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A single-molecule platform for investigation of interactions between G-quadruplexes and small-molecule ligands

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Abstract

Ligands that stabilize the formation of telomeric DNA G-quadruplexes have potential as cancer treatments, because the G-quadruplex structure cannot be extended by telomerase, an enzyme over-expressed in many cancer cells. Understanding the kinetic, thermodynamic and mechanical properties of small-molecule binding to these structures is therefore important, but classical ensemble assays are unable to measure these simultaneously. Here, we have used a laser tweezers method to investigate such interactions. With a force jump approach, we observe that pyridostatin promotes the folding of telomeric G-quadruplexes. The increased mechanical stability of pyridostatin-bound G-quadruplex permits the determination of a dissociation constant K_d of 490 ± 80 nM. The free-energy change of binding obtained from a Hess-like process provides an identical K_d for pyridostatin and a K_d of 42 ± 3 μ M for a weaker ligand RR110. We anticipate that this single-molecule platform can provide detailed insights into the mechanical, kinetic and thermodynamic properties of liganded bio-macromolecules, which have biological relevance.

G-quadruplexes are four-stranded nucleic acid structures arising from the folding of particular guanine (G)-rich DNA and RNA sequences^{1–3}. With hundreds of 5'-TTA GGG repeats in its sequence, the human telomere has the propensity to form G-quadruplexes^{4,5}.

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These quadruplexes have been proposed to play an important role in the maintenance of telomere length as they cannot be extended by telomerase^{6,7}. Such an effect has profound implications in cancer cells, the majority of which have over-expressed levels of telomerase^{6,8}. Small molecules such as telomestatin^{9,10} have been demonstrated to bind to telomeric G-quadruplexes and suppress telomerase activity. This discovery has begun the expansion of telomeric G-quadruplexes into potential therapeutic targets for cancer treatment¹¹⁻¹³.

As a polymerase, telomerase extends the telomere region using an RNA sequence, 3'-CAAUCCCAAUC-5', as a template¹⁴. Our previous studies on human insulin-linked polymorphic region (ILPR) sequence have shown that the mechanical stability of G-quadruplexes¹⁵ is higher than the stall force of RNA and DNA polymerases^{16,17}. This suggests that quadruplexes per se can stall the replication or transcription processes. Telomeric G-quadruplex structures stabilized by a small molecule may have an increased mechanical stability that may further thwart the efforts of telomerase to elongate its substrate, the G-overhang. In previous research, bulk methods such as X-ray crystallography¹⁸ and NMR spectroscopy^{19,20} have been used to determine detailed structural information for the telomeric G-quadruplexes. Other ensemble assays such as thermal melting analysis and surface plasmon resonance (SPR) have provided insights into folding and unfolding kinetics^{21,22}. Fluorescence-based single-molecule methods have revealed the dynamic nature of these structures with and without bound ligands²³⁻²⁵. However, mechanical information regarding the quadruplex-ligand complex has not been investigated. Here, we present the results of studies using a single-molecule mechanical assay to provide kinetic, thermodynamic and mechanical information on quadruplex-ligand interactions.

Our assay makes use of a platform that contains a single telomeric G-quadruplex structure to evaluate its interaction with small-molecule ligands in a laser-tweezers instrument. Based on structural similarities, two ligands have been evaluated, pyridostatin (**PDS**)^{26,27} (Fig. 1a), a highly potent small molecule for telomeric G-quadruplex binding, and a less potent analogue, **RR110** (ref. 28; Fig. 1b). **PDS** has already been shown to induce dysfunctional telomeres in cancer cells with the uncapping of POT1, resulting in DNA damage signalling activation²⁶. In contrast, this phenotype has not been observed upon incubation of **RR110**. Moreover, Förster resonance energy transfer (FRET) melting experiments have shown that, in agreement with *in cellulo* data, the ΔT_m of human telomeric G-quadruplex induced by **RR110** is poor compared to **PDS**. This can be explained by the absence of a third positively charged side chain, which increases the selectivity of **RR110** for the G-quadruplex over dsDNA, but decreases its potency in binding and stabilizing the G-quadruplex²⁸. To investigate the dynamic binding events, we incorporated a 'force jump' approach that allows the observation of events in the range of milliseconds. We further revealed that, following ligand binding, the mechanical stability of human telomeric G-quadruplex increases. This mechanical information has been exploited to retrieve the thermodynamic dissociation constant between the G-quadruplex and the ligand. Previous efforts to obtain the same information from bulk assays have failed due to solubility issues regarding the two ligands. Using analysis similar to the Hess cycle²⁹, we have derived the change in the free energy of binding ($\Delta G_{\text{binding}}$) and used it to calculate the dissociation constant without varying either quadruplex or ligand concentration. The dissociation constants obtained from these two single-molecule calculations agree very well with one another. The generic nature of this single-molecule platform makes it amenable to the investigation of other receptor and ligand systems.

Results and discussion

Ligand binding increases the mechanical stability of human telomeric G-quadruplex

The single-molecule platform comprises a single-stranded fragment of human telomeric sequence 5'-TTA (GGG TTA)₄, or Tel-4G, sandwiched between two dsDNA spacers (see Methods). Two spacers were tethered to two optically trapped beads via digoxigenin-antidigoxigenin and biotin-streptavidin linkages, respectively (Fig. 1c). To investigate the mechanical stability of the DNA structure formed in the Tel-4G sequence in 10 mM Tris buffer (pH 7.4) with 100 mM KCl, we stretched the DNA construct by moving one of the optically trapped beads away from the other with a load rate of 5.5 pN s⁻¹. A rupture event was observed in the force-extension (*F-X*) curve (Fig. 2a, bottom arrow), indicating the unfolding of a secondary structure in the Tel-4G sequence. The contour length of this structure (9.3±0.1 nm, see Methods and Supplementary Fig. S1) indicates a folded structure of 21 nucleotides, which is expected for a telomeric G-quadruplex in this construct. The histogram of the rupture forces (Fig. 2b) shows a population of 21±1 pN for this structure. This suggests that the telomeric G-quadruplex can withstand the load force of polymerases^{16,17}. Using Jarzynski's theorem for non-equilibrium systems^{30,31} (see Methods), the change in free energy of unfolding the quadruplex, ΔG_{unfold} , was found to be 9.8±0.4 kcal mol⁻¹ at 23 °C. This value was consistent with those previously determined for telomeric G-quadruplexes³²⁻³⁵.

The same single-molecule setup was used to evaluate the effect of ligands on the mechanical stability of telomeric G-quadruplex. First, we incubated the Tel-4G construct in the same buffer with 0.5 μM **PDS**, a small molecule specific for G-quadruplex structures^{26,27}. The mechanical stability of the G-quadruplex in the presence of the ligand was revealed by the rupture events in the *F-X* curves (Fig. 2a, top arrow). After each rupture event, we brought the DNA construct back to zero force, waited at least 30 s to allow for ligand binding, and then started the next round of the stretching procedure. When the rupture force histogram was analysed, two populations with 20±1 pN and 41±2 pN were observed (Fig. 2c). The population of 20 pN demonstrated the same rupture force as that without ligand (Fig. 2b). It was therefore assigned as a free G-quadruplex population. The species with an increased rupture force of 41 pN may be due to either specific or non-specific binding of **PDS** to the DNA construct. To rule out the non-specific binding of **PDS** to dsDNA, we mixed 10 μM **PDS** with a DNA construct that contained only two dsDNA spacers (see Methods). The percentage of the unfolding events (~2.5%, see Supplementary Information for the calculation) dramatically reduced compared to that of the rupture events at 41 pN (50%) for Tel-4G with 0.5 μM ligand. This percentage (~2.5%) was identical to that observed for the same construct without ligand. To rule out the binding of the ligand with single-stranded sequences incapable of forming any G-quadruplex, further experiments were performed on the DNA construct that contained 5'-TGT (CCC CAC ACC CCT GT)₂ instead of the Tel-4G (see Methods). This sequence has been shown to remain single-stranded at pH 7 (ref. 36). Mechanical unfolding of this construct yielded a negligible percentage of unfolding events (~2% without **PDS** and ~3% with **PDS**). These results effectively ruled out the non-specific binding of the ligand to the DNA construct and were in accord with the previously observed specificity of **PDS** for G-quadruplex structures^{26,27}. Based on this, we assigned the population with increased rupture force as ligand-bound telomeric G-quadruplexes.

Kinetic measurements indicate that binding of the ligand facilitates the formation of G-quadruplex

To investigate the dynamic binding of **PDS** to the telomeric G-quadruplex, we designed a force jump method (Fig. 3a, see Methods). In this approach, the DNA construct was stretched until the G-quadruplex was unfolded. This was followed by a rapid force jump to 0

pN to allow refolding of the secondary structure for a specific time interval. The refolding of the structure was indicated by a rupture event in the next round of the stretching procedure that started at 7 pN with another force jump process. These two force jumps prevented the refolding of the structure during stretching and relaxing of the DNA at the low force region (<7 pN). Events as fast as 100 ms can be readily measured by this procedure. Using this strategy, we varied the incubation time to measure the folding kinetics of telomeric G-quadruplex with and without $0.5\ \mu\text{M}$ **PDS**. Figure 3b shows force histograms with incubation times ranging from 0–30 s. Except for the 0 s incubation, each histogram demonstrates two populations, similar to those in Fig. 2c. Based on this similarity, we assigned the population with 20 pN rupture force as free G-quadruplex, and the 41 pN species as the G-quadruplex tightly bound with **PDS**. At 0 s incubation, the 41 pN population was not observed (the first histogram in Fig. 3b and the black star in Fig. 3c), indicating that either the ligand is yet to bind to the quadruplex (the first step of pathway B in Supplementary Fig. S2), or the quadruplex-ligand complex has not transformed into a structure with increased mechanical stability (the first step of pathway A in Supplementary Fig. S2).

With this assignment, we calculated and plotted the probability of refolding for each species with a specific incubation time (Fig. 3c, see Supplementary Information for details). Single exponential functions provided excellent fitting to the observed data, from which folding rate constants were obtained. The single-molecule nature of the method allowed us to simultaneously determine the kinetics of individual populations in a mixture of different species. We observed the overall folding rate constant for the G-quadruplexes (including both 20 and 41 pN populations), $k_{\text{fold, ligand}} = 0.20 \pm 0.01\ \text{s}^{-1}$, in a solution with $0.5\ \mu\text{M}$ **PDS**. Further deconvolution analysis (see Supplementary Information for details) of these two populations allowed us to simultaneously determine the folding rate constant of the ligand-free or loosely bound quadruplex (the 20 pN population), $k_1 = 0.48 \pm 0.06\ \text{s}^{-1}$, and for the quadruplex tightly bound with the ligand (the 41 pN population), $k_2 = 0.13 \pm 0.02\ \text{s}^{-1}$ (see Supplementary Fig. S2). Compared to the rate constant for the G-quadruplex in a solution without **PDS**, $k_{\text{fold, no-ligand}} = 0.11 \pm 0.01\ \text{s}^{-1}$ (Supplementary Fig. S2), the value of k_1 ($0.48 \pm 0.06\ \text{s}^{-1}$) was much faster. This indicates that **PDS** increases the formation kinetics of the telomeric G-quadruplex, probably because of the lowered activation energy given that **PDS** participates in the folding of the quadruplex (see the first step of pathway A in Supplementary Fig. S2). Such kinetics information also rules out the scenario that **PDS** binds to a pre-formed G-quadruplex (pathway B in Supplementary Fig. S2), which predicts the ligand has no effect on the folding kinetics of the free G-quadruplex. The fact that k_2 ($0.13 \pm 0.02\ \text{s}^{-1}$) is much slower than k_1 suggests that the rate-limiting step for **PDS** binding is the process in which the loosely associated ligand searches for a tightly bound state (the second step in pathway A in Supplementary Fig. S2). It is noteworthy that with a continuous flow of $0.5\ \mu\text{M}$ **PDS**, the ligand concentration is effectively constant during binding to the single-molecule DNA substrate. Therefore, the first step in pathway A follows a pseudo first-order reaction kinetics, which validates our kinetic analysis discussed here.

To reveal the time to reach equilibrium between free quadruplexes and ligand-bound structures in the presence of **PDS**, we plotted the ratio of these two species with time. Figure 3d shows that equilibrium is reached within 30 s, with a time constant of $\tau = 10$ s (see Supplementary Information for calculation). In the following experiments, a 30 s incubation time was maintained to ensure equilibrated binding between the ligands and the G-quadruplex.

Determination of dissociation constant without varying the concentration of either ligand or receptor

Our finding that **PDS** substantially increases the mechanical stability of telomeric G-quadruplex (Fig. 2c) correlates well with the previous finding that the melting temperature is increased by ~35 K for the telomeric G-quadruplex in the presence of **PDS**²⁶. To quantify the stabilization effect of the ligand, we evaluated the unfolding force histogram of G-quadruplex in the presence of 20 nM to 2 μ M **PDS** (Fig. 4a-e). By integrating the total rupture events of each population in the rupture force histogram using a randomized deconvolution method (see Supplementary Information for details), we analysed the ratio of free G-quadruplex versus ligand-bound structure at each concentration. As shown in Fig. 4f, the ratio clearly follows a Langmuir isotherm³⁷ for a single binding site (see Supplementary Information for the equation). With this model, the dissociation constant K_d was found to be 490 ± 80 nM. This value is similar to the IC_{50} value of 200 nM previously evaluated for a competition assay between **PDS** and **POT1**²⁶.

We further evaluated the stabilization effect of the **PDS** from a free energy perspective. After deconvoluting the rupture events associated with the ligand-bound or free G-quadruplexes described above, we combined the rupture events for each species at different concentrations and calculated the change in free energy (ΔG_{unfold}) using Jarzynski's theorem for non-equilibrium systems (see Methods)^{30,31}. The values of ΔG_{unfold} for the ligand-bound and free quadruplexes were 16.6 ± 0.5 and 8.0 ± 0.5 kcal mol⁻¹, respectively. This result demonstrates that binding of the G-quadruplex with ligand increases the thermodynamic stability of the complex. As a state function, the change in free energy of binding, $\Delta G_{\text{binding}}$, could be calculated using a process analogous to the Hess cycle (Fig. 5a):

$$\Delta G_{\text{binding}} = \Delta G_{Q-\text{unfold}} - \Delta G_{Q-L-\text{unfold}} \quad (1)$$

Here, $\Delta G_{Q-\text{unfold}}$ is the free energy change associated with unfolding of a free quadruplex and $\Delta G_{Q-L-\text{unfold}}$ is associated with the unfolding of a ligand-bound quadruplex. The dissociation constant was then calculated using

$$\Delta G_{\text{binding}} = -RT \ln K_d \quad (2)$$

where R is the gas constant, T is absolute temperature and $\Delta G_{\text{binding}} = -8.6$ kcal mol⁻¹ is calculated from equation (1). This calculation provided the dissociation constant of 460 ± 40 nM, which was identical within experimental error with the value obtained from the Langmuir isotherm. Compared to current dissociation constant assays in which experiments must be performed with different ligand or receptor concentrations, this new method dramatically simplifies the process by requiring only one concentration of ligand or receptor. This is especially beneficial for screening purposes in which large quantity of ligands is often not available.

We applied this new method to obtain the dissociation constant of a related **PDS** analogue, **RR110** (Fig. 1b). In our estimation, this ligand displays >20-fold weaker binding than **PDS**. A practical limitation of the weak binding is that it prevents the accurate determination of the dissociation constant due to the solubility problem for this small molecule. We performed mechanical unfolding of the Tel-4G in 1 μ M **RR110**. The rupture force histogram in Fig. 5b shows a predominant peak at 20 ± 1 pN with a shoulder at 34 ± 1 pN. The 20 pN species was assigned as free G-quadruplex based on the identical rupture force between the two. The population with 34 pN was assigned to the ligand-bound quadruplex. To confirm this assignment, we performed the same experiment in the presence of 10 μ M **RR110**. The unfolding force histogram (Fig. 5c) now shows an increased 34 pN population.

This result is expected as the population of the ligand-bound quadruplex increases with increasing ligand concentrations. After obtaining $\Delta G_{Q\text{-unfold}}$ for the free quadruplex ($8.7 \pm 0.2 \text{ kcal mol}^{-1}$) and $\Delta G_{Q\text{-L-unfold}}$ for the ligand-bound structure ($14.6 \pm 0.3 \text{ kcal mol}^{-1}$), we obtained a $\Delta G_{\text{binding}}$ of $-5.9 \pm 0.4 \text{ kcal mol}^{-1}$ using equation (1) derived from the Hess-like cycle. From equation (2), this yielded a dissociation constant of $42 \pm 3 \text{ }\mu\text{M}$ for **RR110**, which was much lower than that for **PDS** determined above. It is noteworthy that the mechanical stability of the **RR110**-bound quadruplex (34 pN) is also significantly lower than that of the **PDS**-bound quadruplex (41 pN).

Conclusions

Using a force-based single-molecule platform, we have demonstrated that pyridostatin binds to human telomeric G-quadruplex and promotes the folding of this G-quadruplex. By a process analogous to the Hess cycle, we have obtained the dissociation constants of pyridostatin and **RR110**, neither of which has been accurately determined in ensemble assays due to solubility issues. This method simplifies the dissociation constant assay without the requirement for ligand or receptor titration and offers a general platform that can be applied to other biologically relevant ligand–receptor systems. Specifically, this study highlights that G-quadruplexes are important dynamic structures involved in the mechanism of telomere elongation through the action of the enzyme complex telomerase. Furthermore, we believe that the mechanical information acquired by this system could provide novel perspectives for drug testing and design in the future. In addition, the extra forces required to unfold ligand-bound structures shows that, in principle, the small-molecule pyridostatin could well interfere with RNA and DNA polymerases during the processes of transcription and replication *in vivo*.

Methods

Synthesis and characterization methods of **PDS**^{26,27} and **RR110**²⁸ have already been reported. The DNA construct for single-molecule mechanical unfolding studies was prepared using a similar protocol described elsewhere^{15,36}. Briefly, the Tel-4G sequence was sandwiched between two dsDNA spacers (2,028 bp and 2,690 bp). The 2,028 bp spacer was labelled at one end with biotin using a 5′-end biotinylated primer, 5′-GCA TTA GGA AGC AGC CCA GTA GTA GG (IDT, Coralville) during the PCR amplification of a pBR322 plasmid (New England Biolab, NEB). The PCR product was digested by the XbaI restriction enzyme (NEB). This biotinylated handle was then ligated with a dsDNA-ssDNA hybrid, 5′-CTA GAC GGT GTG AAA TAC CGC ACAGAT GCG **X** GCC AGC AAG ACG TAG CCC AGC GCG TC, where **X** represents the ssDNA section. This hybrid consists of an XbaI overhang at the 5′-end and an EagI overhang at the 3′-end. The ssDNA section (**X**) is either the Tel-4G sequence, 5′-TTA (GGG TTA)₄ or a control sequence, 5′-TGT (CCC CAC ACC CCT GT)₂, or contains no nucleotides. The second spacer (2,690 bp) was constructed by the SacI (NEB) and EagI (NEB) digestions of a pEGFP plasmid (Clontech) and subsequently labelled with digoxigenin (Dig) at the SacI end using 18 μM Dig-dUTP (Roche) and terminal transferase (Fermentas). It was finally ligated with the biotinylated DNA spacer prepared above through the EagI site using T4 DNA ligase (NEB). The final construct was ethanol precipitated, dissolved in water and stored at -80°C .

A detailed description of the laser tweezers instrument has been reported elsewhere³⁸. In brief, *P*- and *S*-polarized laser beams from the same source (a diode pumped solid-state (DPSS) laser with a 1,064 nm wavelength, 4 W, continuous-wave (c.w.) mode, BL-106C, Spectra-physics) constituted two traps. The position of each trap was detected separately using two position-sensitive photodetectors (PSD) (DL100, Pacific Silicon Sensor). The *S*-polarized light was controlled by a steerable mirror (Nano-MTA, Mad City Laboratories) at

the plane conjugate to the back focal plane of a focusing objective (Nikon CFI-Plan-Apochromat $\times 60$, NA = 1.2, water immersion, working distance $\sim 320 \mu\text{m}$). During measurement of the folding kinetics, 'jump up' or 'jump down' of the force was achieved by the sudden movement of the steerable mirror using the LabView 8 program (National Instruments Corporation) within a time resolution of 100 ms.

To start the single-molecule unfolding experiments, the DNA construct was first immobilized on the surface of anti-Dig antibody-coated polystyrene beads (diameter, $2.10 \mu\text{m}$, Spherotech) via the Dig/anti-Dig linkage in 10 mM Tris buffer (pH 7.4 with 100 mM KCl) with a specific ligand concentration. Beads coated with streptavidin (diameter, $0.97 \mu\text{m}$; Bangs Laboratory) were also dispersed in the same buffer and injected into the reaction chamber. After separate trapping of these two types of beads by the two laser traps, the DNA construct was tethered between the two beads. Once the DNA was tethered, the bead, controlled by the steerable mirror, was moved away at a constant load speed of $\sim 5.5 \text{ pN s}^{-1}$. This rate allowed us to collect significant amount of data in a reasonable timescale at a condition close to the unfolding equilibrium of a DNA secondary structure. This process allowed a gradual increase in the tension of the DNA construct, which unfolded the G-quadruplex in the DNA molecule. An unfolding event was identified as a sudden change in the end-to-end distance during the process. The unfolding force was directly measured from the F - X curves, and the change in contour length (ΔL) was calculated from the two data points flanking the rupture events using an extensible worm-like chain (WLC) model³⁹,

$$\frac{x}{L} = 1 - \frac{1}{2} \left(\frac{k_B T}{FP} \right)^{\frac{1}{2}} + \frac{F}{S}$$

where x is the end-to-end distance, k_B is the Boltzmann constant, T is absolute temperature, P is the persistent length (51.95 nm)³⁹, F is the force, and S is the elastic stretch modulus ($1,226 \text{ pN}$)³⁹. The stretching and relaxing force-extension curves were used to calculate the change in free energy (ΔG_{unfold}) associated with the unfolding of the G-quadruplex according to Jarzynski's equation^{30,31} for non-equilibrium systems,

$$\Delta G_{\text{unfold}} = -k_B T \ln \sum_{i=1}^N \frac{1}{N} \exp \left(-\frac{W_i}{k_B T} \right)$$

Here, N is the number of pulling experiments and W is the non-equilibrium work done during unfolding of the folded structure, which is equal to the hysteresis area between the stretching and relaxing force-extension curves⁴⁰. The areas under the curve (AUC) of the stretching and relaxing F - X curves (for example, the solid and dotted curves in Fig. 2a, respectively) were measured separately. The difference in AUC between these two curves provided the hysteresis area.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematic of the experimental setup (not to scale)

a,b, Ligands **PDS** (**a**) and **RR110** (**b**). **c**, The force-based single-molecule platform for investigation of the ligand-quadruplex interaction. Human telomeric fragment with a G-quadruplex forming sequence (Tel-4G) is sandwiched between two dsDNA spacers. The overall construct is tethered between two optically trapped beads via a digoxigenin (Dig)-antidigoxigenin (anti-Dig) linkage at one end and a biotin-streptavidin linkage at the other end. The mechanical stability of the G-quadruplex structure in the presence or absence of a ligand is then evaluated by mechanical unfolding of the quadruplex structure.

Figure 2. Mechanical unfolding of Tel-4G with and without PDS

a, Typical F - X curves showing unfolding events (marked by arrows) during stretching for ligand-bound (grey) and ligand-free (black) G-quadruplex structures. The dotted trace represents the relaxing F - X curve for both cases. **b,c**, Histograms of unfolding forces for the structure in Tel-4G in the absence (b) and presence (c) of 0.5 μ M PDS. Black curves in **b** and **c** represent Gaussian fittings. The 21 ± 1 pN population in **b** or **c** represents free G-quadruplex, whereas the 41 ± 2 pN species represents G-quadruplex tightly bound with PDS.

Figure 3. Folding kinetics of quadruplex structure in the presence or absence of PDS

a, Profile of force over time for the force jump method (see text for details). **b**, Histograms of unfolding force for the telomeric G-quadruplex structure with incubation time in a solution with $0.5 \mu\text{M}$ PDS, indicating that the fraction of PDS-bound population increases over time. Black curves represent Gaussian fittings. Vertical dotted lines represent the range (average $\pm \sigma$) of the peak centre for the left population (ligand-free G-quadruplex). **c**, Refolding probability of all quadruplex species versus incubation time in a solution with (filled circles with dotted curve) and without (open circles with a solid curve) $0.5 \mu\text{M}$ PDS. Open diamonds with a dotted curve (ligand-free) and filled diamonds with a solid curve (ligand-bound) are the refolding probabilities for free and ligand-bound G-quadruplexes in a solution containing PDS, respectively. The sum of these two curves is equivalent to that represented by the filled circles with a dotted curve ('in ligand'). Curves represent single exponential fittings, which yield reaction rate constants of $0.20 \pm 0.01 \text{ s}^{-1}$, $0.11 \pm 0.01 \text{ s}^{-1}$, $0.48 \pm 0.06 \text{ s}^{-1}$ and $0.13 \pm 0.02 \text{ s}^{-1}$ (from top to bottom), respectively. The black star indicates little formation of the 41 pN population for 0 s incubation in the solution with PDS, which suggests the presence of free or loosely bound G-quadruplexes before being tightly bound with PDS (see text for details). **d**, Ratio of ligand-bound quadruplex to free quadruplex versus incubation time indicates that equilibrium has been reached at ~ 30 s. The solid curve represents single exponential fitting (see Supplementary Information). Error bars in **c** and **d** are standard deviations from three independent measurements.

Figure 4. Measurement of the dissociation constant between PDS and the telomeric G-quadruplex

a-e, Histograms of unfolding force for the G-quadruplex in various concentrations of **PDS**. The left Gaussian population in each histogram corresponds to the free quadruplex structure, and the right population represents the ligand-bound quadruplex species. **f**, Fraction of G-quadruplex bound with **PDS** versus concentration of the ligand. The solid curve is a fit according to the single site binding model (see Supplementary Information), which provides a dissociation constant of 490 ± 80 nM for **PDS** binding. Error bars are standard deviations from three independent measurements.

Figure 5. Determination of dissociation constant from a single ligand concentration
a, Schematic representation of the process analogous to the Hess cycle for calculation of $\Delta G_{\text{binding}}$. **b,c**, Histograms of the unfolding force for the G-quadruplex structure with 1 μM (**b**) and 10 μM (**c**) **RR110**. Each histogram is fit with a two-population Gaussian curve (grey). The 20 $\pm$ 1 pN population fit with a solid black Gaussian curve represents the ligand-free G-quadruplex, and the 34 $\pm$ 1 pN population fit with a dotted black Gaussian curve depicts the G-quadruplex bound with a weaker ligand, **RR110**, which has a dissociation constant of 42 $\pm$ 3 μM .