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

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G-Quadruplexes are noncanonical structures formed by certain guanine-rich sequences of DNA.[1] The G-quadruplex formed by the human telomeric DNA sequence has been of particular interest, owing to the importance of telomere maintenance for cellular proliferation.[2] Small molecules that bind and stabilize the G-quadruplex can inhibit cell growth via mechanisms that may involve disruption of the telomere and/or the prevention of telomere extension.[3] Consequently, the G-quadruplex formed by telomeric DNA is under investigation as a potential molecular target for anticancer drugs.[4]

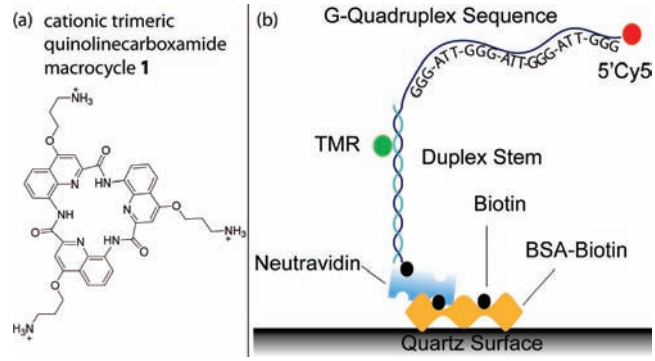
Human telomeric DNA is intrinsically dynamic,[5] and the effect of quadruplex-binding ligands on these dynamics is not known. We studied the effect of quinolinecarboxamide macrocycle **1** (Scheme 1a),[6] a potent quadruplex stabilizing ligand with potential anticancer properties, on the structural dynamics of human telomeric DNA using single molecule fluorescence resonance energy transfer (FRET) spectroscopy.

Single molecule FRET can report on the conformations and dynamics of DNA.[7] A donor (tetramethylrhodamine) and acceptor (Cy5) labeled human telomeric DNA sequence $(GGGTTA)_3$-GGG, *h-telo*, was tethered to a quartz surface via a biotin−Neutravidin interaction and illuminated using total internal reflection excitation (Scheme 1b).[8] FRET, which is defined as the ratio of acceptor emission to the combined donor and acceptor emission, was calculated for each single molecule. This experimental configuration allowed resolution of the intrinsic structural dynamics between the various conformations adopted by *h-telo*.[5,9]

An unfolded *h-telo* molecule results in the greatest distance between the fluorophores and should display a low FRET value while the decreased distance between the fluorophores in a folded G-quadruplex should result in higher FRET. Monovalent cations, especially potassium, are efficient at inducing and stabilizing folded G-quadruplex structures,[10] and consistent with our previous single molecule FRET studies,[5] unfolded *h-telo* (**U**) can be folded into two resolvable conformations, **F1** and **F2** by 2 mM K^+ (FRET efficiency $E = 0.43$, 0.63, and 0.80 respectively, Figure 1a). At 100 mM K^+ concentration, only **F2** is observed (Figure 1b). In contrast, at the saturating concentration of **1** (1 μM) and without K^+, *h-telo* is found primarily in **F1** (Figure 1c). Therefore, **1** drives the human telomeric DNA to a conformation that is not well populated under physiological K^+ concentrations.

The K^+ stabilized *h-telo* can be completely unfolded in less than 1 min by flushing the sample chamber with a buffer without K^+ (data in Supporting Information, SI). In contrast, binding of 1 μM **1** to *h-telo* was essentially irreversible. After incubating *h-telo* with 1 μM **1** and obtaining the FRET histogram (Figure 1c), we removed free **1** by repeated flushing of the sample chamber. We imaged *h-telo* molecules, 4 h after removal of free **1**, in a buffer with no **1** or K^+ and observed no change in the FRET histogram. We then flowed in 100 mM K^+ in an attempt to facilitate the release of **1** and change the *h-telo* conformation to **F2** but did not detect any change (Figure 1d). Though to a lesser extent, the **1** bound to *h-telo* at lower concentrations (10 and 100 nM **1**) was also resistant to immediate removal via buffer exchange (SI).

Scheme 1[a]



[a] (a) Macrocycle **1**. (b) The $(GGGTTA)_3$-GGG oligonucleotide is hybridized to the complementary stem strand and immobilized via a biotin−Neutravidin interaction to a bovine serum albumin-biotin coated quartz surface. TMR (green) and Cy5 (red) are the donor and acceptor fluorophores, respectively.

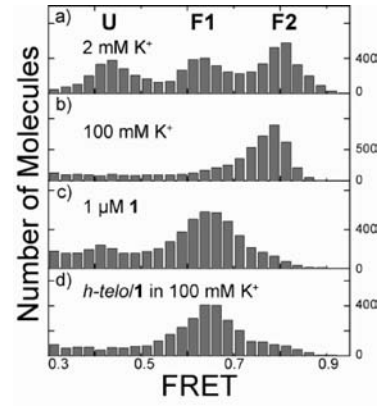


Figure 1. (a) FRET histogram of *h-telo* with 2 mM K^+ showing an unfolded (**U**) and two distinct intramolecular folded states (**F1** and **F2**). (b) *h-telo* with 100 mM K^+, folded into **F2**. (c) *h-telo*/**1** (1 μM **1**) complex, primarily in **F1**. (d) *h-telo*/**1** complex in 100 mM K^+, 4 h after removal of free **1** (1 μM) (all measurements at 25 °C).

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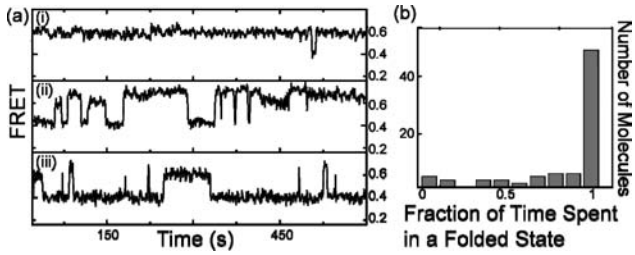

Figure 2. (a) FRET time traces of a *h-telo*/**1** (1 μM **1**) complex (with no free **1** in solution) (i) primarily in **F1**; (ii) transitioning between **U**, **F1**, and **F2**; and (iii) primarily unfolded with transitions to both **F1** and **F2**. (b) A histogram representing the fraction of time spent folded by *h-telo*/**1** complexes.

Given the tight binding of **1** to *h-telo*, we expected each *h-telo*/**1** complex to display a constant FRET value, representing a fixed folded conformation. Instead, we found the *h-telo*/**1** complex (formed by incubating *h-telo* with 1 μM **1**) in a solution with no free **1** to be dynamic (Figure 2a). A histogram of the fraction of time spent folded (Figure 2b, obtained from 10 min time traces of 110 *h-telo*/**1** (1 μM **1**) complexes) showed that over 30% of the complexes spent at least 10% of the time in the unfolded conformation. We observed similar dynamics with 100 and 10 nM **1** as well (SI).

As the concentration of immobilized *h-telo* and bound **1** is in the picomolar range, potential rebinding of dissociated **1** in the absence of free **1** in solution is extremely unlikely, and hence, **1** must remain associated with *h-telo* during the folding and unfolding transitions. That is, *h-telo* can undergo conformational changes as dramatic as unfolding and refolding without complete dissociation of **1**. Although our previous energetics analysis indirectly indicated that a different ligand (hemicyanine-peptide) may allow a partial and transient unraveling of *h-telo*,[11] our result here demonstrates that *h-telo* can be completely unfolded for an extended period of time while still being associated with the ligand.

We previously observed six *h-telo* conformations in the presence of 2 mM K^+: long-lived and short-lived species of **U**, **F1**, and **F2** that differed in their lifetimes by an order of magnitude.[5] To test if the *h-telo*/**1** complex also possessed conformational subspecies, we incubated *h-telo* with 100 nM **1** (*h-telo*/**1** at this concentration of **1** shows both folded states, Figure 3a inset) and washed away the free ligand. The FRET time traces showed that *h-telo*/**1** complexes can access the same molecular conformations as K^+ stabilized *h-telo* — long- and short-lived **U**, **F1**, and **F2** (Figure 3b).

We quantified this by measuring the dwell times of **F2** (from 110 individual complexes over 758 transitions) to obtain the survival probability of a complex in **F2** remaining in **F2** after time *t* (Figure 3a). Fitting the curve with a double exponential decay function gave decay times of 99.5($\pm$1.3) and 8.5($\pm$0.11) s. These decay times differ by a factor of 12 and show that **F2** of the *h-telo*/**1** complex is composed of at least two species with significantly different stabilities. The decay times for **F2** stabilized with 2 mM K^+ differed by a factor of 9 (188($\pm$2) s and 20($\pm$0.1) s),[5] and the comparable ratio of the **F2** decay times for both **1** and K^+ stabilized *h-telo* suggests that K^+ and **1** induce *h-telo* conformational subspecies that are similar in their relative stabilities.

In conclusion, we provide fundamental insights into the dynamic properties of a ligand-stabilized human telomeric DNA G-quadruplex. **1** binds extremely tightly to an unfolded telomeric strand and

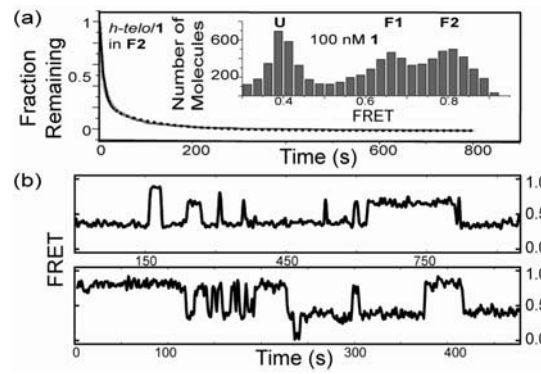

Figure 3. (a) Fraction of *h-telo*/**1** complexes in **F2** remaining in **F2** after time *t*. (Inset) *h-telo* stabilized with 100 nM **1** showing **U**, **F1**, and **F2**. (b) *h-telo*/**1** complexes showing both short (<100 s) and long (>100 s) **U**, **F1**, and **F2** states in the absence of free **1**.

selectively stabilizes the conformation that is not normally favored under physiological conditions. Without involving full dissociation of the ligand, the ligand-quadruplex complex displays conformational diversity and structural dynamics similar to the G-quadruplex stabilized by potassium. Our observation that **1** promotes G-quadruplex folding without preventing the intrinsic intramolecular dynamics of the telomeric DNA may have implications for this synthetic ligand's potential to interfere with telomere function *in vivo*.

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Supporting Information Available: Details of the experimental scheme, FRET data analysis method, **1** and K^+ removal studies, control experiments with a mutant sequence and time traces of *h-telo*/**1** without free **1** in solution. This material is available free of charge via the Internet at http://pubs.acs.org.

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