

Ensemble Force Spectroscopy by Shear Forces

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Abstract

Single-molecule techniques based on fluorescence and mechanochemical principles provide superior sensitivity in biological sensing. However, due to the lack of high throughput capabilities, the application of these techniques is limited in biophysics. Ensemble force spectroscopy (EFS) has demonstrated high throughput in the investigation of a massive set of molecular structures by converting mechanochemical studies of individual molecules into those of molecular ensembles. In this protocol, the DNA secondary structures (i-motifs) were unfolded in the shear flow between the rotor and stator of a homogenizer tip at shear rates up to 77796/s. The effects of flow rates and molecular sizes on the shear forces experienced by the i-motif were demonstrated. The EFS technique also revealed the binding affinity between DNA i-motifs and ligands. Furthermore, we have demonstrated a click chemistry reaction that can be actuated by shear force (i.e., mechano-click chemistry). These results establish the effectiveness of using shear force to control the conformation of molecular structures.

Introduction

In single-molecule force spectroscopy¹ (SMFS), the mechanical properties of individual molecular structures have been studied by sophisticated instruments such as the atomic force microscope, optical tweezers, and magnetic tweezers^{2,3,4}. Restricted by the same directionality requirement of the molecules in the force-generating/detecting setups or the small field of view in magnetic tweezers and the miniature centrifuge force microscope (MCF)^{5,6,7,8}, only a limited number of molecules can be investigated simultaneously using SMFS. The low throughput of SMFS prevents its wide application in the molecular

recognition field, which requires the involvement of a large set of molecules.

Shear flow provides a potential solution to apply forces to a massive set of molecules⁹. In a liquid flow inside a channel, the closer to the channel surface, the slower the flow rate¹⁰. Such a flow velocity gradient causes shear stress that is parallel to the boundary surface. When a molecule is placed in this shear flow, the molecule reorients itself so that its long axis aligns with the flow direction, as the shear force is applied to the long axis¹¹. As a result of this reorientation, all the molecules of the same type (size and length of handles) are

expected to align in the same direction while experiencing the same shear force.

This work describes a protocol to use such a shear flow to exert shear force on a massive set of molecular structures, as exemplified by the DNA i-motif. In this protocol, a shear flow is generated between the rotor and stator in a homogenizer tip. The present study found that the folded DNA i-motif structure could be unfolded by shear rates of 9724-97245 s⁻¹. Besides, a dissociation constant of 36 μM was found between the L2H2-4OTD ligand and the i-motif. This value is consistent with that of 31 μM measured by the gel shift assay¹². Further, the current technique is used to unfold the i-motif, which can expose the chelated copper (I) to catalyze a click reaction. This protocol thus allows one to unfold a large set of i-motif structures with low-cost instruments in a reasonable time (shorter than 30 min). Given that the shear force technique drastically increases the throughput of the force spectroscopy, we call this technique ensemble force spectroscopy (EFS). This protocol aims to provide experimental guidelines to facilitate the application of this shear force-based EFS.

Protocol

NOTE: All the buffers and the chemical reagents used in this protocol are listed in the **Table Materials**.

1. Preparation of the shear force microscope

NOTE: The shear force microscope contains two parts, a reaction unit (homogenizer) and a detection unit (fluorescence microscope). The magnification of the eyepiece is 10x, and the magnification of the objective lens (air) is 4x.

1. Assemble the homogenizer and the microscope on a mounting table. Wear goggles and turn on

the fluorescence microscope, and then adjust the homogenizer to make sure that the excitation light beam of an appropriate wavelength (here, 488 nm was used) passes through the center of the dispersing tip of the homogenizer.

2. Prepare a flat-bottomed reaction chamber that is 5 cm in height and 1.5 cm² x 1.5 cm² in cross-section. To reduce the background, ensure that the selected chamber material does not fluoresce, such as glasses (some plastics have fluorescence).
3. Choose an appropriate homogenizer dispersing tip that can provide the desired shear force.

NOTE: The shear rate is dependent on the distance between the rotor and stator at a fixed rotating speed¹¹ according to the following equation:

$$\text{Shear rate} = \mu \times \pi \times \left(\frac{D}{(D-d)/2} \right) \times V$$

where μ is the dynamic viscosity of water at 20 °C; D is the inner diameter of the stator; d is the outer diameter of the rotor, and V is the shear speed (rpm/s).

4. Clamp the reaction chamber on the sample stage of the fluorescence microscope, and then adjust the chamber to hold the dispersing tip of the homogenizer (**Figure 1**). Make sure the dispersing tip is slightly above (~1 mm) the bottom surface of the reaction chamber.
5. Adjust the vertical position of the homogenizer and the chamber together to ensure the focus of the microscope is on the surface of the dispersing tip. Then, adjust the horizontal position of the dispersing tip to ensure that the detection area (field of view) is set between the rotor and stator (**Figure 1**).
6. Switch on the fluorescence channels according to the fluorescent dye used in the experiment.

- Before the high-speed shearing experiment, use deionized (DI) water to test the shearing with a low shearing speed (e.g., 2,000 rpm) to ensure the dispersing tip can work appropriately without touching the reaction chamber.

2. Unfolding i-motifs with and without ligands

- Prepare a human telomeric i-motif DNA (**Table of Materials**) labeled with a dye and a quencher at its two ends, respectively, in DI water, as described in Hu et al.¹¹.

NOTE: i-motif containing sequence: 5'-TAA CCC TAA CCC TAA CCC TAA CCC TAA.

- Dilute the DNA to 5 μM in the 30 mM MES buffer at pH 5.5 or pH 7.4. To the DNA solution, add ligand L2H2-4OTD, which was synthesized according to Abraham Punnoose et al.¹³, in a concentration range of 0-60 μM . Mix the solution gently for 10 min to fold the i-motif structures without light.
- Check and minimize the background fluorescence intensity of the reaction chamber that is filled with deionized DI water using the fluorescent microscope without shearing. An easy way to minimize the background fluorescence is to wash the reaction chamber with DI water. The background fluorescence value needs to be subtracted in the data analyses later.
NOTE: Stray light should be avoided from this step onwards.
- Set the parameters of the CCD camera using the software. The recommended parameters are as follows: exposure time = 0.5 s, CCD sensitivity = 1600, and recording time = 20 min.

- Using a long pipet, add the DNA solution into the empty and clean reaction chamber. Cover the reaction chamber with a black box. Then, start the homogenizer to perform shearing at a selected shear rate ranging from $9,724\text{ s}^{-1}$ to $97,245\text{ s}^{-1}$ (selected using the software associated with the homogenizer) for 20 min with the CCD camera turned on to record the data.
- After the experiment, remove the chamber and wash it with DI water.

3. Shear force-actuated click reaction

- Prepare i-motif DNA in DI water. Incubate 10 μM i-motif DNA in 300 μL of 30 mM Tris buffer (pH 7.4) supplemented with 150 μM CuCl and 300 μM ascorbic acid for 10 min to fold the i-motif structures (all concentrations are final concentrations in the solution).
NOTE: CuCl is the catalyst of the click reaction. Ascorbic acid will prevent copper (I) oxidization.
- Ultra-filtrate the solution with an ultrafiltration device at a centrifugal force of $14,300 \times g$. Replenish the solution to $\sim 500\text{ }\mu\text{L}$ with 30 mM Tris buffer (pH 7.4) supplemented with 300 μM ascorbic acid after each filtration.
- Repeat the filtration 3x.
- Collect the residual solution and make a final volume of 300 μL by adding 30 mM Tris (pH 7.4) supplemented with 300 μM ascorbic acid along with 20 μM Calfluor 488 azide, 20 μM HPG, and 10 μM TBTA. Once the reagents are added, move the solution to the darkroom.
NOTE: Light should be avoided after this step.
- Check and minimize the background fluorescence intensity of the reaction chamber filled with DI water using the microscope before the shearing experiments.

An easy way to minimize the background fluorescence is to wash the reaction chamber with DI water.

6. Add the DNA solution into the empty reaction chamber with a long pipet, and then start the homogenizer shearing at a shear rate of $63,209\text{ s}^{-1}$ for 20 min with the CCD camera turned on.
7. After the experiment, remove the chamber and wash it with DI water.

Representative Results

Figure 1 outlines the mechanical unfolding and real-time sensing of ensemble molecules in EFS. In **Figure 1B**, the fluorescence intensity of i-motif DNA was observed to increase with the shear rate ranging from $9,724\text{ s}^{-1}$ to $97,245\text{ s}^{-1}$ in a pH 5.5 MES buffer. As a control, fluorescence intensity was not increased when the same i-motif DNA was sheared at a rate of $63,209\text{ s}^{-1}$ in a pH 7.4 MES buffer. This is because the i-motif does not fold at pH 7.4¹⁴. In the previous work, we found that the higher the shear rate, the more the

unfolding of the i-motif¹¹. Based on the unfolding of the i-motif in the optical tweezers¹¹, we calibrated shear force vs. shear rate as shown in **Figure 1C**, which depicted that up to 41 pN could be applied to the FRET-pair labeled i-motif at a shear rate of $77,796\text{ s}^{-1}$. In **Figure 2B**, the ligand (L2H2-4OTD) binding to the DNA i-motif molecules subjected to the shear flow was evaluated, which showed that the increment of the fluorescence intensity became less obvious with increasing L2H2-4OTD concentration (0-60 μM). From the binding curve (i.e., a plot of the bound fractions of the ligand vs. the free ligand concentration) in **Figure 2C**, a dissociation constant (K_d) of 36 μM was determined for the interaction between the L2H2-4OTD ligand and the i-motif¹¹. As a practical application of shear force for chemical reactions, **Figure 3** shows a mechano-click reaction that can be triggered by the shear flow. In **Figure 3B**, the Cu(I) chelated i-motif was unfolded at the $63,209\text{ s}^{-1}$ shear rate, and the released copper (I) triggered the fluorogenic click reaction shown in **Figure 3A**, resulting in increased fluorescence intensity over time (**Figure 3C**).

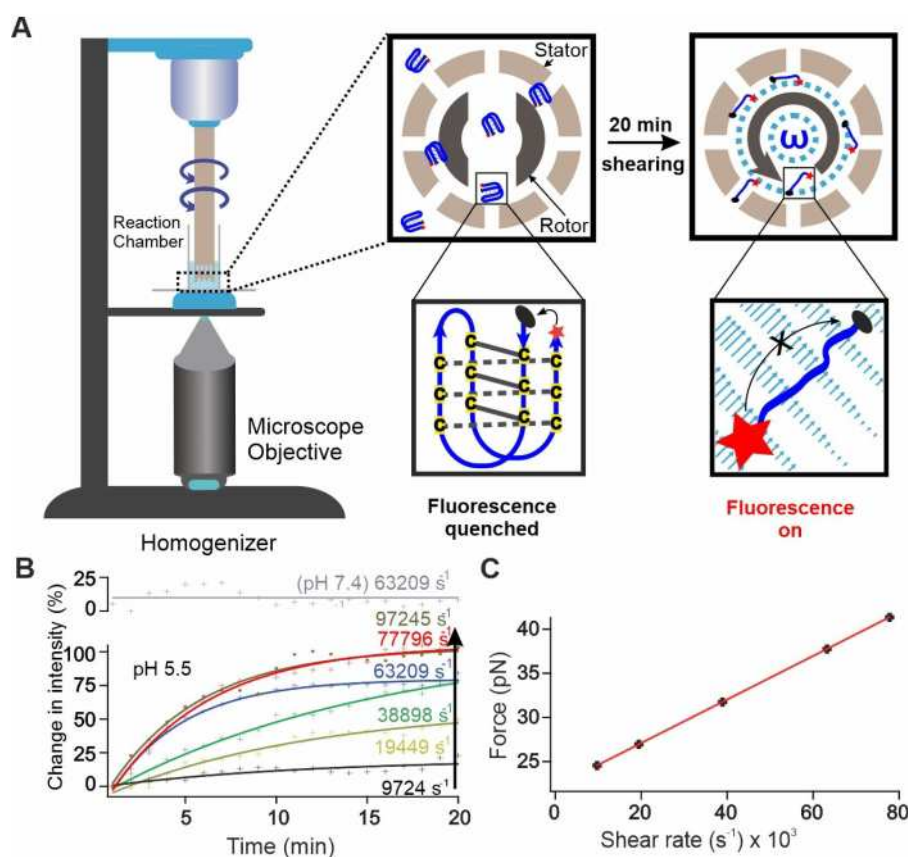


Figure 1: Unfolding of the i-motif DNA structure by shear flow in the EFS. (A) Schematic of the unfolding of an i-motif DNA structure labeled with a FRET pair (Cy5 and quencher) by the shear flow generated in a benchtop homogenizer. Left inset: the light brown indicates the stator, and the dark brown indicates the rotor. Right inset: the dotted cyan circle between the rotor and stator indicates the buffer flow under shear conditions. The dark brown circular arrow indicates the direction of the rotor. The cyan arrows in the zoomed view of the right inset indicate the buffer flow under shearing. The length of each arrow depicts the velocity of a particular flow stream (longer arrows indicate higher velocities). (B) The percentage change in the fluorescence intensity in real-time sensing is due to the unfolding of the i-motif at different shear rates from 9,724 s⁻¹ to 97,245 s⁻¹. Solid curves indicate the exponential fitting. The fluorescence intensity is normalized with respect to the maximum value observed in the experiment. (C) Diagram of the shear rate vs. the shear force for the i-motif shown in (A). The red line depicts a linear fitting ($R^2 = 1.00$). This figure has been modified from Hu et al.¹¹. [Please click here to view a larger version of this figure.](#)

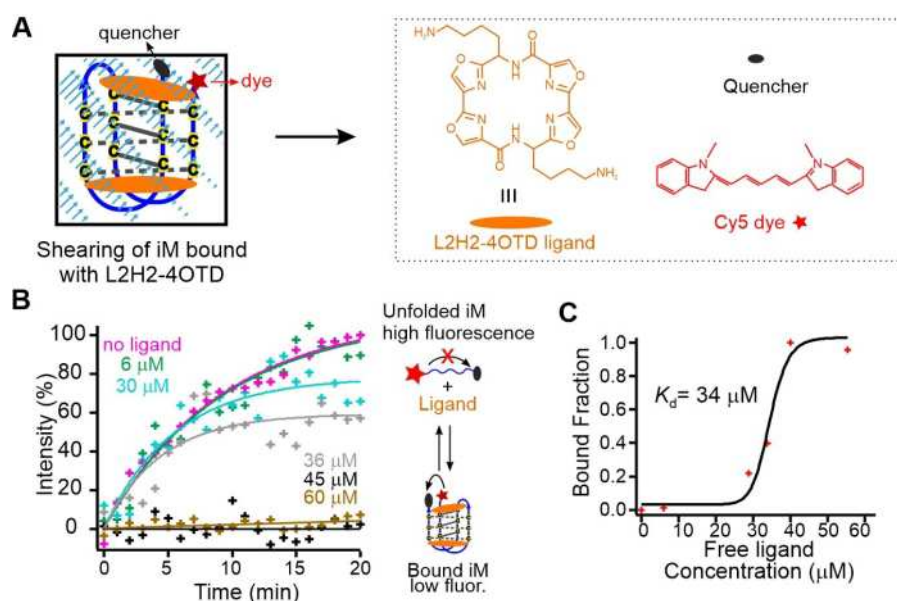


Figure 2: Unfolding of i-motifs bound with ligands. (A) Schematic of the i-motif labeled with a FRET pair (Cy5 and quencher) bound with the L2H2-4OTD ligand¹³. (B) The decreased fluorescence intensity of the i-motif with increasing concentrations of the L2H2-4OTD ligand (pink: 0 μM , green: 6 μM , cyan: 30 μM , gray: 36 μM , black: 45 μM , and light brown: 60 μM). The fluorescence intensity is normalized with respect to the maximum value observed in the experiment. (C) Binding curve of the i-motif at different free L2H2-4OTD concentrations. This figure has been modified from Hu et al.¹¹. [Please click here to view a larger version of this figure.](#)

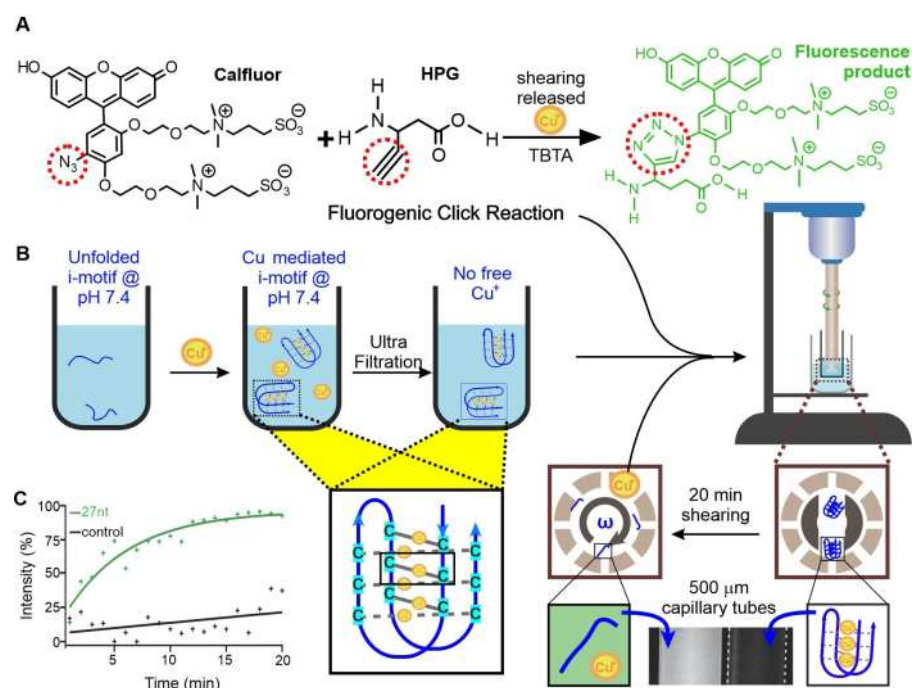


Figure 3: Mechano-click reactions. (A) Fluorogenic click reaction between Calfluor 488 and HPG. The compound shown in green is the fluorescent reaction product. (B) The i-motif DNA structure chelated with copper (I) at pH 7.4 was filtered to remove unbound copper (I) in the solution. The copper (I) chelated i-motif was unfolded by shear flow in EFS. The released copper (I) catalyzed the fluorogenic click reaction, which was shown in the capillary tubes (bottom right). (C) Percentage increase of the fluorescence intensity of the click reaction at a shear rate of $63,209 \text{ s}^{-1}$ for 20 min. The dark curve indicates the fitting of the fluorescence intensity from a control click-reaction without DNA. The green curve represents the exponential fitting. The fluorescence intensity is normalized with respect to the maximum value observed in the experiment. This figure has been modified from Hu et al.¹¹. [Please click here to view a larger version of this figure.](#)

Discussion

The protocol described in this manuscript allows real-time investigation of the unfolding of an ensemble set of biomolecular structures by shear force. The results presented here underscore that DNA i-motif structures can be unfolded by shear force. The unfolding of the ligand-bound i-motif and the shear force-actuated click reactions were proof-of-concept applications for this ensemble force spectroscopy method.

Figure 1 presents the instrument setup. The homogenizer generator tip and reaction chamber must not contact with each other, which is critical to allow a stable shear flow between the rotor and stator without bubble formation. For the commercial homogenizer tip, it is better to choose one with an air hole to reduce bubble formation. In addition, the size of the reaction chamber should not be too large, since a large chamber requires more sample solution. On the contrary, a certain height of the shearing chamber is required to cover

the main body of the homogenizer tip. To reduce systematic error, it is suggested to maintain the same alignment in the setup to carry out all the relevant experiments. It is important to point out that DNA maintains integrity, as evidenced by gel electrophoresis after shearing¹¹.

From **Figure 1B**, the saturating fluorescence increased with the shear rate ranging from 9724 s^{-1} to 97245 s^{-1} . After plotting the plateau fluorescence intensity vs. shear rate, the fluorescence intensity did not increase anymore at the shear rate above 97245 s^{-1} , suggesting that the associated shear force supplied by the shear rate (97245 s^{-1}) was already higher than the required force to unfold the i-motif. Therefore, the plateau fluorescence intensity at 97245 s^{-1} was taken as a reference point (i.e., corresponding to 100% unfolding) to calculate the percentage of unfolded i-motif at any shear rate¹¹. **Figure 1C** shows the shear force generated on the telomeric i-motif at a particular shear rate. To establish the relationship between the shear force and the shear rate, mechanical unfolding of the same telomeric i-motif structure was performed in optical tweezers, and a cumulative plot of the unfolding percentage of the i-motif vs. force was plotted based on the unfolding force histogram of the i-motif in the optical tweezers experiments¹¹. For different DNA structures, force calibration using optical tweezers is recommended. Based on the assumption that the percentages of unfolded i-motif are equivalent between laser tweezers-based mechanical unfolding and shear force-based unfolding at a particular force (i.e., given that the direction of the force experienced by the i-motif is similar in these two methods, which is along the stretched axis of the two DNA handles [overhangs]), we calibrated the shear force vs. shear rate.

Although the homogenizer instruments are readily accessible in any lab, there are a few key steps that should be performed with care. Firstly, to detect accurate fluorescence values, the path of the light should be set between the rotor and the stator. Secondly, when performing the shearing experiments according to the steps of this protocol, it is important to check the fluorescence before the experiment. It is necessary to reduce high background fluorescence intensity by cleaning the whole setup well, especially the homogenizer tip and the reaction chamber. The experiment should be performed in a dark room to further reduce stray light.

One of the most significant benefits of the EFS technique is its flexibility and versatility for the mechanical unfolding of an ensemble set of biomolecular structures, including protein, RNA, and DNA structures. Traditionally, ensemble mechanical unfolding experiments require complex experimental setups. The advantage of EFS is that it reduces complexity in the instrument design, with much-reduced cost. Although EFS can perform high throughput experiments in a shorter time compared with single-molecule techniques, there are some limitations, which include the fact that the force magnitude is determined by the size of the molecular structure. In addition, fluorescent labeling of the detected molecule is required. To achieve larger shear forces, the attachment of shearing handles to the molecule of interest may become necessary. For future applications, the shear force-based EFS can be potentially used to study various mechanochemical phenomena in which chemical reactions, such as analyte recognitions¹⁵ and the polymerization of synthetic polymers, are influenced by mechanical forces.

Disclosures

The authors have no conflicts of interest.

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