DNA Structures

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Tri-G-Quadruplex: Controlled Assembly of a G-Quadruplex Structure from Three G-Rich Strands**

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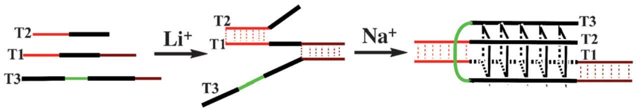
Guanine-rich oligonucleotides are able to self-associate to form G-quadruplexes, a higher-order DNA structure, in the presence of certain cations (typically Na^+ and K^+ ions).[1–3] G-quadruplex structures have drawn the attention of researchers in areas such as medicinal chemistry, supramolecular chemistry, and nanotechnology.[4–7] There are many possible G-quadruplex folding schemes and applications. For example, a recent report showed that long oligonucleotides of the human telomere sequence form (3+1) G-quadruplex structures, with one or more GGGTTA repeats within a single loop.[8] In addition, G-quadruplexes have been used as basic units in the formation of nanostructures.[6] All G-quadruplex structures investigated to date have been formed by one, two, or four G-rich strands.[7] G-quadruplex structures formed by three strands, leading to a tri-G-quadruplex species have yet to be shown. Herein, we demonstrate the formation of an unprecedented tri-G-quadruplex structure by polyacrylamide gel electrophoresis (PAGE), UV absorbance, circular dichroism (CD), and electrospray mass spectrometry (ESI-MS). Our results complete the set of G-quadruplex structures, which can now be formed by one, two, three, or four strands. Our current tri-G-quadruplex design may also provide a new avenue for creating nanoscale materials.

The principle of our design is that G-quadruplex formation requires the presence of a G-quadruplex-compatible cation such as sodium, whereas duplexes do not. Therefore, we used short duplexes as guide strands to preposition the G-rich tracts in close spatial proximity, then we induced the formation of a defined G-quadruplex structure by adding Na^+ (Scheme 1). Three DNA oligonucleotides were designed and synthesized:



Scheme 1. Schematic illustration of the two-step tri-G-quadruplex formation. The three strands first form a duplex in the presence of Li^+ ions and leaving unstructured G-tracts (middle). The formation of the G-quadruplex is induced by the addition of Na^+ ions (right). Red and brown = duplex, thick black lines = G-tracts, green = loop in oligo T3.

T1: **TGAGATGTACTATGA**GGGGG*TGTCATGGTAGAAGT*
T2: GGGGG**TCATAGTACATCTCA**
T3: *ACTTCTACCATGACA*GGGGGTTTGGGGG

The Watson–Crick complementary regions used as linkers between T1 and T2 are in bold and between T1 and T3 are in italics; the G-rich tracts are underlined. The three strands T1, T2, and T3 together form the "T" system (for "tetraplex"). We also designed control sequences in which we removed the G blocks from the T1, T2, and T3 sequences. These four short strands include the Watson–Crick duplex-forming regions, indicated in bold and italics, from the sequences that form the "T" system. These four oligonucleotides are referred to as the "L" system (for "linker") with the same bold/italics designation for the duplex regions as the "T" system:

L1a: **TGAGATGTACTATGA**
L1b: *TGTCATGGTAGAAGT*
L2: **TCATAGTACATCTCA**
L3: *ACTTCTACCATGACA*

As shown in Scheme 1, the desired tri-G-quadruplex structure is formed by three guanine-rich strands. However, a number of alternative G-quadruplex structures could also form, for example resulting from the association of four T1 strands, four T2 strands, two or four T3 strands, or two T1 or T2 strands with one T3 strand, among others. To induce formation of the tri-G-quadruplex structure, we first incubated the three strands in a buffer containing only Li^+ cations, to induce duplex formation, followed by Na^+ ion addition to favor tri-G-quadruplex formation. Non-denaturing PAGE revealed a single band when equimolar amounts of T1, T2, and T3 were annealed in 10 mM cacodylate buffer containing 110 mM Li^+ ions, followed by addition of 100 mM Na^+ ions

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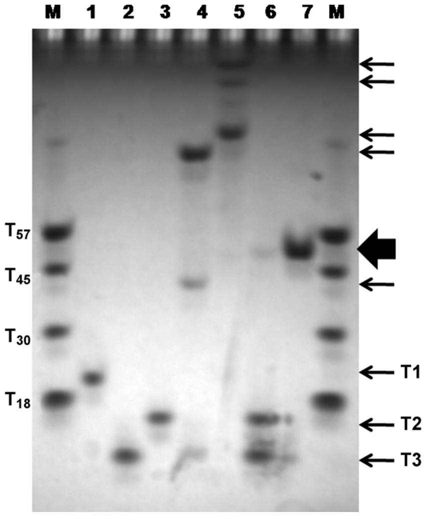

Figure 1. Binding of oligonucleotides from the "T" system (T1, T2, and T3) in 10 mM cacodylate buffer (pH 7.0) containing 110 mM Li^+ and 100 mM Na^+ on a non-denaturing gel shown by UV shadowing. Oligonucleotides were mixed in equimolar amounts. M = DNA markers. Lane 1: T1; lane 2: T2; lane 3: T3; lane 4: T1 and T2; lane 5: T1 and T3; lane 6: T2 and T3; lane 7: T1, T2, and T3. Thick arrow = tri-G-quadruplex; thin arrows = potential byproducts.

(lane 7, thick arrow in Figure 1). This single band migrated between the 45-base and 57-base oligothymidylate markers. The migration of these markers only provides an internal migration standard but does not directly allow identification of single-stranded or higher-order structures.[9] The DNA isolated from the band on the non-denaturing gel ran as three clear bands under denaturing conditions, suggesting that the single band seen in the non-denaturing PAGE consisted of the three oligonucleotides T1, T2, and T3 (Supporting Information, Figure S1). Although bands revealed by UV shadowing are difficult to quantify, the presence of a single band in lane 7 implies an equimolar complex; given the initial equal concentrations of T1, T2, and T3, some free strands should remain if the major band corresponded to a complex with a ratio other than 1:1:1.

Although a single species was formed when all strands were mixed in equal amounts, other structures were expected to form with unequal molar ratios. For example, when T2 was omitted and T1 and T3 were mixed in equimolar amounts, we observed smeared bands of slower mobility than the complex formed from all three strands (lane 5, Figure 1). When samples were incubated without Na^+ ions, these slow mobility bands disappeared from the non-denaturing gel (results not shown). Hence, T1 and T3 likely form both duplex and G-quadruplex structures, as reported in a previous study.[10] Elucidation of the exact structures of these byproducts is beyond the scope of this study. Overall, as shown by non-denaturing PAGE, the single band resulting from the association of the three strands is different from any potential byproducts. We concluded that T1, T2, and T3 mixed at an equimolar ratio form a single structure containing three strands.

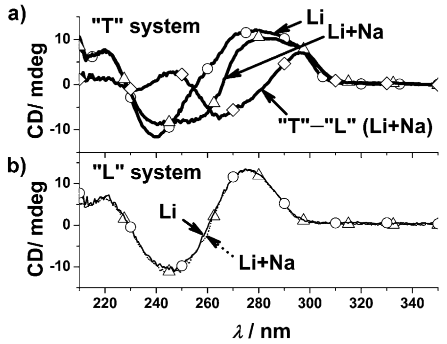

Figure 2. a) CD spectra at 5 °C of the "T" system (2 μM each T1, T2, and T3) and b) "L" system (2 μM each L1a, L1b, L2, and L3) in 10 mM cacodylate buffer (pH 7.0) containing 110 mM Li^+ ions in the presence (△) or absence (○) of 100 mM Na^+ ions. The line (◇) in (a) resulted from subtraction of the CD spectrum of the "L" system from the CD spectrum of the "T" system; both spectra were collected in the presence of Na^+ ions.

To analyze the structure of the "T" complex, classical CD spectroscopy was used. As shown in Figure 2 a, when the three strands were incubated in 10 mM cacodylate buffer containing 110 mM Li^+ ions (pH 7.0), the CD spectrum exhibited positive and negative peaks around 276 nm and 240 nm, respectively; this spectrum is similar to that of a Watson–Crick duplex.[11] Upon addition of 100 mM Na^+ ions, the CD spectrum changed. Both the positive and negative peaks were red-shifted to 280 nm and 250 nm, respectively. We hypothesize that this change in the CD spectrum reflects the Na^+-dependent formation of a G-quadruplex. The CD spectra of the "L" system in 10 mM cacodylate buffer containing 110 mM Li^+ ions and in the presence or absence of 100 mM Na^+ ions are superimposable; these spectra exhibit a positive peak around 276 nm and negative peak around 245 nm under all conditions, indicative of duplex formation (Figure 2 b).[11] These CD results suggest that the short duplexes are formed in 110 mM Li^+ ions and demonstrate that our strategy results in a tri-G-quadruplex. The CD spectrum of the G-quadruplex should correspond to the differential CD spectrum attained by subtracting the spectrum of the "L" complex from that of the "T" complex under the same conditions. As shown in Figure 2 a (diamonds), the subtracted spectrum shows positive and negative peaks around 295 nm and 260 nm, respectively, characteristic of a Type II CD signature, suggesting an antiparallel G-quadruplex structure, in agreement with the formation of the predicted tri-G-quadruplex.

To further demonstrate that the complex of T1, T2, and T3 is a tri-G-quadruplex, we performed UV melting experiments. G-quadruplexes show a hypochromic sigmoidal transition at 295 nm and a hyperchromic sigmoidal transition at 240 nm, whereas Watson–Crick duplexes only display a hyperchromic transition at around 260 nm.[15] Figure 3 a depicts the melting profile of the "T" system in 10 mM cacodylate buffer containing 110 mM Li^+ ions, in the presence or absence of 100 mM Na^+ ions. The melting curves at 260 nm both exhibited a single sigmoidal shape in the presence or absence of Na^+ ions, and the melting temperatures (T_m) were estimated to be 50 °C and 61 °C, respectively (Figure 3 a). At

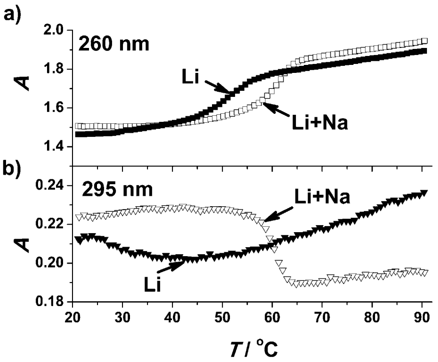

Figure 3. UV melting curves of the "T" system (2 μM each T1, T2, and T3) at a) 260 nm and b) 295 nm in 10 mM cacodylate buffer (pH 7.0) containing 110 mM Li^+ ions in the presence (□ and ▽) or absence (■ and ▼) of 100 mM Na^+ ions.

295 nm, a hypochromic transition was observed in the presence of Na^+ ions with an estimated melting temperature of 61 °C (Figure 3b). In the absence of Na^+ ions, only a very low temperature transition was observed at 295 nm (Figure 3b). For the control "L" system, no transition was observed at 295 nm (Figure S2a, Supporting Information). The transition at 260 nm had a melting temperature around 53 °C (Figure S2b). Therefore, the melting results, in agreement with previous reports,[14] demonstrate that a G-quadruplex is formed by the "T" system but not by the "L" system.

Thermal difference spectra (TDS) can be used as a fingerprint, indicative of particular nucleic acid structures.[16,17] The absorbance TDS of a G-quadruplex exhibits a negative peak around 295 nm and two positive peaks around 275 nm and 243 nm.[16] Figure 4a displays the TDS of the "T" system in 10 mM cacodylate buffer (pH 7.0) containing 110 mM Li^+ ions in the presence or absence of 100 mM Na^+ ions. The two spectra show some similarities, except for a negative peak at 295 nm only found when the sample is incubated in the presence of Na^+ ions. When the TDS in the absence of Na^+ ions was subtracted from the one in the presence of Na^+ ions, the ΔTDS shown in Figure 4b was obtained. The ΔTDS is characterized by positive peaks around 275 nm and 243 nm and a negative peak around 295 nm. Therefore, the ΔTDS results also support the model based on PAGE, CD, and UV-melting results of a G-quadruplex formed by three strands.

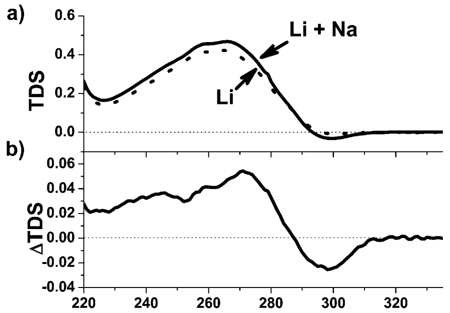

Figure 4. a) TDS of the "T" system (2 μM each T1, T2, and T3) in 10 mM cacodylate buffer (pH 7.0) containing 110 mM Li^+ ions in the presence (solid line) or absence (dotted line) of 100 mM Na^+ ions. b) The difference in TDS obtained by subtracting the TDS of the "T" system in the absence of Na^+ ions from the one in the presence of Na^+ ions.

We recorded the CD spectra of the "T" system at different temperatures (Figure S3). In the absence of Na^+ ions, the T_m of the "T" system is about 49 °C at 276 nm, in excellent agreement with the value found by UV melting (Figure S3a). In the presence of Na^+ ions, the T_m is around 61 °C at 295 nm (and also at 276 nm; data not shown). Figure S3b depicts the CD intensity at 276 nm of the "L" system at various temperatures; we found that in the presence and absence of Na^+ ions it exhibits a thermal transition at 53 °C. The comparison of "T" and "L" melting temperatures indicates that, in the absence of sodium, the unstructured G-tracts in the "T" oligonucleotides slightly destabilize the duplex (T_m of 49 °C versus 53 °C). In contrast, in the presence of Na^+ ions, the obvious increase in melting temperature (61 °C versus 53 °C) suggests that the sodium-dependent "T" quadruplex positively contributes to the structural stability.[12–14]

To provide unambiguous evidence of tri-G-quadruplex formation, we measured the masses of the complexes by electrospray mass spectrometry (ESI-MS). Experiments were carried out with NH_4^+ counterions.[18–21] Because electrospray requires the use of a volatile salt (ammonium acetate), we evaluated tri-G-quadruplex formation with NH_4^+ ions by native PAGE and CD. The "T" system ran as only one band on a non-denaturing gel in the presence or absence of NH_4^+ as found in Na^+ ions (Figure 1, Figure S4a). The difference in the CD spectra by subtracting the "L" from the "T" spectra collected with NH_4^+ ions showed positive and negative peaks around 295 nm and 260 nm, respectively, demonstrating that the G-quadruplex structure was formed (Figure S4b). By ESI-MS, we detected three main peaks belonging to a single charge-state distribution (Figure 5), which demonstrated that a single complex was present with a mass corresponding to the sum of the masses of the three stands (vertical lines). The slight offset to higher masses is due to specific (that is, inter-quartet) NH_4^+ ions retained and, for charge states 10− and 9−, further non-specific NH_4^+ and Na^+ adducts. The data from the NH_4^+ solution shown here further demonstrate that our strategy of using Li^+ ions to induce duplex formation first,

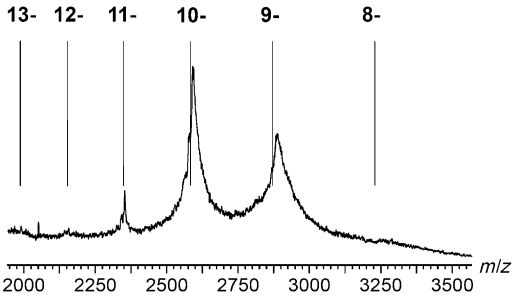

Figure 5. ESI-MS spectrum of the "T" system (T1, T2, and T3) in 100 mM NH_4OAc. The vertical lines indicate the simulated charge-state distribution of the heterotrimeric "T" complex without any adducts ($C_{819}H_{1021}N_{327}O_{495}P_{80}$).

and then Na^+ or NH_4^+ ions to induce tri-G-quadruplex formation was successful.

In summary, we demonstrated, for the first time, formation of a G-quadruplex structure by three G-rich strands. This tri-G-quadruplex was constructed by using conventional Watson–Crick duplexes as guides for spatial orientation of the G-rich tracts. Alternative templated strategies have been proposed to pre-organize the G-units for the formation of G-quartets or G-quadruplexes.[22–27] The duplex approach we used herein is simple and versatile; it relies on commercially available products and could be easily transposed to different sequences. In fact, we found that linker duplexes as short as six base pairs are sufficient for guiding the tri-G-quadruplex formation (Figure S5). Nevertheless, some may argue that the tri-molecular quadruplex is "contaminated" by these two duplex tails and that it is difficult to observe the "real" features of a tri-quadruplex structure. Therefore, efforts are now being made to design "pure" tri-G-quadruplex species, by removing the duplex region after assembly.

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