Effect of loop length variation on quadruplex-Watson Crick duplex competition

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ABSTRACT
The effect of loop length on quadruplex stability has been studied when the G-rich strand is present along with its complementary C-rich strand, thereby resulting in competition between quadruplex and duplex structures. Using model sequences with loop lengths varying from T to T5, we carried out extensive FRET to discover the influence of loop length on the quadruplex-Watson Crick duplex competition. The binding data show an increase in the binding affinity of quadruplexes towards their complementary strands upon increasing the loop length. Our kinetic data reveal that unfolding of the quadruplex in presence of a complementary strand involves a contribution from a predominant slow and a small population of fast opening conformer. The contribution from the fast opening conformer increases upon increasing the loop length leading to faster duplex formation. FCS data show an increase in the interconversion between the quadruplex conformers in presence of the complementary strand, which shifts the equilibrium towards the fast opening conformer with an increase in loop length. The relative free-energy difference (ΔG') between the duplex and quadruplex indicates that an increase in loop length favors duplex formation and out competes the quadruplex.

INTRODUCTION
The fundamental unit of the G-quadruplex (1,2) is a square planar arrangement of guanines, where each guanine accepts and donates two Hoogsteen hydrogen bonds, placing four electronegative carbonyl groups towards the interior of the tetrad. These quartets are then stabilized by monovalent cations (3), which form cation-dipole interactions with the eight guanines, reduce the repulsion of central oxygen atoms, enhance hydrogen bond strength and stabilize quartet stacking. Interest in these structures has increased markedly in the past decade, as the evidence for their possible functional roles in vivo has accumulated. These guanine-rich segments are found in biologically significant regions of the genome such as telomeres (4), immunoglobulin switch regions (5) and promoter regions of eukaryotic cells (6–8). The interconversion between double- or single-stranded DNA and G-quadruplex in cells is dependent upon a number of cellular proteins (9). Some of these proteins include the β-subunit of the Oxytricha telomere-binding protein (10), Saccharomyces cerevisiae Rap1 (11), Sgs1 (12), Hop1 (13), Mre11 (14), Kem1 (15) proteins and human nuclease with G4 selectivity (16). A number of proteins interacting specifically with G-quadruplexes have been implicated in diseases like Bloom’s (17) and Werner’s syndromes (18).

Recent bioinformatics studies have found that sequences which can fold into G- quadruplex structures are widely dispersed in the human genome (19,20). The prevalence of potential quadruplex forming sequences will depend on the guanine stretches and the nature of the loops. Trends in the sequence of the loops derived from a genome-wide survey of potential quadruplex sequences reveal that the loop length and its composition may determine the functionality of the quadruplexes and may modulate their role between genomes. Lately, a highly conserved potential quadruplex sequence upstream of c-kit has been found in human, mouse, rat and chimpanzee (21). G-stretches of potential quadruplex forming sequences in various protooncogenes are conserved but the regions interspersing these G-stretches, which form the loop of the quadruplex are not conserved. However, variation in loop length and composition affects quadruplex topology, stability (22–28) and its molecular recognition. This may modulate the function of the gene that harbors the potential quadruplex motif. In a genomic context, the guanine stretches are present along with their C-rich complementary strand, which generates a competition between a Hoogsteen bonded quadruplex and a Watson–Crick hydrogen bonded duplex. This quadruplex–duplex competition has been studied for

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Table 1. Dual-labeled (5′ fluorescein and 3′ TAMRA) quadruplex forming sequences and their complementary sequences used in the study

| NTG | 5′ F TGGG T GGT TGG TGGT TGGT | 3′ |
|-----|--------------------------------|----|
| G3T | 5′ F TGGG T GGT TTG GGT TGG | 3′ |
| G3T2 | 5′ F TGGG TTT TTG TGG TGGT | 3′ |
| G3T3 | 5′ F TGGG TTG TTG TGG TGGT | 3′ |
| G3T4 | 5′ F TGGG TTT TTG TGG TGGT | 3′ |
| G3T5 | 5′ F TGGG TTGTT TGG TGGT | 3′ |

Different sequences under various experimental conditions (29–41). Using telomeric oligonucleotides d[AGGG(TTA GGG)]$_3$ and d[TCCC(AATCC)]$_3$, it has been shown that pH, temperature and salt concentration, controls the quadruplex-Watson Crick competition (33). Recently, we have reported the role of osmolytes and small molecule on quadruplex-Watson Crick competition (40). We found that these perturbants stabilize quadruplex and shift the equilibrium towards quadruplex formation.

Quadruplex-Watson Crick competition and the influence of the loops on quadruplex stability has been investigated separately, however, the role of loop length in modulating this competition still remains unaddressed. Herein, we address the role of loop length on the competition between quadruplex and duplex DNA. In the present study, we have used intramolecular quadruplex forming sequences, which have similar G-tracts but vary in their thymine loop length (T–T5). We compared the stability of these quadruplexes having different loop lengths with their respective duplexes, estimated the binding affinity and hybridization kinetics of these preformed quadruplexes towards their respective complementary strands, and observed the conformational fluctuations in the quadruplex conformers in the absence and presence of their respective complementary strands.

**MATERIALS AND METHODS**

**Oligonucleotides and nomenclature**

Unlabeled and dual-labeled (5′ Fluorescein and 3′ TAMRA) G-rich oligonucleotides and their respective complementary strand were procured from Sigma-Aldrich, Singapore. All the oligonucleotides were HPLC purified. The oligonucleotides used in this study are listed in Table 1.

The concentration of unlabeled oligonucleotide solutions was determined from the absorbance at 260 nm using a molar extinction coefficient for G-rich and C-rich strands, calculated by extrapolation of tabulated values of the monomers and dimers (42) at 25°C, using procedures reported earlier (43). The concentration of the labeled oligonucleotide was determined by measuring the absorbance of the attached fluorescein moiety at 496 nm using a molar extinction coefficient of 4.1 × 10$^{4}$/M/cm$^{-1}$ (44). All experiments were done in 50 mM MES buffer pH 7.4, 100 mM KCl. CD spectra were recorded in a Jasco spectropolarimeter (model 715, Japan) equipped with a thermoelectrically controlled cell holder and a cuvette with a path length of 1 cm. CD spectra for quadruplexes (5 μM) were recorded between 220 and 325 nm at 25°C in 50 mM MES buffer pH 7.4, 100 mM KCl. Equimolar concentration of quadruplexes (5 μM) and their respective complementary strands were incubated for 2 h at 25°C and CD spectra were recorded between 220 and 325 nm at 25°C in 50 mM MES buffer pH 7.4, 100 mM KCl.

**Steady-state experiments**

Fluorescence experiments were performed using Fluoromax 4 (spex) spectrofluorimeter and FLUostar OPTIMA from BMG labtech (Germany). The excitation wavelength was set at 480 nm and the emission spectra were recorded from 500 to 700 nm. We collected temperature-dependent fluorescence cooling and heating curves for the quadruplexes alone and for equimolar mixture of each quadruplex and with its complementary strand at the rate of 0.2°C/min. These curves showed no hysteresis suggesting that the process is in thermodynamic equilibrium (SI). For clarity, we have only presented the cooling curves in the main text. Normalized donor (fluorescein) emission ($I_{520}$ = $F_b/F_0$) at 520 nm was plotted as a function of temperature, where $F_b$ is the fluorescence at any temperature and $F_0$ is the fluorescence at 95°C. The van’t Hoff analysis was performed using the cooling profile of quadruplexes using a two state model for unstructured–structured transition to obtain the thermodynamic parameters for quadruplex formation (45).

A FLUostar OPTIMA fluorescence plate reader was used to determine the binding affinity of each G-quadruplex to its complementary strand. The plate reader provides the advantage of working with many samples at very dilute concentrations and on systems that suffer from thermodynamic and kinetic inertia, and require prolonged incubation. The experiments were done in 384-well black plates, using excitation (480 nm) and emission (520 nm) filters for fluorescein. The wells were loaded with the solution of fixed concentration of preformed quadruplex (12 nM) and increasing concentrations of complementary strand (0–300 nM). Sample mixtures were incubated for a period of 24 h at 25°C, to ensure attainment of equilibrium and the plate was read at 520 nm. For analysis of the data, the observed fluorescence intensity was considered as the sum of the weighted contributions from a folded G-quadruplex strand and an extended G-strand:

$$F = (1 - \alpha_b)F_0 + \alpha_bF_b$$

where $F$ is the observed fluorescence intensity at each titrant concentration, $F_0$ and $F_b$ are the respective fluorescence intensities of initial and final states of titration, $\alpha_b$ is the mole fractions of quadruplex in duplex form. Assuming 1:1 stoichiometry for the interaction in case of complementary strand binding, it can be shown that:

$$[Q]_b \frac{\alpha_b^2}{1 + K_A} = [Q]_b[Q]_0 + [C]_0 + 1/K_A \alpha_b + [C]_0 = 0$$
where $K_A$ is the association constant, $[Q]_0$ is the total G-strand concentration and $[C]_0$ is the added complementary strand concentration.

From Equations (1) and (2), it can be shown that:

$$
\Delta F = \left( \frac{\Delta F_{\text{max}}}{2Q_1} \right) \left\{ [Q]_0 + [C]_0 + 1/K_A \right\} - \sqrt{\left\{ [Q]_0 + [C]_0 + 1/K_A \right\}^2 - 4[Q]_0[C]_0} 
$$

where $\Delta F = F - F_0$ and $\Delta F_{\text{max}} = F_{\text{max}} - F_0$.

**Kinetic experiments**

The kinetic experiments were performed in a Fluoromax 4 (spx) spectrofluorimeter. The opening up of the quadruplex (30 nM) upon addition of an equivalent concentration of the complementary strand was monitored as the increase in the fluorescence intensity at 520 nm as a function of time. The data obtained for G3T were fit by a single exponential Equation (4a), whereas the kinetics data for other quadruplexes (G3T2–G3T5) required a double exponential Equation (4b) with good residuals (SI). The equation used was:

$$
\Delta F = A_1 e^{-t/\Gamma_1} + A_2 e^{-t/\Gamma_2} \tag{4a}
$$

$$
\Delta F = A_1 e^{-t/\Gamma_1} + A_2 e^{-t/\Gamma_2} \tag{4b}
$$

where $\Gamma_1$ and $\Gamma_2$ are the time constants of the kinetics and $A_1$ and $A_2$ are their respective weighted amplitudes.

**Fluorescence correlation spectroscopy**

Fluorescence correlation spectroscopy (FCS) experiments were performed with a home-built spectrometer using single-photon excitation as described previously (46). Briefly, the experimental setup consists of a high-numerical aperture objective lens (60 x, 1.2 NA, water immersion, Nikon, Melville, NY, USA), which focuses a continuous wave blue laser (Argon ion Laser 1.2 mW at 488 nm from LG Laser GmbH, Germany) into the sample. The laser power at the objective back aperture is kept typically <100 µW. Fluorescence is collected through the microscope objective separated from the excitation light by a dichroic mirror (DC 514, Chroma Inc., Brattleboro, VT, USA) and an emission filter (S25DF30 DTM for the fluorescein and S85DF40 for Tamra, respectively Chroma Inc.), and focused with an achromatic lens ($F = 150$ mm, Newport, Irvine, CA, USA) onto a multimode fiber (25 µm core diameter, Newport). The other end of the fiber is connected to a fiber-coupled APD detector (SPCM-AQR-150, Perkin-Elmer, Fremont, CA, USA). The detector signal is then processed with an autocorrelator card (ALV5000E, ALV Laser VmbH, Langen, Germany). All FCS experiments were performed in 50 mM MES buffer pH 7.4, 100 mM KCl at 25°C at 100 mM concentration in presence and absence of equimolar concentration of complementary strands after 24 h of incubation. Autocorrelation profiles obtained for quadruplex for G3T fits to a single conformer model as shown in Equation (5), whereas quadruplexes G3T2–G3T5 fits to two conformer model as shown in Equation (6) (47,48). The autocorrelation function obtained for quadruplex G3T with its complementary strand fits to Equation (7), and quadruplexes (G3T2–G3T5) in presence of complementary strand fits to Equation (8).

$$
G(\tau) = G_0 \left( \frac{1}{1 + (\tau/\tau_D)} \right) \left( \frac{1}{1 + a^2(\tau/\tau_D)} \right)^{1/2} \tag{5}
$$

$$
G(\tau) = \frac{1 - F + F\exp(\tau/\tau_R)}{N(1 - F)} \left( \frac{1}{1 + (\tau/\tau_D)} \right) \left( \frac{1}{1 + a^2(\tau/\tau_D)} \right)^{1/2} \tag{6}
$$

$$
G(\tau) = G_1 \left( \frac{1}{1 + (\tau/\tau_{D1})} \right) \left( \frac{1}{1 + a^2(\tau/\tau_{D1})} \right)^{1/2} + G_2 \left( \frac{1}{1 + (\tau/\tau_{D2})} \right) \left( \frac{1}{1 + a^2(\tau/\tau_{D2})} \right)^{1/2} \tag{7}
$$

$$
G(\tau) = \left[ \frac{1-F+F\exp(\tau/\tau_R)}{N(1-F)} \right] G_1 \left( \frac{1}{1+(\tau/\tau_{D1})} \right) \left( \frac{1}{1+a^2(\tau/\tau_{D1})} \right)^{1/2} + G_2 \left( \frac{1}{1+(\tau/\tau_{D2})} \right) \left( \frac{1}{1+a^2(\tau/\tau_{D2})} \right)^{1/2} \tag{8}
$$

where $F$ is the dark fraction, $N$ is the number of particles, $\tau_R$ is the time scale of internal conformational fluctuation, $\tau_D$ and $\tau_{D1}$ is the diffusion time of quadruplex, $\tau_{D2}$ is the diffusion time of duplex and $a$ is the ratio of radial to axial distance of the probe volume. $G_1$ and $G_2$ are the fraction of population weighted by the square of the brightness.

**RESULTS**

CD spectra provide valuable information about the structural transitions in nucleic acids. We therefore recorded the spectra of quadruplexes with similar G stretches but varied loop length (T–T5) in the absence and presence of their respective complementary strands. The G3T quadruplex has a parallel conformation with characteristic positive and negative peaks at 262 and 240 nm, respectively (Figure 1a). In case of G3T2 and G3T3, there was a positive peak at 262 nm, a small positive peak at 292 nm and a negative peak at 237 nm, suggesting the presence of a predominant antiparallel quadruplex and a small fraction of antiparallel population (Figure 1b and c). Further increase in the loop length for G3T4 and G3T5, resulted in a positive peak at 292 nm, a relatively small positive peak at 260 nm, and a negative peak at 238 nm, indicating the presence of a predominant antiparallel quadruplex with a small parallel population (Figure 1d and e). The addition of an equimolar concentration of the complementary strand to the preformed G3T quadruplex,
resulted in only a moderate shift in the positive and negative peaks to 267 and 245 nm, respectively, suggesting minor duplex formation. Similar additions of equimolar concentrations of respective complementary strands to preformed G3T2 and G3T3 quadruplexes led to the substantial loss of the small band at 292 nm, and a prominent shift in positive and negative peaks. The broad positive peak obtained at 277 nm and the negative peak at 246 nm suggests duplex formation with some contribution from a residual parallel quadruplex population (Figure 1b and c). Addition of equimolar complementary strands to preformed G3T4 and G3T5 quadruplexes resulted in the prominent shift in the positive peak from 292 to 280 nm and a shift of the negative peak from 238 to 246 nm, which indicates duplex formation. However, a broad shoulder base around 290 nm still exists, suggesting the presence of certain fraction of quadruplex in the solution (Figure 1d and e).

Next, we assessed the influence of loop length on the quadruplex to duplex transition by determining the binding affinity of quadruplexes towards their respective complementary strand through FRET. When a quadruplex opens in presence of its complementary strand, the distance between the donor and acceptor increases. This results in reduced energy transfer from the donor to the acceptor and an increase in donor signal. We monitored the fluorescence of fluorescein with increasing complementary strand concentration for all the sequences used in this study. Figure 2 represents the relative fluorescence intensity change ($\Delta F$) as function of the complementary strand concentration. The difference in profile of relative fluorescence intensity change ($\Delta F$) obtained for quadruplexes with different loop length reflects the amount of duplex formed upon hybridization of quadruplex to its complementary strand. We observed that the quadruplex formed by G3T required approximately 25 times excess of complementary strand for maximum duplex formation (Inset Figure 2), whereas other quadruplexes G3T2–G3T5 required approximately 6 times excess of complementary strand for maximum duplex formation. The plot represented in Figure 2 was used to calculate the binding affinity of quadruplexes to their complementary strands using Equation (3). The binding affinity towards the complementary strands at 25°C is shown in Table 2. Next, we followed the kinetics of duplex formation, which involves the unfolding of quadruplex and hybridization to its complementary strand at 25°C in 100 mM KCl. Data analysis shows that the kinetics of duplex formation for G3T follows a single exponential with mean time constant of 9896 s. However, for quadruplexes G3T2–G3T5, best fitting results required a double exponential kinetics model, which reveals the presence of at least two quadruplex conformers having different duplex formation kinetics (SI). Using Equation (4b), we obtained the relative proportions of quadruplex conformers and their respective time constant values (Table 2). Data analysis shows that duplex formation involves a contribution from a predominant slow opening conformer and small population of fast...
The kinetics of duplex formation for G3T quadruplex fits to single exponential kinetics, whereas data for quadruplexes G3T2–G3T5 fits to biexponential kinetics. 

| Quadruplex | A₁ | A₂ | Γ₁ (s) | Γ₂ (s) | Kᵢ (M⁻¹) | Qᵢ (nM) | Dᵣ (nM) |
|------------|----|----|--------|--------|-----------|---------|---------|
| G3T        | 0.94 | –  | 9896 ± 100 | –      | 1.0 × 10⁷ ± 0.5 | 24.2    | 5.8     |
| G3T2       | 0.88 | 0.12   | 9614 ± 115 | 1415 ± 135 | 3.4 × 10⁷ ± 0.5 | 18.6    | 11.4    |
| G3T3       | 0.70 | 0.30   | 6510 ± 125 | 1080 ± 98  | 9.6 × 10⁷ ± 0.7 | 13.2    | 16.8    |
| G3T4       | 0.72 | 0.28   | 6120 ± 108 | 747 ± 50   | 1.3 × 10⁸ ± 0.2 | 11.7    | 18.3    |
| G3T5       | 0.66 | 0.34   | 2920 ± 150 | 364 ± 65   | 1.7 × 10⁸ ± 0.3 | 10.7    | 19.3    |

The amount of duplex at equilibrium, A is the equilibrium constant for duplex formation. A₁ and A₂ are weighted fractions of quadruplex population hybridizing to complementary strand with their respective Γ₁ and Γ₂ time constant values. Kᵢ is the equilibrium constant for duplex formation. Qᵢ and Dᵣ is free quadruplex and duplex concentration, when equimolar concentration of quadruplex and complementary strand (30 nM) are mixed together in 50 mM MES buffer pH 7.4, 100 mM KCl. The amount of duplex at equilibrium, Dᵣ, was calculated from the binding affinity towards the complementary strand obtained from different experimental conditions, using the equation Kᵢ = Dᵣ(Q₀ – Dᵣ) × (C₀ – Dᵣ), where Q₀ and C₀ (Q₀ = C₀) are the initial quadruplex and complementary strand concentration.

The kinetics of quadruplex formation for G3T quadruplex fits to single exponential kinetics, whereas data for quadruplexes G3T2–G3T5 fits to biexponential kinetics. A₁ and A₂ are weighted fractions of quadruplex population hybridizing to complementary strand with their respective Γ₁ and Γ₂ time constant values. Kᵢ is the equilibrium constant for duplex formation. Qᵢ and Dᵣ is free quadruplex and duplex concentration, when equimolar concentration of quadruplex and complementary strand (30 nM) are mixed together in 50 mM MES buffer pH 7.4, 100 mM KCl. The amount of duplex at equilibrium, Dᵣ, was calculated from the binding affinity towards the complementary strand obtained from different experimental conditions, using the equation Kᵢ = Dᵣ(Q₀ – Dᵣ) × (C₀ – Dᵣ), where Q₀ and C₀ (Q₀ = C₀) are the initial quadruplex and complementary strand concentration.

We next investigated the dynamic behavior and conformational heterogeneity in a quadruplex–duplex population using FCS (46). This technique allows analysis of spontaneous fluctuations in the fluorescence signal arising in a microscopic volume of fluorescent sample. These fluctuations arise due to hydrodynamic processes like diffusion and biophysical processes like conformational fluctuations (49–55). Therefore, in principle this technique can be employed to understand the influence of loop length on dynamic fluctuations in quadruplex structure during the quadruplex-Watson Crick duplex transition. We performed FCS measurements for quadruplexes with similar G-stretches with varying loop length (T–T5). The autocorrelation function for G3T fits to a single diffusing conformer Equation (5) with diffusion time (τᵣ) value of 0.095 ms. Interestingly, the autocorrelation function for quadruplexes (G3T2–G3T5) did not fit to a single diffusion equation, suggesting the existence of more than one conformer undergoing conformational transformations. These conformational motions between interconverting conformers may not substantially affect the overall hydrodynamic radius, but demonstrate variation in FRET efficiency due to difference in donor and acceptor distance. Therefore, we used Equation (6), which allows estimation of reaction time (τᵣ) and diffusion time (τᵣD), and fits an autocorrelation function with reasonable residuals, as shown in Figure 3a–e. The τᵣ in Equation (6) corresponds to the time scale for conformational fluctuations in the quadruplex and τᵣD corresponds to the diffusion time of the quadruplex through a small observation area. The analysis of FCS data shows an increase in the diffusion time (τᵣD) from 0.133 to 0.164 ms when the loop length is increased from T2 to T5 with a corresponding increase in the conformational fluctuation (τᵣ) from 0.062 to 0.093 ms (Table 3). The τᵣ and τᵣD values obtained from Equation (6) were derived with the assumption...
that $R \ll D$. But this is not observed here, as $R$ and $D$ values show a difference of ~2-fold. We accept that if $D$ and $R$ are close, then the diffusion and the reaction kinetic region in the autocorrelation function are not easily separable. However, for the case when the diffusion constants of the two species are nearly equal, the separation remains a reasonable approximation, as pointed out by Torres and Levitus (54). Further, FCS experiments were performed to investigate the influence of complementary strand on the interconversion amongst the quadruplex conformers while driving duplex formation. An equimolar solution of dual-labeled quadruplex and its complementary strand would have two fluorescent populations corresponding to the quadruplex and duplex species, which are expected to have different FRET efficiencies and diffusion time (Figure 3f–j). G3T adopts a single quadruplex conformation and its autocorrelation function in presence of complementary strand fits to Equation (7). Here, we assume that the duplex has only one conformation and the estimated $D_{D1}$ and $R_D$ value correspond to the diffusion time of the quadruplex and duplex structure, respectively. We observed that the $D_{D1}$ value was 0.088 ms and $R_D$ value was 0.198 ms. However, the autocorrelation function obtained for G3T2–G3T5 quadruplexes in presence of their respective complementary strands fits to Equation (8) which provides $R$, $D_{D1}$ and $R_{D2}$ values that correspond to the conformational fluctuation within the conformers in quadruplex structure, diffusion time of the quadruplex and duplex structure, respectively. We observed that the $D_{D1}$ values increased from 0.105 to 0.172 ms and the $R_{D2}$ values increased from 0.232 to 0.298 ms with increase in loop length from T2 to T5. Interestingly, the $R$ values, which correspond to the conformational fluctuations in the quadruplex structure decreased from 0.059 to 0.030 ms with increase in the loop length. This suggests the increase in fluctuations among the quadruplex conformers in presence of complementary strand, especially for quadruplex with longer loop length.

We also analyzed the role of loop length on the relative stability of quadruplex and duplex, which affect the fate of the competition. The fluorescence cooling profile would involve the contribution from quadruplex, duplex and random coil populations with the relative trend in the donor fluorescence signals as duplex $> \text{random coil}$. G3T quadruplex adopts only one conformer and data obtained for G3T in absence and presence of complementary strand were fit to Equations (5) and (7) (Materials and methods section), respectively. Quadruplexes G3T2-G3T5 adopts two conformers, and the data obtained in absence and presence of their complementary strands were fit to Equations (6) and (8) (Materials and Methods section), respectively. Where $R$ is the time scale of conformational fluctuation in quadruplex, $D_{D1}$ and $D_{D2}$ is the diffusion time of quadruplex, $D_D$ is the diffusion time of duplex.

### Table 3. Fluorescence correlation spectroscopy measurements conducted in 50 mM MES buffer pH 7.4, 100 mM KCl at 25°C

|        | Quad | Quad + Complementary strand |
|--------|------|-----------------------------|
|        | $R_D$ (ms) | $D_{D1}$ (ms) | $D_{D2}$ (ms) |
| G3T    | 0.062 ± 0.003 | 0.133 ± 0.008 | 0.088 ± 0.008 | 0.198 ± 0.010 |
| G3T2   | 0.093 ± 0.003 | 0.164 ± 0.002 | 0.140 ± 0.003 | 0.285 ± 0.006 |
| G3T3   | 0.070 ± 0.003 | 0.138 ± 0.011 | 0.135 ± 0.003 | 0.253 ± 0.003 |
| G3T4   | 0.082 ± 0.002 | 0.152 ± 0.002 | 0.140 ± 0.003 | 0.285 ± 0.006 |
| G3T5   | 0.093 ± 0.003 | 0.164 ± 0.002 | 0.172 ± 0.004 | 0.298 ± 0.005 |

Figure 4. Fluorescence cooling curves for quadruplexes formed (a) G3T, (b) G3T2, (c) G3T3, (d) G3T4 and (e) G3T5 in absence (open square) and presence (filled square) of equimolar concentration (30 nM) of complementary strand in 50 mM MES buffer, pH 7.4, 100 mM KCl with cooling rates of 0.2°C/min.
lower temperature (20°C) for an equimolar mixture of G-rich and C-rich strand varies for quadruplexes with different loop length. We observed an increase in the fluorescence intensity at 20°C with increase in loop length (T2–T5). This increase in the intensity was found to be maximum for G3T5 and minimum for G3T, indicating maximum duplex formation for G3T5 and minimum for G3T.

We determined the thermodynamic profile of quadruplex formation through fluorescence cooling curves (SI). The thermodynamic parameters for quadruplex formation were obtained using a two-state model for unstructured–structured transition, assuming that a major contribution is from the predominant population only. Since it is difficult to obtain the thermodynamic parameters for quadruplex from spectroscopic measurements with high precision, therefore, we have drawn qualitative trends from this analysis. The thermodynamic parameters for the G3T quadruplex could not be determined due to high thermal stability of structure, which melts at temperatures >85°C. $T_m$ values are within ±1°C error. van’t Hoff analysis performed for fluorescence cooling profile to obtained thermodynamic parameters. $\Delta H^\circ$, $\Delta S^\circ$ and $\Delta G^\circ$ is within 10% error.

Predicted thermodynamic parameters for the duplex formed by all sequences obtained through HYTHER (ozone2.chem.wayne.edu) using experimental conditions of 30 nM strand concentration and 100 mM monovalent cations.

| Quadruplex\(a\) | $T_m$\(^\circ\) | $\Delta H^\circ$ kcal/mol | $\Delta S^\circ$ cal/mol/K | $\Delta G_{25}$ kcal/mol | Duplex\(b\) |
|-----------------|-----------------|--------------------------|--------------------------|------------------------|---------|
| G3T             | -               | -                        | -                        | -                      | 60.4    |
| G3T2            | 82              | -44                      | -121                     | -8.0                   | 61.7    |
| G3T3            | 71              | -36                      | -105                     | -4.7                   | 62.7    |
| G3T4            | 63              | -33                      | -97                      | -4.1                   | 63.4    |
| G3T5            | 46              | -31                      | -95                      | -2.7                   | 64.0    |

\(a\)Fluorescence cooling profile of quadruplex (30 nM) obtained in 50 mM MES buffer, 100 mM KCl, pH 7.4. The thermodynamic parameters for the G3T quadruplex could not be determined due high thermal stability of structure, which melts at temperatures >85°C. $T_m$ values are within ±1°C error. van’t Hoff analysis performed for fluorescence cooling profile to obtained thermodynamic parameters. $\Delta H^\circ$, $\Delta S^\circ$ and $\Delta G^\circ$ is within 10% error.

\(b\)Predicted thermodynamic parameters for the duplex formed by all sequences obtained through HYTHER (ozone2.chem.wayne.edu) using experimental conditions of 30 nM strand concentration and 100 mM monovalent cations.

### DISCUSSION

To understand the role of loop length on the quadruplex–duplex competition, we determined the binding affinity of preformed quadruplexes to their complementary strands, and obtained the following trend G3T < G3T2 < G3T3 < G3T4 < G3T5. The change in binding affinity obtained upon increasing the loop length from T to T5 is not very large (1.0 \times 10^7 M^{-1} to 1.7 \times 10^6 M^{-1}), resulting in a difference of ~1.7 kcal/mol in $\Delta G^\circ$ value for duplex formation. However, the $\Delta G^\circ$ for quadruplex formation varied from ~8.0 kcal/mol to ~2.7 kcal/mol with increase in loop length from T2 to T5 (Table 4). As the loop length has opposite effects on the thermodynamic stability of quadruplexes and duplexes, the important parameter that would dictate the predominance of either of the population (duplex or quadruplex) is the relative free energy difference, the $\Delta \Delta G^\circ$ between the duplex and quadruplex structure, and not the $\Delta G^\circ$ alone. $\Delta \Delta G^\circ$ increases in magnitude upon increasing the loop length from ~2.2 kcal/mol to ~8.5 kcal/mol for G3T2 and G3T5, respectively. The greater negative magnitude of $\Delta \Delta G^\circ$ implies that the competition favors duplex formation, as in case of T5. Further, this predominance of duplex is strongly competed by the quadruplex on decreasing the loop length.

Our kinetic data reveal the presence of one conformer for G3T quadruplex and two conformers for quadruplexes G3T2–G3T5, which unfold at different rates in presence of their complementary strands. The presence of two conformers (parallel and antiparallel structures) is also supported by our CD study. The conformers contributing towards duplex formation include a predominant slow opening conformer and a small population of fast opening
CONCLUSION

Loop length and its composition have vital influence on conformation, stability and on the structural competition between the quadruplex and Watson–Crick duplex. The relative stability of the duplex and quadruplex structure would dictate the predominance of either of these structures at equilibrium. Our results unambiguously reinforce that the increase in the loop length of the potential quadruplex forming sequence favors duplex formation and outcompetes the quadruplex.
SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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