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Effect of Loop Sequence and Loop Length on the Intrinsic Fluorescence of G-Quadruplexes

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S Supporting Information

ABSTRACT: Guanine quadruplex structures (GQSs) exhibit unique spectroscopic features, including an inverse melting profile at 295 nm, distinctive circular dichroism features, and intrinsic fluorescence. Herein, we investigate effects of loop sequence and loop length on the intrinsic fluorescence of 13 DNA QSs. We report label-free fluorescence enhancements upon intramolecular QS formation of up to 16-fold and a shift in the emission maximum to the visible portion of the spectrum. Effects can be understood in the context of available nuclear magnetic resonance QS structures. The intrinsic fluorescence of QSs may be useful for nucleic acid studies and for the development of label-free detection methods.

Guanine quadruplex structures (GQSs) have been of great interest for decades. They are found in DNA telomere repeats and the promoter region of certain proto-oncogenes, such as *c-myc*, *c-kit*, and *bcl-2*.^{1–3} Guanine-rich sequences in DNA or RNA can potentially form a QS if they contain the pattern $G_xL_aG_xL_bG_xL_cG_x$, where $x \geq 2$ and loops *a*, *b*, and *c* consist of at least one nucleotide. The four stretches of G's interact with one another to form two or more quartets that stack upon each other, with the backbone in a parallel or antiparallel geometry. Formation of the QS requires dehydrated K^+ or Na^+ to be present between or within the stacked quartet planes, which stabilizes charge accumulation from having four adjacent strands.⁴ Detailed ion binding studies have been performed on diverse QS contexts under various ionic strength conditions, and the general consensus is that they prefer K^+ over other ions.^{5–7}

The unique structure and folding of QSs lead to distinctive spectroscopic features as compared to those of other nucleic acid motifs. For example, the QS has a characteristic “inverse” or hypochromic melting profile when monitored at 295 nm, in which absorbance decreases as the structure unfolds.⁸ The QS also has characteristic patterns in circular dichroism (CD) experiments: for a parallel topology, there is a positive peak near 260–265 nm with a minimum near 245 nm, whereas for an antiparallel topology, there are positive peaks at 245 and 295 nm with a minimum near 260–265 nm.⁹ The intrinsic fluorescence of DNA QSs was reported recently¹⁰ and is also likely due to the extended conjugation of the four-base quartet. Fluorescence studies of DNA QSs to date have been conducted primarily on the telomeric QS sequence.^{11–13} Our lab recently reported that

RNA QSs exhibit intrinsic fluorescence as well. We showed that the folding cooperativity of a QS, which can be tuned by adjusting the loop sequence or number of guanines in each stretch,^{14,15} can be assessed by monitoring QS fluorescence as a function of K^+ concentration.¹⁵

There is, however, little knowledge of what parameters influence the intrinsic fluorescence of QSs. It seemed likely that loop sequence and length would play important roles in fluorescence given the key roles that these structural features play in QS folding and stability.^{6,16,17} In this study, we conduct fluorescence measurements to investigate the effect of loop sequence and length on the intrinsic fluorescence of dG_2 and dG_3 QSs upon K^+ titration. We then characterize the molecularity and folding cooperativity of a highly fluorescent sequence, dG_3T , compare it to its A-loop counterpart and available structures, and discuss advantages and potential applications of intrinsic QS fluorescence.

To investigate the effects of loop sequence and length on QS fluorescence, we designed 13 DNA QSs with various single-base loops (A, C, or T),¹⁸ loop lengths (1–3 nucleotides), and G-stretch lengths.^{2,3} To simplify the study, the loops within each QS were chosen to comprise a single base but be of variable length, following the pattern “ dG_xL_y ”, where *x* and *y* represent the number of quartets and loop nucleotides, respectively. The DNA QS motifs used in this study and their abbreviations are listed in Table 1.⁶

The QS topology was determined by monitoring the ellipticity at 5 μ M DNA and 10 mM lithium cacodylate (pH 7.0) at 1 M K^+ . The CD and fluorescence spectra for all 13 sequences are provided in Figures S1–S13 of the Supporting Information, and the QS topologies are summarized in Table 1. The CD results reveal that QSs with loops with one nucleotide (i.e., dG_2L and dG_3L) have parallel topologies as expected.^{6,18} QSs with longer loops were found by comparison to CD to be parallel, antiparallel, or a combination of the two topologies (Table 1).

To compare fluorescence intensities in unfolded, physiological K^+ , and saturated K^+ states, we collected fluorescence data on each sequence at 0, 100, and 1000 mM K^+ (Figure 1A). We found that the wavelength of maximal emission varies by ~80 nm among the QSs, ranging from 340 to 420 nm (Table 1 and Figures S1–13 of the Supporting Information). In general, dG_3L_y QSs are more fluorescent than their dG_2L_y counterpart (Figure

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Table 1. DNA GQS Sequences, Topologies, and Emission Wavelength Maxima

dG _x L _y	sequence	topology ^a	λ _{em} (nm) ^b
dG ₂ A	GGAGGAGGAGG	parallel	400
dG ₂ A ₂	GGAAGGAAGGAAGG	parallel	410
dG ₂ A ₃	GGAAAGGAAAGGAAAGG	parallel	420
dG ₂ T	GGTGGTGGTGG	parallel	385
dG ₂ T ₂	GGTTGGTTGGTTGG	antiparallel	385
dG ₂ T ₃	GGTTTGGTTTGGTTTGG	antiparallel	NA ^c
dG ₃ A	GGGAGGGAGGGAGGG	parallel	385
dG ₃ A ₂	GGGAAGGGAAGGGAAGGG	antiparallel	340, 420
dG ₃ A ₃	GGGAAAGGGAAGGGAAGGG	antiparallel	390
dG ₃ T	GGGTGGGTGGGTGGG	parallel	390
dG ₃ T ₂	GGGTTGGGTTGGGTTGGG	parallel	380
dG ₃ T ₃	GGGTTTGGGTTTGGGTTTGGG	mixed ^d	340
dG ₃ C	GGGCGGGCGGGCGGG	parallel	390

^aTopology judged by CD spectroscopy at 1 M K⁺. ^bValues determined by peak maxima in the emission spectrum at 1 M K⁺ with excitation at 260 nm. ^cThe fluorescence was weak, and the peak wavelength was not available (NA). ^dAssigned as mixed because CD has positive ellipticity at both 260 and 295 nm.

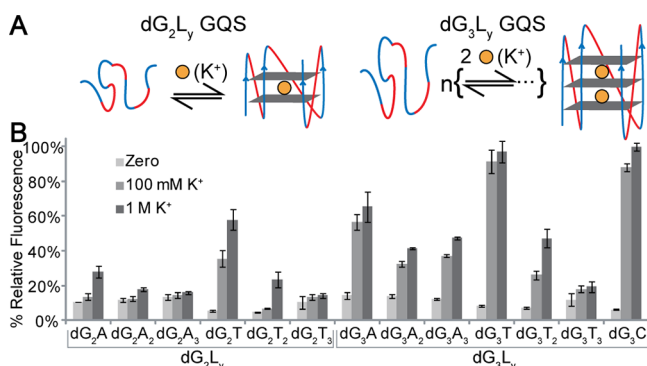


Figure 1. Experimental design and fluorescence titration results. (A) GQS folding model for the dG₂L_y GQS (left) and dG₃L_y GQS (right). The guanine stretches (G_x) are colored blue and the loops (L_y) red. dG₂L_y and dG₃L_y GQSs with parallel topologies are depicted in panel A, although antiparallel quartets are found (Table 1). (B) Relative fluorescence intensity of an individual GQS upon K⁺ titration. The fluorescence intensity for each GQS was obtained at the λ_{em} shown in Table 1; for dG₂T₃, we chose the fluorescence intensity at 340 nm (see Figure S6B of the Supporting Information). Experiments were performed at 5 μM, with identical instrument settings, and the highest fluorescence intensity was normalized as 100%.

1B). In addition, GQSs with loops with one nucleotide have significantly higher fluorescence emission under saturating K⁺ conditions than loops with two or three nucleotides. Indeed, for T loops, fluorescence decreases markedly with increasing loop size, for both dG₂ and dG₃ sequences, to the point where there is almost no fluorescence for the T₃ loop sequences (Figure S14–S15 of the Supporting Information). Of the dG₂L_y GQSs tested, dG₂T is the most fluorescent, being ~2–6 times more fluorescent than the other GQS-forming dG₂L_y sequences. Among all DNA GQSs tested, dG₃T and dG₃C achieve the highest overall fluorescence in 1 M K⁺, as well as the largest enhancement in fluorescence, ~12- and ~16-fold, respectively, upon GQS formation (Figure 1B).

We selected dG₃T for further spectroscopic and thermodynamic evaluation because nuclear magnetic resonance (NMR) structural information is available for it.¹⁹ Full K⁺ titrations were

performed, which led to a K⁺_{1/2} value of 1.2 ± 0.4 mM and a Hill coefficient (*n*) of 0.57 ± 0.02 (Figure 2A,B). The low value of *n*

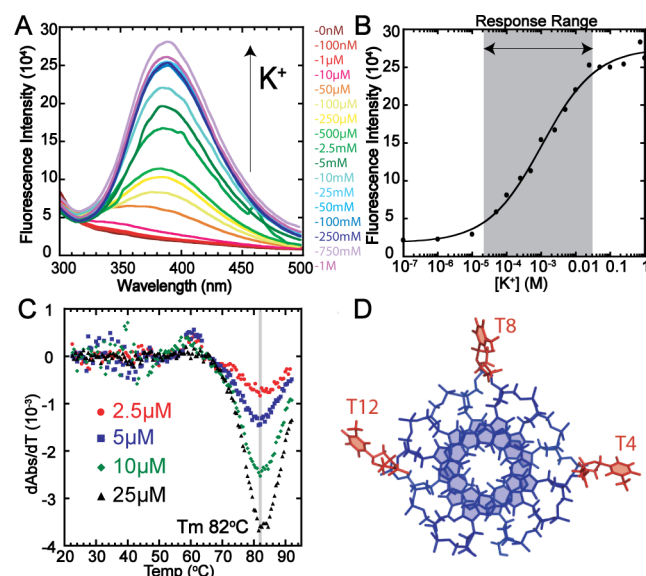


Figure 2. Characterization of dG₃T. (A and B) Emission spectra and K⁺ titration plot monitored at 390 nm for dG₃T. The K⁺_{1/2} and the Hill coefficient (*n*) are 1.2 ± 0.4 mM and 0.57 ± 0.02 using eq S1. The average was obtained from three experiments, and the error is the standard deviation. The response range (10–90% signal) of dG₃T is shaded gray. (C) UV melting of dG₃T monitored at 295 nm at various concentrations. The vertical bar shows the position of the T_m. (D) NMR structure of d(GGGT)₄ (PDB entry 2LE6).¹⁹ The 3′-terminal T (T16) is not shown. Loops and G-quartets are colored red and blue, respectively.

indicates that GQS folding is anticooperative, which is also reflected in the wide, ~1000-fold analyte response range. Such anticooperativity is consistent with our recent GQS folding studies with long RNA GQSs.^{14,15} In addition, UV melting was monitored at 295 nm at dG₃T concentrations ranging from 2.5 to 25 μM, and the melting temperature (*T*_m) was found to be independent of concentration (Figure 2C), indicating that the fluorescence signal observed at 5 μM dG₃T K⁺ titration is due to intramolecular GQS folding.

Our results indicate that the increase in fluorescence observed upon K⁺ titration is due to formation of GQSs, as GQS-specific signals were observed in the CD under these conditions (Figure S1–S13 of the Supporting Information). Likewise, non-GQS-forming sequences did not exhibit an increase in fluorescence upon K⁺ titration.^{10,b} We also tested different anions (fluoride and acetate) and found that fluorescence intensity changes were similar to those with chloride (data not shown), suggesting absence of anion induced quenching. Comparison of fluorescence intensity among GQS candidates at 1 M K⁺ is justified, as plateaus were observed at higher K⁺ concentrations, indicating that the GQS is in its fully folded states (Figure S14 of the Supporting Information). The origin of GQS intrinsic fluorescence is proposed to be contributed by a ¹G*G excimer.¹⁰ It is likely that the loop sequence and length alter the GQS, thereby affecting the excited states and energy transfer properties of GQSs.

It is of interest to correlate the spectroscopic observations herein with GQSs. An NMR structure is available for 5′-d(GGGT)₄,¹⁹ which has the same CD profile and *T*_m within 2 °C of that of dG₃T.²⁰ In the structure, the T-loops are extruded and

do not interact with the G-quartets (Figure 2D). The flipped-out nature of the loop bases is likely responsible for the stronger fluorescence of this GQS, as T's are known to quench fluorescence.²¹ Because dG₃C had a relative fluorescence intensity and an emission wavelength maximum similar to those of dG₃T, and the same topology as dG₃T (both parallel) (Table 1), it is likely that the one-pyrimidine loops of dG₃C and dG₃T are extruded. The strong decrease in fluorescence with T-loop length (Figure S15 of the Supporting Information) likely arises because lengthening the extruded loops leads to enhanced collisional quenching by the thymine.

As compared to dG₃C and dG₃T, dG₃A has significantly weaker fluorescence. Moreover, among the dG₂L sequences, dG₂A has significantly lower fluorescence than dG₂T. Interestingly, an NMR structure of 5'-d(GGA)₄, similar in sequence to dG₂A, reveals that the A's interact with the G-quartet to form a heptad structure (Figure S16 of the Supporting Information).²² It is likely that such direct GQS interactions quench fluorescence in dG₂A and dG₃A.

The dependence of the maximal emission wavelength on loop sequence can also be correlated with GQSs. In particular, dG₂A₁₋₃ emits at the longest wavelengths, with emission maxima of 400–420 nm, whereas dG₂T₁₋₂ has emission maxima of ~385 nm (Table 1 and Figure S15 of the Supporting Information). Apparently, the heptads present in the A-loop GQS extend the conjugation of the G-quartet leading to the shift in fluorescence into the visible light portion of the spectrum. Notably, two peaks were observed in the fluorescence emission of dG₃A₂, one at 340 nm and the other at 420 nm (Figure S8 of the Supporting Information), which suggests that the quartets in dG₃A₂ may experience different environments, one with a heptad and one without.

The strong fluorescence enhancement and wide dynamic range of K⁺ detection of dG₃T upon GQS folding demonstrate its merit for sensor development and broad response range K⁺ detection. The intrinsic fluorescence of GQSs can be used in fluorescence melting, kinetics, or quadruplex primer amplification (QPA) readouts²⁰ without the incorporation of unnatural bases such as 2-aminopurine (2-AP), which have been found to affect the T_m of the dG₃T GQS²³ and to interfere with certain biological studies. Moreover, the intrinsic fluorescence of dG₃T could be utilized as a readout to replace a GQS ligand binding reporter such as ZnPPIX in the G-quadruplex integrated hybridization chain reaction (GQ-HCR).²⁴

In summary, we have investigated the intrinsic fluorescence of DNA GQSs with various loops sequences and lengths. We observe a wide dynamic range of emission intensity and maxima, and these can be understood in light of available NMR structures. The label-free fluorescence of the GQS motif described provides a tool for nucleic acid studies and sensor development where fluorescence output is desired.

■ ASSOCIATED CONTENT

■ Supporting Information

Materials and methods and Figures S1–S16. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contributions

C.K.K. and M.E.S. are joint first authors.

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Notes

The authors declare no competing financial interest.

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■ ADDITIONAL NOTES

^aWe did not study loops of G's as these would lead to heterogeneous mixtures of states.

^bAccording to CD, dG₂C, dG₃C₂, and dG₃C₃ were not found to form GQSs presumably because of competitive Watson–Crick structures (data not shown). It appears that for dG_xC_y to form a GQS, *x* and *y* should be no less than two.

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