

## Research Article

# Structure and Stability of a Dimeric G-Quadruplex Formed by Cyclic Oligonucleotides

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We have studied the structure and stability of the cyclic dodecamer d<pGGGTTAGGGTTA>, containing two copies of the human telomeric repeat. In the presence of sodium, NMR data are consistent with a dimeric structure of the molecule in which two cycles self-associate forming a quadruplex with three guanine tetrads connected by edgewise loops. The two macrocycles are arranged in a parallel way, and the dimeric structure exhibits a high melting temperature. These results indicate that cyclization of the phosphodiester chain does not prevent quadruplex formation, although it affects the global topology of the quadruplex.

## 1. Introduction

One of the most studied noncanonical DNA motifs is the G-quadruplex, where four guanines are paired through their Watson-Crick and Hoogsteen sides [1–3]. These structures are receiving substantial attention in research areas ranging from molecular biology to structural and analytical chemistry [4–6]. It has been suggested that G-quadruplexes play a role in several biological processes, such as telomere integrity, genetic recombination, transcription, or replication. In addition, they are attractive targets for drug design, especially in cancer chemotherapy [7–11]. Clear evidence of quadruplex formation *in vivo* has been found recently [12–14].

G-quadruplexes can fold in many ways that differ in their chain number and orientation. Whereas single  $G_n$  tracks arrange in parallel structures, multiple  $G_n$  repeats fold with different topologies that are influenced mainly by the nucleotide sequence between  $G_n$  repeats as well as by the kind of counterion. In occasions, different topologies have been found for the same oligonucleotide in solid and solution studies.

On the other hand, cyclic oligonucleotides have emerged as interesting molecules in research for diagnosis and as therapeutic agents due to their increased nuclease resistance relative to their linear analogues [15, 16]. These

molecules are also interesting for structural studies since the conformational constraint induced by cyclization of the chain may increase the relative stability of the structure of interest [17–21]. G-quadruplexes have been used as templates for enhancing the efficiency of the synthesis of cyclic oligonucleotides. This approach takes advantage of the proximity between the two oligonucleotide termini in some quadruplex topologies to improve phosphodiester ligation [22–24].

G-quadruplex forming cyclic oligonucleotides may be interesting in a number of applications. For example, these nuclease resistant oligonucleotides can be very useful probes to study G-quadruplex interacting proteins. However, the conformational constraint induced by cyclization affects the range of structures that a G-quadruplex can adopt. For example, diagonal loops or double-chain-reversal loops are not possible in quadruplexes formed by cyclic oligonucleotides.

To gain insight on the effect of cyclization on the structure of G-quadruplexes, we have studied the structure and stability of the cyclic dodecamer d<pGGGTTAGGGTTA>, containing two copies of the human telomeric repeat. The analogous linear oligonucleotide d(TAGGGTTAGGGT) forms two interconverting dimeric structures in solution: a parallel quadruplex with double-chain-reversal loops, and

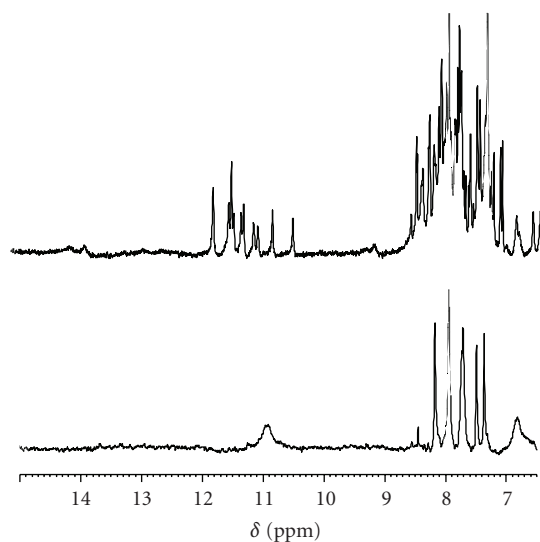


FIGURE 1: One-dimensional NMR spectra of d<pGGGTTA-GGGTTA> in H<sub>2</sub>O at 60 μM (bottom) and 600 μM (top) oligonucleotide concentrations (buffer conditions: 100 mM NaCl, 25 mM sodium phosphate pH 7,  $T = 5^{\circ}\text{C}$ ).

an antiparallel quadruplex with edgewise loops [25]. On the other hand, the same sequence forms a parallel quadruplex with double-chain-reversal loops in the crystal (in presence of  $\text{K}^+$ ) [26]. A similar structural diversity has also been observed in an oligonucleotide containing four copies of the human telomeric repeat, which in  $\text{K}^+$  forms an intramolecular parallel quadruplex in the crystal [26] and an antiparallel quadruplex with a diagonal and two edgewise loops in  $\text{Na}^+$ -containing solution [27]. Other quadruplex topologies have been observed in a variety of oligonucleotides containing different number of telomeric repeats [28–31] (see [32] for a recent review on these studies).

## 2. Materials and Methods

The synthesis of d<pGGGTTAGGGTTA> was carried out following previously reported methods [33]. Samples for NMR experiments were prepared in 100 mM NaCl, 25 mM sodium phosphate buffer pH 7, with an oligonucleotide concentration ranging from 60 to 600 μM. NMR spectra were acquired in a Bruker AVANCE spectrometer operating at 600 MHz and equipped with a cryoprobe. Two-dimensional experiments (NOESY, TOCSY, and DQF-COSY) were carried out at  $5^{\circ}\text{C}$  in either D<sub>2</sub>O or in H<sub>2</sub>O/D<sub>2</sub>O 9:1. NOESY spectra were acquired with mixing times of 50, 100, and 200 ms, and TOCSY spectra were recorded with standard MLEV-17 spin-lock sequence, and 80 ms mixing time. NOESY spectra in H<sub>2</sub>O were acquired with 50 and 150 ms mixing times. In 2D experiments in H<sub>2</sub>O, water suppression was achieved by including a WATERGATE [34] module in the pulse sequence prior to acquisition. The spectra were processed with Topspin software and analyzed with the program Sparky [35].

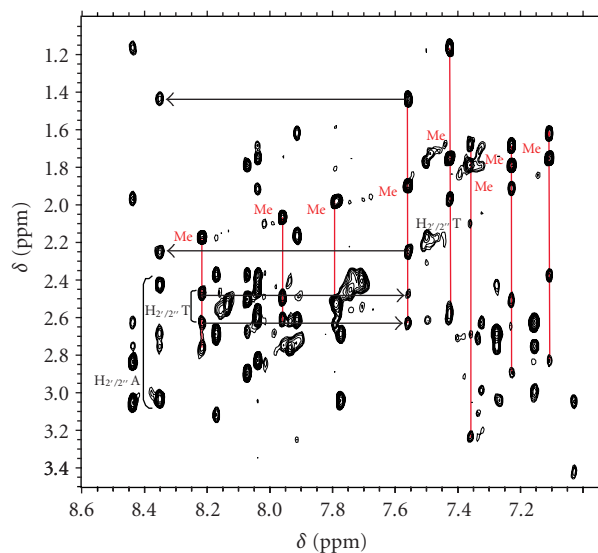


FIGURE 2: NOESY spectra of d<pGGGTTAGGGTTA> in D<sub>2</sub>O (200 ms mixing time) at 600 μM oligonucleotide concentration (same buffer conditions as in Figure 1). The eight thymine signals are indicated together with some informative sequential NOE contacts.

CD spectra were obtained following the change of ellipticity from 220 nm to 320 nm at different temperatures on a Jasco spectropolarimeter equipped with a Peltier temperature control used to set the temperature between  $5^{\circ}\text{C}$  and  $90^{\circ}\text{C}$ . The changes in ellipticity versus temperatures were plotted and used to obtain the melting temperature. Melting experiments were recorded at  $0.5^{\circ}\text{C}/\text{min}$  at the maximum wavelength. CD spectra were recorded at oligonucleotide concentrations ranging from 5 to 50 μM. The spectra were normalized to facilitate comparisons.

## 3. Results and Discussion

NMR spectra change dramatically upon oligonucleotide concentration. At low concentration, only six H6/H8 aromatic signals are detected (as a logical consequence of the repetitive sequence), and the exchangeable proton spectrum is very broad (see Figure 1). However, at high oligonucleotide concentration the exchangeable proton spectra shows 12 narrow signals between 10.0 and 12.0 ppm, and 24 aromatic signals (corresponding to H6/H8 protons) are observed in the non-exchangeable proton spectrum (see Figure 2). These data indicate the formation of an asymmetric dimer. Two fragments of the NOESY spectra in D<sub>2</sub>O are shown in Figures 2 and 3. The NMR spectrum in these conditions exhibits narrow and well dispersed signals, which indicates that the oligonucleotide adopt a well-defined structure. However, the NMR spectra of this molecule could not be unambiguously assigned due to its highly repetitive sequence. In spite of this, many structural features can be spotted from this spectrum. First, the cross-peaks of the imino and amino protons are consistent with the presence of three G-tetrads. Secondly, as shown in Figure 3, six

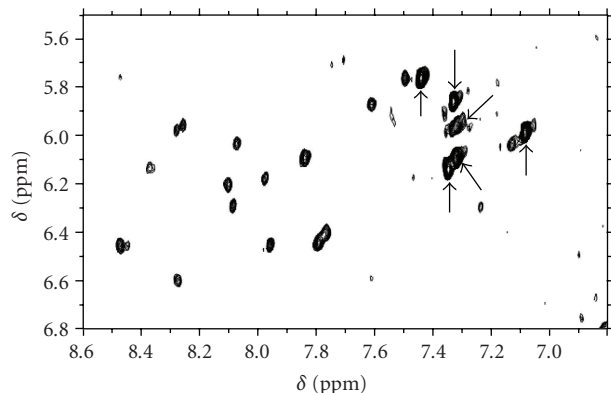


FIGURE 3: NOESY spectra of  $d\langle pGGGTTAGGGTTA \rangle$  in  $D_2O$  (200 ms mixing time) at  $600 \mu M$  oligonucleotide concentration (same buffer conditions as in Figure 1). Strong  $H1'-H8$  cross-peaks, characteristic of *syn* guanines, are indicated.

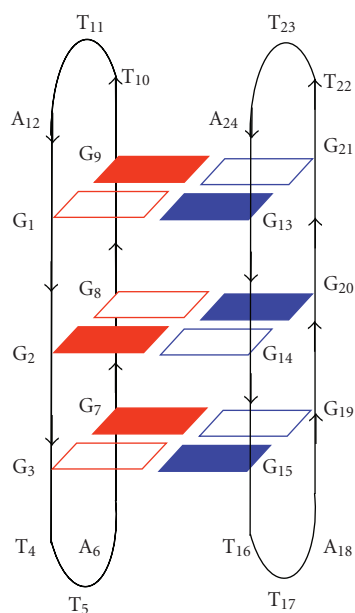


FIGURE 4: Schematic model of the dimeric structure of  $d\langle pGGGTTAGGGTTA \rangle$ , consistent with the experimental data in  $Na^+$  buffer. Guanines *syn* and *anti* are indicated with open and solid rectangles, respectively.

strong  $H1'-H8$  cross-peaks are observed, indicating that the glycosidic angle of the corresponding guanines is in a *syn* conformation (Gs). The six remaining guanines are in an *anti* conformation (Ga). Moreover, the occurrence of four steps Gs-Ga can be established from the sequential  $H1' \rightarrow H8$  NOEs steps. Finally, no Ga-Ga steps are present in this structure since no sequential NOEs are observed between guanines in *anti*.

All these data, together with symmetry considerations, led us to suggest a model for this structure in which two cyclic dodecamers self-associate forming an antiparallel quadruplex with three G-tetrads (Figure 4). The two macrocycles are arranged in a parallel way. Overall, the structure is similar

to antiparallel quadruplexes resulting from a head-to-head association of two hairpins with “edgewise” loops.

It is interesting to compare these results with other structures of quadruplexes formed by linear oligonucleotides containing repeats of the human telomeric sequence. Different groups have shown that linear oligonucleotides containing two repeats tend to adopt antiparallel quadruplex structures in sodium buffer [25, 36]. In the case of  $d(UAGGGT^{Br}UAGGGT)$  the structure is an asymmetric dimer [25], but the relative orientation of the two molecules is different than in the dimeric structure of  $d\langle pGGGTTAGGGTTA \rangle$ . The distribution of *syn* and *anti* guanines is also different in both cases. In the presence of  $K^+$ , linear oligonucleotides containing two repeats of the human telomere have the propensity to adopt parallel-stranded structures [25, 36], which are obviously impossible in the case of the cyclic analogues. We must conclude that the conformational constraint induced by cyclization of the phosphodiester chain affects the topology of the quadruplex. This result is not surprising since cyclization is formally equivalent to introducing an additional nonnative loop in the sequence. The effect of loop variations in the structure and topology of quadruplex has been extensively studied by several groups [37–39].

Since many oligonucleotides containing human telomeric repeats tend to adopt different structures in presence of  $K^+$  or  $Na^+$  cations, we tackled the study of the effect of these two cations on the structure of  $d\langle pGGGTTAGGGTTA \rangle$ . The profile of the CD spectra in  $Na^+$  buffer was consistent with an antiparallel G-quartet architecture characterized by a positive band at 295 nm, a positive maximum at 265 nm, and a negative band at 248 nm, a positive maximum at 295 nm, and a negative maximum at 265 nm (Figure 5(a)). However, in presence of  $K^+$  the CD spectra of  $d\langle pGGGTTAGGGTTA \rangle$  changes dramatically (Figure 5(b)). The negative band at 265 nm disappears, and the minimum around 235 nm is more pronounced. In these experimental conditions the CD spectrum is not consistent with a pure antiparallel or parallel G-quadruplex, the latter presenting a characteristic positive maximum at 265 nm [36]. The experimental CD spectrum suggests the presence of several conformations in equilibrium. NMR spectra conducted at  $500 \mu M$  oligonucleotide concentration in  $K^+$  buffer exhibit very broad signals (data not shown), in agreement with the occurrence of multiple conformations or aggregation. This result is not unexpected since it is well documented that  $K^+$  cations favour the parallel-stranded structures, which in this case are impeded by the cyclization of the phosphodiester chain.

The thermal stability of this structure has been studied by NMR and CD experiments. CD spectra of  $d\langle pGGGTTAGGGTTA \rangle$  in  $Na^+$  are characteristic of antiparallel quadruplexes (see Figure 5). Melting curves were recorded at different oligonucleotide concentrations, and thermodynamic parameters were obtained from the variation of the melting temperature with the concentration [40]. Thermodynamic parameters for dimer formation in 100 mM NaCl buffer solution are  $\Delta H^0 = -35$  kcal/mol,  $\Delta S^0 = -92$  cal/mol, and  $\Delta G_{298}^0 = -8$  kcal/mol. It is interesting to compare these parameters with the values for the unimolecular quadruplex formed by analogous linear oligonucleotides

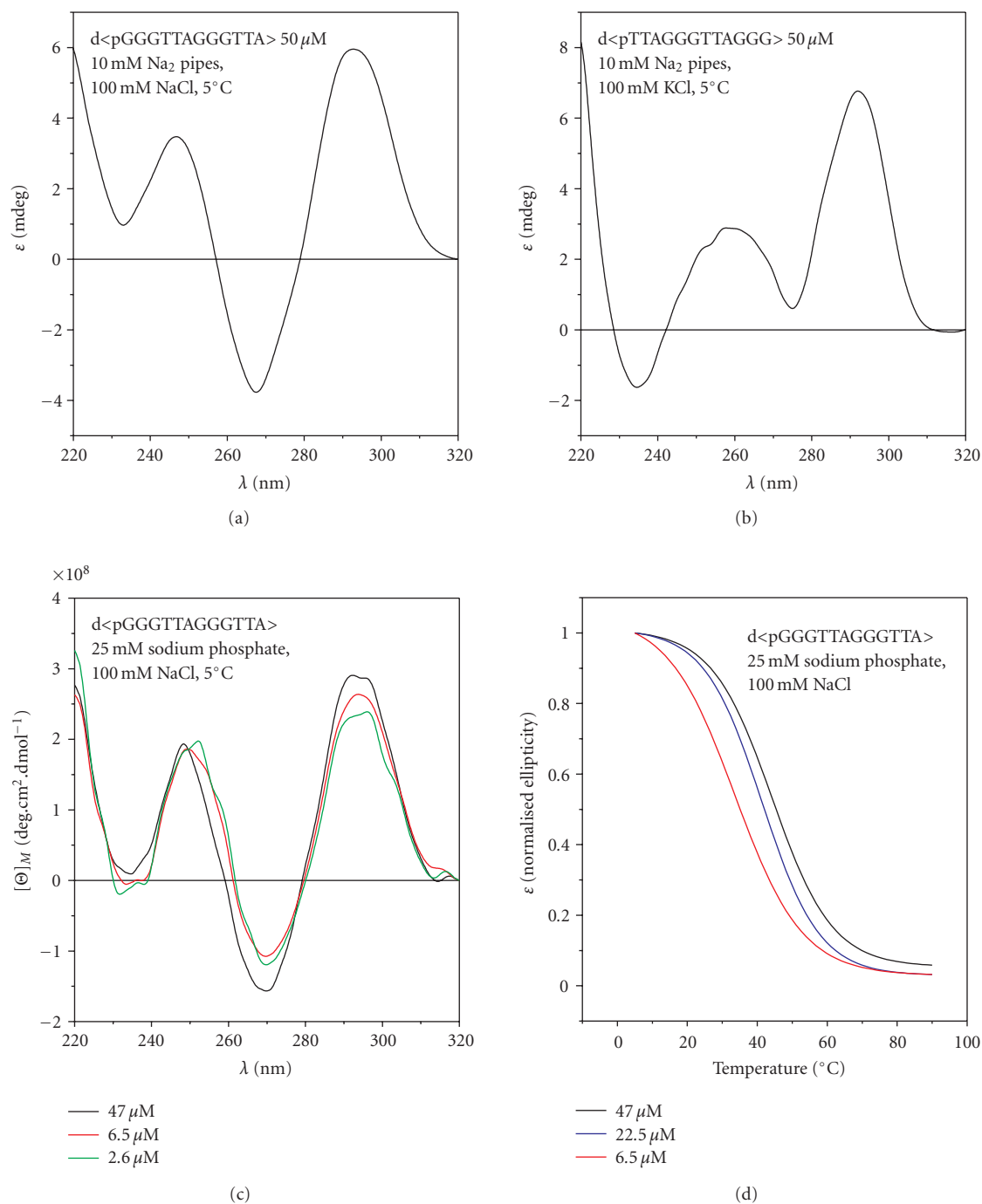


FIGURE 5: CD spectra of d<pGGGTTAGGGTTA> in media containing  $\text{Na}^+$  (a) or  $\text{K}^+$  (b). Converted to molar ellipticity CD spectra (c) and normalized melting curves (d) at different oligonucleotide concentrations in the same buffer conditions as NMR experiments.

containing four repeats of the human telomere. For example, the thermodynamic parameters for d(AGGGTTAGGGTTAGGGTTAGGG), under the same buffer conditions, are  $\Delta H^0 = -54$  kcal/mol,  $\Delta S^0 = -163$  cal/mol, and  $\Delta G_{298}^0 = -5.4$  kcal/mol [41]. Interestingly, formation-free energy is lower for the quadruplex formed by two cyclic oligonucleotides than for the quadruplex formed by the “native” sequence with four telomeric repeats. The larger stability

of the former is entropic in nature. The lower formation enthalpy in the dimer is probably a consequence of the constraint in the loops induced by the cyclization. We can conclude that “native” loops are enthalpically more stable. However, the entropic cost of forming the quadruplex through the self-association of two cyclic oligonucleotides with two repeats is lower than in the case of the folding of a linear oligonucleotide with four telomere repeats.

## 4. Conclusions

In summary, we have shown that guanine-rich cyclic oligonucleotides can form dimeric quadruplex structures. The conformational constraint induced by cyclization of the chain does not prevent quadruplex formation but has a profound influence in the global topology and stability of the structure. Such effect must be taken into account in the potential application of cyclic G-quadruplex as molecular probes.

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