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Probing the Structure and Dynamics of Telomeric G-Quadruplexes with Thienoguanosine, a Fluorescent Analogue of Guanosine

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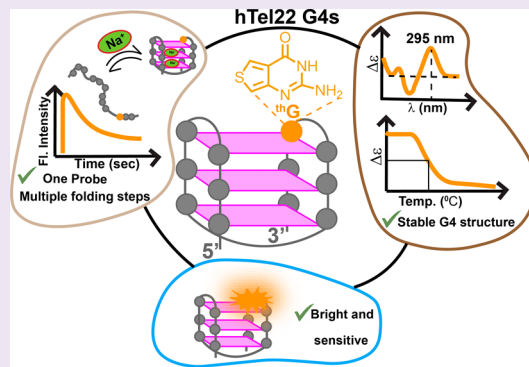


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ABSTRACT: The potential of thienoguanosine (thG), an isomorphous fluorescent analogue of guanosine, was evaluated as a reporter for probing the structure and dynamics of hTel22, a human telomeric G-quadruplex (G4) sequence. Nine of the 12 guanines directly participating in G-tetrads were individually substituted with thG, and their impact on the hTel22 structure was examined by circular dichroism, thermal melting, and ¹H NMR spectroscopy. In Na⁺ buffer, substitutions at external tetrads maintained the native antiparallel topology and thermal stability, while substitutions within the middle tetrad significantly disrupted the G4 structure. Molecular dynamics simulations supported that thG incorporation in external tetrads is well tolerated, whereas it alters base stacking and Na⁺ coordination in middle tetrad positions. In K⁺ buffer, external substitutions favored the antiparallel over the hybrid topology, resulting in a modest destabilization. thG-labeled sequences at external tetrads in both Na⁺ and K⁺ buffers exhibited high fluorescence quantum yields, long fluorescence lifetimes, and high sensitivity to local conformation. As an application, thG fluorescence signal proved instrumental in accurately characterizing the kinetics of Na⁺-induced G4 folding, enabling all kinetically resolved folding steps to be captured from a single fluorescence observable. Overall, thG serves as a remarkably sensitive, minimally perturbing fluorescent guanosine analogue for structural and dynamic studies of G4s. Moreover, its longest lifetime (18.5 to 28.5 ns), at least twice that in DNA duplexes, is suitable for time-gated detection with high signal-to-noise ratio and easy distinction of G4s from duplexes, offering a key advantage for G4-targeting drug discovery.



1. INTRODUCTION

G-quadruplexes (G4s) are noncanonical nucleic acid secondary structures formed by guanine-rich DNA and RNA sequences, which are ubiquitously distributed throughout genomes, including telomeres, promoter regions of protein-coding genes, untranslated regions, replication origins, and other regulatory loci.^{1–3} They play important roles in gene regulation, DNA replication, transcription, translation, and chromosome stability.^{4,5} Consistent with these widespread functions, dysregulation of G4 formation, resolution, or recognition has been linked to genetic disorders, hereditary diseases, cancer, and aging-related pathologies.^{6–8} G4-forming sequences are also implicated in the replication, transcription, and survival of pathogens, highlighting their relevance in infectious diseases.⁹

G4s consist of stacks of planar G-tetrads stabilized by Hoogsteen base pairing through Hydrogen bonds (H-bonds) and electrostatic interactions with cations such as K⁺ or Na⁺. Based on their sequence and the nature of the cation involved, G4s can adopt diverse topologies that differ with their strand

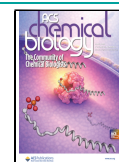
orientation and loop arrangement.¹⁰ Since G4s perform essential functions in cells and are promising therapeutic targets, it is essential to investigate their structural dynamics, including their folding, unfolding and the population of partially folded intermediates, in order to understand their biological role and guide the development of strategies targeting G4s. In this context, intense research efforts have notably been devoted to the development of small-molecule G4 binders acting either as stabilizers or destabilizers, with applications spanning cancer, viral infections, and other G4-mediated pathologies.^{11–19} Various biophysical techniques have been used for structural studies of G4s *in vitro*, including

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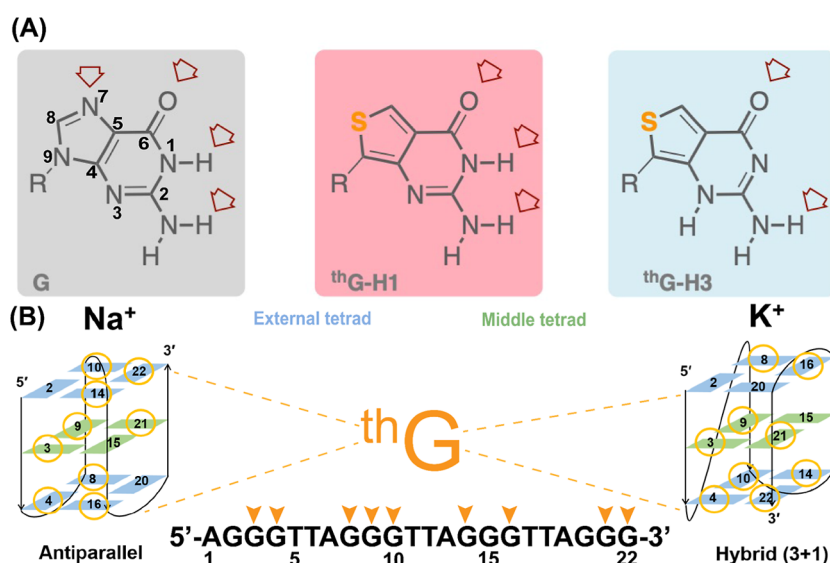


Figure 1. (A) Chemical structures of guanosine (G) and tautomers of thienoguanosine (thG-H1 and thG-H3). Atoms involved in Hoogsteen hydrogen bonding are indicated by red arrows. (B) hTel22 sequence labeled with thG at selected positions. G residues indicated by arrows in the sequence and circled in the folded G4s were individually replaced with thG. hTel22 adopts an antiparallel topology in the presence of Na⁺ ions³⁹ and predominantly, a hybrid topology in the presence of K⁺ ions.⁴⁰

circular dichroism (CD), nuclear magnetic resonance (NMR), X-ray crystallography, and cryogenic electron (cryo-EM) microscopy.^{20–22} However, these methods generally require relatively large sample quantities, are not easily applicable to studies in complex cellular environments, and provide only limited insight into the dynamic nature of G4s. These limitations are circumvented by fluorescence techniques, which allow dynamic studies with high sensitivity and temporal resolution under physiological conditions.

Since natural nucleosides are poorly emissive, labeling by external fluorophores is required. A class of early developed probes is based on fluorescent ligands that show enhanced fluorescence upon noncovalently binding to G4 structures. Although valuable, these ligands often suffer from limited selectivity, as they bind G4 DNA and double-stranded DNA with comparable affinities.^{23–25} Another class is based on tagging with donor–acceptor pairs for Förster resonance energy transfer (FRET), which allow ensemble and single molecule measurements. As the chromophores are not part of the G4 core, they only provide indirect information on the structural changes occurring at this level.^{26,27} Moreover, both ligand binding and terminal labeling can perturb G4 topology and stability.^{28–30} To overcome these limitations and achieve truly site-specific interrogation of the G4 core, fluorescent nucleoside analogues (FNAs) represent a particularly attractive alternative.^{31–34} FNAs can substitute the natural nucleobases within the G-tetrads, enabling direct optical readout of the local nucleic-acid environment without the use of bulky external fluorophores and with reduced risk of structural perturbation.³⁵ Several G-based FNAs, such as 8-vinyl-2'-deoxyguanosine (VdG),³⁶ 8-(2-phenylethenyl)-2'-deoxyguanosine (StG), 8-(2-pyridyl)-2'-deoxyguanosine (2PyG),³⁷ 6-methylisoxanthopterin (6-MI),³⁸ and 8-[2-(pyrid-4-yl)-ethenyl]-2'-deoxyguanosine (4PVG),³⁷ have already been used to replace G residues in G-tetrads. Though these FNAs have been shown to provide useful information on G4s, none of them met the requirements of an ideal analogue of G, preserving G4 stability while maintaining high fluorescence quantum yield

(ϕ) and lifetime (τ), as well as high sensitivity to the environment.

Among the possible, minimally perturbing candidates, thienoguanosine (thG) an isomorphous analogue of G seems of high interest.^{41–45} thG differs from G only in that the purine's N7 and N9 are replaced by CH and C, respectively, and a sulfur atom is introduced at position 8 (Figure 1A). thG has been shown to perfectly replace G residues in DNA duplexes,^{46,47} while maintaining reasonable emission quantum yields, along with long-lived mean fluorescence lifetimes.^{46,47} Interestingly, in a buffered solution, thG exists as an equilibrium mixture of thG-H1 and thG-H3 (Figure 1A), its keto-amino tautomers with nearly equal percentage.^{41,48} In fully complementary dsDNA, this equilibrium shifts to the thG-H1 tautomer, enforced by Watson–Crick base pairing with an opposite C,^{41,48} while in mismatched duplexes, the shift is only partial.⁴¹ As compared to matched duplexes, thG fluorescence increases in mismatched duplexes⁴⁷ and also during protein-induced base flipping that decreased thG stacking with its neighboring nucleobases.^{46,49} Due to this strong sensitivity to base stacking, we hypothesized that, despite the lack of a N atom at the N7 equivalent position, thG incorporated in G4-forming oligonucleotides should be sensitive to G4 topology and tetrad position (middle vs external) in the folded structures.

To investigate thG's ability to act as a sensitive reporter of G4 structure and dynamics, we have systematically replaced nine out of the 12 G residues of the human telomere repeat sequence hTel22 by thG (Figure 1B). This sequence is widely accepted as a model system for studying G4s due to its high biological relevance.^{50–53} Being located at chromosome ends, hTel22 sequence plays an important role in chromosome end protection by regulating the accessibility of telomerase and telomere-binding proteins. Importantly, high-resolution structural studies have established that hTel22 folds into an antiparallel topology in Na⁺ buffer and predominantly into a hybrid (3 + 1) topology in K⁺ buffer, both comprising three stacked G-tetrads.^{39,40} Here, CD and NMR spectroscopies together with thermal denaturation studies and molecular

dynamics (MD) simulations were used to characterize the folding and stability of the ^{13}C -labeled sequences, complemented by Quantum Mechanical (QM) calculations to simulate their CD signature. In Na^+ buffer, ^{13}C substitution was found to preserve the antiparallel topology, whereas in K^+ buffer the topology became predominantly antiparallel too. Steady-state and time-resolved fluorescence spectroscopy further revealed that ^{13}C maintains high quantum yield and long fluorescence lifetime within G4 environments in both ionic conditions. Finally, we utilized ^{13}C 's fluorescence as a tool to monitor the Na^+ -induced folding kinetics of hTel22. Together, our data show that ^{13}C , when incorporated in external G-tetrad positions, represents a promising and sensitive fluorescent G analogue for monitoring the structure and dynamics of G4s.

2. MATERIALS AND METHODS

2.1. Materials

$\text{O}^3'-(2\text{-Cyanoethyl-diisopropylphosphoramidite})-\text{O}^5'-\text{DMT}-2'-\text{deoxy-N}^2\text{-DMF-}^{13}\text{C}$ was synthesized as previously described^{43,45} and used to specifically replace G residues at different positions in hTel22 (Figure 1B). The ^{13}C -labeled sequences were prepared using solid-phase chemical synthesis on an ABI 394 RNA/DNA synthesizer and purified by reverse-phase HPLC. Several labeled sequences were prepared and HPLC-purified by Eurogentec (Belgium). Native and ^{13}C -labeled sequences were folded into G4s by dissolving in 10 mM Tris–HCl buffer, 100 mM NaCl or KCl, pH 7.5, then heating at 95 °C for 3 min, and slow cooling to RT.

2.2. UV–Visible Absorption and Circular Dichroism (CD) Spectroscopy

Absorption spectra were recorded on Cary 4000 (Agilent) and UV-2700 (Shimadzu) UV–visible spectrophotometers using quartz cuvettes of 1 cm optical path length. An extinction coefficient at 260 nm of $228,500 \text{ M}^{-1} \text{ cm}^{-1}$ was used for the unlabeled hTel22 sequence and $229,430 \text{ M}^{-1} \text{ cm}^{-1}$ for ^{13}C -labeled sequences.

CD spectra were recorded in the 220–330 nm range on a Jasco J-820 spectropolarimeter in 1 mm path length quartz cuvettes. A Peltier controller was used for temperature control, and nitrogen gas was purged at a flow rate of 5 L/min to remove oxygen and moisture from the optical path and to prevent ozone generation. Ellipticity, absorbance and high-tension voltage (HT) were recorded simultaneously. To ensure accurate data, the absorbance was kept below 1.2 and the HT voltage was maintained below 600 V. All spectra were corrected for the buffer contribution. Ellipticity (θ) in mdeg was converted to molar circular dichroism ($\Delta\epsilon$) ($\text{M}^{-1} \text{ cm}^{-1}$) using

$$\Delta\epsilon = \frac{\theta}{32980 \times c \times l} \quad (1)$$

where c is the concentration of oligonucleotides and l is the path length of the cuvettes.

Melting temperatures were determined by CD spectroscopy (Jasco J-820) and UV-absorption (Cary 4000 HP). Signals for both techniques were recorded at 295 nm in the temperature range 20–100 °C with 10 °C/min speed in CD and 1 °C/min speed in UV-absorption. Mineral oil was added on the top of the samples to avoid evaporation. The melting temperature was determined from the minimum of the first derivative of the observed signal change as a function of temperature.

2.3. Native Polyacrylamide Gel Electrophoresis (PAGE)

Unlabeled and ^{13}C -labeled G4s prepared in Na^+ and K^+ buffers as described in Section 2.1 were analyzed using native PAGE. Four μg of DNA was loaded on 20% native polyacrylamide gels supplemented with 10 mM of KCl or NaCl. Electrophoresis was carried out at 120 V for 1 h at RT. The gel was then scanned using UV shadowing and a ChemiDoc imager (Bio-Rad), taking advantage of the ^{13}C fluorescence signal (excitation at 300 nm).

2.4. NMR Experiments

One-dimensional (1D) ^1H NMR experiments were performed on 600 and 800 MHz Bruker spectrometers equipped with a cryoprobe. NMR spectra were recorded at 298 K. DNA samples (100 μM to 1 mM) were in 10 mM sodium or potassium phosphate buffer (pH 7.0), supplemented with 100 mM NaCl or KCl and 10% D_2O . A jump-and-return pulse sequence was used to suppress the water signal.

2.5. MD Simulations and QM Calculations

The first model of the hTel22 NMR solution structure (PDB-ID: 143D)³⁹ in antiparallel topology was used as a reference structure for the generation of hTel22 labeled by ^{13}C -H1 tautomer at individual positions 8, 9, and 10. The antiparallel hTel22 was complemented with three Na^+ ions in the inner part of the cavity formed by G-tetrads.

The OL15 force field was used for the parametrization of the DNA.⁵⁴ ^{13}C parameters and the MD protocol were retrieved from previous works.^{47–49} ^{13}C -labeled hTel22 structures were included in a rectangular box of TIP3P type water molecules buffering 10 Å from the G4 surface. To replicate experimental high ionic strength, an excess of Na^+ ions was added to the solvent up to doubling the system charges. The solvent was initially energy minimized for 500 steps with the steepest descent algorithm (SD) followed by 2500 steps with the conjugate gradient algorithm (CG) while keeping the solute as frozen. The solvated solute was then energy minimized for 1000 steps with the SD followed by 5000 steps with the CG before being heated to 300 K at constant volume with the Langevin thermostat for 1 ns. System density was equilibrated with the Berendsen barostat at constant pressure for 1 ns, before 50 ns of unrestrained MD simulations were produced at constant pressure. Finally, 500 ns of MD trajectories were produced for analysis purposes. MD simulations were run with AMBER25⁵⁵ using a time step of 2 fs with the GPU-accelerated pmemd. In MD simulations, a positional restraint of 10 kcal/(mol·Å²) was applied to the Na^+ ions within the inner cavity formed by the G-tetrads. MD trajectories were then analyzed by cpptraj.⁵⁶ A hierarchical agglomerative (bottom-up) approach was used to cluster MD frames.

The ground state geometries of hTel22 sequences labeled by ^{13}C at positions 8 and 10 have been optimized at the Quantum Mechanics/Molecular Mechanics (QM/MM) level, by resorting to the ONIOM⁵⁷ procedure, starting from the reference geometries issued by MD simulations. In the geometry optimizations, the QM layer (the 11/12 G bases, ^{13}C and the inner Na^+ ions) has been fully optimized, whereas the MM part (the other bases and the phosphor-deoxyribose backbone) has been constrained to the starting MD structure. We used the AMBER⁵⁸ force field for the MM part, and the cost-effective M052X⁵⁹/6-31G(d) Density Functional Theory (DFT) calculations for the QM part, including solvent effect with the Polarizable Continuum Model (PCM).⁶⁰ The CD spectrum has been simulated by computing the 80 lowest energy excited states of the full system (TD/M052X/6-31G(d)/MM), and convoluting each electronic transition with a Gaussian with half-width-half-maximum of 0.25 eV. Each electronic transition has been red-shifted by 0.8 eV, which is the average error in simulating the absorption spectrum of the G monomer with the same level of theory. This computational approach has been already profitably used to investigate the photophysics and the spectral properties of DNA sequences, including G4s.^{61–65}

2.6. Steady-State Fluorescence Spectroscopy

Excitation and emission spectra were recorded on a Fluoromax Plus (Horiba) fluorimeter equipped with a thermostated cell compartment to perform all measurements at 20 °C. All spectra were corrected for lamp fluctuations, Raman scattering, instrumental wavelength dependent bias and buffer contribution. The fluorescence quantum yield (ϕ) of ^{13}C -labeled sequences was calculated using quinine sulfate (QS) in sulfuric acid (0.5 M) as a reference ($\phi_{\text{QS}} = 0.546$)⁶⁶

$$\phi = \phi_{\text{QS}} \frac{A_{\text{QS}}}{A} \frac{I}{I_{\text{QS}}} \frac{n^2}{n_{\text{QS}}^2} \quad (2)$$

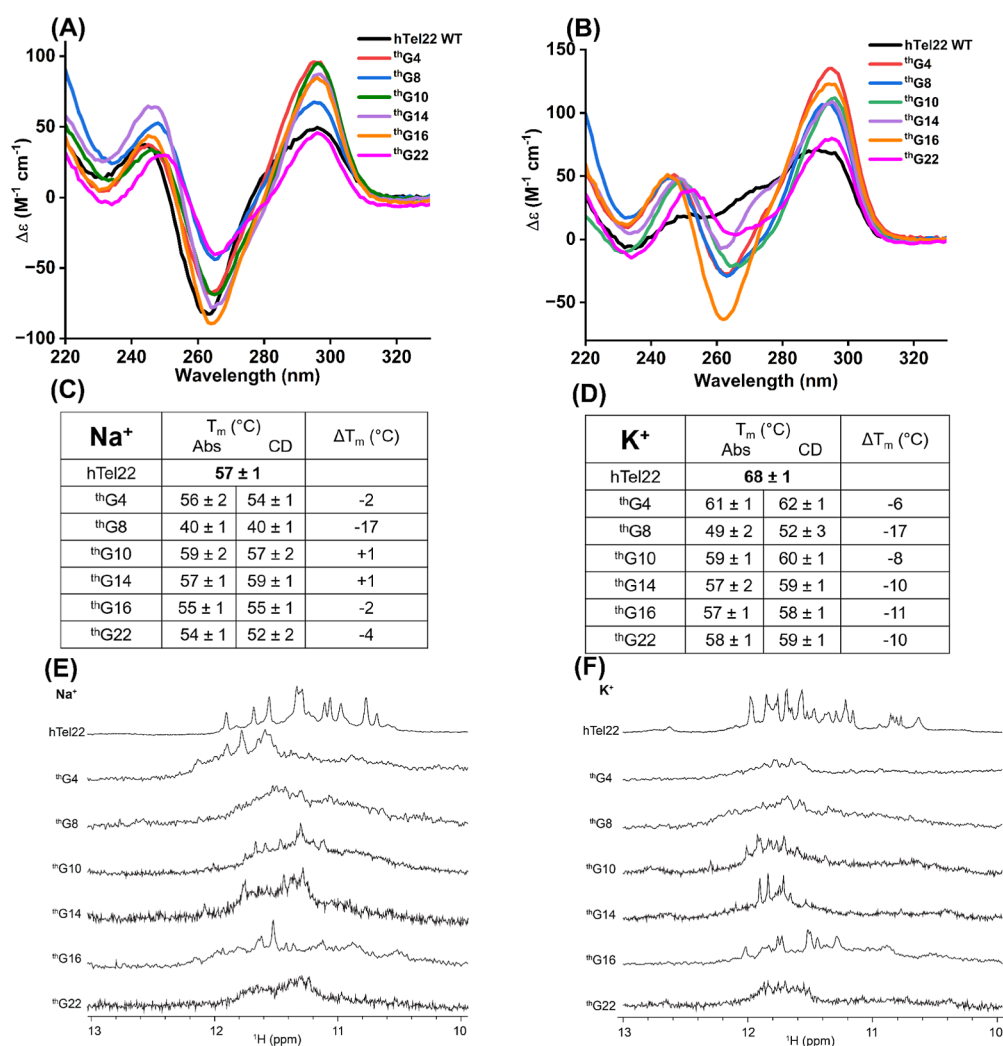


Figure 2. Structural characterization of hTel22 G4s labeled by ¹³G at external tetrads. CD spectra in 1 mm cuvette of 35 μ M unlabeled hTel22 WT (black) and ¹³G-labeled hTel22 sequences in (A) Na⁺ and (B) K⁺ buffers. Melting temperature (T_m) of hTel22 G4s measured using absorption and CD spectroscopy in (C) Na⁺ and (D) K⁺ buffers. ΔT_m was calculated by $\Delta T_m = T_m(\text{labeled}) - T_m(\text{WT})$, where $T_m(\text{labeled})$ corresponded to the mean of the values determined by absorption and CD techniques. 1D ¹H NMR spectra showing the imino proton region (10–13 ppm) of (E) Na⁺- and (F) K⁺-folded hTel22 G4s. CD and melting experiments were performed in 10 mM Tris–HCl buffer (pH 7.5), supplemented with 100 mM NaCl or KCl at 20 °C, while NMR experiments were performed in 10 mM sodium or potassium phosphate buffer (pH 7.0), supplemented with 100 mM NaCl or KCl at 25 °C.

where A and A_{QS} are the absorbance at the excitation wavelength; I and I_{QS} are the area under the emission curve and, n and n_{QS} are the solvent refractive indexes for the sample and QS, respectively. The quantum yields are reported as means $\pm$ standard error of the mean for three independent experiments.

2.7. Time-Resolved Fluorescence Measurements

Time-resolved measurements were performed using the time-correlated single photon counting (TCSPC) technique. The fluorescence intensity decays of ¹³G-labeled G4s were recorded on a homemade setup, having a pulse-picked supercontinuum white light laser (EXR-20, NKT photonics) with an UV extension (SuperK Extend UV, NKT Photonics). Excitation was at 370 nm with a 10 MHz repetition rate. Emission was collected at 460 nm with a 16 nm band-pass monochromator (Jobin–Yvon H10) and an LBW 450 filter. A microchannel plate Hamamatsu R3809U photomultiplier coupled to a pulse preamplifier (HFAH 20, Becker & Hickel) was used for photon detection. Data were then recorded on a TCSPC counting board (SPC 130, Becker & Hickel). The instrumental response function (IRF) with a full width at half-maximum of 50 ps was recorded with a polished aluminum reflector.

Time-resolved decays were first fitted using the maximum entropy method (MEM) to obtain the distribution of decay times without making any assumption on the number of decay components and distribution shape. MEM maximizes the Skilling-Jayne's entropy function^{67,68}

$$S = \int_0^\infty \alpha(\tau) - m(\tau) - \alpha(\tau) \log \frac{\alpha(\tau)}{m(\tau)} d\tau \quad (3)$$

where $\alpha(\tau)$ and $m(\tau)$ are the reconstructed distribution and primary model, respectively. This method helps to determine the number of lifetime components needed to fit the decays.

Next, the fluorescence decays were fitted with the DecayFit software⁶⁹ that uses a nonlinear least-squares fitting procedure to fit the decay curves to a multiexponential decay function convolved with the experimental IRF. The quality of the fits was evaluated by the reduced χ^2 and the distribution of residuals. The amplitude-weighted mean lifetime was calculated as $\langle \tau \rangle = \sum \alpha_i \tau_i$, where α_i are the preexponential factors and τ_i are the characteristic lifetimes.

Time-resolved anisotropy measurements were performed on the same instrument using excitation pulses at 355 nm with a 10 MHz repetition rate. Decays were collected at 460 nm using long pass filter

KV 389 and polarizers set at magic angle ($I_{MA}(t)$), parallel ($I_{\parallel}(t)$) and perpendicular ($I_{\perp}(t)$) directions. Magic angle decay ($I_{MA}(t)$) is related to parallel ($I_{\parallel}(t)$) and perpendicular decay ($I_{\perp}(t)$) as follows

$$I_{MA}(t) = \frac{1}{3}(I_{\parallel}(t) + 2I_{\perp}(t)) \quad (4)$$

Fluorescence anisotropy ($r(t)$) can be expressed as

$$r(t) = \frac{I_{\parallel}(t) - I_{\perp}(t) \times G(\lambda)}{I_{\parallel}(t) + 2I_{\perp}(t) \times G(\lambda)} \quad (5)$$

where $G(\lambda)$, the grating factor at emission wavelength λ , was given a value of 1.15.⁷⁰

Rotational correlation times have been determined by simultaneous fitting of $I_{MA}(t)$, $I_{\parallel}(t)$ and $I_{\perp}(t)$ through following equations

$$I_{MA}(t) = N(t) \quad (6)$$

$$I_{\parallel}(t) = N(t) \times (1 + 2r(t)) \quad (7)$$

$$I_{\perp}(t) = N(t) \times (1 - r(t)) \quad (8)$$

The fitting function is built with a convolution of a sum of exponential decaying functions with a normalized Gaussian function of standard deviation σ standing for the IRF and a Heavyside function. The anisotropy decay function $r(t)$ is defined as

$$r(t) = r_0 \sum_i \beta_i \times \exp\left(-\frac{t}{\theta_i}\right) \quad (9)$$

where r_0 is the fundamental anisotropy and θ_i are the rotational correlation times with their fractional contribution β_i . The time-resolved fluorescence decays were associated with a weighting corresponding to the standard deviation of the number of emitted photons and were fitted globally using a custom algorithm that combined eqs 6–9. Fluorescence lifetimes and correlation times were used as shared parameters, while amplitudes were allowed to freely vary. The fitting function was built in OriginPro 2024 (OriginLab). The number of correlation time components was determined based on the χ^2 value and the distribution of residuals.

2.8. Fluorescence and Stopped-Flow Kinetic Experiments

The kinetic traces of Na⁺-induced hTel22 folding were obtained by combining fluorescence traces acquired from the Fluorolog fluorimeter (Horiba) and the SFM-300 stopped-flow apparatus (Bio-Logic, France) equipped with a Xe–Hg lamp. In the fluorimeter measurements, the kinetic trace is recorded by exciting at 350 nm and monitoring the emission at 460 nm at 1 s intervals at 20 °C. thG-labeled hTel22 sequence was used at 1 μ M concentration, and folding was initiated by adding 100 mM NaCl to the 10 mM Tris buffer, pH 7.5. The time interval between NaCl addition and the start of data acquisition was recorded manually with a stopwatch. This delay was subsequently added to align the kinetic trace with the stopped-flow trace that was recorded using an excitation at 365 nm, one of the main emission lines of the Xe–Hg source. thG's emission was collected using a 420 nm long-pass filter (ITOS GmbH, Germany). High voltage during the measurement was maintained at 750 V to ensure an optimal signal-to-noise ratio. The dead time of the stopped-flow instrument was determined as 2.2 ms and added to the time axis of the recorded traces. Buffer contribution was subtracted from all fluorescence traces prior to analysis.

The traces obtained by the stopped-flow setup and the fluorimeter were merged by scaling their fluorescence intensities in their temporal overlap zone. This results in a continuous kinetic trace that was fitted using

$$I_t = I_f - (I_f - I_{ini}) \sum_i a_i e^{-k_{obs,i}t} \quad (10)$$

where I_t and I_f are the fluorescence intensities at time t and after full folding of the G4 structure, respectively. I_{ini} represents the initial fluorescence intensity. a_i are the fractions of the total resolved

amplitude ($I_f - I_{ini}$) associated with each kinetic step (with $\sum_i a_i = 1$) and $k_{obs,i}$ are the corresponding observed rate constants. During the fitting process with the Origin software, the number of components was progressively increased until the χ^2 value approached 1 and the distribution of residuals became random around the value 0.

3. RESULTS

3.1. Position-Dependent Impact of thG on hTel22 G4 Structure and Stability

G-tetrads can be categorized as external and middle tetrads, with external tetrads being stacked at only one side and middle tetrads being sandwiched between two other tetrads. In our individually hTel22 labeled sequences, G residues were replaced by thG at positions 4, 8, 10, 14, 16, and 22 in the external tetrads and positions 3, 9, and 21 in the middle tetrad (Figure 1B). The hTel22 sequences were folded in both Na⁺ and K⁺ buffers.

hTel22 wild type (WT) sequence in Na⁺ buffer is characterized by a positive CD peak at ~295 nm and a negative peak at ~265 nm (Figure 2A, black line), typical of antiparallel topology.⁷¹ In contrast, hTel22 in K⁺ buffer shows a CD spectrum with a positive peak at ~288 nm, a shoulder near 270 nm, and a negative peak at ~240 nm (Figure 2B, black line), which corresponds to a dominant hybrid topology.^{29,71} The topology of hTel22 WT in both buffers was confirmed by the 1D ¹H NMR spectra of hTel22 WT displaying imino proton peaks between 10.4 and 12.6 ppm similar to those previously described⁷¹ (Figure 2E,F).

In Na⁺ buffer, the CD spectra of thG-labeled sequences at external tetrad positions indicated that the labeling preserved the antiparallel topology (Figure 2A). Native PAGE (Figure S1) further showed that the sequences labeled in the external tetrads migrated to positions comparable to hTel22 WT, in line with preserved compact G4 conformations. Melting temperatures determined by absorption and CD measurements at 295 nm revealed that thG substitution at the external tetrads led to marginal changes in T_m , with the exception of position 8, where a substantial decrease is observed (17 °C) (Figure 2C). The ~10–12 ppm region of the ¹H NMR spectrum is characteristic of imino protons involved in G4 formation. The presence of imino proton resonances between 10.4 and 12.6 ppm supports the formation of the G4 structures in all modified sequences (Figure 2E). Although relatively sharp peaks are observed at positions 10, 14, and 16, the broader signals in thG-labeled sequences compared to hTel22 WT suggest partial destabilization and/or increased conformational heterogeneity.

Interestingly, the CD spectra of the same labeled sequences in K⁺ buffer differed markedly from that of the hTel22 WT (Figure 2B) and instead resembled those in Na⁺ buffer (Figure 2A), suggesting a decrease in the hybrid population to the benefit of the antiparallel topology. This change in population was accompanied by a 6–11 °C decrease of the T_m value as compared to hTel22 WT. Similar to Na⁺ buffer, among the external tetrad positions, the largest decrease in T_m value was observed at position 8 (17 °C) (Figure 2D). Native PAGE (Figure S1) further showed that the labeled sequences migrated at positions comparable to hTel22 WT, indicating the preservation of compact G4 conformations. As in Na⁺ buffer, the 1D ¹H NMR imino protons peaks (Figure 2F) in K⁺ buffer confirmed G4 formation in all sequences modified by thG at external tetrad positions. Consistent with observations in Na⁺ buffer, the 1D ¹H NMR spectra in K⁺ buffer (Figure 2F)

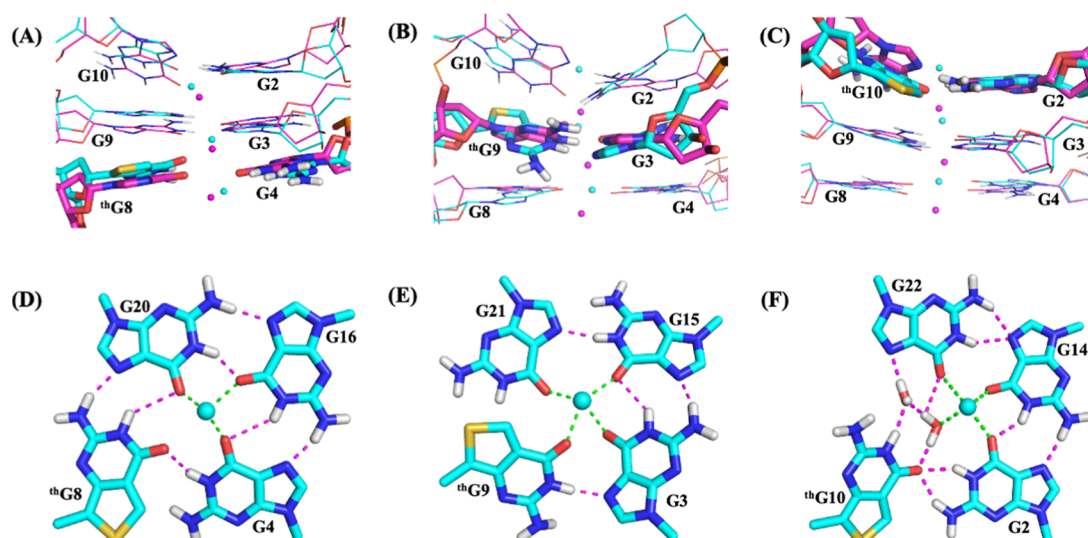


Figure 3. Structural impact of thG in position 8 (A,D), 9 (B,E), and 10 (C,F) in representative MD frames, and comparison with the unlabeled WT sequence. thG-labeled sequences are in cyan, while the unlabeled WT sequence is in magenta. The nucleotides involved in the G-tetrad bearing thG are shown as cyan sticks, nucleotides from other G-tetrads are shown as lines. The Na⁺ ion within the tetrad is shown as a small sphere (colored cyan in thG-labeled hTel22, colored magenta in hTel22 WT). Metal ion coordination and H-bond interactions are highlighted by cyan and magenta dashed lines, respectively. For clarity, hTel22 nucleotides not included in G-tetrads are omitted, as well as nonpolar H atoms.

Table 1. Comparison between Interatomic Key Distances within Tetrads of the WT and thG-Labeled hTel22 as a Consequence of thG Inclusion at Different Positions^a

key distances in Å within the G-tetrad bearing th G in position 8 (th G-labeled/wild-type)		key distances in Å within the G-tetrad bearing th G in position 9 (th G-labeled/wild-type)		Key distances in Å within the G-tetrad bearing th G in position 10 (th G-labeled/wild-type)	
th G8(O6) - G4(N1H1)	2.2/2.4	th G9(O6) - G21(N1H1)	4.7/2.4	th G10(O6) - G2(N1H1)	2.1/2.3
th G8(C7) - G4(N2H21)	2.8/2.0	th G9(C7) - G21(N2H21)	4.2/1.9	th G10(C7) - G2(N2H21)	2.7/1.8
G20(O6) - th G8(N1H1)	2.6/2.1	G21(O6) - G15(N1H1)	3.8/2.2	G22(O6) - th G10(N1H1)	4.6/2.4
G20(N7) - th G8(N2H21)	2.0/2.0	G21(N7) - G15(N2H21)	2.7/2.0	G22(N7) - th G10(N2H21)	4.6/2.0
G16(O6) - G20(N1H1)	1.9/2.4	G15(O6) - G3(N1H1)	2.4/2.6	G14(O6) - G22(N1H1)	2.7/2.4
G16(N7) - G20(N2H21)	2.0/1.9	G15(N7) - G3(N2H21)	2.0/1.9	G14(N7) - G22(N2H21)	2.1/1.9
G4(O6) - G16(N1H1)	2.7/2.1	G3(O6) - th G9(N1H1)	3.5/2.1	G2(O6) - G14(N1H1)	2.0/2.4
G4(N7) - G16(N2H21)	2.0/1.9	G3(N7) - th G9(N2H21)	3.3/1.9	G2(N7) - G14(N2H21)	2.0/2.1

^aDistances are calculated in the MD representative frame. Atom names follow the AMBER force field.

display imino proton signals indicative of G4 formation for all sequences modified at external tetrad positions, albeit with evidence of partial destabilization and/or conformational heterogeneity.

In sharp contrast to the external positions, labeling at positions 9 and 21 in the middle G-tetrads provided strongly altered CD spectra (Figure S2A,B) and large drops in *T_m* values (Figure S2C,D) as well as broad peaks in the ~10–12.7 ppm region of the 1D ¹H NMR spectra (Figure S2E,F), suggesting that thG at these positions largely alters G4 folding. The strongest impact was at position 21, where the CD and NMR spectra resembled those of unfolded sequences, and no *T_m* value could be determined, suggesting that thG at this position prevented the folding of G4 structures. However, hTel22 labeled with thG at position 3 and folded in K⁺ buffer retains partial folding into a hybrid G4 topology, as indicated by a characteristic CD spectrum (Figure S2B) and relatively sharp imino peak protons in the NMR spectrum (Figure S2F), despite a 28 °C drop in its *T_m* value. On native PAGE gels, the corresponding bands migrated more slowly than hTel22 WT bands in both Na⁺ and K⁺ buffers (Figure S1A–D), in line with less compact or partially unfolded structures.

Overall, our data suggest that thG incorporation into external tetrads preserves the G4 antiparallel topology and stability in Na⁺ buffer, while it is less well tolerated in the hybrid topology formed in K⁺ buffer. Substitutions within the middle tetrad result in a markedly larger destabilization penalty than those in the external tetrads, and this effect is strongly position dependent. Thus, the structural and thermodynamic impact of thG incorporation in hTel22 is both position- and topology-dependent.

3.2. MD Simulations and QM Calculations of hTel22 G4 Labeled by thG

To structurally interpret our experimental observations, we performed MD simulations for the unlabeled hTel22 WT and the hTel22 sequences bearing thG substitution within external tetrads (at positions 8 and 10) and the middle tetrad (at position 9). The NMR solution structure of hTel22 in antiparallel topology (PDB-ID: 143D)³⁹ was used as a structural template to generate thG-labeled forms in Na⁺ buffer. Conformational stability was evaluated by monitoring the root mean-square deviation (RMSD) along MD simulation time, while cluster analysis of MD frames was used to select a representative structure of the most populated cluster of MD

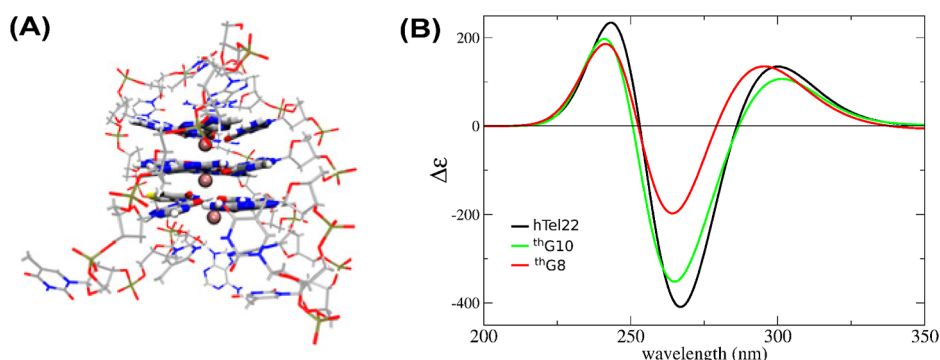


Figure 4. QM/MM prediction of the Circular Dichroism spectra of thG8- and thG10-labeled hTel22. (A) Schematic description of the computational model adopted to study the thG10-labeled hTel22. Nucleobases treated at the QM level are depicted as sticks, whereas the MM system is depicted with lines. (B) Computed CD spectra for hTel22 WT (black line), hTel22 with thG in position 10 (green line) and hTel22 thG in position 8 (red line), according to PCM/AMBER/M052X/6-31G(d) QM/MM calculations.

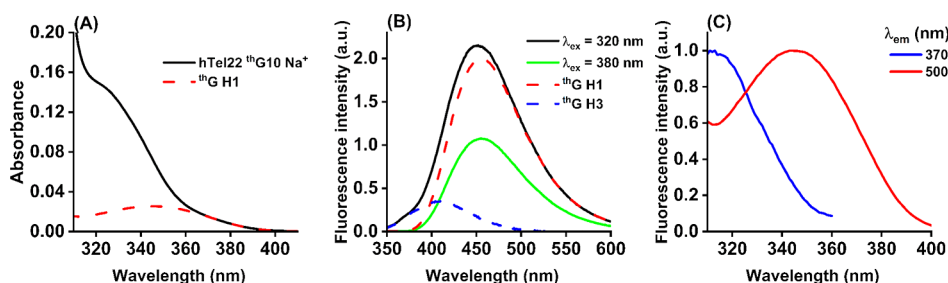


Figure 5. Spectroscopic characterization of hTel22 labeled by thG at position 10 in Na⁺ buffer. (A) Absorption spectra of 35 μM thG10-labeled hTel22 G4 (black). The excitation spectrum at λ_{em} = 500 nm (red spectrum in panel C) was normalized at the red edge to determine the contribution of thG-H1 tautomer (dashed red), as previously described.⁴⁶ (B) Emission spectra recorded at λ_{ex} = 320 nm (black) and λ_{ex} = 380 nm (green). By normalizing the red edge of the two spectra, the contributions of thG-H1 (dashed red) and thG-H3 (dashed blue) were determined. (C) Excitation spectra were recorded at λ_{em} = 370 and 500 nm, and normalized at their fluorescence intensity maxima. All spectra were recorded in 10 mM Tris–HCl buffer (pH 7.5), 100 mM NaCl at 20 °C.

frames for speculating about the impact of thG on the G4 structure.

For hTel22 labeled at position 8 with thG, visual inspection of the representative MD frame highlighted a slight distortion of the lower tetrad planarity, because of the bending of thG toward the middle Na⁺ ion within the G-tetrads (Figure 3A). Comparison with the native G in hTel22 WT revealed that this thG movement is triggered by the loss of H-bonding with the G residue at position 4 in the G-tetrad (due to N7 replacement by CH in thG) and by the steric hindrance of the thG sulfur atom in position 8 (Figure 3D). This also results in a subtle change of the canonical Hoogsteen H-bonds within the G-tetrad (compare Figure 3D with Figure S4A, Table 1). However, thG preserves the conformational stability of the three G-tetrads for the entire MD simulation time (Figure S3A).

In contrast, a pronounced destabilization was observed when thG was substituted to G in position 9, which triggered a marked distortion of the middle tetrad, as well as the flanking external tetrads (Figure 3B). The representative MD frame exhibited significant positional oscillations as compared to hTel22 WT, leading to a loss of tetrad planarity and partial unfolding of the two other tetrads, as also highlighted by the RMSD profile of the G-tetrads (Figure S3B). Besides a dramatic change in the Hoogsteen H-bond network (compare Figure 3E with Figure S4B), thG inclusion in position 9 increases the distance between G bases within the G-tetrads (Table 1), which further contribute to destabilization and unfolding. This predicted strong impact of thG on hTel22 G4

structure appears fully consistent with its experimentally observed impact on CD and NMR spectra, as well as on *T*_m values.

For thG10-labeled hTel22 structure, the major fluctuations are confined to the upper tetrad bearing thG10, while only very minor fluctuations are observed for the middle and lower tetrads (Figure S3C). Visual inspection of the representative MD frame revealed that thG10 is positioned away from both the coordinating Na⁺ ion and the neighboring G22 residue (compare Figure 3F with Figure S4C). This displacement created space for two water molecules that form a hydrogen bond network connecting thG10 to the Na⁺ ion and G22 residue, thereby attenuating the thG10-induced displacement (Figure 3F) and changes in interatomic distances within the thG10-containing tetrad (Table 1).

The differential stability of hTel22 bearing thG in position 8 and 10 can be related to changes in the local loop environments.^{72,73} Indeed, position 8 is proximal to a lateral loop that exposes thG to a dynamic environment which can weaken G-tetrad stacking and reduce local stabilization. In contrast, position 10 is adjacent to the diagonal loop that imposes topological constraints and promotes a compact arrangement within G-tetrads. This correlates with the relatively higher stability of hTel22 labeled with thG in position 10 compared to position 8. It should be noted that no MD simulations were performed in K⁺ buffer, as the mixture of topologies observed for hTel22 WT and the modification of the populations of this mixture induced by thG labeling compromised the identification of the representative structure/

Table 2. Fluorescence Properties of thG-Labelled hTel22 G4s in Na⁺ and K⁺ Buffers^a

	Na ⁺					K ⁺				
	H1 maxima (nm)	H1 (%)	ϕ	$\langle\tau\rangle$ (ns)	θ (ns)	H1 maxima (nm)	H1 (%)	ϕ	$\langle\tau\rangle$ (ns)	θ (ns)
th G4	461 ± 1	84 ± 2	0.20 ± 0.01	9.4	2.0	459 ± 1	87 ± 1	0.24 ± 0.03	10.6	2.1
th G8	463 ± 1	90 ± 2	0.45 ± 0.02	24.7	3.0	465 ± 1	85 ± 1	0.47 ± 0.05	23.3	3.0
th G10	456 ± 1	89 ± 1	0.35 ± 0.03	14.2	2.6	451 ± 1	96 ± 2	0.33 ± 0.02	13.7	3.0
th G14	461 ± 2	88 ± 1	0.42 ± 0.06	22.9	3.0	461 ± 0	92 ± 1	0.52 ± 0.03	22	3.0
th G16	458 ± 2	89 ± 2	0.28 ± 0.03	10.6	3.0	455 ± 2	91 ± 1	0.29 ± 0.01	11.4	3.2
th G22	463 ± 2	86 ± 1	0.31 ± 0.02	16.6	3.2	462 ± 2	90 ± 1	0.41 ± 0.03	19.2	3.3

^ahTel22 sequences were labelled by thG at the external tetrads at the positions indicated. For Na⁺ and K⁺ buffers, the wavelength of emission maximum (nm) and contribution (as percentage) of thG-H1 tautomer to the total emission, as well as the fluorescence quantum yield ϕ , amplitude-weighted mean fluorescence lifetime $\langle\tau\rangle$ and rotational correlation time θ were determined. The $\langle\tau\rangle$ values were determined from the time-resolved fluorescence parameters (Table S1) of the fits of the decays in Figure S7. Excitation wavelength was 370 nm. The θ values were obtained from the fits of the anisotropy decay curves in Figure S8.

topology to be used in computational structural studies for reliable correlation between *in silico* and experimental data.

Furthermore, we have computed the absorption and CD spectra of thG8- and thG10-labeled hTel22 in Na⁺ buffer, based on the reference structures issued by MD simulations (Figure 4A). As shown in Figure 4B, our computational approach reproduces the shape of hTel22 CD spectrum, typical of antiparallel G4. Incorporation of thG at both positions does not change the shape of the spectrum and does not provide any CD signal in the spectral region where thG absorbs. Overall, MD and QM calculations support our experimental data, showing that replacing G residues by thG in the external tetrads preserves hTel22 G4 integrity more effectively than replacing G residues within the middle G-tetrad.

3.3. Spectroscopic Properties of thG in hTel22 G4s in Na⁺ and K⁺ Buffers

We next investigated the photophysical properties of thG incorporated within the external tetrads of hTel22, at positions 4, 8, 10, 14, 16, and 22. The absorption spectra of all labeled hTel22 sequences exhibited a main band around 330 nm and a red-shifted shoulder extending beyond 360 nm (Figures S5A; S5 and S6, panels A, D, G, J, M; and Figure S6P). Furthermore, the emission (Figures S5B; S5 and S6, panels B, E, H, K, N; and Figure S6Q) and excitation (Figures S5C; S5 and S6, panels C, F, I, L, O; and Figure S6R) spectra further revealed two emissive species, a blue-shifted one with an excitation maximum at ~315 nm and emission maximum at ~400 nm (blue spectra) and a red-shifted one with excitation maximum at ~350 nm and emission maximum at ~460 nm (red spectra). By analogy to the free thG nucleoside^{48,74} and thG-labeled mismatched DNA duplexes,⁴⁸ the blue- and red-shifted emissive species could be attributed to the thG-H3 and thG-H1 tautomers, respectively (Figure 1A). Deconvolution of the emission spectra upon 320 nm excitation, where both tautomers absorb, indicated that 85–96% of the total emission intensity originates from the thG-H1 tautomer (Table 2), far above the nearly equal contribution of the thG-H1 and thG-H3 tautomers in the emission spectrum of free thG in buffer.⁴⁸ This stabilization of thG-H1 tautomer in hTel22 G4 is somewhat less pronounced than in matched thG-labeled duplexes, where the Watson–Crick base pairing with opposite C completely shifts the equilibrium toward the thG-H1 tautomer, abolishing thG-H3 emission.⁴¹ As for the thG-labeled duplexes, nonemissive charge transfer (CT) states of the thG-H1 tautomer^{46,47,49} may also contribute to the absorption spectrum of the labeled hTel22 G4s, but because the

absorption spectra of these CT states and thG-H3 tautomer overlap, their relative contribution is difficult to establish and will be investigated in future work. Importantly, the emission maxima of the thG-H1 tautomer (451–465 nm) in G4s are very similar to those observed in matched DNA duplexes. In both cases, the emission is blue-shifted by ~10 nm compared to free thG-H1 in water (470 nm), and closely resembles the emission maxima of free thG-H1 in methanol and ethanol,⁴⁸ suggesting that the thG-H1 tautomer is well protected from aqueous solvent and therefore, is efficiently stacked in the hTel22 G4 structure.

Since the thG-H1 tautomer is by far the predominant emissive species of thG-labeled hTel22 G4s and can be selectively excited at >360 nm, all subsequent photophysical measurements were performed using excitation at 370 nm. The ϕ values of the hTel22 sequences with thG incorporated into external tetrads were found to be highly dependent on the context, ranging from 0.20–0.45 in Na⁺ and 0.24–0.52 in K⁺ (Table 2). This is in marked contrast to the thG-labeled matched duplexes where the ϕ values were substantially lower (~0.15) and almost independent of the sequence context.⁴⁷ The lowest ϕ values (0.2–0.29) were observed for positions 4 and 16 in the lower tetrad of the major antiparallel G4 topology in Na⁺ and K⁺ buffers. In contrast, the highest ϕ values (0.45–0.52) were observed for position 8 and 14 in the upper tetrad, approaching the value of the free nucleoside in water.^{41,43} The strong emissive character of thG and its dependence on the context were mirrored by the amplitude-weighted mean fluorescence lifetime $\langle\tau\rangle$ obtained from the time-resolved fluorescence decays (Figure S7). For positions 4 and 16, the mean lifetime was shortest (~10 ns), as a result of the contribution of an additional fast decay component (τ_1 , 1.1–1.6 ns) likely translating an additional nonradiative decay pathway, absent at other positions (Table S1). In contrast, the largest values of $\langle\tau\rangle$ (22–25 ns) were observed for positions 8 and 14, which exceed the lifetime (20 ns) of free thG H1 tautomer in water.^{31,41} Importantly, very high values were observed for the long-lived lifetime (18.5–28.5 ns) in the two- or three-exponential decays of all labeled G4s (Table S1). These values are substantially longer than the corresponding lifetime values (10–12 ns)⁴⁷ observed in thG-labeled DNA duplexes, indicating that thG-labeled G4s can be readily distinguished from thG-labeled duplexes through time-resolved measurements.

Next, we measured the time-resolved fluorescence anisotropy decays of the labeled hTel22 sequences to investigate the mobility of thG within the G4s (Figure S8). At all positions, the

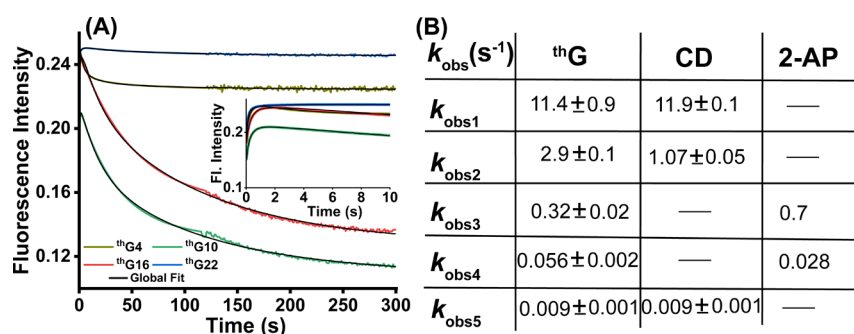


Figure 6. Na^+ -induced folding kinetics of hTel22 labeled by $^{\text{th}}\text{G}$ at positions 4, 10, 16 and 22. (A) Folding kinetic traces of $1 \mu\text{M}$ hTel22 labeled by $^{\text{th}}\text{G}$ at positions 4, 10, 16, and 22 within the external tetrads, following rapid mixing with 100 mM NaCl in 10 mM Tris buffer (pH 7.5). Solid black lines describe the global fits of the kinetic traces using eq 10 and the k_{obsi} values given in panel (B). Inset: zoom of the kinetic traces within the first 10 s. (B) Comparison of the observed rate constants (k_{obsi}) obtained from the global fit with those previously determined on the same system by CD spectroscopy⁷⁶ and 2-aminopurine (2-AP) fluorescence.⁷⁵

time-resolved anisotropy decays of $^{\text{th}}\text{G}$ were well fitted with a single correlation time in both Na^+ and K^+ buffers. For five positions, the rotational correlation time (θ) value ranged between 2.6–3.2 ns, whereas for position 4 its value was slightly shorter (~ 2.0 –2.1 ns). These rotational correlation times are in reasonable agreement with the theoretical rotational correlation time of the hTel22 sequence at 25 °C calculated by the HYDROPRO program (~ 4 ns)⁷⁵ using the PDB-143D structure of hTel22 in its antiparallel topology. Introducing an additional exponential component did not improve the χ^2 value or the distribution of the residuals, indicating the absence of a short-lived correlation time and therefore, the absence of any local $^{\text{th}}\text{G}$ motion in the G4. These data support the conclusion that $^{\text{th}}\text{G}$ is stably stacked in hTel22 G4s labeled by $^{\text{th}}\text{G}$ in external tetrads and thus, only reports about the tumbling motion of hTel22.

3.4. hTel22 Folding Kinetics Monitored by $^{\text{th}}\text{G}$ Fluorescence

To demonstrate its utility, we leveraged $^{\text{th}}\text{G}$'s fluorescence to monitor the Na^+ -induced folding kinetics of the hTel22 sequence (Figure 6). For this experiment, we selected four external positions (4, 10, 16, 22) distributed across the four G-tracts of hTel22, in order to probe the folding dynamics over the full structure. Since telomeric G4 folding was previously shown to involve both fast and slow kinetic components,^{26,75–77} we combined the kinetic data recorded with a stopped-flow instrument and a fluorimeter to obtain a combined kinetic trace covering a range from few ms to hours (Figure 6A). In these traces, the initial fluorescence increase caused by the addition of 100 mM NaCl remained unresolved, indicating that it corresponds to a very fast step occurring during the 2.2 ms dead time of the stopped-flow set up. This unresolved fluorescence increase was followed by a marked resolved increase within 1 s (Figure 6A inset), followed by a slow decrease. All four kinetic traces were globally fitted to a five-exponential model with shared rate constants (k_{obsi}) across all traces, yielding an excellent χ^2 value (>0.99). The obtained k_{obsi} values are in excellent agreement with those previously reported on the same system using CD spectroscopy⁷⁶ or the fluorescence of 2-aminopurine (2-AP) replacing an A residue at position 13 in hTel22⁷⁵ (Figure 6B). Consequently, our data show that the fluorescence signal from $^{\text{th}}\text{G}$ in any of the G-tracts allows accurate monitoring of the folding kinetics of hTel22, providing, based on a single

signal, all the k_{obsi} values previously determined by a combination of techniques.

4. DISCUSSION

The main objective of this study was to establish thienoguanosine ($^{\text{th}}\text{G}$) as a fluorescent reporter for probing telomeric G4s. By substituting nine different guanine positions across both external and middle tetrads of the hTel22 sequence, we clearly identified position-dependent effects. In Na^+ buffer, all substitutions within external tetrads (G4, G8, G10, G14, G16, and G22) preserved the native antiparallel topology, as confirmed by CD spectroscopy, with only marginal changes in melting temperature, except for position 8 where a significant drop was observed. Although CD provides strong evidence that the topology is preserved in Na^+ buffer, a more detailed structural investigation would be required to determine whether the exact native structure is maintained upon $^{\text{th}}\text{G}$ substitution and to fully elucidate the role of $^{\text{th}}\text{G}$ within the folded structure. This could, in principle, be addressed using NMR-derived structural restraints obtained from 2D NMR spectroscopy. However, such experiments were not feasible due to the poor spectral resolution of highly concentrated samples.

In K^+ buffer, $^{\text{th}}\text{G}$ substitution at the external tetrads shifted the native hybrid topology into a predominant antiparallel topology (Figure 2B). $^{\text{th}}\text{G}$ substitution likely disrupts hybrid G-tetrads, favoring a stable antiparallel G4 topology ($T_m > 50$ °C). In both the buffers, the proper stacking of $^{\text{th}}\text{G}$ in the external tetrads was evidenced by their blue-shifted emission maximum and the absence of any short rotational correlation time, associated with a local motion of $^{\text{th}}\text{G}$ in the G4 structure.

In contrast, substitutions within the middle tetrad caused major perturbations. CD spectra showed large deviations from the native antiparallel form, sharp drops in T_m , and broadening or disappearance of the imino proton resonances, indicating substantial G4 destabilization. MD simulations provided a detailed structural rationale in this context. At external tetrads, $^{\text{th}}\text{G8}$ and $^{\text{th}}\text{G10}$ retained the global G4 fold and required only local adjustments. In our simulations, $^{\text{th}}\text{G8}$ bent inward toward the central Na^+ ion, whereas $^{\text{th}}\text{G10}$ shifted slightly outward, generating a small cavity stabilized by a structured network of two water molecules bridging a Na^+ ion and G22. These local adaptations remained confined to the substituted tetrad, which is fully consistent with the low degree of destabilization and CD spectra close to that of hTel22. In contrast, $^{\text{th}}\text{G9}$ in the

Table 3. Overview of Fluorescent Guanine Analogues Incorporated Into Various G4 Structures^a

Nucleobase	Structure	Sequence	Positions	ΔT_m		Topology		λ_{ex} and λ_{em}	QY(ϕ)	
				NaCl	KCl	NaCl	KCl		NaCl	KCl
8-Vinyl-2'-deoxyguanosine (VdG)		hTel22	G14	+4.0	0.0	NA	NA	277 and 400 nm	0.12	0.12
			G15	+2.0	0.0				0.12	0.12
			G16	+3.0	+5.0				0.12	0.12
C8-furyl-dG (^{fur} dG)		hTel22	G3	+6.7	+3.3	Antiparallel	Antiparallel	290 and 379 nm	NA	NA
			G4	+0.6	-1.1	Antiparallel	Antiparallel			
			G8	+7.8	+12.0	Antiparallel	Hybrid 2			
			G10	+2.8	+0.5	Antiparallel	Hybrid			
8-(4"-cyanophenyl)-deoxyguanosine (CNP ^h dG)		mTBA	G5	+8.0	+7.1	NA	NA	325 and 452 nm	NA	NA
			G8	NA	-6.3	NA	NA			
8-(2-pyridyl)-2-deoxyguanosine (2PyG)		hTel23	G9	+8.3	+9.8	NA	Hybrid	300 and 415 nm	0.08	0.09
			G23	-1.9	-8.4	Antiparallel	Antiparallel		0.10	0.05
			G17	-9.4	-4.4	NA	Parallel		0.03	0.02
8-[2-(pyrid-4-yl)-ethenyl]-2-deoxyguanosine (4PVG)		hTel23	G9	+7.6	+8.2	NA	Hybrid	355 and 490 nm	0.03	0.04
			G23	-6.0	-6.2	Antiparallel	Hybrid		0.06	0.04
8-(2phenyl ethenyl)-2-deoxyguanosine (StG)		hTel23	G9	+7.1	+5.4	NA	Hybrid	340 and 450 nm	0.37	0.44
			G23	-0.8	-8.2	Antiparallel	Hybrid		0.37	0.33
6-methyl isoxanthopterin (6MI)		hTel26	G8	-10.0	-13.0	NA	NA	350 and 430 nm	0.02	0.04
Thienoguanosine (th G)		hTel22	G4	-2.0	-6.0	Antiparallel	Antiparallel	370 and 460 nm	0.20	0.24
			G8	-17.0	-17.0				0.45	0.47
			G10	+1.0	-8.0				0.35	0.33
			G14	+1.0	-10.0				0.42	0.52
			G16	-2.0	-11.0				0.28	0.29
			G22	-4.0	-10.0				0.31	0.41

^aAll G4 sequences were telomeric sequences, with the exception of the modified thrombin-binding aptamer (mTBA) used with CNP^hdG. For each G analogue, the labelled positions, the impact of the analogues on melting temperature (ΔT_m) and G4 topology, their excitation (λ_{ex}) and emission maxima (λ_{em}), and their quantum yield (ϕ) in Na⁺ and K⁺ buffers are given. Position dependent changes in ϕ values highlight the environmental sensitivity of the probes. To facilitate direct comparison, the corresponding data obtained on thG-labelled hTel22 sequences in this study are shown in the last row. NA indicates data not available.

middle tetrad disrupted Hoogsteen hydrogen bonds, induced large-scale oscillations and loss of tetrad planarity, leading to partial unfolding that propagated to adjacent tetrads.

This positional hierarchy, where external tetrads are tolerant and middle tetrads are highly sensitive, is fully consistent with the literature on guanine analogues and guanine damage products.^{78–81} Studies with 7-deazaguanine (7dzG), a close analogue of thG that also lacks the N7 atom, oxidative lesions (8-oxoguanine, O6-methylguanine), and natural base substitutions (G → A/T/C) reported the same trend. Although any of the substitution impacts G4 stability, the degree of destabilization is consistently greatest when the middle tetrad is modified.^{78–81} It should be noted that in the seminal work studying the impact on G4s of replacing G residues with a large set of G analogues,⁸¹ 7dzG was found to have one of the most detrimental impact. However, this study was conducted on tetramolecular G4s, where consequently all G residues in a tetrad were replaced by 7dzG. In contrast, in excellent agreement with our data, single substitutions of 7dzG were found to marginally impact the affinity of an intramolecular telomeric G4 for the BG4 antibody.⁸⁰ Another interesting example is that of 8-oxoguanine, which contains an O-substitution at C8 and protonation at N7, eliminating the

hydrogen bond acceptor function at this position, as with thG. Despite the loss of a hydrogen bond and potential steric interference associated with the N7–H group, 8-oxoguanine is tolerated at certain positions in a modified hTel22 sequence, inducing a distorted G tetrad.⁷⁹ Similar to 8-oxoguanine, thG contains a C–H group at position 7, which may introduce steric hindrance, as well as a sulfur atom at position 8 that alters its hydrogen-bonding properties. It is therefore not surprising that thG substitution can be accommodated in a position-dependent manner, albeit with local structural distortions, in agreement with MD simulations (Figure 3). In addition, alternative hydrogen-bonding arrangements, such as base triads involving thG, may also form. A more detailed structural investigation will be required to fully elucidate the structure of thG within the folded structure.

Beyond structural compatibility, thG offers major spectroscopic advantages over other probes. When thG is incorporated in the external tetrads, thG retains high quantum yield in both Na⁺ and K⁺ buffers. It was found that thG fluorescence was largely dominated by the thG-H1 tautomer, which is likely favored over the thG-H3 tautomer due to its ability to form an additional Hoogsteen base pair (Figure 1A). The thG labeled hTel22 G4s are also characterized by remarkably long

fluorescence lifetimes. At all positions, the long-lived lifetime value in the range 18.5–28.5 ns is suitable for selective detection with high signal-to-noise ratio, even in complex media such as the cellular context, by using a time-gated detection scheme or a fluorescence lifetime-based setup. Moreover, this long-lived lifetime value is at least twice higher than that of thG in matched DNA duplexes, which will help to easily distinguish G4s from duplexes. This distinction is particularly important in the context of G4-targeted drug discovery, where a major and long-standing challenge is achieving high selectivity for G4s over DNA duplexes, as many reported G4 ligands bind both structures with comparable affinity.^{23,24,29} Moreover, ϕ and $\langle\tau\rangle$ values are highly dependent on the position occupied by thG in the G4, indicating an exquisite sensitivity of thG fluorescence to its environment. Full understanding of the photophysical processes governing this sensitivity in relation with G4 topology and identity of thG neighbors will require further spectroscopic characterization with QM calculations as part of a future study currently underway.

To further illustrate the relevance of using thG as a substitute for G residues in G4s, we compare it to fluorescent nucleoside analogues currently used to study G4s, with a particular focus on G analogues. Most of these analogues^{35,37,82} are substituted at the C8 position of guanine, as this site on the outer face does not participate directly in Hoogsteen hydrogen bonding or cation coordination.^{83,84} Similar to thG, 8-vinyl-2'-deoxyguanosine (VdG) (Table 3) only minimally destabilizes G4s, but its ϕ value (~ 0.12) was found to be lower than for thG and identical at the three positions tested, suggesting a lower sensitivity to its microenvironment.³⁶ Another example is the 8-furyl-2'-deoxyguanosine (^{Fur}dG). When incorporated in hTel22, it induces position-dependent changes in T_m value, ranging from ± 1 °C to $+12$ °C. Moreover, like thG, it preserves the antiparallel topology in Na⁺ buffer at all positions and favors the antiparallel topology at two positions in K⁺ buffer. ^{Fur}dG was reported to exhibit a “turn-on” response in G4s compared to duplexes, with a ~ 10 -fold increase in emission intensity. However, since its ϕ values were not reported, its comparison to thG is difficult in this critical point.^{35,82,84–86} Push–pull analogues of 8-aryl guanine such as ^{CNPh}dG were also developed. While ^{CNPh}dG was found to be well emissive in duplexes, it was strongly quenched in G4s.^{35,84} Several 8-substituted guanosine analogues such as 2PyG, 4PVG, and StG were also introduced (Table 3).^{35,37} Incorporated in telomeric G4 sequences, they induced a larger change in stability than thG in Na⁺ buffer and a comparable change in stability in K⁺ buffer. While 2PyG changed the G4 topology at several positions in K⁺ buffer, 4PVG and StG preserved the G4 topology in both Na⁺ and K⁺ buffers. Moreover, while 2PyG and 4PVG display rather low ϕ values (0.02–0.10) in G4s, StG achieves ϕ values ($\phi \sim 0.37$ –0.44) comparable to thG. However, due to the limited number of positions tested, the dependence of StG's ϕ values on its substitution site is difficult to appreciate. Finally, other guanine analogues modified at the less favorable C6 position, such as the 6MI analogue have also been tested, but their incorporation into G4s causes large decreases in T_m value and low ϕ values.³⁸

Based on this comparison, thG appears as a highly suitable probe which, thanks to its isostericity with guanine, can replace the latter in G4 external tetrads with limited structural alteration, while exhibiting high ϕ , a very long $\langle\tau\rangle$ and high

sensitivity to the environment. Moreover, the absence of any bulky side groups at positions 2 or 8 in thG is likely a key advantage over other G analogues for accurately and unbiasedly monitoring the interaction of G4s with ligands or target proteins. Although it can be anticipated that isothiazologuanosine, which is identical to thG but possesses the N7 atom to form the complete Hoogsteen bond network,⁸⁷ could preserve the structure of G4s even better, it is difficult at this stage to predict its fluorescence properties and sensitivity to the environment in G4s. Furthermore, its tedious synthesis will require considerable effort to obtain it in the phosphoramidite form necessary for the chemical synthesis of labelled oligonucleotides.

To further demonstrate the potential of thG in monitoring G4 structural and dynamic changes, we used thG as a fluorescence reporter for monitoring G4 folding kinetics. To the best of our knowledge, no guanosine analogue has yet been reported to monitor the folding dynamics of G4s as direct participant of the tetrads. In our folding kinetics, we examined four external positions (4, 10, 16, and 22) distributed across the four G-tracts of hTel22, all of which display an initial fast fluorescence increase followed by a slower decrease that can be fitted with a five-exponential function. The observed rate constants were found to be in full agreement with the observed rate constants previously determined by using CD spectroscopy and 2-AP fluorescence in the loop. A key advantage of thG is that a single fluorescence observable reports on all kinetically resolved steps of G4 folding in a single experiment. In contrast, CD spectroscopy and 2-aminopurine (2-AP) fluorescence are sensitive only to certain aspects of the folding process—primarily global secondary-structure formation or loop rearrangements, respectively—and therefore provide only part of the complete kinetic information. Due to its high compatibility with G4 structures and its sensitivity to the environment, thG appears to be sensitive to both local changes resulting from limited structural modifications and more global changes associated with the formation of the folded G4. Therefore, thG appears as a highly promising reporter for studying folding and unfolding dynamics of G4s.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acscchembio.6c00168>.

Native PAGE of wild-type and thG-labeled hTel22 sequences in Na⁺ and K⁺ buffers; Structural characterization of hTel22 G4s labeled by thG in middle tetrads; RMSD profiles from MD simulations; interaction network within G-tetrads in hTel22 WT; Spectroscopic characterization of hTel22 labeled by thG at external tetrad positions in Na⁺ buffer; Spectroscopic characterization of hTel22 labeled by thG at external tetrad positions in K⁺ buffer; Time-resolved fluorescence decays of thG-labeled hTel22 G4s, Time-resolved decay parameters for thG-labeled hTel22 G4s and Time-resolved fluorescence anisotropy decays of thG-labeled hTel22 G4s (PDF) (PDF)

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ABBREVIATIONS

G4s, G-quadruplexes; hTel22 WT, wild-type Human telomeric sequence; thG, thienoguanosine; ϕ , quantum yield; CD, Circular dichroism; PAGE, Polyacrylamide gel electrophoresis; NMR, Nuclear magnetic resonance; cryo-EM, cryogenic electron microscopy; FRET, Förster resonance energy transfer; FNAs, Fluorescent nucleoside analogues; VdG, 8-vinyl-2'-deoxyguanosine; StG, 8-(2-phenylethenyl)-2'-deoxyguanosine; 2PyG, 8-(2-pyridyl)-2'-deoxyguanosine; 6-MI, 6-methylisoxanthopterin; 4PVG, 8-[2-(pyrid-4-yl)-ethenyl]-2'-deoxyguanosine; ^{Fur}dG, 8-furyl-2'-deoxyguanosine; ^{CNPh}dG, 8-(4'-cyano-phenyl)-dG; MD, Molecular dynamics; QM, Quantum Mechanical; QM/MM, Quantum Mechanics/Molecular Mechanics; DFT, Density Functional Theory; TCSPC, Time-correlated single photon counting; IRF, Instrumental response function; CT, charge transfer; T_m , Melting temperature; 7dzG, 7-deazaguanine; mTBA, modified thrombin-binding aptamer

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