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.[9] 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:

\[
\text{T1: } \text{TGAGATGTACATAGGGGGTGTCA} \text{AGTGAAGT} \\
\text{T2: } \text{GGGGTCATAGTACATCTCA} \\
\text{T3: } \text{ACTTCTACCAGAC} \text{GGGGTGGGG} 
\]

The Watson–Crick complementary regions used as linkers between T1 and T2 are in bold and between T1 and T3 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:

\[
\text{L1a: } \text{TGAGATGTACATGA} \\
\text{L1b: } \text{TGTCATAGGTAAGT} \\
\text{L2: } \text{TCATAGTACATCTCA} \\
\text{L3: } \text{ACTTCTACCAGAC} \text{GGGG} 
\]

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.
OMITTED and T1 and T3 were mixed in equimolar amounts, we observed slow mobility bands and negative peaks around 276 nm and 245 nm under all conditions, indicative of duplex formation (Figure 2b).[11] 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 2b).[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 2a (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 sigmoidal transition at around 260 nm.[15] Figure 3a 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 (Tm) were estimated to be 50°C and 61°C, respectively (Figure 3a). At
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.

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$$^+$ ions.[18–21] Because electrospray requires the use of a volatile salt (ammonium acetate), we evaluated tri-G-quadruplex formation with NH$_4$$^+$ ions by native PAGE and CD. The “T” system ran as only one band in the presence of NH$_4$OAc. The vertical lines indicate the simulated charge-state distribution of the heterotrimeric “T” complex without any adducts (C$_{519}$H$_{1232}$N$_{177}$O$_{495}$P$_{80}$).

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.

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.

Figure 5. ESI-MS spectrum of the “T” system (T1, T2, and T3) in 100 mM NH$_4$OAc. The vertical lines indicate the simulated charge-state distribution of the heterotrimeric “T” complex without any adducts (C$_{519}$H$_{1232}$N$_{177}$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 proposed to pre-organize the G-units for the formation of G-quadruplexes.

features of a tri-G-quadruplex. Therefore, efforts are now being made to design “pure” tri-G-quadruplex species, by removing the duplex region after assembly.

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