. In the table below, the results of these two assays are listed and compared. Based on the results of these two measurements, the average number of dsDNA loaded per particle indicated a densely packed DNA surface.

DNA duplex length	16 bp	21 bp	33 bp	45 bp
Number of dsDNA/AuNP using the OliGreen assay	93.3 ± 3.7	86.6 ± 4.5	60.0 ± 2.2	53.3 ± 3.9
Number of dsDNA/AuNP using Alexa488 fluorescence	95.0 ± 1.3	94.3 ± 0.5	81.2 ± 0.8	57.0 ± 0.8
Average	94.1	90.3	70.5	55.1

Table S1. Determination of dsDNA loading per AuNP using the OliGreen assay and the standard Alexa488 fluorescence calibration curve assay.

Image analysis

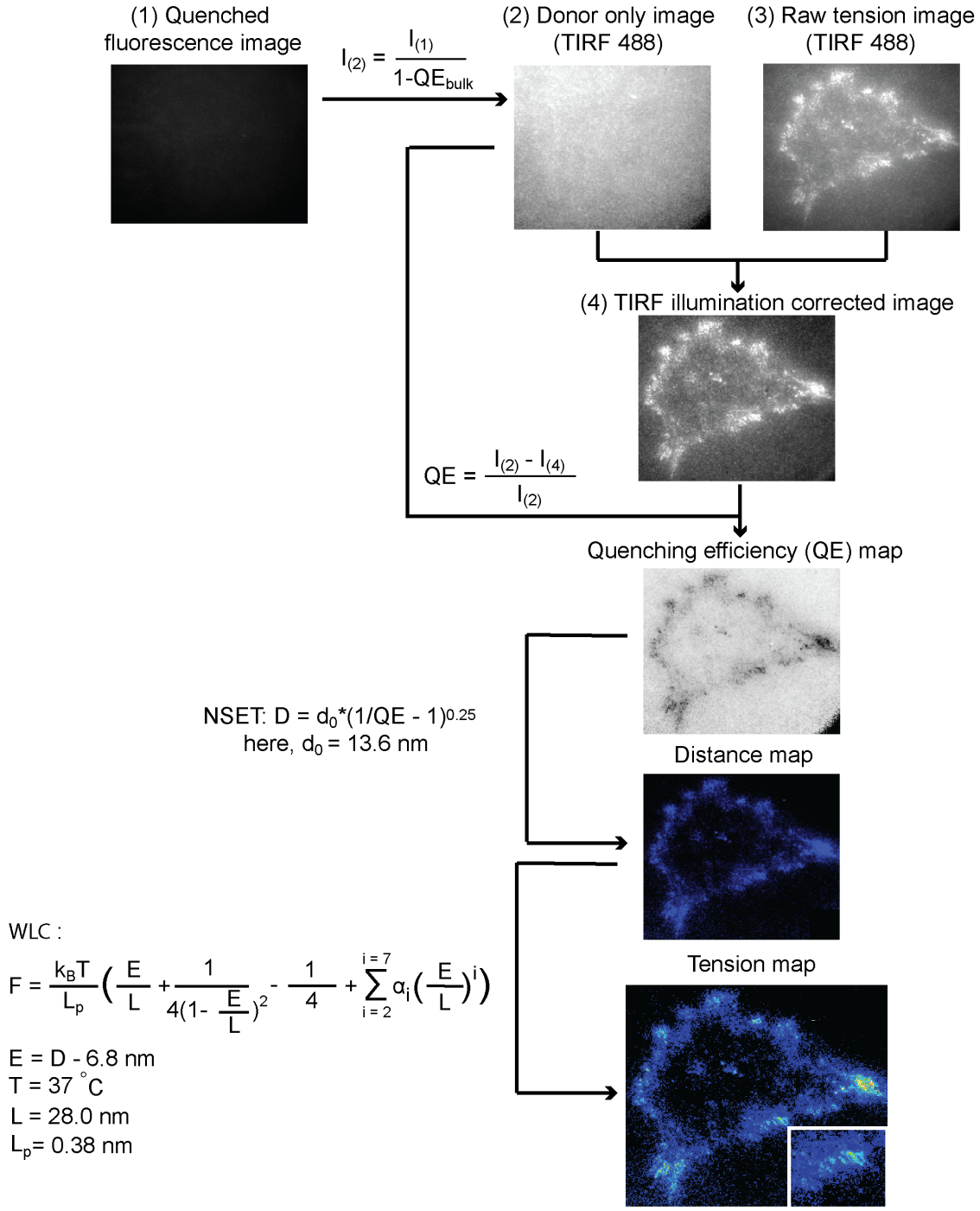

Figure S6. Stepwise image analysis of cell tension by using NSET and WLC models.[7]

Cell Control experiments

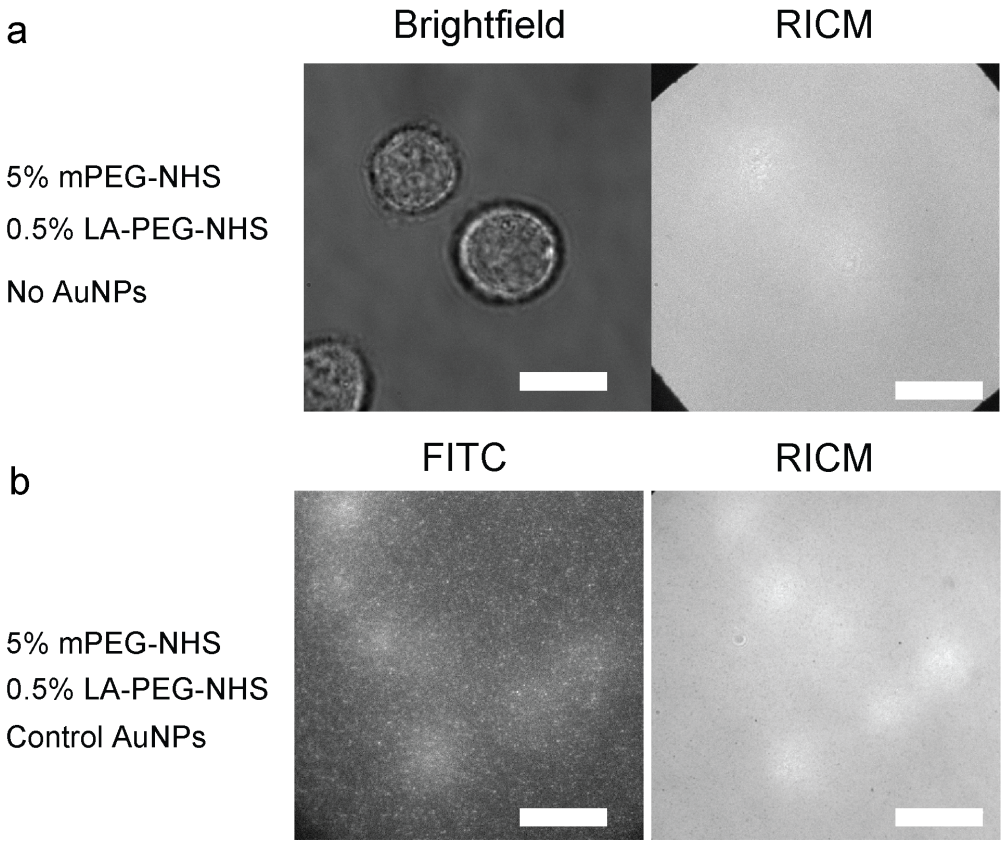

Figure S7. Control experiments for non-specific interactions between cells and the substrate

To demonstrate that cells exclusively interact with the (cRGDfK)C peptide at the terminus of the AuNP-sensor within the experimental time frame of 1 h, we performed two control experiments. In the first control experiment, the surface was functionalized with 5% w/v mPEG-NHS and 0.5% w/v lipoic acid-PEG-NHS but not incubated with the AuNP-sensor. In this case, cells failed to interact or engage with the surface as shown in the BF and RICM images in Figure S6a. Therefore, cells do not bind to defect sites nor the PEG passivation layer.

In the second set of controls, we incubated the same substrate as in Figure S7a along with AuNPs that were modified with 100% COOH-EG8-SH (rather than 99% COOH-EG8-SH and 1% **3** that are typically used in experiments). In this case, we wanted to investigate whether cells engaged the AuNP or the terminal COOH groups presented on the AuNP. As in the previous control, cells were unable to engage the surface within the 1 h experimental time window (Figure S7b). These experiments also allowed us to determine the amount of background fluorescence (including instrumental noise and AuNP scattering) and cellular auto-fluorescence.

Taken together, this data shows that cell surface integrin receptors directly interact with the cRGDfK(C) peptide with molecular specificity.

Sensor reversibility

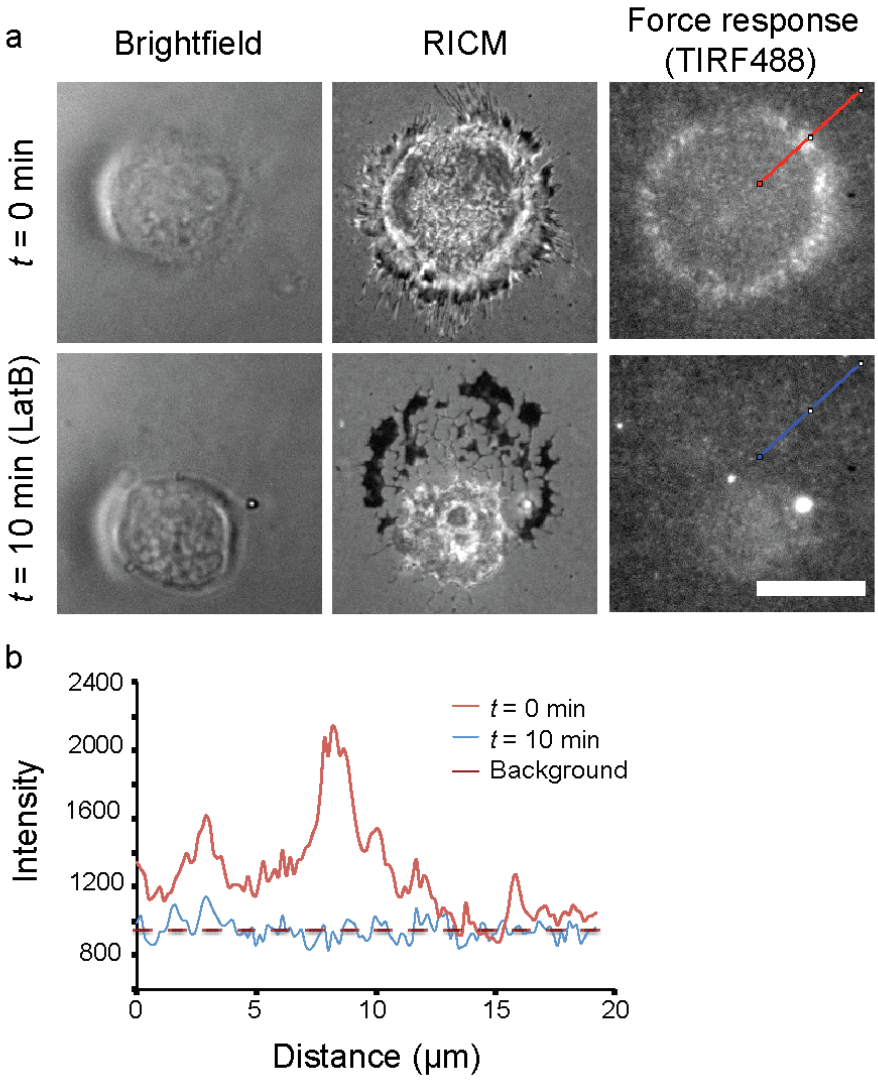

Figure S8. (a) Tension signal was monitored before and after treatment with 25 μM LatB. At *t* = 0 min, the cell adhered to the surface (upper panel) and triggered the force response. After 10 min incubation with LatB, the cell moved (lower panel) and the force response also disappeared and recovered to background level. Scale bar, 15 μm. (b) Plot showing line profiles of tension before (red) and after (blue) treatment with 25 μM LatB. The dotted line represents the background fluorescence intensity of the quenched sensor (500 ms acquisition time).

Real time imaging of cell contraction

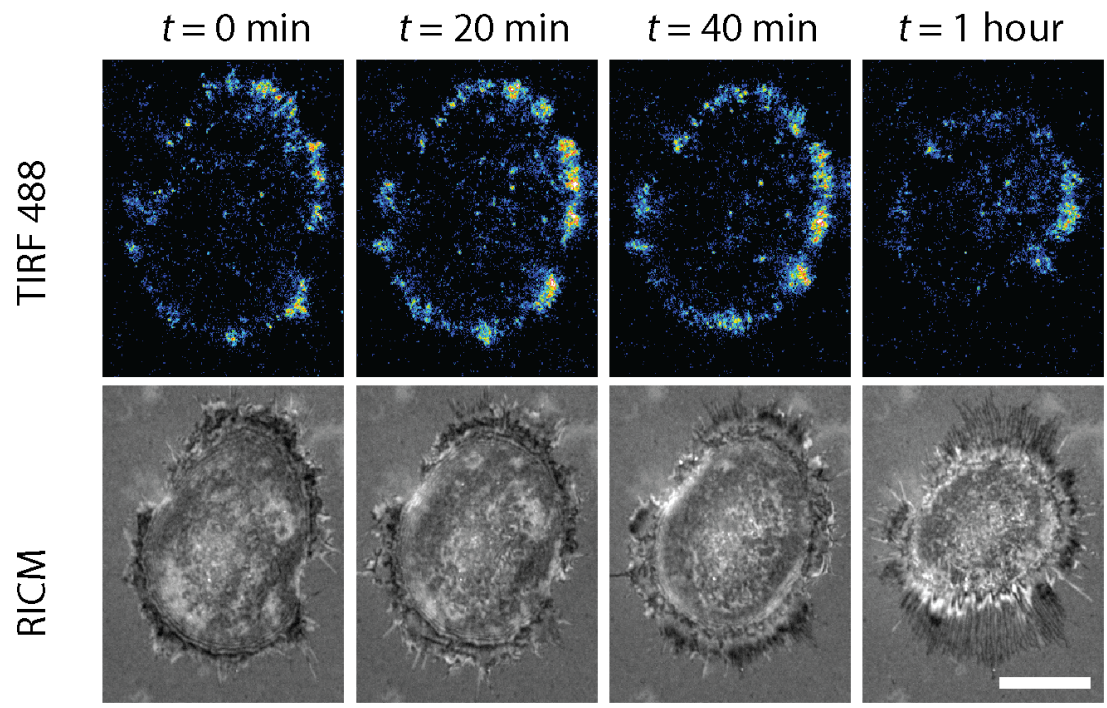

Figure S9. Time-lapse imaging of cell contraction over 1 h. Full video available as a supporting file. Scale bar, 20 μm

Histogram analysis of tension within adhesions

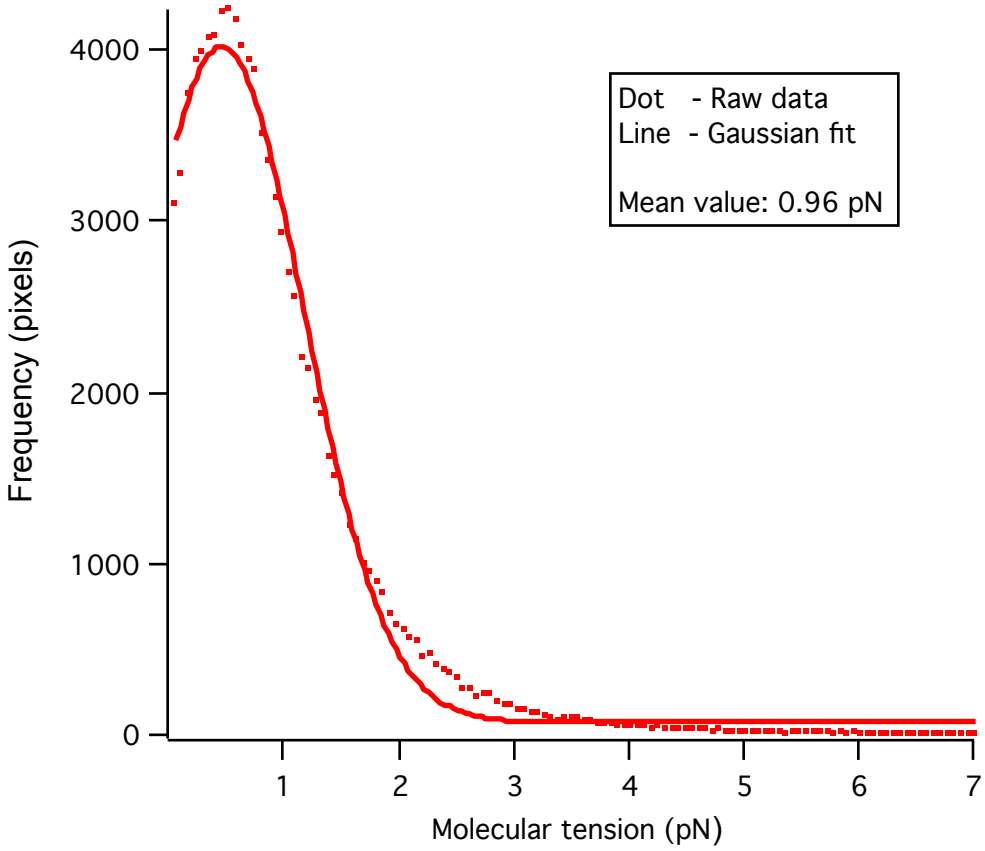

Figure S10. Histogram analysis showing the frequency distribution of tension within cellular adhesions. The data was accumulated from three different cells and displayed after background subtraction and conversion as shown in Figure S6. Most cell adhesions display a tension of 0.5-3 pN, in agreement with literature estimates using bulk polymer traction force microscopy methods.

Reference:

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