Supporting Information

G-quadruplex DNA Bound by a Synthetic Ligand is Highly Dynamic

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Materials and Methods

I. Single Molecule Total Internal Reflection Microscopy Setup

Prism type total internal reflection microscopy was performed with a 532 nm Nd:YAG laser (Crystal Laser, GCL-025-M) and the fluorescence emission from both TMR and Cy5 was collected with a back-illuminated electron-multiplying charge coupled device (CCD) camera (iXON, Andor Technology, South Windsor, CT). Data acquisition and analysis was done with in-house software, available on request. (Further details are provided in Reference 8.)

II. Materials used for experiments

The DNA sequences used in the experiments were oligonucleotides custom-synthesized by Integrated DNA Technologies, (Coralville, Iowa):

5’Cy5-(GGG TTA GGG TTA GGG TTA GGG) AGA GGT AAA AGG ATA ATG GCC ACG GTG CG-3’
Biotin
5’- CGC ACC GTG GCC ATT ATC CTT (amino-C6 dT)TA CCT CT-3’

The amino-modified C6 dT was labeled with tetramethylrhodamine and annealed with the telomere-repeat containing strand, resulting in the construct shown in Scheme 1b: a 21-base long four-repeat telomeric sequence attached to a 29-basepair partial duplex stem. The annealed DNA was stored in 20 mM Tris 50 mM NaCl pH 8.0 (T50). For immobilizing the DNA, each channel was first treated with 50 µL 1 mg/ml Bovine Serum Albumin-Biotin (Sigma-Aldrich) for 5 minutes, and then incubated for 1
minute with 50 µL 0.2 mg/ml Neutravidin (Pierce). DNA was diluted in T50 to 50 – 200 pM final concentration and 50 µL was added to the channel and allowed to bind to immobilized Neutravidin for 5 minutes. Free DNA was removed by flushing the channel with 100 µL T50. This results in approximately 250 molecules in each 100 µm by 50 µm imaging area of the slide surface. We then incubated the molecules in 50 µL of 20 mM Tris pH 8.0 (T0) buffer for five minutes to allow complete unfolding of the quadruplex. This was confirmed by observing h-telo molecules in the Imaging Buffer (10 mM Tris pH 8.0, 0.8% (wt/wt) dextrose monohydrate, 0.1 mg/ml glucose oxidase, 0.02 mg/ml Catalase (Sigma) and 1 mM Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), IB) to be completely unfolded.

To change the salt concentration of the h-telo sample, a 100 mM and 1 M KCl stock was diluted by direct addition into the imaging buffer. The trimeric quinolinecarboxamide macrocycle I ligand was stored at 4°C and added directly to the Imaging Buffer for h-telo/ligand experiments.

III. FRET data analysis methodology

The data obtained from a single molecule FRET experiment is a collection of donor and acceptor emission intensities as a function of time for each immobilized h-telo molecule. FRET for each molecule is defined as: 

\[ \text{FRET} = \frac{I_{\text{Acceptor}}}{I_{\text{Donor}} + I_{\text{Acceptor}}} \]

Approximately 18% of the donor emission leaks into the acceptor detection channel and this was corrected in all of the FRET data. All the molecules in each FRET time trace that contained an active donor and acceptor fluorophore and emitted at a combined intensity consistent with a single molecule (multiple molecules within a diffraction limited spot are correspondingly brighter than the average intensity from single molecules) contributed to the data used in plotting the FRET histograms. For the data obtained from individual traces, molecules had to satisfy an additional criteria of single step photo-bleaching of both the donor and the acceptor fluorophores. These molecules were manually selected.

To calculate the FRET values that were used to create the histograms in Figure 1, we averaged the FRET value displayed by a particular molecule over 20 frames. At the recording time resolution of 100 ms/frame, this corresponds to 0.2 seconds. For the histograms in Figure 1, 20 – 25 0.1 s time resolution movies (containing 200 – 300 h-telo molecules each) were recorded and a frequency histogram of the FRET values was plotted for each buffer condition. The ten minute long traces in Figure 2a were recorded at 0.9 s time resolution and the time spent folded was calculated by manually selecting the regions in each FRET trace that corresponded to a folded state. For Figure 3a, time spent in F2 was obtained by manually selecting the high FRET regions in the single molecule traces from individual
h-telo molecules. The survival probability for a molecule in F2 remaining in F2 after time $t$ was fit to:

$$A_1 e^{-t/t_1} + A_2 e^{-t/t_2}$$

**IV. Potassium stabilization of h-telo is reversible**

Immobilized h-telo was folded with 100 mM K$^+$ and we obtained FRET histogram (Figure S1.a) showing almost no unfolded h-telo ($E = 0.4$). We then flowed in 100µL of Imaging Buffer containing no K$^+$. After 1 minute, we started data acquisition to obtain the second histogram (Figure S1.b). Clearly, 100 mM K$^+$ stabilization of h-telo can be quickly and completely reversed by flowing in a buffer without K$^+$.  

![Figure S1 a) h-telo stabilized by 100 mM K$^+$. b) 1 minute after removal of K$^+$.](image)

Figure S1 a) h-telo stabilized by 100 mM K$^+$, b) 1 minute after removal of K$^+$.  


V. Ligand stabilization of h-telo at low concentrations of I is long lasting

Unfolded h-telo molecules were incubated with 1 in the Imaging Buffer. We obtained FRET histograms until no further change in the histograms was observed, and then flushed the channel with 100 µL T0. One hour after removing free 1 from the solution, we added 100 µL of the Imaging Buffer and took single molecule time traces to create the histograms shown in Figure S2. As seen from both sets of histograms, a fraction of h-telo/I remains folded after removal of free 1 from the sample channel.

![Histograms](image)

Figure S2 a) left: h-telo stabilized by 100 nM 1. right: 60 minutes after removal of free 1.

b) left: h-telo stabilized by 10 nM 1. right: 60 minutes after removal of free 1.

A Job analysis (see JACS, 2007, 129, 11890 for details) suggested that 1 can bind to h-telo in a 2:1 stoichiometry. Our FRET data suggests that F2 is formed at a low concentration of the ligand while both F1 and F2 can be observed at 100 nM 1. Increasing ligand concentration to the saturating value of 1 µM 1 results in completely folded h-telo in the F1 state only.
While the binding of ligand 1 to h-telo is very tight, we observed a 50% reduction in the fraction of folded h-telo molecules that were first incubated with 100 nM 1, before free ligand was removed from the solution. After flushing the sample channel with a buffer that did not contain any ligand or K+, we obtained FRET histograms over the period of several hours that showed dissociation of the ligand from h-telo. At the more relevant concentration of 1 µM 1, the three hour observation period was not long enough (fluorophores in solution at room temperature photobleach over time) to observe dissociation. The presence of ligand dissociation implies a reversible, non-covalent binding between h-telo and ligand 1.

![FRET histograms showing dissociation of ligand from h-telo](image)

Figure S3: top: h-telo stabilized by 100 nM 1. bottom: 60 minutes after removal of free 1. The observed dissociation implies a reversible, non-covalent binding of 1 to h-telo.

VI. Mutant control for quadruplex-specific ligand interaction

We designed a h-telo mutant sequence that contained 4 point mutations. This sequence was labeled with the same fluorophores as h-telo and assembled into an identical partial duplex construct. We treated this sample with varying concentrations of the ligand and obtained the data in Figure S4. It appears evident from the histograms that the ligand interacts specifically with the G-quadruplex structure formed by the h-telo sequence and not with either of the fluorescent probes or the partial duplex junction.

h-telo: 5’ Cy5 – GGG TTA GGG TTA GGG TTA GGG

mutant: 5’ Cy5 – GTG TTA GTG TTA GTG TTA GTG
Figure S4: FRET histograms for the ligand interacting with (a) *h-telo* and (b) the mutant sequence. The difference is due to 1 interacting specifically with the G-quadruplex structure formed by *h-telo* and not with the fluorophores or the partial duplex junction.

**VII. FRET time traces showing *h-telo*/1 dynamics in the absence of free 1**

Immobilized *h-telo* was incubated with a given concentration of 1. After reaching equilibrium, free 1 was removed from the solution by flushing the channel with 100 µL of T0 twice. Twenty minutes later, imaging buffer containing no K⁺ or 1 was added and single molecule FRET time traces were obtained, showing the folding-unfolding dynamics of individual *h-telo*/1 complexes in the absence of free 1 in solution.
Figure S5: (h-telo/1) complexes formed by incubating h-telo with 10 nM 1 were imaged 20 minutes after removal of free 1 from the sample. These three complexes fluctuate between U, F1 and F2 states.

Figure S6: (h-telo/1) complexes formed by incubating h-telo with 100 nM 1 were imaged 20 minutes after removal of free 1 from the sample. These three complexes fluctuate between U, F1 and F2 states.