Supplementary information

Mechanical Unfolding of Ensemble Biomolecular Structures by Shear Force

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Materials

All the chemicals, unless specified, were purchased from VWR (Radnor, PA) with >99% purity. All the oligos were purchased from Integrated DNA Technologies (IDT, Coralville, IA). The streptavidin- or antidigoxigenin-coated polystyrene beads were purchased from Spherotech (Lake Forest, IL, USA). The i-motif binding ligand, L2H2-4OTD was a gift from Professor Kazuo Nagasawa from Tokyo University of Agriculture and Technology.

Synthesis of the human telomeric i-motif construct

The construct was synthesized by tethering the ssDNA hosting i-motif forming sequence, 5'-TAA$(CCCTAA)_4$, between 1558-bp (base pairs) and 2391-bp dsDNA handles. The 2391-bp dsDNA handle was prepared by digestion of the PCR amplified λ-DNA (primers: 5′-AAA AAA AAG AGC TCC TGA CGC TGG CAT TCG CAT CAA AG-3′ and 5′-AAA AAA AAG GTC TCG CCT GGT TGC GAG GCT TTG TGC TTC TC-3) using SacI and BsaI-HFv2 restriction enzymes (NEB). The digested product was gel purified and labeled with digoxigenin (Dig) at the SacI digested end using terminal transferase (Thermo Fischer) in presence of Dig-dUTP (NEB).

The other 1558-bp dsDNA handle was obtained from the PCR amplification of PBR322 plasmid (NEB) using 5′-end biotinylated reverse primer, 5′-/Biotin/GCA TTA GGA AGC AGC CCA GTA GTA GG and forward primer, 5′-AAA ATC TAG AGG CTA CAC TAG AAG GAC AGT ATT TG. The amplified product was digested with BsaI-HFv2 restriction enzyme and purified using agarose gel.

Next, the ssDNA containing the i-motif forming sequence (Oligo 8, Table S2) was annealed with the Oligo 6 and Oligo 7 (Table S2) from 95-25 °C, which generated the i-motif forming sequence sandwiched between two short duplex adapters. The preparation was finally ligated between the 1558-bp and 2391-bp DNA handles through a one-pot ligation reaction using T4 DNA ligase (NEB).

Mechanical unfolding experiment in optical tweezers

Four-channel microfluidic chamber was used to perform the single-molecule mechanical unfolding experiments using laser tweezers. The detailed description of the laser tweezers instrument and methods have been reported elsewhere.[S1] Briefly, the Dig-labeled DNA construct was immobilized on the bead surface through Dig-anti-Dig complex formation by incubating DNA construct (~1 ng) with 1 μL of 0.1% solution of digoxigenin (Dig)-antibody coated polystyrene beads (diameter: 2.10 μm, Spherotech) for about 30 min at room temperature (25 °C). The incubated i-motif DNA sample was diluted in 1 mL of 10 mM Tris (pH 7.4, supplemented with 100 mM KCl). One ml of buffer containing streptavidin-coated polystyrene beads (1 μL, diameter: 1.87 μm, Spherotech) was injected into the microfluidic chamber. The buffers without and with the L2H2-4OTD ligand (a gift from Prof Kazuo Nagasawa from Tokyo University of Agriculture and Technology) were flowed in the top (buffer) and bottom (ligand) channels, respectively.

The streptavidin coated bead was trapped in the ligand channel and moved to the buffer channel where the anti-Dig coated bead was trapped by another laser beam. The DNA construct initially on the surface of the antibody-coated bead was tethered between the two types of

beads in the buffer channel by escorting one of the beads closer to another using a steerable mirror (Madcity Laboratories Inc., Madison, WI). In the force-ramping experiments, the streptavidin-coated bead was moved away from the anti-Dig coated bead with a loading rate of ~5.5 pN/s (in the range of 10-30 pN) by using the steerable mirror. The construct structure was unfolded when the tension inside the tether was gradually increased. The unfolding of the i-motif structure was depicted by a sudden change in the end-to-end distance during the process. Single-molecule tether was confirmed by a DNA overstretching plateau at 65 pN or a single-step breakage event in the F-X curves. The F-X curve for each tether was recorded in a Labview 8 program (National Instruments Corp., Austin, TX), and data treatment was performed using Matlab (The MathWorks, Natick, MA) and Igor (WaveMetrics, OR, U.S.A.) programs. The unfolding force was measured directly from the F-X curves, while the change-in-contour-length (ΔL) due to the unfolding was calculated by the two data points flanking a rupture event using an extensible worm-like chain (WLC) model (eqn S1)),[S2, 3]

$$\frac{\Delta x}{\Delta L} = 1 - \frac{1}{2}\left(\frac{k_b T}{FP}\right)^{1/2} + \frac{F}{S} \qquad \text{……..(eqn S1)}$$

where Δx is the change in extension between the data points of the stretching and relaxing curves at the same force (F), k_B is the Boltzmann constant, T is absolute temperature, P is the persistent length (50.8 ± 1.5 nm)[S2], and S is the elastic stretch modulus (1243 ± 63 pN).[S2]

Calculation of the shear force at a particular shear rate

To establish the relationship between the shear force and the shear rate, a cumulative plot of unfolding percentage of the i-motif versus force was plotted based on the unfolding force

histogram of the i-motif (Figure 1F, blue curve, right axis). The percentage of unfolded species at each force from the cumulative force plot was equivalent to the percentage of unfolded species by shearing at 20 min, when a steady state was achieved (Figure 1B). Based on this equivalency, the plot of the shear force *vs* the shear rate was obtained (Figure 1G).

Unfolding of the i-motifs with and without L2H2-4OTD

DNA i-motif was labeled by the Cy5 and Iowa Black® RQ quencher FRET pair at the 5' and 3' respectively (IDT). In the shearing cuvette of 200 μL, 5 μM of i-motif DNA in 30 mM MES buffer at pH 5.5 or 7.4 was mixed with the L2H2-4OTD ligand in the concentration range of 0-60 μM. The shearing with a shear rate of 63209/s was performed inside a Polytron® Biotrona® homogenizer (PT-MR 3000, with a generator PT-DA 07/2, EC-B101) for 20 min on top of an inverted Nikon TE2000-U fluorescence microscope. Increase in the fluorescence intensity during shearing of the FRET pair labelled i-motif under different conditions were recorded over time. These temporal plots were fit with single exponential curves.

The plateau values from these fitting curves (around 20 min of the shearing) were used as the steady-state signals. These plateau values with and without L2H2-6OTD were used to calculate the ligand-bound percentage of the i-motif given that the curve without ligand as 0% binding and that with 45 μM ligand as 100% binding (see main text).

Dissociation constant was retrieved from the Hill' equation (eqn S2),[S4]

$\phi=\phi_{max}\times [C]^n/(K_d^n +[C]^n)$……. (eqn S2)

$[C]= C- (\phi\times[S])$ ……. (eqn S3)

where,ϕ is the bound fraction, ϕ_{max} represents the maximal bound fraction, [C] represents free

ligand concentration, C depicts total ligand concentration, K_d is the dissociation constant, n is the Hill coefficient, and [S] is the substrate (i-motif- 5 μM) concentration.

Free ligand concentration ([C]) is calculated using eqn S3 as shown in the Table S1 below.

Table S1. *Relation of total ligand concentration and free ligand concentration.*

Total ligand concentration- C	*Bound fraction- ϕ*	*Free ligand concentration- [C]*
0.0	*0.00*	*0.0*
6.0	*0.01*	*5.9*
30.0	*0.22*	*28.9*
36.0	*0.40*	*34.0*
45.0	*1.00*	*40.0*
60.0	*0.96*	*55.2*

Shear force actuated click reactions

Ten μM of i-motif DNA in 300 uL of Tris buffer (pH 7.4) supplemented with 150 μM CuCl and 300 μM ascorbic acid was incubated for 10 min to fold the i-motif structure. The solution was ultrafiltrated 3 times with 3K MWCO Amicon® (Millipore Sigma, (St. Louis, MO)) filter at relative centrifugal force (RCF) of 14300 ×g. Each time, 30 mM Tris buffer (pH 7.4) supplemented with 300 μM ascorbic acid was used to replenish the solution to ~500 μL. To the final residual solution, 30 mM Tris (pH 7.4) supplement with 300 μM ascorbic acid was added along with 20 μM Calfluor® (Click Chemistry Tools, (Scottsdale, AZ)), 20 μM HPG (Santa Cruz Biotechnology (Dallas, Texas)), and 10μM TBTA (Tokyo Chemical Industry (Montgomeryville, PA)) in the final volume of 300 μL. After the solution was sheared in the homogenizer, Cu+ chelated inside the i-motif was exposed to the solution to catalyze the fluorogenic click reaction measured by the inverted Nikon TE2000-U fluorescence

microscope.

CD experiments

The CD spectroscopy was performed with the 10 µM i-motif forming sequence in a 30 mM Tris buffer in presence of 300 µM ascorbic acid at pH 5.5 or pH 7.4 (without and with 150 µM cuprous chloride). After the samples were heated to 95˚C for 10 minutes and cooled to room temperature, they were placed inside a quartz cuvette with 1 mm optical path length to obtain the CD spectra using Jasco-810 spectropolarimeter (Easton, MD). Average of three scans on the wavelength of 220-320 nm were taken at the rate of 50 nm/min (Figure S8). The obtained spectra were baseline corrected and smoothed by Savitzky-Golay function. After the cuprous chloride and ascorbic acid were added, solution was purged with N_2 for 20 mins to avoid oxidation of the copper.

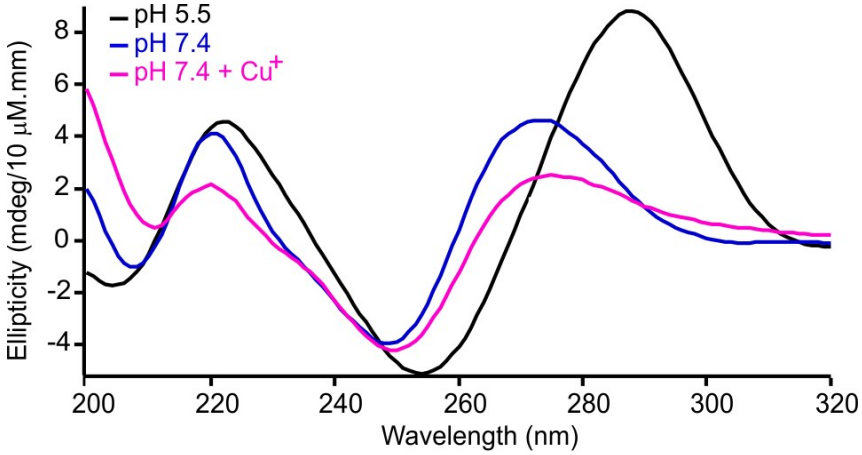

Figure S1. *CD measurements of 10 µM DNA that contains a telomeric i-motif sequence, 5'-TAA CCC TAA CCC TAA CCC TAA CCC TAA (Oligo 3 in Table S2), in 10 mM MES (2-(N-morpholino) ethanesulfonic acid) buffer (pH 5.5-black trace) and 30 mM Tris buffer (pH 7.4) with (pink trace) and without 150 µM (blue trace) Cu+. All the solutions were supplemented with 300 µM ascorbate acid. The black trace depicts the formation of the i-motif, whereas the*

blue trace shows ssDNA. The pink trace shows the signatures characteristic of the Cu^{+}-chelated i-motif.[S5]

Expected change-in-contour-length (Δ*L*)

The expected change-in-contour-length (Δ*L*) during the unfolding of the i-motif structure was calculated by the equation S4,

$\Delta L = N \times L_{nt} - x$……. (eqn S4)

where *N* (number of the nucleotides involved in the i-motif formation) = 21, L_{nt} (contour length of each nucleotide) = 0.43 nm[S6, 7], and *x* (end-to-end distance of the folded i-motif) = 1nm[S8-10]. This yielded an expected $\Delta L = 21 \times 0.43$ nm – 1nm = 8.0 nm.

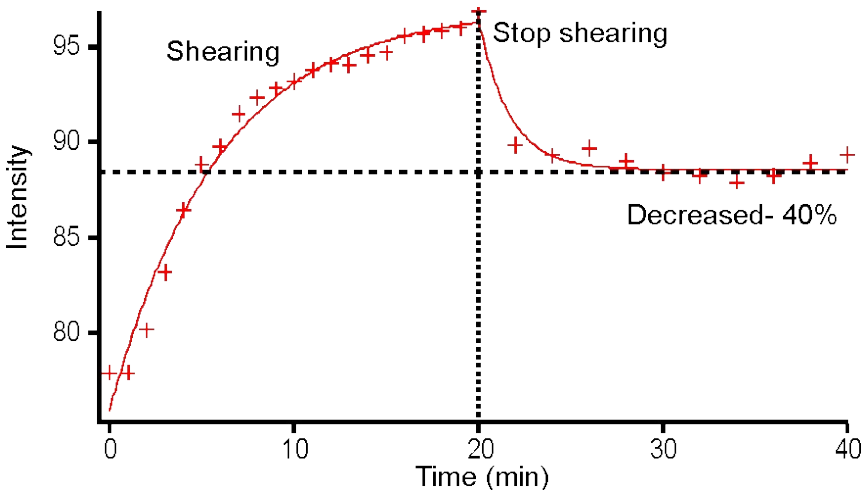

Figure S2. *Plot of fluorescence intensity of the telomeric i-motif (Oligo 5 in Table S2) versus time during and after the shearing. The intensity decreased after the shearing was stopped. The curves have been fitted with exponential functions.*

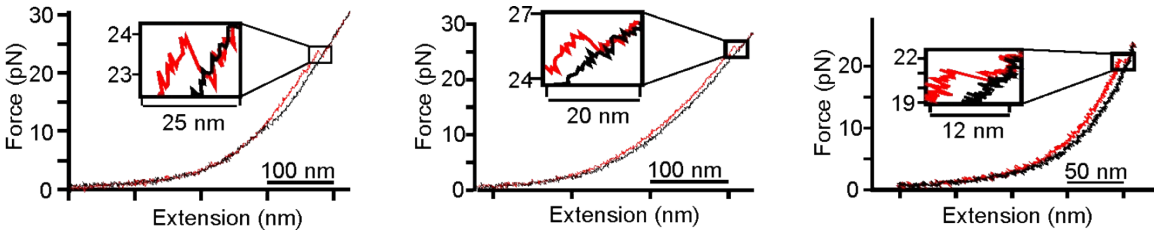

Figure S3. *Typical force-extension curves of unfolding the human telomeric DNA i-motif (Oligo 3 in Table S2) in 50 mM MES buffer (pH 5.5) supplemented with 100 mM KCl at room temperature.*

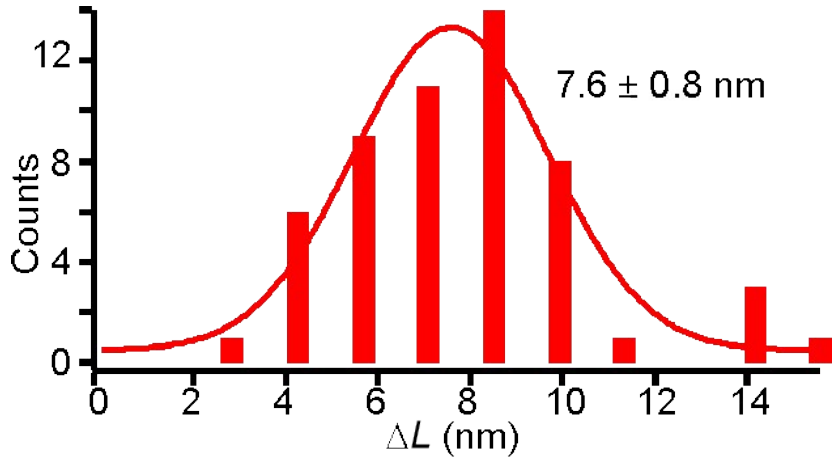

Figure S4. *Histogram of change-in-contour-length (ΔL) accompanied during unfolding of the human telomeric DNA i-motif (Oligo 3 in Table S2) in 50 mM MES (pH 5.5) supplemented with 100 mM KCl at room temperature.*

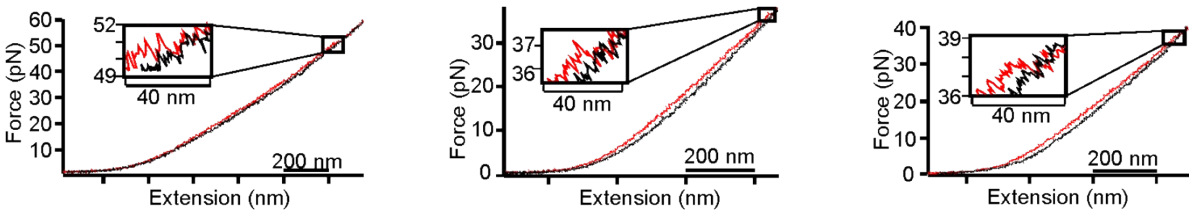

Figure S5. *Typical force-extension curves of unfolding the human telomeric DNA i-motif (Oligo 3 in Table S2) in presence of 60 μM L2H2-4OTD in 50 mM MES buffer (pH 5.5) supplemented with 100 mM KCl at room temperature.*

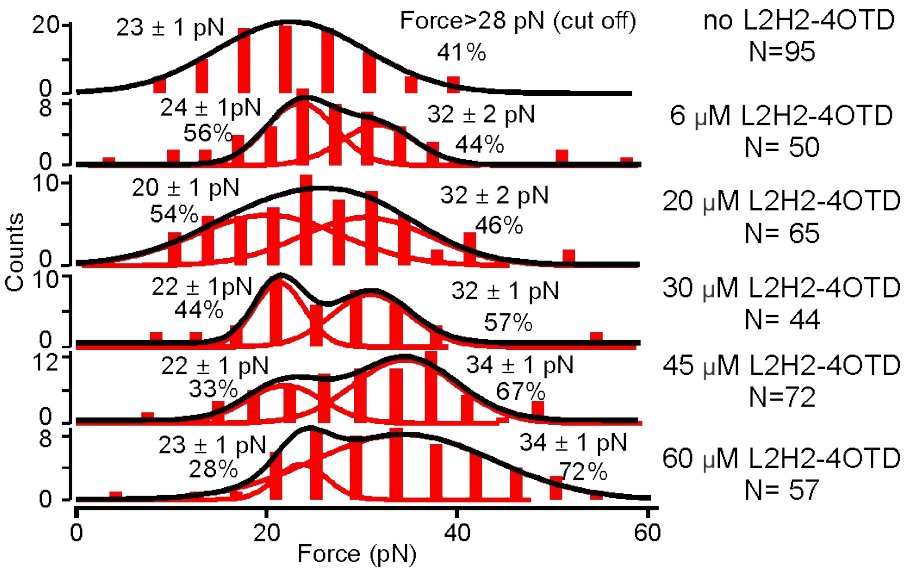

Figure S6. *Unfolding force histograms of the human telomeric DNA i-motif (Oligo 3 in Table S2) in 50 mM MES (pH 5.5) supplemented with 100 mM KCl at room temperature in presence of different concentrations (0 - 60 μM) of the L2H2-4OTD. Forces greater than the cut-off point (28 pN) were considered as the high force (ligand bound) populations. The ligand bound fraction was calculated by subtracting the high force population at each L2H2-4OTD concentration by the high force population without ligand (top panel). Black and red curves represent Gaussian fitting's.*

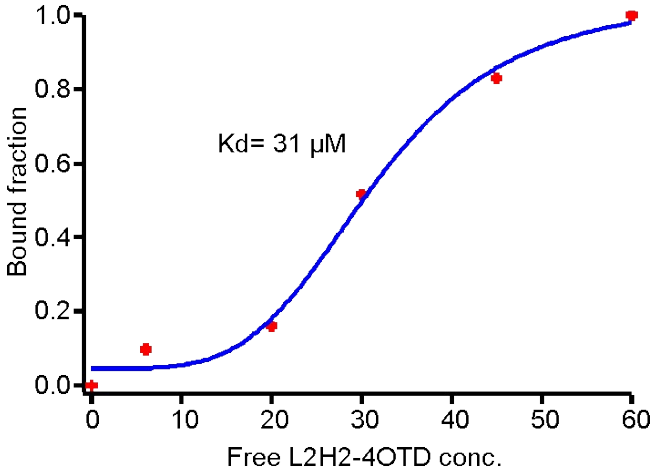

Figure S7. *Binding curve of the i-motif at different concentrations of the L2H2-4OTD ligand obtained from single-molecule mechanical unfolding experiment in optical tweezers (Figure S6). Blue curve represents the Hill's fitting.*

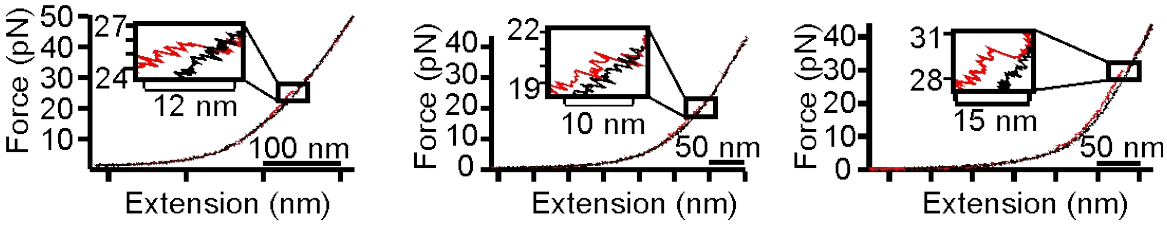

Figure S8. *Typical force-extension curves of unfolding the human telomeric DNA i-motif (Oligo 3 in Table S2) in 30 mM Tris buffer (pH 7.4) supplemented with 100 mM KCl, 150 μM CuCl and 300 μM of ascorbic acid at room temperature.*

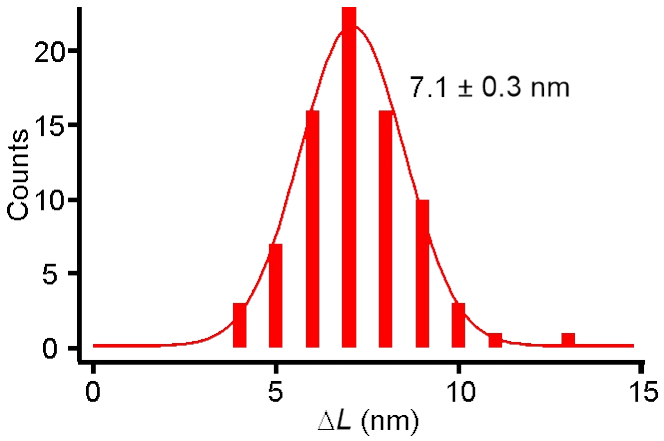

Figure S9. *Change-in-contour-length (ΔL) histogram during unfolding of the 27-nt human telomeric DNA i-motif (Oligo 3 in Table S2) in 30 mM Tris buffer (pH 7.4) supplemented with 100 mM KCl, 150 μM CuCl, and 300 μM of ascorbic acid at room temperature.*

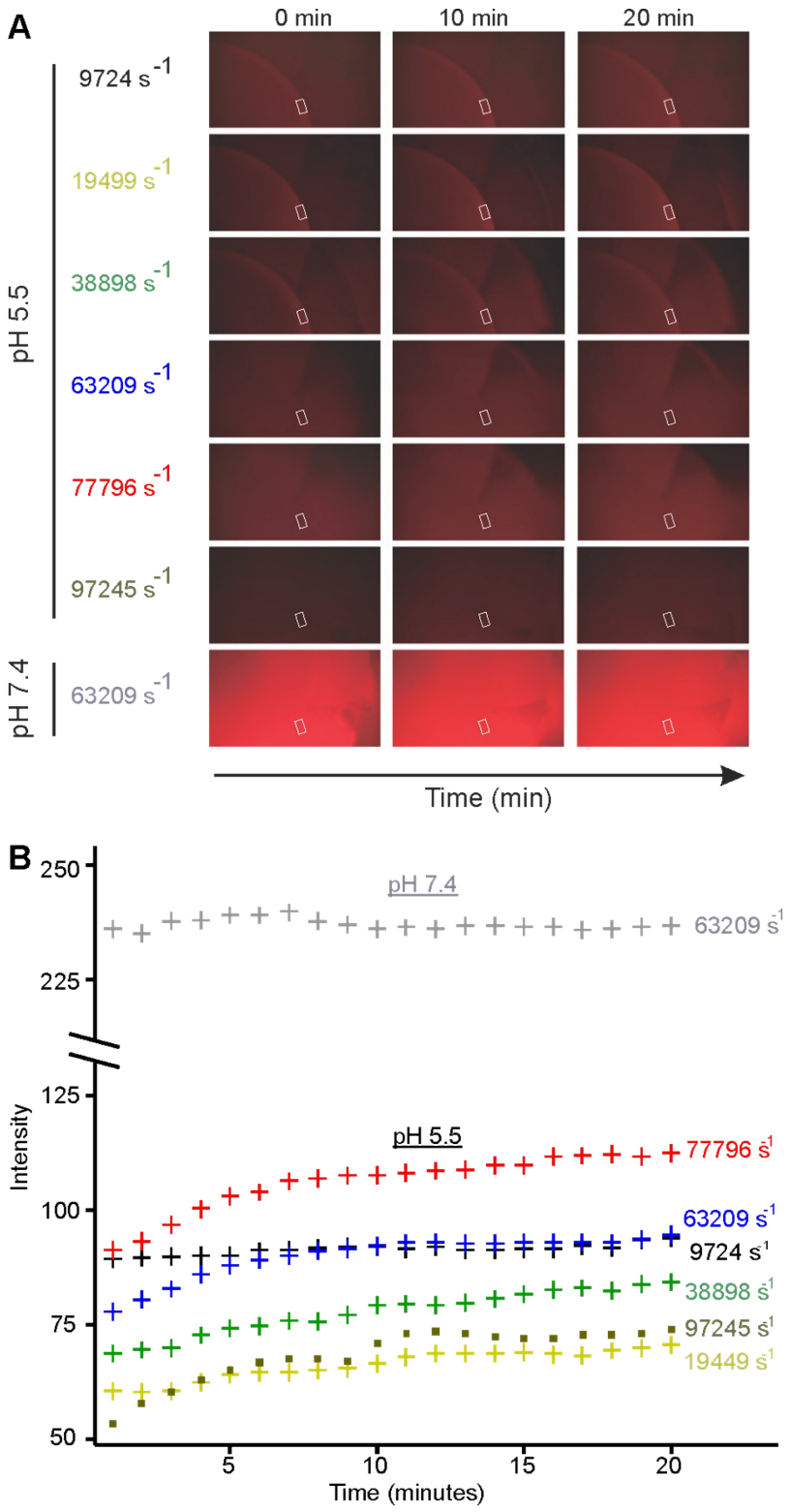

Figure S10. *Raw data for the fluorescence imaging of real time shearing experiment. A) Real time fluorescence images at different time intervals (0, 10, and 20 minutes) at different*

shearing rates in pH 5.5 and pH 7.4. The white box in each photo (length × width = 827 × 465 μm,) indicates the location between the rotor and stator in which the fluorescence intensity was measured. B) Real time changes in fluorescence intensity due to the unfolding of the i-motif at the shearing rate of 63209s^{-1} at pH 7.4 (top) and at different shear rates at pH 5.5 (bottom).

Table S2. DNA Sequences:

Oligos	Sequences
1	5’CCC TAA CCC TAA CCC TAA CCC
2	5’TAA CCC TAA CCC TAA CCC TAA CCC
3	5’TAA CCC TAA CCC TAA CCC TAA CCC TAA
4	5’-TAA TAA CCC TAA CCC TAA CCC TAA CCC TAA TAA
5	5’Cy5/TAA CCC TAA CCC TAA CCC TAA CCC TAA/ Iowa Black® RQ/
6	5’-CGC ATC TGT GCG GTA TTT CAC ACC GT
7	5’-CAG GGA CGC GCT GGG CTA CGT CTT GCT GGC
8	5’CGG TAC GGT GTG AAA TAC CGC ACA GAT GCG TAA CCC TAA CCC TAA CCC TAA CCC TAA GCC AGC AAG ACG TAG CCC AGC GCG TC
9	5’-TTA GGG TTA GGG TTA GGG TTA GGG TTA
10	5’-TGA GTG TGA GTG TGA GTG TGA GTG TAT

Reference

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