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Following G-quartet formation by UV-spectroscopy

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Abstract Oligodeoxynucleotides which include stretches of guanines form a well-known tetrameric structure. We show that the recording of reversible absorbance changes at 295 nm allows to precisely monitor intramolecular guanine (G)-quartet formation and dissociation. Accurate T_m and thermodynamic values could be easily extracted from the data, whereas classical recordings at 260 nm led to a much larger uncertainty and in extreme cases, to completely inaccurate measurements. This inverted denaturation profile was observed for all G-quartet-forming oligonucleotides studied so far. This technique is very useful in all cases where intramolecular or intermolecular quadruplex formation is suspected.

Key words: Oligonucleotide; Guanine; G-quartet; Telomere; Tetrad; Unusual DNA structure; Ultraviolet absorbance; Ultraviolet melting

It is well known that guanine-rich polymers may adopt a non-B structure at physiological concentrations of monovalent ions. A number of techniques have been proposed to monitor G-quartet formation: NMR [1–3], crystallography [4,5], circular dichroism [6], Raman spectroscopy [7], gel electrophoresis [8,9], nuclease sensitivity, photocrosslinking [10], chemical probing [11,12], calorimetry [13] and ultraviolet absorption. In the latter case, absorbance of a guanine-rich oligonucleotide is recorded vs. temperature. In most cases, the profiles do not provide a precise determination of the melting temperature [14]. This situation led several laboratories to record absorbance at different wavelengths (272 or 273 nm [15,16], rather than 260 nm). Only in these cases were thermodynamic parameters succesfully determined from UV-melting measurements. Most of the $\Delta H°$ and $\Delta S°$ values compiled in the literature were rather the result of CD-melting experiments.

We wanted to optimize the quality of the melting profiles. Unmodified oligodeoxynucleotides were obtained and purified as previously described [17]. All concentrations were estimated by UV absorption [18] and expressed in strand molarity. The absorbance spectrum of the guanine-rich $d^{5'}$(AGGGTTAGGGTTAGGGTTAGGG)$^{3'}$ oligonucleotide was recorded at 1 and 90°C (Fig. 1A). As previously noted by Scaria and colleagues [15], the difference spectrum exhibits an isosbestic point around 280 nm, whereas a net hyperchromism is observed upon G-quartet formation at 285 nm or higher wavelength. This difference is maximal at 295 nm. For these reasons, we investigated whether the formation of the G-quartet structure could be followed at 295 nm. In parallel, we recorded the absorbance profile at 260 nm and 295 nm (Fig. 1B). A nice sigmoidal curve was obtained at 295 nm with a melting temperature of 56°C in a 0.1-M NaCl, 10-mM sodium cacodylate buffer. A smaller variation (4%) of absorbance was recorded at 260 nm upon G-quartet dissociation. The melting temperature at 295 nm was independent of pH (in the pH 5–7.5 range) and also independent of oligonucleotide concentration (in the 0.5–50-μM strand concentration range, not shown). The later results demonstrated that the formation of this structure was an intramolecular process. The profile was perfectly *reversible* (cooling and heating curves were superimposed) and *reproducible* (two cooling profiles were also superimposed). As expected for the dissociation of a G-quartet, the T_m was dependent on the nature of the monovalent ion. A T_m of 63°C was obtained in a 10-mM sodium cacodylate buffer with 0.1 M KCl, as compared to 37°C in a 10-mM sodium cacodylate buffer with 0.1 M LiCl. Thus, as expected, the effect of ions on the stability of this structure could be summarized as $K^+ > Na^+ >> Li^+$.

A thermodynamic analysis of the denaturation profile was then performed. From the classical relation $\Delta G° = -RT \ln(K) = \Delta H° - T\Delta S°$ one can deduce that $\ln(K) = -(\Delta H°/R)*(1/T) + (\Delta S°/R)$. Thus, $\ln(K)$ can be expressed as a linear function of $1/T$. The equilibrium constant K can be written as $K = \theta/(1-\theta)$ for an *intra*molecular equilibrium (θ is the fraction of folded oligodeoxynucleotide). θ (in the range 0.04–0.96), and thus K, can be easily determined at each temperature from the denaturation profiles. The all-or-none model which is in agreement with the experimental data, led to a straight line ($r > 0.995$; not shown), the slope of which is $-\Delta H°/R$ and y-intercept $\Delta S°/R$. In the case of intramolecular equilibria, the $\Delta H°$ could not be derived from the classical concentration dependency of the T_m (the melting temperature is concentration-independent). Thus, for the dAGGGTTAGGGTTAGGGTTAGGG oligonucleotide in 0.1 M KCl, a $\Delta H°$ of −54 kcal/mol and a $\Delta S°$ of −163 cal/mol/K were determined. *Intra*molecular G-quartet formation was fast, as shown by a reversible denaturation profile, and even for temperature changes as fast as 3°C/min (15 times faster than in the experiment reported in Fig. 1B), no hysteresis was observed. However, the fact that two distinct species were observed by 1D-NMR at all temperatures (no line broadening is evidenced) showed that the folded-to-unfolded transition was slow in the NMR scale ($\sim 10^{-2}$ s).

To confirm that the signal measured at 295 nm was in relation to the G-quartet formation/dissociation, we performed several control experiments. (i) A control oligonucleotide, with a similar sequence, but with 5 guanines substituted by 7-deaza guanines, gave no large variation of absorbance at 295 nm in a 0–90°C temperature range. (ii) No clear transition was obtained with a control oligonucleotide containing only 3 stretches of 3 guanines $d^{5'}$(TTAGGGTTAGGGTTAGGGTTA)$^{3'}$. (iii) The denatura-

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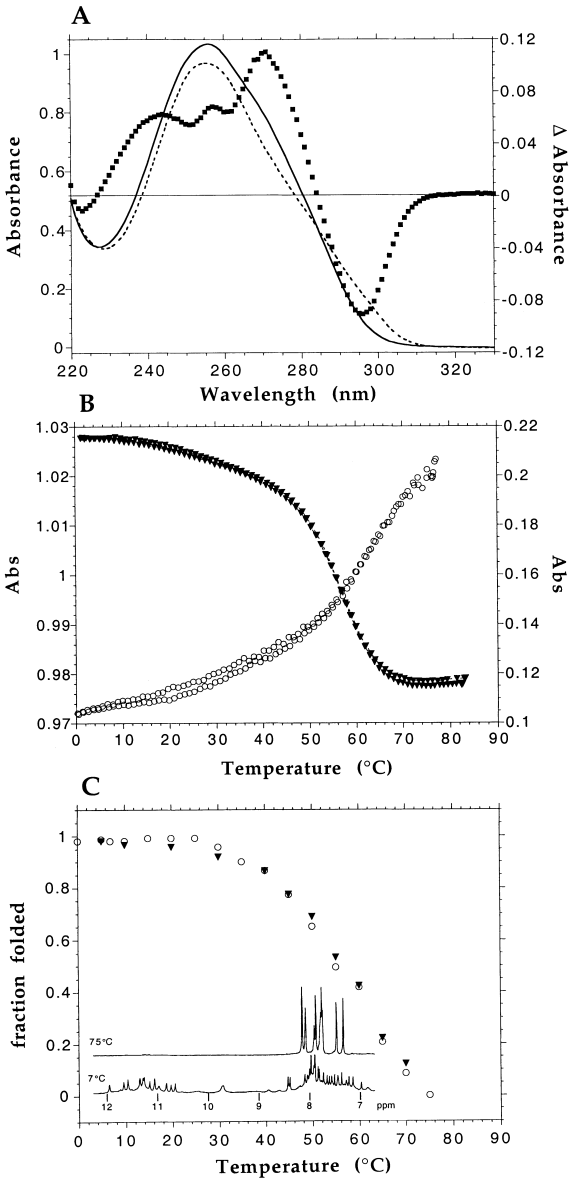

Fig. 1. A: Absorbance spectra of the dAGGGTTAGGGTTAGGGTTAGGG oligonucleotide at 90°C (full line) and 1°C (dotted line). Absorbance measurements were obtained using a Kontron-Uvikon 940 spectrophotometer. The solutions were vortexed, introduced in quartz optical cells and overlaid with a thin layer of paraffin oil to prevent evaporation. The optical pathlength was 1 cm. The squares represent the differences in absorbance of the high vs. low temperature spectra (right scale). B: Denaturation profiles obtained at pH 7.0 for the $d^{5'}(AGGGTTAGGGTTAGGGTTAGGG)^{3'}$ oligodeoxynucleotide at two different wavelengths: 260 nm (open circles) and 295 nm (black triangles). Left vertical axis: absorbance values at 260 nm; right vertical axis: absorbance values at 295 nm. Experimental conditions: 10 mM sodium cacodylate buffer, 0.1 M NaCl. The profiles were reversible, as indicated by the superimposition of the denaturation and the renaturation profiles for each wavelength. The temperature of the 6-cells holder was regulated by a circulating liquid (90% water/10% glycerol) using a Haake cryothermostat and monitored by a thermoresistance immersed in an accompanying cell containing only buffer. Constant heating or cooling rates (dT/dt) were obtained using a Haake PG20 temperature programmer. The rate of temperature changes was set at 12°C/h. Absorbance and temperature were recorded every 6 min. Water condensation on the cell walls at low temperatures was prevented by gently blowing a stream of dry air in the cell compartment. C: Fraction of oligonucleotide engaged in G-quartet formation, as a function of temperature, derived from the measurement of the intensity of T12H6 (circles) or T17 CH_3 (triangles) (proton assignments from [20]). Insert: 1D-NMR spectrum of the exchangeable and aromatic proton region at 7°C and 75°C. Experimental conditions: 90% H_2O/10% D_2O, pH 7.0, 0.1 M NaCl, 0.5 mM DSS Strand concentration 1.5 mM. The NMR experiments were performed on a 500-MHz varian spectrometer using the JR sequence [33] with a repetition delay of 5 s. The chemical shifts were referenced to DSS and the spectra were normalized to the intensity of the DSS peak.

tion of an oligonucleotide with 4 stretches of 3 guanines $d^{5'}(AGGGTTAGGGTTAGGGTTAGGG)^{3'}$ was followed by one-dimensional H-NMR spectroscopy in the same experimental conditions, except for the strand concentration which was in the millimolar range. The proton NMR spectra showed two sets of peaks corresponding to two species in slow exchange on the NMR time scale. At low temperature, the dominating species is that previously assigned to the folded G-quartet. The species which dominates at high temperature is ascribed to the unfolded form. The melting profile was followed by the measurement of the intensity of several well-resolved aromatic, methyl or exchangeable imino protons of the G-quartet. As shown in Fig. 1C, the shape of the melting profile was similar to the one presented in Fig. 1B, giving a T_m of 56°C. This result confirms that the melting curve at 295 nm corresponds to the denaturation of an intramolecular G-quartet structure.

We wanted to confirm that the 295-nm profile was a general phenomenon for G-quartet formation, and not specific to our oligonucleotide. A total of eight different sequences, susceptible of forming an intramolecular G-quartet have been tested: $d^{5'}(GGTTGGTGTGGTTGG)^{3'}$ (15apt, aptamer against thrombin); $d^{5'}(GGGTTAGGGTTAGGGTTAGGG)^{3'}$ (21h, human telomeric repeat); $d^{5'}(GGGTGTGGGTGTGGGTGTGGG)^{3'}$ (21s, *S. cerevisiae* telomeric repeat); $d^{5'}(AGGGTTAGGGTTAGGGTTAGGG)^{3'}$ (22h, human telomeric repeat); $d^{5'}(TTTAAGGGTTAGGGTTAGGGTTAGGG)^{3'}$ (26h); $d^{5'}(TTTAAGGGTGTGGGTGTGGGTGTGGG)^{3'}$ (26s); $d^{5'}(GGGTGTGTGGGTGTGTGGGTGTGTGGG)^{3'}$ (27s); and $d^{5'}(GGGGTTTTGGGGTTTTGGGGTTTTGGGG)^{3'}$ (28mer, oxytricha telomeric repeat). Several of these oligonucleotides have been shown to form an intramolecular quadruplex by NMR [19–22]. An increase of absorbance at 295 nm was systematically observed upon G-quartet formation, and the results are presented in Table 1. Intramolecular folding leading to the formation of 2, 3 or 4 guanine quartets could always be followed at 295 nm. This is in contrast with the absorbance recorded at 260 nm, where G-tetrad dissociation could lead to an increase (Fig. 1B), a decrease (Fig. 2A) or no cooperative change (Fig. 2B) of absorbance. A general trend was observed for the eight oligonucleotides: the T_m was higher in KCl than in NaCl (a well known property of G-quadruplex), and this is a result of more negative (i.e. more favorable) enthalpies of G-quartet formation, which are only partially compensated by more negative (i.e. less favorable) entropies of G-quartet formation. It should be noted that the exact impact of Na^+ to K^+ substitution is strongly dependent on the oligonucleotide (ΔT_m between +7 and +30°C).

Table 1
List of the oligonucleotides tested in this study

Oligonucleotide length	Sequence	Cation	T_m (°C)	$\Delta H°$ (kcal/mol)	$\Delta S°$ (cal/mol/K)	ΔG (310 K) (kcal/mol)
15apt	GGTTGGTGTGGTTGG	Na^+	20	−28	−95	+1.5
		K^+	50	−43.5	−135	−1.5
		Li^+	< 0	n.d.	n.d.	n.d.
21h	GGGTTAGGGTTAGGGTTAGGG	Na^+	58	−51	−155	−3.0
		K^+	65	−60.5	−179	−5.1
22h	AGGGTTAGGGTTAGGGTTAGGG	Na^+	56	−54	−163	−3.5
		K^+	63	−57	−169	−4.6
		Li^+	37	−35.5	−115	+0.1
21s	GGGTGTGGGTGTGGGTGTGGG	Na^+	51	−45	−139	−1.9
		K^+	67	−64	−189	−5.4
26h	TTTAAGGGTTAGGGTTAGGGTTAGGG	Na^+	44	−38.5	−121	−1.0
		K^+	55	−63	−193	−3.2
26s	TTTAAGGGTGTGGGTGTGGGTGTGGG	Na^+	37	−37.5	−121	+0.0
		K^+	63	−66	−197	−4.9
27s	GGGTGTGTGGGTGTGTGGGTGTGTGGG	Na^+	37	−41	−132	+0.0
		K^+	62	−57	−171	−4.0
28	GGGGTTTTGGGGTTTTGGGGTTTTGGGG	Na^+	67	−82	−240	−6.9
		K^+	84	n.d.	n.d.	n.d.

The abbreviated name is followed by the primary sequence of the oligonucleotide. Two or three different monocations were tested in each case (K^+: 0.1 M KCl; Na^+: 0.1 M NaCl; Li^+: 0.1 M LiCl) in a pH 7.0, 10 mM sodium cacodylate buffer. The $\Delta H°$ and $\Delta S°$ were calculated from the ln(K) vs. 1/T plots, which gave linear fits ($r > 0.995$). As the equilibrium is intramolecular, at T_m, $K = 1$, thus $\Delta G = 0 = \Delta H° - T_m \Delta S°$, therefore $T_m = \Delta H°/\Delta S°$. The ratio of the calculated $\Delta H°$ and $\Delta S°$ gives T_m values in good agreement with the measured values. Last column: ΔG of intramolecular quadruplex formation at 37°C or 310 K (the quadruplex is the predominant form at physiological temperature whenever $\Delta G < 0$). n.d.: not determined (melting temperature too high or too low).

For most oligonucleotides, a ΔH of roughly −20 kcal/mol was calculated for the formation of each G-quartet: for example, the 15apt and oligonucleotides which may, respectively, form two and three G-quartets, have $\Delta H°$ values of −43.5 and −60.5 kcal/mol in 0.1 M KCl. This value is a bit lower than some values reported in the literature, especially for tetraplexes formed with four independent short strands [23]. Thermodynamic parameters have been compiled recently [24]. Both CD-spectroscopy and/or calorimetry were used to obtain these parameters [25]. For example, Sugimoto et al., using CD spectroscopy, reported a $\Delta H°$ of −25 kcal/mol per tetrad for the $d(TTGGGG)_4$ sequence [26]. Nevertheless, similar values have been reported for the intramolecular folding of different oligonucleotides [13,16], and even lower values were estimated for the $d(TA_2G_3)_4$ sequence [9]. The addition of a 5′-terminal overhang has a negative impact on the stability of the quadruplexes (compare 21h, 22h, and 26h, or 21s and 26s) as deduced from lower T_m values. For all oligonucleotides the ΔG of quadruplex formation was negative in a potassium buffer at physiological temperature (37°C), indicating that the predominant species was the folded form in these conditions (Table 1). The conversion from the sodium to the potassium form is associated with a net free energy change ($\Delta G°$) of −1.1 to −4.9 kcal/mol, depending on the oligonucleotide (a value of −1.7 kcal/mol was reported for the G3T4G3 sequence [27], but larger differences have been determined for the $d(TTGGGG)_4$ sequence [26]). Finally, preliminary differential scanning calorimetry measurements for the 22h oligonucleotide (at higher strand concentration: 100–400 μM) gave a profile in good agreement with the UV-spectroscopy model-dependent calculated values (to be presented elsewhere).

There are several major differences between intra- and intermolecular G-quartet formation. Intermolecular quartets involving four independent strands are usually parallel-stranded [3,5,28,29], and in many cases, all guanines are in the *anti* conformation. This leads to a completely different CD spectrum, with the notable absence of a positive CD peak at 295 nm. To test whether *inter*molecular G-quartet dissociation also led to a decrease in absorbance at 295 nm, we followed the denaturation of the $(dTGGGG)_4$ preformed tetraplex [30] at 20-μM strand concentration in a 10 mM sodium cacodylate buffer containing 1 mM KCl. The T_m was determined to be 73°C, from the absorbance recordings at 295 nm, but the transition was absolutely not reversible (i.e. once dissociated, tetraplex reassociation was very slow at a strand concentration of 20 μM) (Fig. 2C). At 295 nm, a 60% increase of absorbance was obtained upon intermolecular G-quartet formation. However, the complete irreversibility of the phenomenon is a major obstacle to the determination of thermodynamic parameters. In 10 mM KCl, the T_m was shifted to 88°C. No dissociation of the quadruplex could be observed at 0.1 M KCl even at 95°C. Finally, the dGGGTTAGGG oligonucleotide was also shown to form a quadruplex by absorbance changes at 295 nm (T_m of 39°C in 0.1 M KCl, at 7 μM strand concentration). The melting temperature was concentration dependent (in agreement with a bimolecular process) but a small hysteresis was observed, leading to non-superimposable heating and cooling curves (not shown).

It is rather surprising that, to our knowledge, this property of the G-quartet has not been exploited. G-quartets have major applications in several distinct fields of molecular biology: many oligonucleotide aptamers may fold into a G-quartet,

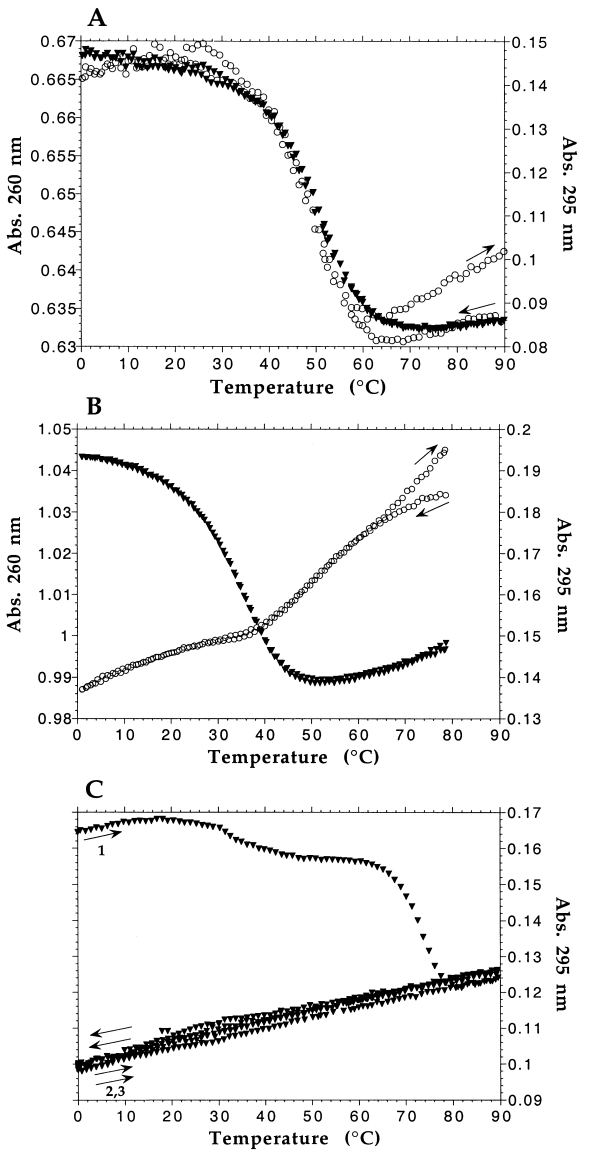

Fig. 2. A: Denaturation profiles obtained at pH 7.0 for the dGGTGGTGTGGTGG oligodeoxynucleotide at two different wavelengths: 260 nm (open circles) and 295 nm (black triangles). Left vertical axis: absorbance values at 260 nm; right vertical axis: absorbance values at 295 nm. Experimental conditions: 10 mM sodium cacodylate buffer, 0.1 M KCl. The discrepancy at high temperatures between the heating and cooling curves is the result of evaporation (the volume change is in the order of 1%): the experiment started with a cooling cycle (i.e. starting at high temperature, and slowly cooling) followed by a heating cycle. Note that the scales for the 260- and 295-nm readings are notably different. B: Denaturation profiles obtained at pH 7.0 for the dGGGTGTGTGGGTGTGTGGG oligodeoxynucleotide (27mer) at two different wavelengths: 260 nm (open circles) and 295 nm (black triangles). Left vertical axis: absorbance values at 260 nm; right vertical axis: absorbance values at 295 nm. Experimental conditions: 10 mM sodium cacodylate buffer, 0.1 M NaCl. As in A, the experiment started with a cooling cycle (i.e. starting at high temperature, and slowly cooling) followed by a heating cycle. C: Denaturation profiles obtained at pH 7.0 for the dTGGGG oligodeoxynucleotide at 295 nm (black triangles). For clarity reasons, the absorbance changes at 260 nm are not presented for this oligonucleotide. Experimental conditions: 10 mM sodium cacodylate buffer, 1 mM NaCl. The intermolecular tetraplex was prepared at 0°C, in a 0.1-M KCl pH 7 buffer, 2.2-mM strand concentration for 72 h, then diluted at 0°C to 20-μM strand concentration. The small variation of absorbance around 30–40°C is reproducible, and is probably the result of the presence of several different tetraplex species. Contrary to A and B, the experiment starts with a heating cycle (in order to start with the preformed tetraplex). After the first melting curve (indicated by '1') no reassociation of the tetraplex was observed for the second ('2') and third ('3') heating and cooling cycles. ←

several 'antisense' oligonucleotides contain runs of guanines [31], a G-quartet might be involved in HIV RNA dimerization [32], and the G-rich strand of telomeres may fold into a G-quartet structure. Thus, many people are interested in measuring the stability of G-rich oligonucleotides in vitro. This simple modification of a well-known technique (recording the absorbance at 295 nm instead of 260 nm to monitor G-quartet formation) offer several major advantages. (i) The variation of absorbance is important: G-quartet formation leads to a 50–80% increase of absorbance, whereas a much weaker relative variation was recorded at shorter wavelengths (see Fig. 1B or Fig. 2B). This change in absorbance is thus quantitatively important in the micromolar concentration range, and allows accurate determination of T_ms at concentrations as low as 0.1 μM. For very concentrated samples, up to 300 μM, apart from using a shorter pathlength, it is possible to record absorbance changes at 305 nm, where the extinction coefficient of the unfolded oligonucleotide is very low. It should also be noted that, due to the small variation of absorbance at 260 nm upon G-quartet formation, accurate concentration measurements may be performed at this wavelength, independently of the structure (i.e. folded or unfolded) of the oligonucleotide. (ii) The upper and lower baselines are flat; i.e. both the folded and unfolded form do not show a large variation of absorbance over a wide temperature range; thus the variation of absorbance is mostly the result of a sole phenomenon: i.e. G-quartet dissociation. (iii) At this longer wavelength most UV spectrometers show a better stability, thus limiting experimental artifacts. Furthermore, any drift of the instrument may also be limited by recording the absorbance at a neighboring wavelength (325 nm), where the extinction coefficient of DNA is negligible. These three advantages allow a precise determination not only of melting temperature, but also of thermodynamic parameters, provided that the profile is reversible. It is thus possible to extrapolate ΔG values at physiological temperature with a good precision ($\Delta G = -4.6$ kcal/mol for the quadruplex formation by $d^{5'}(AGGGTTAGGGTTAGGGTTAGGG)^{3'}$ human telomeric G-strand in a pH 7.0 sodium cacodylate buffer containing 0.1 M KCl). (iv) The transition at 295 nm is inverted as compared with the melting profile of a classical DNA duplex, i.e. G-quartet dissociation leads to an *increase* instead of a *decrease* in absorbance. Thus, the variation of absorbance at this wavelength is sufficient to monitor whether the G-strand of telomeres is involved in G-quartet formation or in a duplex with the complementary C-strand (manuscript in preparation).

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