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Interdependence of Pyrene Interactions and Tetramolecular G4-DNA Assembly

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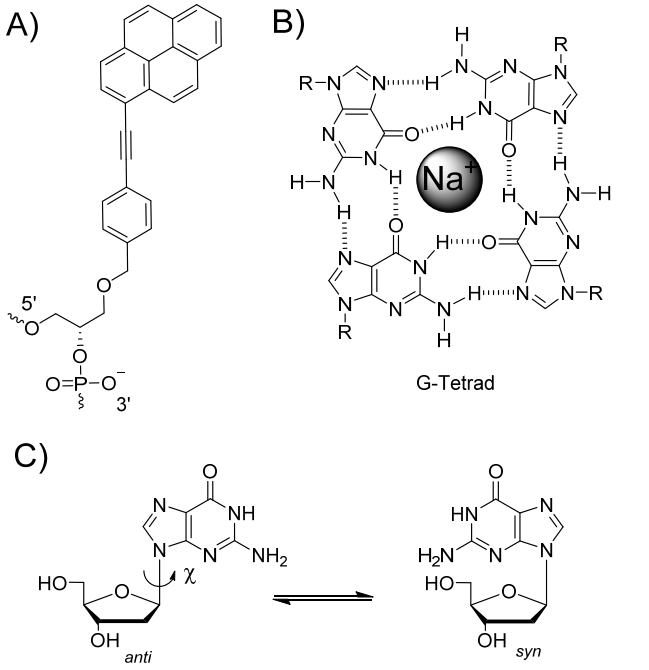
Abstract: Controlling the arrangement of organic chromophores in supramolecular architechtures is of primary importance for the development of novel functional molecules. Insertion of a twisted intercalating nucleic acid (TINA) moiety, containing phenylethynylpyren-1-yl derivatives, into a G-rich DNA sequences alters G-quadruplex folding, resulting in supramolecular structures with defined pyrene arrangements. Based on CD, NMR and ESI-mass-spectra, as well as TINA excited dimer (excimer) fluorescence emission we propose that insertion of the TINA monomer in the middle of a dTG_4T sequence (*i.e.* dTGG**X**GGT, where **X** is TINA) converts a parallel tetramolecular G-quadruplex into an assembly composed of two identical antiparallel G-quadruplex subunits stacked via TINA-TINA interface. Kinetic analysis showed that TINA-TINA association controls complex formation in the presence of Na^+ but barely competes with guanine-mediated association in K^+ or in the sequence with the longer G-run (dTGGG**X**GGGT). These results demonstrate new perspectives in the design of molecular entities that can kinetically control G-quadruplex formation and show how tetramolecular G-quadruplexes can be used as a tuneable scaffold to control the arrangement of organic chromophores.

Introduction

Guanine-rich sequences are known to form highly stable structures called G-quadruplexes.[1] There is growing experimental evidence that cellular proteins recognise and bind to such G-rich sequences with high-affinity and specificity.[2] Unfortunately, guanine-rich oligonucleotides (GROs) are highly polymorphic and can adopt various topologies depending on the presence of metal ions, the oligonucleotide concentration in solution etc.[1e, 3] Therefore, it is becoming important to characterise newly discovered folding mechanisms of guanine-rich oligonucleotides (GROs). This information can further be exploited to direct GROs folding into desired assemblies for their application in biology,[4] bio/nanotechnology[5] and medicine.[6] In this regard, chemistry offers the means of manipulating GROs assemblies in ways not possible using standard nucleotides.[7]

Recently, we[8] and others[9] reported that the assembly of a GRO sequence can be affected by the covalent introduction of an intercalating moiety. When a pyrene moiety in the structure of intercalating nucleic acids was inserted on the top or the bottom of G-quartet assemblies, the resulting G-quadruplex structures were more thermally stable and more biologically active than unmodified complexes.[8a, 8c, 8e, 9c, 9d] In contrast, significant destabilisation of G-quadruplexes was observed when a TINA monomer was inserted in the middle of G-runs in DNA triplex-forming oligonucleotides (TFOs).[8b, 8d]]



Scheme 1. A) Structure of twisted intercalating nucleic acid monomer (TINA, monomer **X**); B) Hydrogen bond formation in a G-tetrad; C) drawing of *anti* and *syn* conformations of glycosidic bonds for 2′-deoxyguanosine.

In order to gain further insight into the effect of the TINA moiety on G-rich sequences we decided to investigate constructs of TINA in a well-studied sequence dTGGGGT (dTG_4T) from the *Tetrahymena* telomere. In the presence of Na^+ and K^+ ions the unmodified sequence forms a tetramolecular parallel G-quadruplex with all guanines arranged in *anti* glycosidic conformation (Scheme 1C) and it is characterised by slow association kinetics.[10] To our surprise, a covalent insertion of a TINA monomer in the middle of the dTG_4T sequence (*i.e.* dTGG**X**GGT, where **X** is TINA monomer) does not prevent G-quadruplex formation. Instead, based on circular dichroism (CD), electrospray ionisation mass spectrometry (ESI-MS) and NMR, a novel G-quadruplex topology is proposed in which two G-quadruplex subunits are stacked via a TINA-TINA interface in Na^+. Kinetic analysis reveals that TINA-TINA directed association is prevalent in the presence of Na^+ for the TGG**X**GGT sequence whereas G-mediated association is dominant in the sequence with a longer G-tract or in K^+ solutions. Analysis of CD spectra shows that TINA's position in the dTG_4T sequence determines the orientation of TINA chromophores which leads to significant changes in fluorescence emission spectra. These results highlight the capabilities of G-quadruplex scaffolds in directing the arrangement and subsequent electronic interactions of organic chromophores. This is also an exciting example of a new G-quadruplex topology governed by non-guanine moieties which may ultimately lead to the expansion of the G-quadruplex alphabet.

Results and Discussion

We inserted the TINA moiety at the 5'-end of the dTG_4T sequence, in the middle of the G-tract (once and twice), and between T and dG at the 5'-end (Table 1). In line with previous research on tetramolecular G-quadruplexes bearing organic chromophores at the 5'-end of the sequence,[8e, 11] our synthetic constructs **XTG** and **TXG** formed more thermally stable G-quadruplexes which also assembled significantly faster and at lower oligonucleotide concentration in comparison with the unmodified sequence (Figure 1, Table 1 and Figure S4 in the Supporting Information). According to native gel electrophoresis, **XTG** also formed higher order aggregates in K^+ but not in Na^+ solution (Figure S1 and Table S2 in the Supporting Information). Circular dichrosim (CD) spectra of **XTG** and **TXG** were characteristic for parallel G-quadruplexes[12] (group I according to classification proposed by Karsisiotis *et al*[13]) with a positive ellipticity at 262 nm and a negative ellipticity at 238 nm in which all guanosines adopt *anti* glycosidic conformation[14] (Figure 1A, B). In contrast, CD spectra of the dTG_4T constructs with single and double TINA insertion in the middle of the sequence were drastically different, especially in the presence of 110 mM NaCl. Maxima at 294 and 244 nm and a minimum at 265 nm seen for **GXG** and **GXX** in NaCl are typical signatures of antiparallel G-quadruplexes (group III)[13] in which guanosines adopt both *syn* and *anti* glycosidic conformation (grey lines in Figures 1C and D).[14] In 110 mM KCl, both sequences exist in equilibrium between parallel (predominant) and antiparallel topology resembling group II G4-DNAs.[13] However, insertion of the TINA monomer in the middle of the sequence with six guanosines (**G3X**) led to the formation of a complex with CD spectra typical for parallel G-quadruplexes (Figure 1E, Table S2 in the Supporting Information).

Table 1. Sequences synthesised, thermal stability[a] and the association rate constant[b] for the selected assemblies.

Abbreviation	Sequence, DNA, 5'-3'	$T_{½}$ (°C), NaCl melting / annealing	$T_{½}$ (°C), KCl melting / annealing	k_{on} (M^{-3} s^{-1})
TG_4T	TGGGGT[c]	57.5 / < 20	> 90 / < 20	~2.4×10^7
XTG	**X**TGGGGT[d]	85.0[e]/n.d[f]	> 90 / n.d	-[g]
TXG	T**X**GGGGT	83.0 / 75.0	> 90 / n.d	-
GTG	TGGTGGT	< 20	< 20	-
GXG	TGG**X**GGT	58.0 / 54.5	57.0 / 55.5	7.30×10^{11}
GXX	TGG**XX**GGT	32.0 / 31.5	35.5 / 38.5	-
G3X	TGGG**X**GGGT	> 90 / 68.5	>90 / 69.5	9.38×10^{11}
TG_6T	TGGGGGGT	-	-	8.90×10^{10}

[a] $T_{½}$ values of G-quadruplexes at 10 μM strand concentration obtained during melting and annealing processes monitoring absorbance at 260 nm for unmodified and 373 nm for modified assemblies using 0.18 °C/min temperature ramp in the presence of 110 mM NaCl or KCl in 10 mM Li cacodylate at pH 7.2. $T_{½}$ values are reported within the uncertainty of ± 1.0 °C as determined by repetitive experiments. [b] k_{on} values (± 30%) of selected assemblies were experimentally obtained by monitoring ellipticities at 265 (**G3X**, TG_6T) and 295 nm (**GXG**) using CD spectrometer in 10 mM Li cacodylate buffer and 110 mM NaCl at 20 °C, pH 7.2 (see Supporting Information). [c] Parallel G-quadruplex of TG_4T was formed at 200-300 μM strand concentration at +4 °C and then diluted to 10 μM to perform melting experiments. k_{on} value for TG_4T at 20 °C was estimated from the literature.[6] [d] **X** refers to TINA monomer. [e] a transition at lower temperatures is observed that reflects melting of aggregates (Supporting Information, Figure S3), [f] n.d. – could not be determined due to broad annealing profile, [g] not performed.

We assessed the molecularity of our complexes using ESI-MS[15] (Figure 2) and PAGE (Figure 3, see also Figure S1 and discussion in the Supporting Information). For ESI-MS analysis, complexes formed in Na^+-containing buffer were precipitated from NH_4OAc/ethanol and the pellets obtained were re-suspended in 150 mM NH_4OAc buffer, pH 7.0. CD spectra of these solutions were similar to CD spectra recorded in NaCl (Figure 2, right). According to ESI-MS spectra, single-stranded species with different charge states were detected for **XTG**, **TXG** and **GXG** sequences in the *m/z* region of 500-1200 (see Figure S12 in the Supporting Information). This suggests that a significant amount of the complexes dissociate during ionisation. Nevertheless, the ESI-MS spectra of **XTG** and **TXG** show the tetramers in two charge states (Figure 2, left). The peaks at *m/z* 1551.1 and 1861.4 correspond to the four-stranded structures, at charge states 6- and 5-, respectively. They are accompanied by tetramolecular species with three ammonium adducts. The ESI-MS spectrum of **GXG** is notably different; the peak at *m/z* 1554.0 at the charge state 3- corresponds to the **GXG** dimer: $[(\mathbf{GXG})_2 - 3H]^{3-}$ (calculated *m/z* = 1553.96). However, the peak at *m/z* 1559.9 with the 6- charge state shows that the tetramolecular complex with two ammonium adducts is formed by the **GXG** sequence (calculated *m/z* = 1559.7). The peaks at *m/z* 1871.8 and 1875.2, both with a charge state of 5-, correspond to the four-stranded complex with two and three ammonium adducts, respectively. Unfortunately, complexes formed by the **G3X** sequence did not produce a satisfactory electrospray signal under similar conditions. According to PAGE analysis, **GXG** and **G3X** assemblies have similar mobility in the native gel, both in Na^+ and K^+, which suggests that the **G3X** complex is also composed of four strands (Figure 3, see also Figure S1 and discussion in the Supporting Information).

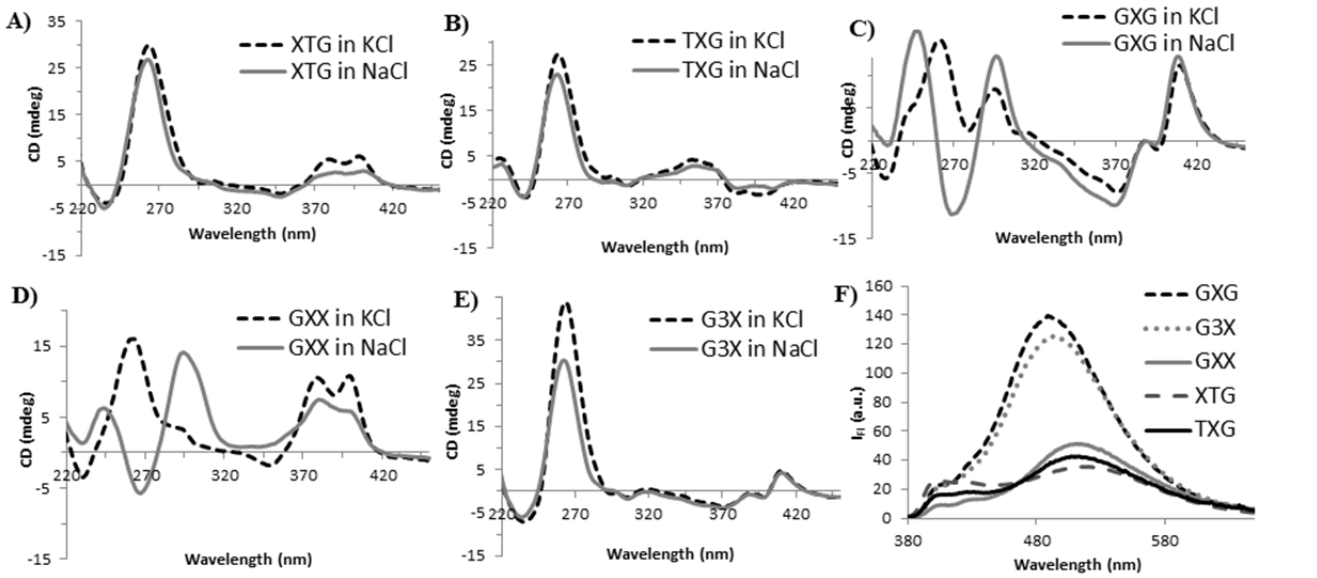


Figure 1. a)-e) Circular dichroism spectra of modified G-quadruplexes at 10 μM strand concentration in the presence of 110 mM KCl (dashed line) or NaCl (grey line) in 10 mM Li cacodylate buffer at pH 7.2, 20 °C. f) Steady-state fluorescence emission spectra of TINA-dTG$_4$T assemblies at 10 μM strand concentration in the presence of 110 mM NaCl in 10 mM Li cacodylate buffer at pH 7.2, λ_{ex} = 373 nm (all samples were adjusted to an identical optical density at an excitation wavelength of 373 nm).

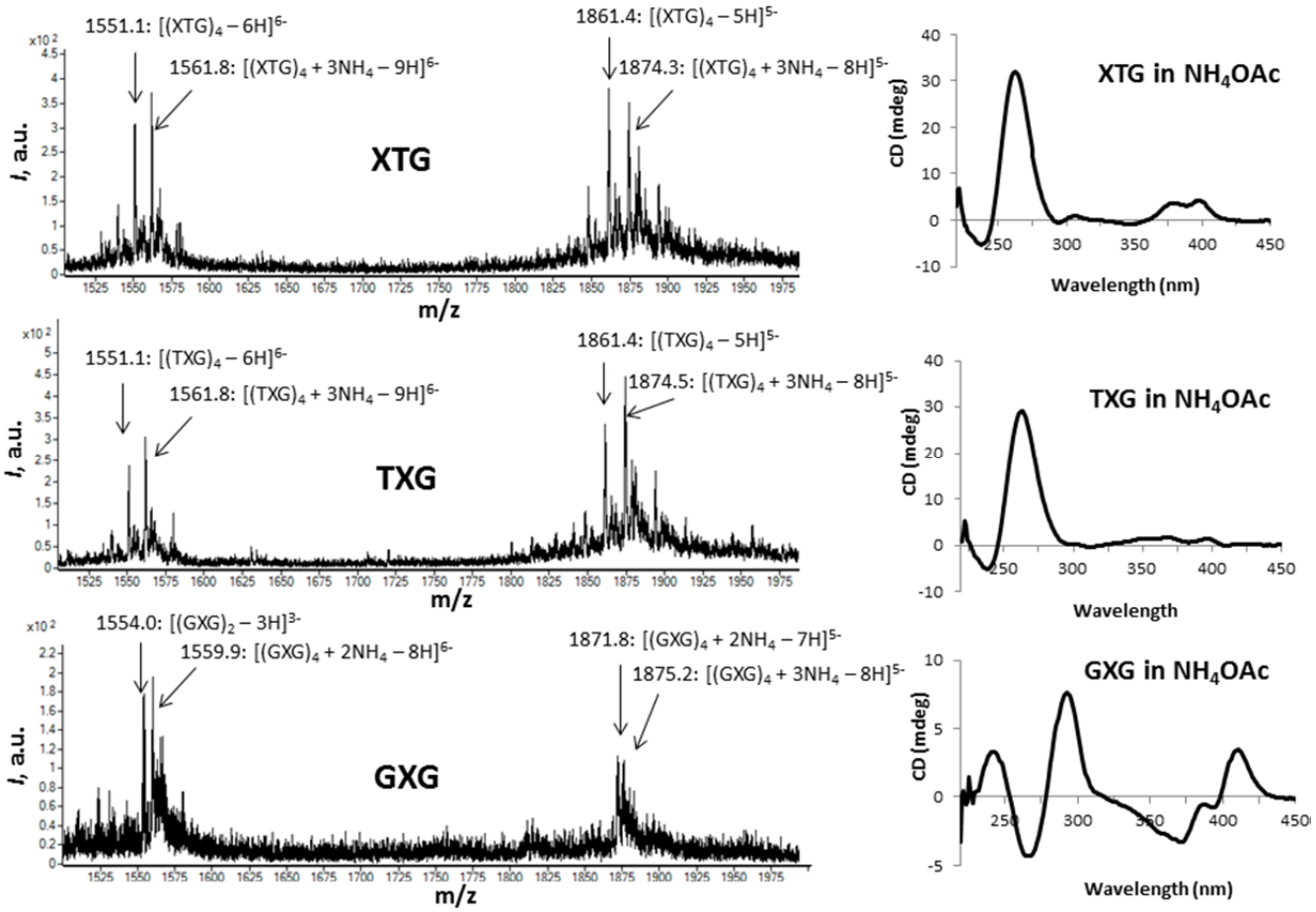


Figure 2. ESI-MS spectra (left) and CD spectra (right) of G-quadruplexes formed by **XTG**, **TXG** and **GXG** sequences in 150 mM ammonium acetate buffer (pH 7.0). CD spectra were recorded at 20 °C. The same stock solution of G4-DNAs (200-300 μM) was diluted for ESI-MS (20 μM) and CD spectroscopy (10 μM DNA) in 150 mM ammonium acetate buffer (pH 7.0).

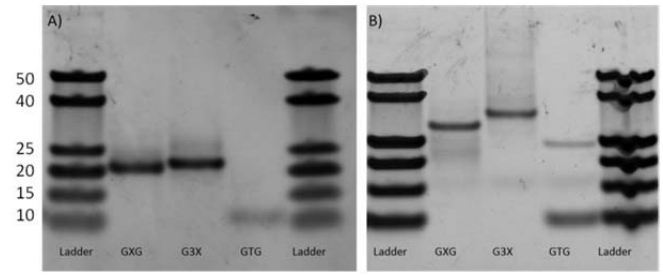


Figure 3. Native PAGE (20 %) analysis of oligonucleotides (100 μM) in the presence of 110 mM NaCl (A) or 110 mM KCl (B) in 50 mM HEPES buffer at pH 7.2. Ladder contains 10-, 15-, 20-, 25-, 40- and 50-mer oligothymidylates.

These results demonstrate that tetramolecular complexes are formed by TINA-TG_4T sequences and that **GXG** forms a stable dimer that may either be a product of G4-DNA dissociation or an intermediate during the assembly into tetramolecular complex. It is worth mentioning that TINA-TG_4T complexes are also detected without any ammonium or sodium adducts in contrast to unmodified tetramolecular assembly of dTG_4T which is only detected as a complex with three ammonium ions in ESI-MS.[15a] This suggests that TINA-TINA hydrophobic interactions hold these assemblies together. The schematic representation of complexes proposed for TINA-TG_4T sequences are presented in Table S2, Supporting Information. The striking difference in the CD spectra in the UV-region of our TINA-TG_4T constructs prompted us to carry out further investigation of this phenomenon.

Incorporation of thymidine in the middle of the G-tract of dTG_4T destabilises G-quadruplexes (**GTG**, Table 1). This sequence partially formed G-quadruplex only in the presence of 110 mM KCl and at high DNA concentration (100 μM) (Table S2, Figure S1 in the Supporting Information). Single TINA insertion in **GXG** destabilises G-quadruplex assembly in K^+ but not in Na^+ (Table 1). Two TINA insertions in **GXX** led to the least thermally stable G-quadruplex. However, for both **GXG** and **GXX** constructs annealing occurs considerably faster than for the parent G-quadruplex (Table 1, Figure S4, in the Supporting Information). Faster temperature ramps (0.5 and 1.0 °C per min) resulted in the pronounced hysteresis between melting and annealing profiles (Table S3, Supporting Information). It should be noted that at 10 μM concentration the association of unmodified sequence TG_4T is very slow: half-association time is 110 days (calculated at 4 °C, in 110 mM Na^+).[10] All TINA-modified G-quadruplexes could be re-formed at 10 μM concentration as shown by the annealing profiles. To determine the kinetic of TINA-G-quadruplex formation we performed isothermal renaturation experiments[10, 16] using circular dichroism spectroscopy and determined the association rate constant, k_{on} for **GXG**, **G3X** and TG_6T sequences. For TINA-modified sequences we have also determined k_{on} by monitoring TINA absorbance at 373 nm using a UV-Vis spectrometer which gave us values comparable with those obtained by CD (Table S5 in the Supporting Information). We found that the association rate constant, k_{on}, was significantly higher for **GXG** in comparison to the earlier reported k_{on} for the dTG_4T sequence (Table 1). It is interesting that k_{on} values for **GXG** and a sequence that has a longer G-tract (dTG_6T) are quite similar (10^{10} – 10^{11} M^{-3} s^{-1}). TINA inserted in the middle of the dTG_6T sequence (**G3X**) had a slightly increased k_{on} value in comparison to that of dTG_6T. For both **GXG** and **G3X** constructs the order of the reaction was experimentally estimated to be 3.97, which agrees with the values reported in literature for tetramolecular G-quadruplexes (3.6 – 4.0).[10] This suggests that formation of complexes by **GXG** and **GXX** sequences is governed by TINA-TINA association rather than guanosine assembly in Na^+ solutions. It should be mentioned that observation of the **GxG** dimer in ESI-MS suggests that the dimer is an intermediate of the tetrameric structure and it does not affect the order of the reaction determined by UV-Vis spectroscopy.[17]

The formation of TINA G-quadruplexes results in electronic communication between 1-phenylethynylpyrenes[18] (Figure 1). UV-Vis spectra show a 7-9 nm red-shift in the TINA absorption region upon assembly of all TINA-G-quadruplexes indicating that J-aggregates are formed (Figure S2, Supporting Information). It is striking to see a correlation between CD spectra profiles for TINA and fluorescence emission spectra of corresponding G-quadruplexes (Figure 1). In the case of the **XTG** sequence, a positive Cotton effect is observed in CD spectra with two peaks at 375 and 397 nm. This leads to formation of an excited dimer (excimer) with maximum at 517 nm in fluorescence emission spectra (Figure 1F). It should be noted that the excimer maxima for 1-phenylethynylpyrenes upon their arrangement on the duplex scaffold are usually detected at 505-508 nm.[18] In contrast, TINA insertion between 5'-T and G (construct **TXG**) results in negative CD exciton couplet (–/+ pattern, Figure 1B) that indicates the *anticlockwise* orientation of the transition dipoles of chromophores; this causes a significant reduction of monomeric fluorescence at ca. 395-400 nm and leads to excimer formation with maximum at 517 nm (Figure 1F). Moreover, sequences with a single TINA in the middle of the G-tract (**GXG** and **G3X**) exhibit a positive CD exciton couplet (+/– pattern, Figure 1C and F) that indicates the *clockwise* orientation of the TINA transition dipoles. A significant blue-shift in the excimer band from 517 to 490 nm and a 2.5-fold increase in fluorescence intensity are observed for both **GXG** and **G3X** G-quadruplexes as a result of a change in orientation of chromophores in comparison with **TXG** assembly (Figure 1F). Fluorescent excimer bands and CD exciton couplets are significantly reduced or disappear after melting of G4 assemblies (see representative spectra in Figure S11 in the Supporting Information). It is interesting that fluorescence and CD spectra in the TINA region are similar in K^+ and Na^+ solutions suggesting that TINA molecules are in similar arrangements despite the difference in topologies of G-quadruplexes formed (Figure 1 and Figure S6A in the Supporting Information). Fluorescence excitation spectra for all constructs exhibit a similar pattern (Figure S6B in the Supporting Information) revealing that the difference in the position of the excimer bands is a result of a different orientation of the TINA's transition dipoles rather than a result of the exciplex formation between TINA and nucleobases. It should be noted that placement of two TINA monomers opposite each other in the middle of a DNA duplex does not lead to excimer formation – a consequence of a poor overlap between pyrenes (Figure S6A in the Supporting Information).[18d, 19] This shows that tetramolecular G-quadruplexes can provide access to assemblies of chromophores with different orientation of transition dipoles which expands versatility of DNA-chromophore architectures.[20]

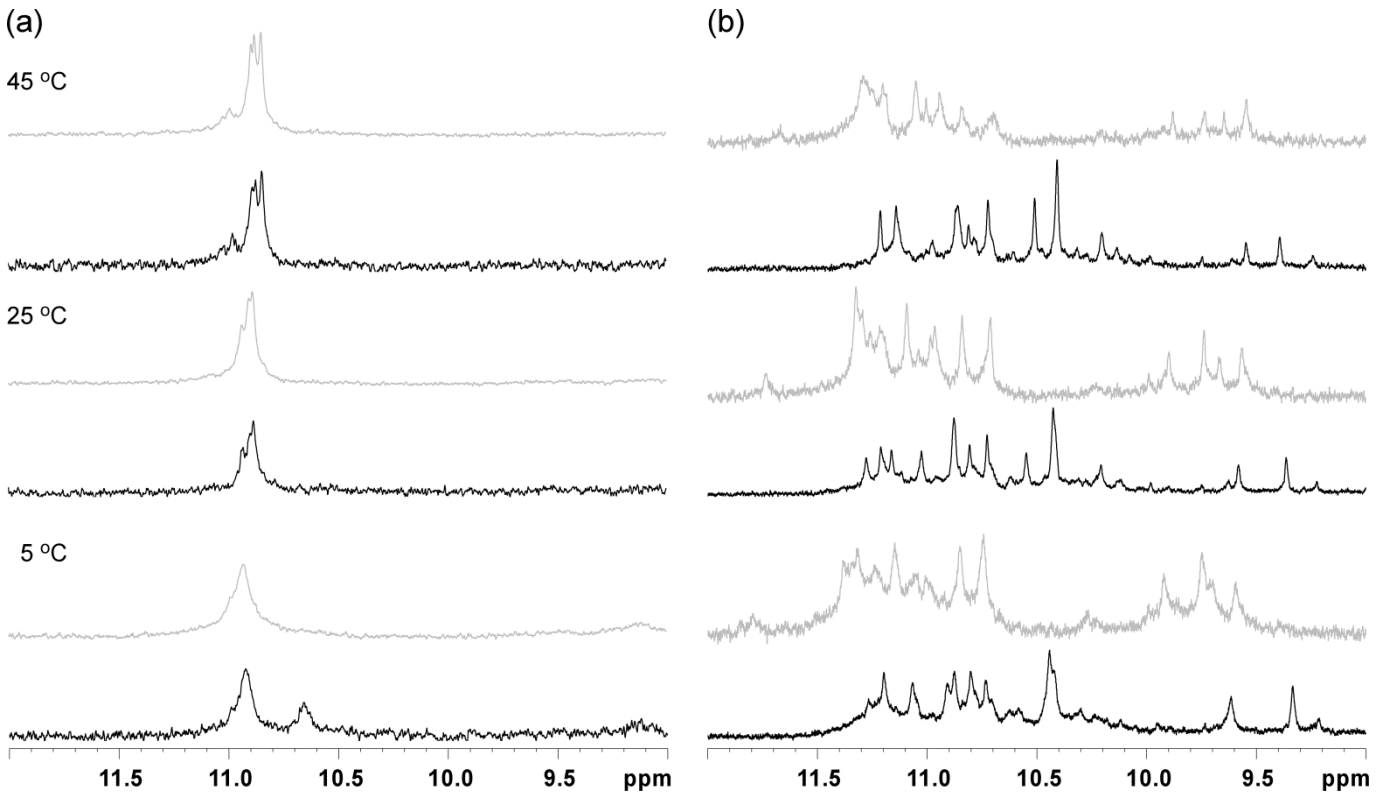


Figure 4. NMR spectra of **TXG** (A) and **GXG** (B) at indicated temperatures in Na^+ containing buffer (black lines) or supplemented with KCl (grey lines). Experimental conditions: 800 MHz spectrometer (**TXG**) and 700 MHz spectrometer (**GXG**); buffer: 10 mM Na^+ phosphate buffer (10% D_2O), pH 7.0; DNA concentrations: 0.5 mM strand concentrations for all complexes except for **TXG** in NaCl (grey line, 0.1 mM); concentrations of salts added: no additional NaCl (black line) and 10 mM KCl (grey line) for **TXG** and 100 mM NaCl (black line) or 100 mM KCl (grey line) for **GXG**.

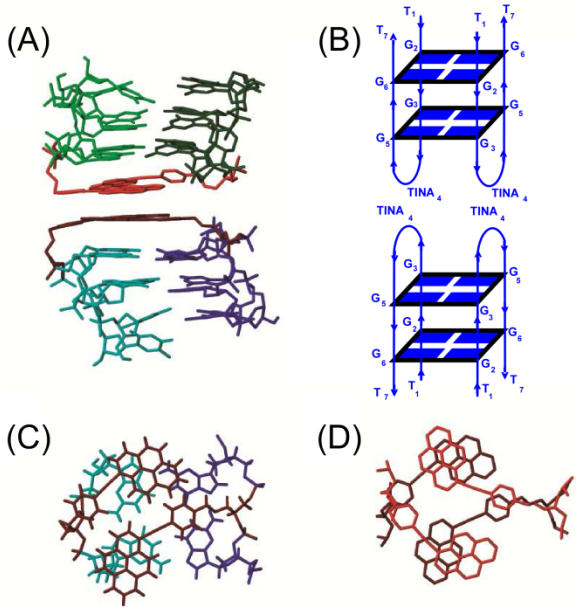


Figure 5. A) Model of the tetrameric structure of **GXG** in Na^+ phosphate buffer showing the four subunits of TGG**X**GGT in different colours. B) Scheme showing the possible arrangement of magnetically equivalent subunits. C) Detail of the interaction between TINA moieties and the G-tetrad underneath. D) Detail of the stacking interaction between TINAs that stabilises the association between two antiparallel G-quadruplexes.

To gain further structural information about TINA-TG_4T assemblies we perfomed NMR analysis of several constructs. NMR spectra of **TXG** exhibit imino signals at around 11 ppm that persist at relatively high temperatures, indicating the formation of stable G-quadruplexes (Figure 4A and Figure S7A in the Supporting Information). Signal dispersion for this complex is rather poor and there are no significant differences between Na^+ and K^+ buffers. However, the scenario for **GXG** is completely different (Figure 4B). The NMR signals are sharper and present a very good dispersion, especially in Na^+ buffer (Figure 4B, black spectra), suggesting a completely different G-quadruplex structure which is consistent with our observations using CD spectroscopy. ^{1}H-NMR spectra in the imino region look identical at high (1000 µM) and low (100 µM) oligonucleotide concentrations suggesting that G-quadruplex formation is independent on strand concentrations (Figure S7C in the Supporting Information). Strong H1'-H8 cross-peaks in the NOESY spectra indicate the presence of guanines in *syn* conformation, characteristic of antiparallel G-quadruplexes (Figure S8). The number of imino signals (Figure 4B) and thymine spin systems observed in the TOCSY and COSY spectra is not consistent with a single symmetric species which is usually observed for the full parallel topology (Figure S9). In such topology, adopted by the unmodified dTG_4T oligonucleotide, protons in each of the subunits are magnetically equivalent. The presence of multiple spin systems in the NMR spectra of **GXG** in Na^+ buffer, together with the high redundancy of the sequence, makes it impossible to carry out an unambiguous assignment of all resonances. In spite of this, interesting structural features can be obtained from the

spectra. The number of spin systems and the presence of guanines in *syn* conformation indicate the formation of antiparallel species in which the different subunits are not magnetically equivalent. In addition, several TINA pyrene spins systems can be distinguished in the TOCSY spectra. In the NOESY spectra, these TINA spin systems exhibit a number of TINA-TINA and TINA-guanine cross-peaks (Figure S9). This indicates the presence of contacts between TINA moieties and an extensive interaction surface between TINA moieties and the G-tetrads.

Although we cannot completely rule out the possibility of G-quadruplex formation with all strands being in parallel orientation while some guanines adopt a *syn*-conformation (see proposed assembly in Table S2, Supporting Information),[21] formation of antiparallel quadruplexes is much more plausible on the light of the NMR, CD and ESI-MS data. This experimental information, together with symmetry considerations, is consistent with the formation of G-quadruplex species, resulting from the self-association of two dimers through TINA-TINA interactions (Figure 5). Each dimer consists of two subunits each of which is formed by head-to-head association of two hairpins arranged in an antiparallel quadruplex stabilised by two G-tetrads. Although not in identical buffer conditions, this **GXG** dimer retains enough stability to be detected by ESI-MS analysis (see above). The association of two hairpins can give rise to G-quadruplexes of different symmetry. Considering the number of *syn* and *anti* guanines observed in the spectra (Figure S8 in the Supporting Information), we propose that several quadruplexes of different symmetry may co-exist under Na^+ conditions. Both symmetrical and asymmetrical configurations are possible (Figure S10 in the Supporting Information). A molecular model of a tetrameric structure resulting from association of two antiparallel dimeric quadruplexes through TINA-TINA interactions is shown in Figure 5A. This model was built with the Sybyl program and refined with the AMBER package. After performing a molecular dynamics, the structure remains stable, indicating that this model is plausible.

Conclusions

We have synthesised and characterised novel G-quadruplexes containing TINA moieties at various positions in the dTG_4T sequence. A discrete intermolecular clustering of pyrene,[22] perylene[23] and porphyrin[24] containing DNAs have been suggested in the past based on observations using CD, UV-Vis and EPR spectroscopies. Here, for the first time, an NMR-based model demonstrates the association of four identical nucleic acid units assisted through interactions of organic chromophores. Kinetic experiments showed that TINA-TINA association is prevalent in Na^+ but not in K^+ solutions and that elongation of the G-track suppresses the effect of TINA in the $dTG_n\mathbf{X}G_nT$ sequence. Several guanosine analogues exist that enhance the kinetics of tetramolecular G-quadruplex formation, especially when they are used instead of the 5'-dG.[25] Recently, a change in the strand orientation in the tetramolecular G-quadruplex was achieved using several 8-methyl-2'-deoxyguanosines in the dTG_4T sequence.[21b] Our example shows that non-nucleosidic moieties can compete with the G-mediated association of G-rich strands and lead to topologies that might differ from that of the parent sequence. In this regard, using non-guanosine molecular entities may become an invaluable means of improving the kinetics of G-quadruplex assemblies. This also shows that various chromophore arrangements can be achieved using G-quadruplex scaffolds. Certainly, we will see new interesting examples of such architectures in the near future.

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Notes and references

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Electronic Supplementary Information (ESI) available: [oligonucleotide synthesis, CD, UV-Vis melting/annealing data, fluorescence spectra, details of isothermal annealing, ESI-MS, NMR spectra]. See DOI: 10.1039/b000000x/

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