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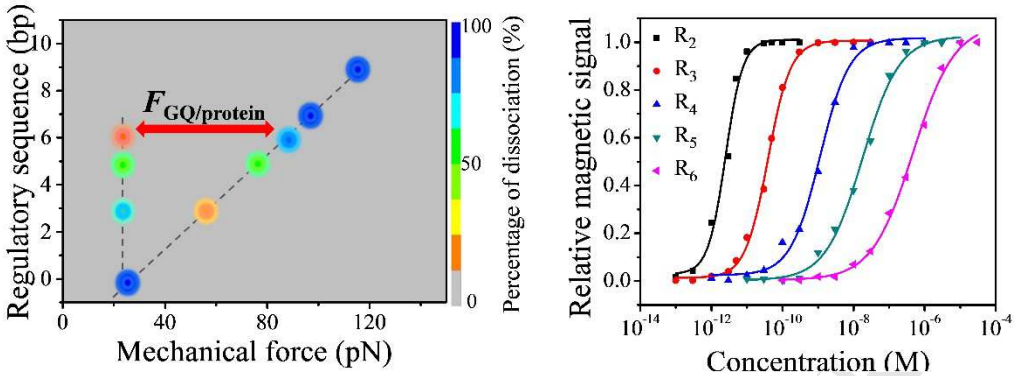
Graphical abstract



Regulatory-sequence mechanical biosensor: a versatile platform for investigation of G-quadruplex/label-free protein interactions and tunable protein detection

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Abstract: Mechanical biosensors can be used to quantitatively explore DNA-protein binding mechanisms by detecting targets at low concentrations or measuring force in single-molecule force spectroscopy. However, restrictions in single-molecule manipulation and labelling protocols have hindered the application for bulk analysis of label-free protein detection. Here, we present the integration of molecular force measurement and finely tunable detection of label-free proteins into one mechanical sensor. Regulatory-sequence force spectroscopy was obtained to investigate the binding force of DNA G-quadruplexes (GQ) and label-free protein. The dual control of regulatory sequences and mechanical forces induces the structure switching from DNA duplex to GQ/protein complex. It exhibits a synergistic effect, enabling the rational fine-tuning of the dynamic range for biosensing protein concentrations over eight orders of magnitude. Furthermore, this method was exploited to estimate the stability of the human telomeric DNA GQ by Ku protein and ligand

methylpyridostatin. The results revealed that human telomeric GQ has two different binding sites for Ku protein and ligand. Force spectroscopy integrating label-free force measurement and tunable target detection holds great promise for use in biosensing, drug screening, targeted therapies, DNA nanotechnology, and fields in which GQ are of rapidly increasing importance.

Keywords: mechanical biosensor, regulatory sequence, force spectroscopy, G-quadruplexes, tunable protein detection

1. Introduction

Nucleic acid-protein interactions play an important role in many biological processes, such as the transport and translation of RNA, packaging of DNA, and genetic recombination, replication and repair [1-3]. The use of biotechnology to modulate these interactions and tune the expression of some virulence genes and proteins is conducive to treating disease [4]. G-quadruplexes are four-stranded nucleic acid structures arising from the folding of particular guanine (G)-rich DNA and RNA sequences. They can form spontaneously at sufficiently high concentrations of guanine. In addition, monovalent cations such as K^+ and Na^+ stabilize these structures. Furthermore, the structural transition of a G-rich region from double-stranded helix to an atypical G-quadruplex structure can be facilitated by interacting with protein or other molecules [5-7]. With tandem TTAGGG repeats in its sequences, the human telomere has the propensity to form GQ [8, 9]. Human telomeric GQ play regulatory roles in telomere extension and can be stabilized by several proteins and small-molecule ligands, emerging as potential therapeutic targets for cancer treatment [10]. Moreover, nucleic acid aptamers, such as GQ aptamers, have been validated in DNA nanotechnology as reporters with high binding specificity and affinity for a

broad range of targets, including metal ions, small organic compounds, and proteins [11, 12]. Therefore, it is crucial to characterize the strength of GQ/protein interactions and quantitatively identify the binding partners of these reporters. The investigation of the binding forces between them could contribute to molecular recognition [13], drug development [14] and potentially mechanical manipulation of biochemical processes [15].

Mechanical biosensing is a useful approach to measure the interaction forces and concentrations of proteins and nucleic acids using force- and mass-based methods. Generally, mechanical biosensors capitalize on attributes that scale advantageously as physical size is reduced. First, mechanical sensors provide exquisite mass resolution. The minimum detectable added mass is proportional to the total mass of the device. Second, mechanical compliance converts an applied force into a measurable signal changes. This force responsivity opens new opportunities for measuring the force of biomolecules interaction that govern transcribe and transport. For example, nanomechanical sensors can resolve forces of ~1.8pN, which enable the binding behavior of drug molecules with different chirality and DNA of various sequences to be distinguished [14, 16-18]. Several mass-based cantilever sensors with surface-stress or dynamic-mode methods [19, 20] have been used to attain diagnostic sensitivities from the picomolar to nanomolar range. The detection of ultra-low DNA concentrations was achieved using an alternative mechanical sensor based on a photonic crystal nanowire array [21]. In addition, single-molecule force spectroscopy further enhances the ability of force-based sensors to manipulate individual molecules and measure microscopic forces with pN resolution [22]. Atomic force microscopy is the most commonly used technique, providing novel insights on GQ melting forces [23], protein/GQ binding strength [24, 25], dynamics [26], and energy landscapes [27]. Optical and magnetic tweezers based on trapped beads have been used to study the mechanical, kinetic and thermodynamic properties of the folding and binding of

telomeric GQ with proteins and small-molecule ligands [28-30]. However, achieving advanced performance for both purposes, force resolution and concentration sensitivity, remains a critical challenge due to the incompatibility of single-molecule force measurement and high-throughput biosensors. DNA origami nanostructures [31], split aptamers [32] and stochastic hairpin hopping [33] have been used as expanded single-molecule sensing templates to detect protein, DNA and small molecules, but they lack fine control of target affinity and dynamic range, limiting applications to target concentrations of typically 3-6 orders of magnitude [34]. Additionally, the target proteins are commonly free for unlabelled detection but require immobilization onto a surface during force measurement. Equally important for real applications are practical considerations: can force measurement and target detection be integrated within one mechanical system?

Recently, Yao and co-workers reported the force-induced remnant magnetization spectroscopy (FIRMS) technique, which uses the mechanical force applied to magnetic beads to distinguish different noncovalent bonds on the basis of their binding strengths [35]. Once the force-induced dissociation occurs, the magnetic beads conjugated with biological molecules undergo Brownian motion, which consequently randomizes the magnetic orientations of the beads, resulting in magnetic signal decrease. The decrease of magnetic signal at a corresponding force value indicates the dissociation of noncovalent bonds with different strengths. The overall magnetic signal of the magnetic beads is detected by a sensitive atomic magnetometer (more details see Fig. S1). The binding forces of noncovalent bonds can be precisely determined by gradually increasing the mechanical force in the form of centrifugal [36], or acoustic input [37]. FIRMS, different from the single-molecule force spectroscopy techniques such as AFM and optical tweezer, can efficiently measure tens of thousands bonds at a time with high force resolution of 2 pN [36], which is practical for binding force measurement and high-throughput force-based biosensor.

Herein, based on FIRMS, we develop a platform to investigate GQ/protein interactions termed regulatory-sequence (R-S) force spectroscopy, which integrates force measurement and protein detection. The resultant R-S force spectroscopy revealed the binding force of GQ with unlabelled protein. FIRMS of immobilized protein was used to confirm the force measurement. The dual control of regulatory sequence and mechanical force enabled fine-tuning of the affinity and dynamic range for protein detection over eight orders of magnitude. We also demonstrated the general applicability of this platform.

2. Experimental

2.1. Chemicals and reagents

DNA oligonucleotides were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). The sequences of the biotinylated G-quadruplex and complementary DNA are depicted in Table S1. Thrombin, bovine serum albumin, 3-aminopropyltriethoxysilane (APTES), glutaraldehyde, sodium borohydride and Tween-20 were purchased from Sigma-Aldrich (USA). M280 and Tirs-HCl buffer were obtained from Invitrogen. Ku protein (Ku70+Ku80) was purchased from Abcam (Cambridge, United Kingdom). Fetal calf serum was obtained from Solarbio (Beijing, China). All other chemical reagents were of analytical reagent grade and were used as received without further purification. High-purity deionized water (18.2 MΩ•cm) purified with a Millipore System was adopted to prepare all aqueous solutions in this work.

2.2. Preparation of aldehyde-terminated glass substrate

The glass sample chamber, with diameter of 3 mm, was first treated with piranha solution (H_2SO_4 (98%)/H_2O_2 (30%) =7:3 in volume) for 30 min at 90 ºC to remove potential organic contaminants on the surface and to generate surface hydroxyl

groups, and then washed thoroughly with high-purity deionized water and dried with high-purity nitrogen gas. The silanization reaction was carried out in liquid phase by reacting the hydroxylated surface with 1% APTES in toluene for 2 h. The resulting substrate were then rinsed with the solvent and dried under a stream of nitrogen. The silanized surface was activated by incubation in a solution of glutaraldehyde (1% v/v) in Tris-HCl buffer (20 mM Tris-HCl, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.4) for 3 h at room temperature and then rinsed with Tris-HCl buffer.

2.3. Immobilization of magnetically labelled DNA duplexes

The GQ DNA and the partially complementary amino-terminated RDNA (each 3 μM) were incubated in TE buffer (10 mM Tris PH 8.0, 1 mM EDTA, 1 M NaCl) at 95ºC for 5 min and then slowly cooled down to room temperature. Afterwards, 20 μL of the formed DNA duplex was added into the fresh aldehyde-terminated sample chamber where the imine bonds were formed after incubation overnight at 4ºC and stabilized by borohydride reduction. The immobilized DNA duplexes were rinsed with buffer and incubated with 1% (w/v) bovine serum albumin and 0.05% Tween-20 for 30 min. Then, the streptavidin-conjugated magnetic particles (M280, Invitrogen) were introduced into the sample chamber. After incubation for 2 h, the physically absorbed magnetic particles were removed from the surface by applying centrifuge with the speed of 1000 rpm for 5 min. The sample was then magnetized for 2 min using a permanent magnet (~0.8 T).

2.4. Quantification of the binding force of GQ and unlabelled thrombin

For the thrombin system, Tris-HCl buffer (20 mM Tris-HCl, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.05% Tween-20, pH 7.4) was used as the base buffer. Thrombin of 300 nM of was introduced to the surface-immobilized DNA duplexes with regulatory sequence $R_{i=0\text{-}15}$ immersed under the buffer solution and incubated at 37ºC for 30 min. The excess unbound thrombin was removed by washing

procedure in this experiment. For FIRMS measurement of each R_i, mechanical force with varying amplitudes was applied on the samples by gradually increasing the speed of the centrifuge (Sigma, 3-30KS) and the magnetic signal was recorded. The force values are calculated from $f = m\omega^2 r$, where $m = 5.8\times10^{-15}$ kg is the buoyant mass of the particles, ω is the angular speed, and r =4.5 cm is the distance from the sample to the center of the centrifuge. The centrifuge time for each speed was 5 min. Magnetization measurements were performed using in-house made scanning magnetic imaging based on an atomic magnetometer with a sensitivity of about 200 fT $Hz^{-1/2}$. The error in the magnetic signals in the experiments carried out in this study is usually ±1.5 pT. Each dissociation force reported by FIRMS was repeated at least three times, with standard deviations less than 2 pN.

2.5. Quantification of the binding force of GQ and immobilized thrombin

The thrombin was immobilized on the fresh aldehyde-terminated glass sample chamber by incubation in the solution (20 mM Tris-HCl, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.4) at 4 ºC overnight and then rinsed extensively with Tris-HCl buffer to remove any unbound thrombin. Then 20 μL of 300 nM GQ DNA was added into the chamber and incubated at 37 ºC. Magnetic beads coated with streptavidin dispersed in the same buffer were injected into the reaction chamber and incubated for 2 h. Then the physically absorbed beads were removed by applying centrifuge with the speed of 1000 rpm for 5 min. The sample was magnetized for 2 min using the same magnet. Forces with varying amplitudes were applied on the samples by gradually increasing the speed of the centrifuge and the magnetizations were recorded. In regulatory-sequence mode, the magnetic signal of complex was measured by FIRMS under a constant force of 26 pN. It is large enough to remove the dissociated signal but much smaller than the binding force of the complex, which can identify the switch.

2.6. Detection of thrombin based on sequence regulation

To determine the observed rate constant k_{Ri} of different DNA duplex with R_i from the kinetics measurements in Fig. 4a, a single exponential equation, $Y = A – B\times\exp(-k_{Ri}\times t)$, was used to fit the curves. Here, Y represents the relative amplitude of signal change, t is the incubation time, and A and B are fitting constants.

Different concentrations of thrombin (20 μL) were added into the surface-immobilized DNA duplexes. The complementary length for the GQ domain was varying from two to six. After being incubated at 37 ºC for 30 min, the excess unbound protein was removed by washing with the buffer. The magnetic signal was recorded while 26 pN was applied to the complex. Here, the force of 26 pN was only able to dissociate the duplex formed by GQ DNA and R_0.

2.7. Detection of thrombin based on force regulation

The sample surface modified by magnetically labelled DNA duplex was immersed in thrombin solution with various concentrations. The R_9 duplex was used in this experiment. After being incubated at 37 ºC for 30 min, the sample was subject to the regulatory force provided by the centrifuge to induce the dissociation of the duplex. The magnetic signal was recorded before and after applying the force.

2.8. Investigation of the interaction between Human telomeric GQ, protein and ligand

The influence of ligand mPDS on telomeric GQ/Ku protein interaction was investigated with Tirs-HCl buffer (10 mM Tris-HCl, 100 mM KCl, and 0.05% Tween-20, pH = 7.5). The measurements of the interaction forces of telomeric GQ with Ku protein, mPDS and Ku protein+mPDS were similar to the thrombin system (Fig. 1). The sequences of the telomeric GQ DNA strand and the regulatory DNA (T_0-T_{16}) complementary to GQ DNA are shown in Table S2. The dynamic range of

mPDS cooperation was measured with 500 nM of Ku protein while a constant force of 26 pN was applied to the T_{11} duplex.

3. Results

3.1. Concept of the method

The scheme of R-S force spectroscopy is shown in Fig. 1. The DNA duplex formed by magnetically labelled GQ DNA and tethered regulatory complementary DNA (denoted by RDNA) was used as an internal force reference to probe the GQ/protein interactions in a competitive assay. We used FIRMS to quantify the magnetically labelled targets and the binding force of DNA duplexes. The thrombin GQ aptamer, a common model system in aptamer-based affinity assays, was chosen to demonstrate proof-of-concept of this method. On the RDNA part, R_0 produced a stable elementary duplex, while the length of GQ complementary sequence (denoted by regulatory sequence, Table S1) was tuned to control the binding force of DNA duplex ($F_{\mathrm{Ri,\ i=0\text{-}15}}$). The presence of target protein will induce the formation of GQ/protein complex via a competing equilibrium with regulatory sequence. When the interaction force of GQ with the target protein is larger than that with the regulatory sequence, the switch from the duplex to GQ structure occurs [38], leading to a shorter stretch of DNA duplex (R_0 duplex) holding the magnetic bead to the surface and hence a decrease in the force needed to release the beads. Otherwise, the duplex endures, abolishing structure switching. During the modulation of regulatory sequence, we assume that, in the presence of thrombin, when the length of regulatory sequence is less than a balanced value at which point RDNA was termed as R_b, the switch from the duplex to GQ structure occurs. The dissociation force of the DNA duplex changes from F_{Ri} to F_{R0}. When one base is added on the balanced regulatory sequence, the switch does not occur. Therefore, the binding force of unlabelled protein with GQ ($F_{GQ/protein}$) can be obtained as following:

$$F_{GQ/protein} = F_{Rb} - F_{R0} \quad (1)$$

where F_{Rb} represents the binding force of the DNA duplex hybridized by thrombin GQ DNA and the balanced RDNA (R_b).

Furthermore, the final dissociation of the DNA duplex induced by an appropriate mechanical force will produce a change in the magnetic signal corresponding to the GQ/protein complex, enabling detection of the target protein. Dual control of sequence and force permitted the integration of force measurement and finely tunable protein detection into one mechanical sensor.

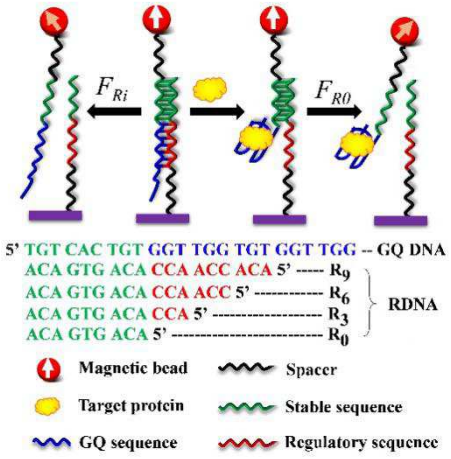

Fig. 1. Design of R-S force spectroscopy. Besides stable sequence R_0 (in green), GQ DNA and RDNA forming a duplex structure contain GQ sequence (in blue) and regulatory sequence (in red), respectively.

3.2. R-S force spectroscopy analysis

To assess $F_{GQ/protein}$, R-S force spectroscopy was obtained from a series of FIRMS spectra. The first FIRMS experiment to validate structure switch of regulatory-sequence DNA duplex was carried out. The typical magnetic field profiles before and after mechanical dissociation of the duplex are included in Fig. S2. With the stable sequence R_0, the GQ sequence binds with thrombin. The F_{R0} did not change (26 pN, Fig. 2a) after the addition of thrombin, indicating that structure switching

didn't affect the binding force of R_0 duplex. However, two transitions appeared at 26 and 56 pN in the FIRMS of R_3 duplex (Fig. 2b) after the addition of thrombin, corresponding to the dissociation of R_0 and R_3 duplex with dissociation percentage 68.3±1.5% and 31.7±1.5%, respectively. It indicates that most aptamer domains were switched to the GQ form, leading to a shorter stretch of DNA duplex. Thus, the length of regulatory sequences is crucial to modulate the balance of the duplex/GQ formation induced by thrombin binding.

In order to find the threshold of structure switch to quantify the binding force of GQ/thrombin, we performed a series of FIRMS spectra under different length of regulatory sequence to analyze the change in the binding force of DNA duplex. The transitions of the magnetic signals are associated with both mechanical forces and structure switching. Therefore, R-S force spectroscopy plot of the percentage of duplex dissociation against mechanical force and regulatory sequence was obtained. In the case of no thrombin, it shows the linear dependency of the length of the regulatory sequence on the force, and the DNA duplex was totally dissociated. These force measurements are consistent with previous reports [39]. However, when thrombin was introduced to separate the GQ strand from its complement, a transition appeared at the force of 26 pN for $R_{0\text{-}6}$ duplex, whose percentage of dissociation decrease with the increasing of the regulatory sequence (Fig. 2d). Importantly, no transition appear at 26 pN when the length of regulatory sequence increased to 7 bp. It indicated that the threshold of structure switching (R_b) was located between R_6 and R_7. Thus, from the measured F_{Ri} (F_{R6}=90 pN, F_{R7}=98 pN), we determined $F_{GQ/protein}$ as 68±4 pN according to Equation 1. These data demonstrate that R-S force spectroscopy has the capacity to resolve the binding forces of multiple DNA/DNA and DNA/protein interactions.

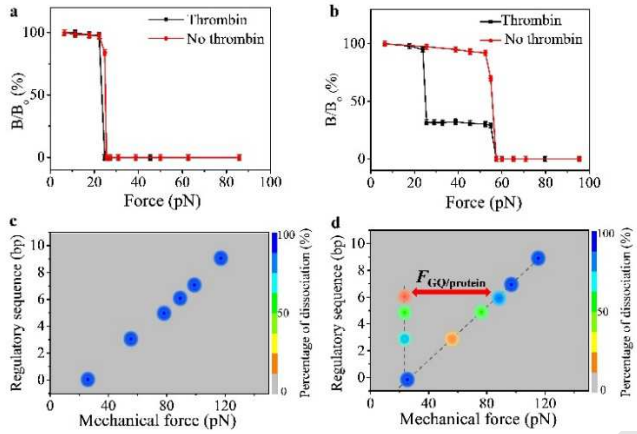

Fig. 2. Binding force of GQ aptamer and label-free thrombin. (a,b) FIRMS spectra of R_0 (a) and R_3 (b) duplexes. The vertical axis of B/B_o is the ratio of the measured signal with the initial signal. (c,d) R-S force spectroscopy without (c) and with thrombin (d), respectively. The results in (a,b) correspond to the data points in (c,d) with regulatory sequence at 0 and 3 bp, respectively. The detailed FIRMS results are shown in Fig. S3. $F_{GQ/protein}$ was deduced from the threshold of structure switching, as indicated by arrow in (d).

3.3. Verification of GQ/thrombin binding force

To verify the results obtained from R-S force spectroscopy, the interaction force of immobilized thrombin and GQ was measured using a conventional direct method (Fig. 3a). The centrifugal force with increasing amplitudes was applied to directly induce the dissociation of the GQ/thrombin complex. As shown in Fig. 3b, the dissociation transition in magnetic signal occurred at 79±1.1 pN, slightly larger than the value obtained from R-S force spectroscopy (68±4 pN). Previous studies indicated that the different thrombin state, immobilized and label-free, resulted in different binding force [26, 27, 40, 41]. To decouple and interpret the effect of thrombin state and measurement method on interaction force, we also employed the regulatory-sequence method to probe the binding force of GQ with the immobilized

thrombin (Fig. 3c). After the GQ and the immobilized thrombin formed the complex, the regulatory sequence was added in solution to induce GQ structure switching and dissociate the GQ/thrombin complex. If the switch from the GQ/thrombin complex to GQ/RDNA duplex structure occurs, only a small centrifugal force (here 26 pN) can remove the dissociated magnetic bead, leading to a decrease in the magnetic signal, which can identify the switch.

From Fig. 3d we can see that there is a sharp transition in magnetic signal when the length of regulatory sequence increases to 8 bp. Therefore, the binding force of immobilized thrombin and GQ aptamer was deduced to be 79±7 pN via the reference of F_{R7} and F_{R8} (Fig. S4). This result was in good agreement with the direct measurement above, confirming that the difference of measured forces mainly results from the change of thrombin state. The immobilization of thrombin may enhance the mechanical stability of GQ/thrombin complex.

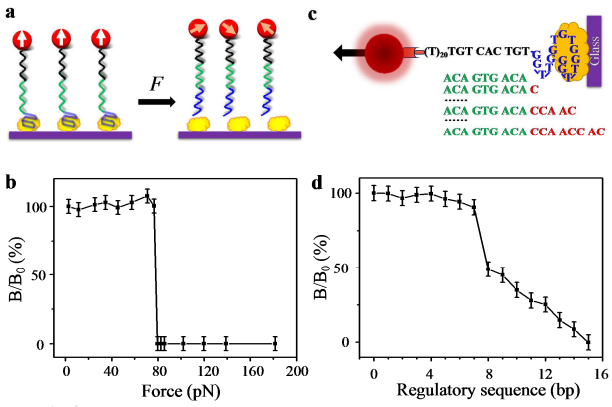

Fig. 3. Verification of the binding force and feasibility of R-S force spectroscopy on immobilized thrombin. (a,b) Illustration and FIRMS spectrum of direct method. (c,d) Confirm the binding force of GQ and immobilized thrombin by R-S force spectroscopy.

Altogether, these results demonstrate the feasibility of R-S force spectroscopy for the measurement of interaction force between GQ and protein, either immobilized or

label-free. The force calibration and resolution were set by the reference of DNA duplex while the performance of FIRMS measurement is better than single base pair [36].

3.4. Thrombin detection based on sequence modulation

From the R-S force spectroscopy of thrombin we can see that the regulatory sequence modulated the binding amplitude of GQ/thrombin complex (i.e. shorter regulatory sequence results higher probability of GQ/thrombin complex). To further investigate the binding kinetics of the complex, we varied the incubation time of thrombin to measure the magnetic signal change under a constant force of 26 pN using the method in Fig.1. Fig. 4a shows dynamic binding of 300 nM thrombin under different regulatory sequence (R_3, R_6 and R_9). The curves of R_3 and R_6 indicate that the equilibrium between free and bound thrombin has been reached in 30 min with different binding amplitude. But no signal change was observed for R_9, consistent with the result of R-S force spectroscopy. Furthermore, the observed rate constants were obtained through the fitting of exponential functions (see Methods). The value of k_{R3} (0.11±0.01 min^{-1}) was faster than that of k_{R6} (0.07±0.01 min^{-1}). It indicates that the regulatory sequence can tune the binding probbility and overall affinity of GQ/thrombin complex.

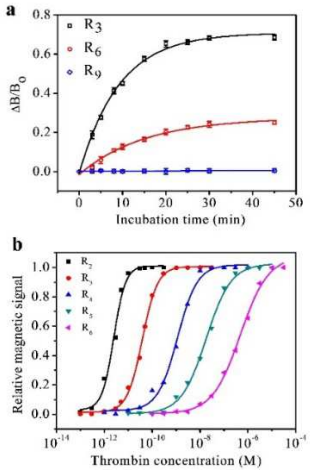

Fig. 4. Binding kinetics and dynamic range of GQ/thrombin complex based on sequence modulation. (a) Relative binding amplitude versus incubation time of thrombin under different regulatory sequence. The vertical axis of $\Delta B/B_o$ is the ratio of the amount of signal decrease with the initial signal. (b) The affinity and dynamic range of GQ aptamer can be efficiently tuned across eight orders of magnitude of protein concentration by modulating the length of regulatory sequence (R_2-R_6).

By DNA-based structure switching, the control of the sequence's overall affinity provides a means of broadening the concentration range over which biosensors respond robustly to changes in the target concentration [42, 43]. Consequently, when we varied the concentration of thrombin solution, the sequence modulation should allow our force spectroscopy to finely tune detection range. Because the structure switching was induced by the regulatory sequence shorter than R_b, we used the sequence R_2-R_6 to detect thrombin binding events via the dissociation signal under the constant force mode of F_{R0}. As expected, the sensor with R_6 shows a typical hyperbolic binding curve with the useful dynamic range [42] (defined here as the interval between 10% and 90% of total activity) reaching almost 2 orders of magnitude (Fig. 4b, pink curve). The binding affinity and probability is related to the length of regulatory sequence complementary to the domain of the GQ aptamer, with shorter (i.e., more looser binding) regulatory sequence responding more sensitively to thrombin. By the sequence modulation from R_6 to R_2, the dynamic range of thrombin detection is finely-tuned over 8 orders of magnitude and the detection limit reaches 1 pM (Fig. 4b). Moreover, because it alters affinity by regulatory sequence, this approach does not change the structure of the aptamer's binding site and thus does not alter the aptamer's specificity [44].

3.5. Thrombin detection based on force modulation.

Mechanical force dissociates DNA duplexes, and thrombin induces the formation of GQ [45]. However, it is unclear whether mechanical force and thrombin have positive cooperativity on structure switching from the duplex to GQ/thrombin complex, especially when neither has an effect on the stability of the duplex. It is noted that the excess unbound thrombin was removed by washing procedure during all the above experiment. If the presence of thrombin can facilitate the mechanical dissociation of stabilized duplex, it would offer the possibility to construct a multifaceted modulation platform for thrombin detection.

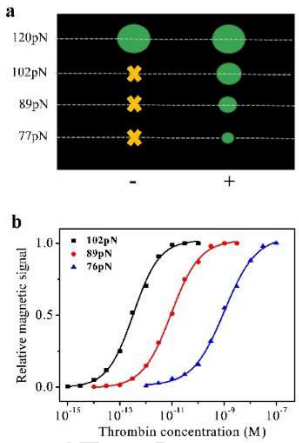

Fig. 5. Mapping of dissociation signal and extending of dynamic range based on force modulation. (a) The signal change of stabilized duplex of R_9 in the absence (-) and presence (+) of free thrombin under different mechanical force was mapped. Cross: No signal change was observed under the corresponding force. Ellipse: Signal change was occurred with the area indicating the relative signal change amplitude. (b) Using the synergistic effect of exogenous force and endogenous thrombin, we engineered the force-based sensor with the stabilized duplex of R_9 and further extended the dynamic range of sensors by exerting different mechanical force.

To test this hypothesis, we exerted different mechanical force on the stabilized duplex of R_9 in the absence and presence of free thrombin using the platform in Fig.1. The measured signal changes before and after exerting the force are mapped in Fig.

5a. Because F_{R9} (119 pN) is stronger than $F_{GQ/thrombin}$, thrombin can't induce structure switching of R_9 duplex. As expected, no dissociation signal occurred with the mechanical force lower than 119 pN when excess unbound thrombin was removed. However, the dissociation signal was observed under a weaker force of 77 pN in the presence of 300 nM thrombin. The amplitude of the dissociation signal became bigger with the increase of mechanical force. These results clearly suggest that thrombin promotes the mechanical dissociation of the stabilized duplex. It provides a space of 77-119 pN for force modulation capable of tuning the thrombin detection. Therefore, we generated a regulatory-force mode for biosensing (Fig. 5b). The dynamic range spans 6 orders of magnitude with the fixed sequence of R_9. The detect limit of 10 fM was much lower than 1 pM achieved using the regulatory-sequence mode. Thus, the use of precise force control provides a more flexible and continuously tunable route. Moreover, through the simultaneous modulation of mechanical force and the stabilized duplex (i.e., 8-mer) the detection limit of the force-based sensor could be further extended.

In order to verify the practicability of the developed force spectroscopy, we prospectively used our method to determine the thrombin concentration in fetal calf serum. The signal response behavior in pure buffer and 10% serum was shown in Fig. S5. It didn't show any significant effect and the same profile was obtained, indicating that our method is robust and performs well even in complex samples.

3.6. General applicability of method

To demonstrate the general applicability of R-S force spectroscopy, we investigated the interaction between human telomeric GQ and Ku protein. Ku proteins are nuclear protein that are highly conserved in eukaryotes and involved in DNA repair [46], maintenance of telomere length and transcription regulation [47]. Previous studies have already shown that Ku proteins have the capacity to interact with

telomeric DNA or GQ-forming sequences [48]. However, the specificity has not been quantified in terms of the binding force, which may be valuable for understanding therapy mechanism and developing novel anticancer agents. Furthermore, we explored the influence of a ligand methylpyridostatin (mPDS, structure shown in Fig. S6), an analogue of pyridostatin, on the interaction force of telomeric GQ/Ku protein complex. Proteins or ligand molecules that induce and stabilize telomeric GQ structures have the potential to inhibit telomerase activity and serve as anticancer agents [49].

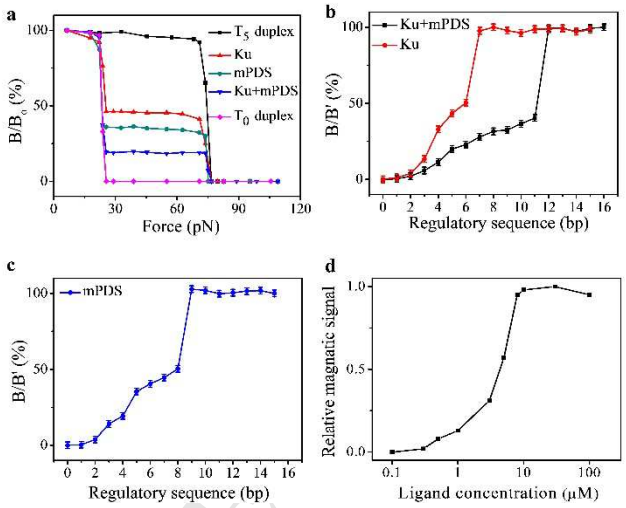

Fig. 6. Interactions of human telomeric GQ with Ku protein and mPDS. (a) FIRMS spectra of the DNA duplex of telomeric GQ with Ku protein or mPDS. (b, c) Determining the interaction forces of telomeric GQ with Ku protein and mPDS by regulatory-sequence mode. The vertical axis of B/B' is the ratio of the measured signal with the strongest signal. (d) Dynamic range of mPDS cooperation with Ku protein of 500 nM stabilizing telomeric GQ.

The experiment method is the same as the thrombin/GQ system (Fig. 1). We firstly probed the interactions of telomeric GQ with Ku protein and mPDS by FIRMS of DNA duplexes with regulatory sequence (T_5 with the sequence shown in Table S2). With the presence of Ku protein (red curve), two transitions were located at 26 pN

and 77 pN, corresponding to the dissociation of the T_0 and T_5 duplex, respectively (Fig. 6a). The similar results were observed with mPDS and a mixture of Ku protein and mPDS. It demonstrates that both Ku protein and mPDS can stabilize the telomeric GQ structure. Interestingly, the amplitude of the first transition is different from each other, which may be ascribed to their difference in binding affinity. To further quantitatively estimate the stability of telomeric GQ, we use regulatory-sequence mode to determine the binding forces (Fig. 6b), which became larger in the presence of both Ku protein and mPDS (61±4 pN and 112±4 pN for Ku protein and Ku protein+mPDS bound to telomeric GQ, the corresponding FIRMS spectra of regulatory-sequence DNA duplexes were shown in Fig. S7). It indicates that Ku protein and mPDS probably bind with telomeric GQ simultaneously. In order to analyze the binding site of Ku protein and mPDS to GQ, we measured the binding force of mPDS/GQ complex (90±7 pN, Fig. 6c), revealing that the interaction with telomeric GQ is Ku protein < mPDS < Ku protein+mPDS. If Ku protein and mPDS were bound to the same site of telomeric GQ, the binding force of Ku protein+mPDS would be coincident with that of Ku protein or mPDS alone. Therefore, our results suggest that telomeric GQ has two different binding sites for Ku protein and mPDS, and can be stabilized cooperatively. Both single-molecule [28] and ensemble experiments [50] have observed similar results from different ligand and protein. However, the cooperative dynamic range is seldom explored. With 500 nM of Ku protein, we observed the maximum cooperative response, which reached 8-10 mM of mPDS (Fig. 6d), which may be guided for the drug interaction in cancer therapy.

4. Discussion

DNA and protein interactions with similar binding strengths and multiple binding partners have been implicated in gene regulatory processes. Here, we demonstrated a versatile and powerful platform to quantify multiple interaction forces

and finely tune the detection range of unlabelled proteins in the integrated force spectroscopy. To our knowledge, there are no other methods that provide such information in a synergistic manner.

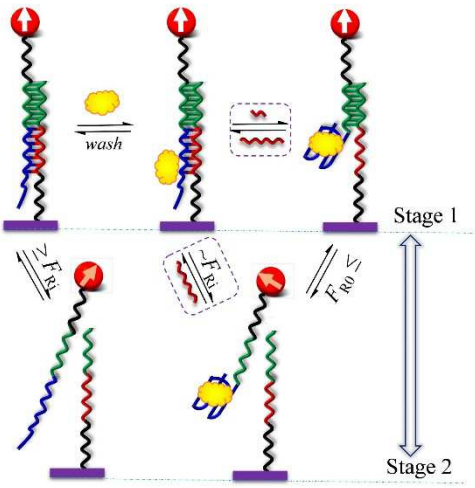

Fig.7. Proposed sensing pathways of synergistic regulation. Schematic illustration includes two stages: thrombin binding and mechanical dissociation with the sequence and force modulation (in dash box), respectively.

The developed force spectroscopy is capable of fine-tuning of detection dynamic range because the target protein can induce the structure switching and promote the mechanical dissociation. We summarize a sensing pathway with dual modulation of sequence and force involving a two-stage process (Fig. 7): thrombin binding and mechanical dissociation, which is a post-binding analysis. In the first stage, the formation of the complex is mediated by nonspecific binding of thrombin with the DNA duplexes. Before the structure switching, an intermediate can form and be stabilized by the long regulatory sequence. The sequence modulation facilitates the determination of the predominant species at the end of this stage and tunes their affinity. In the second stage, an appropriate and tunable force induces the final dissociation of the DNA duplexes. As a result, the thrombin concentration was

deduced from the magnetic signal change of the magnetically labelled complex. The results reveals that the mechanical force and thrombin have positive cooperative effect on the dissociation of DNA duplex, which enable us to extend the detection dynamic range based on force modulation.

In conclusion, our method provides a versatile platform to permit the integration of single-molecular force measurement and finely tunable detection of label-free proteins into one mechanical sensor, which is difficult to achieve using other methods. The dual modulation of force and sequence are reciprocal for mechanical sensing: sequence modulation extends R-S force spectroscopy, and force modulation extends DNA-based sensing. In addition, the novel R-S force spectroscopy can resolve the binding force of multiple interactions beyond prior FIRMS by spreading the dissociation information along multiple axes, yielding a sequence-force correlation spectrum. Moreover, the target proteins can be either immobilized or label-free. This DNA-based sensor shows highly sensitive label-free protein detection, which can be tuned by both the length of the regulatory sequence and the applied force. The platform complementing force spectroscopy with regulatory sequences has many applications for the quantification of binding force, the detection of label-free targets, drug screening, targeted therapies, and fields in which GQ are of rapidly increasing importance. The utilization of this methodology is not limited to protein/GQ systems because it is also valid for general single stranded DNA sensing systems with structure switching between the duplex and other unique secondary or tertiary structures to explore the interaction mechanism of ions, nucleic acids, proteins, and small molecule ligands in a biologically relevant context.

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Highlights

►We present the integration of biomolecular force measurement and fine tunable detection of label-free proteins into one mechanical biosensor.

►The binding force of DNA G-quadruplexes and label-free protein can be determined by Regulatory-sequence force spectroscopy.

►The dual control of regulatory sequences and mechanical forces enable the rational fine-tuning of the dynamic range for detecting thrombin concentrations over eight orders of magnitude.

►The influence of a ligand methylpyridostatin on the interaction force of telomeric GQ/Ku protein complex has been explored by this method.