Quadruplex Formation

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# Combination of i-Motif and G-Quadruplex Structures within the Same Strand: Formation and Application**

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Guanine- and cytosine-rich sequences may fold into tetraplex structures called G-quadruplexes (G4) and i-motifs under certain conditions.[1–3] In 2002, we defined the relationship between the tetraplex and duplex structures and determined which structures predominate under certain conditions.[4] Subsequently, a number of research groups constructed different nanodevices based on switching between structures as induced by changes in environmental factors.[5–7]

Herein, we demonstrate the formation of a "double-quadruplex" structure with i-motif and G4 domains on the same strand. Previous studies have demonstrated that duplexes can be combined with quadruplexes;[8–10] however, to the best of our knowledge, the coexistence of G4 and i-motif structures on the same strand has not been reported previously. G-quadruplex formation requires the presence of a G4-compatible cation, whereas the i-motif demands acidic conditions (Figure 1). Furthermore, we show that the double-quadruplex structure can be visualized by the use of crystal violet (CV; see Figure S1 in the Supporting Information) as an external probe. The structural switching can be employed as a NOTIF logic gate (Figure 1). The simple structure can be constructed readily and economically, and structural changes are fast.

To favor the simultaneous formation of C- and G-tetraplexes, one must choose a sequence unable to form a stable intramolecular duplex. This task is difficult, as i-motif and G4 structures have "mirror" requirements. As a consequence, multiple consecutive CG base pairs can be formed. To avoid competition with the duplex structure as much as possible, we designed a sequence in which different lengths of G tracts and C tracts are present:

$^{5'}$*CCCCTTCCCC*T<u>GGGTGGGTTGGGTGGG</u>T*CCCCTTCCCC*$^{3'}$ (MIX)

The italicized and underlined bases should form i-motif and G-quadruplex structures, respectively. Furthermore, we expected the G-rich region to form a parallel G-quadruplex (as the result of two very short loops). This feature avoids peak overlap in the circular dichroism (CD) spectrum, as the i-motif has a positive peak at 286 nm, and the parallel G-quadruplex has a positive peak at 260 nm.[11,12] We previously demonstrated that the G-quadruplex formed by an oligonucleotide with the underlined sequence is very stable.[13] Since the transition temperature, $T_m$, corresponding to melting of the G-quadruplex should be higher than that for the melting of the i-motif, thermal difference spectra (TDS) and UV melting analysis can be used to distinguish the formation of the two tetraplexes. For comparison, we also designed and synthesized control sequences (see Table S1 in the Supporting Information).

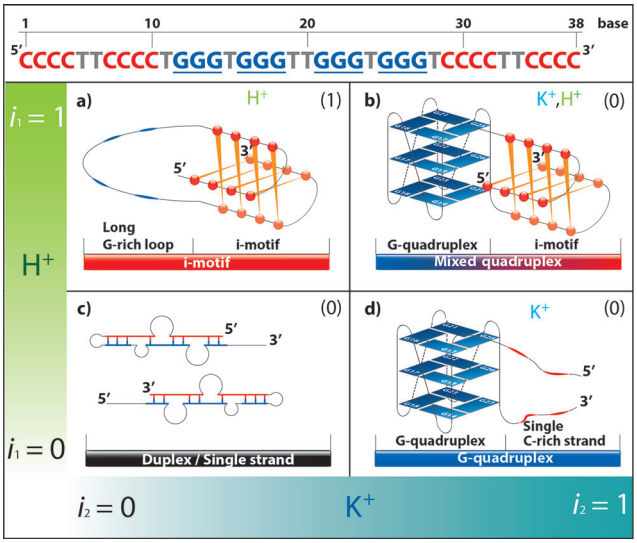


***Figure 1.*** Schematic illustration of the main structures formed by MIX under different conditions and their utility as a NOTIF logic gate in the presence of CV. The fluorescence intensity of CV at a) pH 5.8 in the absence of $K^+$ ($i_1 = 1$, $i_2 = 0$; i-motif form) is considerably enhanced (output signal, 1) relative to the intensities at b) pH 5.8 in the presence of $K^+$ (coexistence of G4 and i-motif structures), c) pH 7.4 in the absence of $K^+$ (duplex/single strand), and d) pH 7.4 in the presence of $K^+$ (G-quadruplex; output signals, 0). Thus, NOTIF gate behavior is observed.

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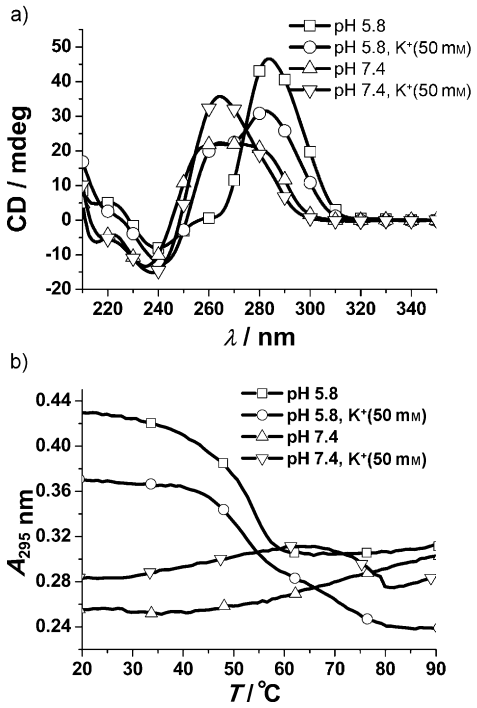


***Figure 2.*** a) CD spectra and b) UV melting profiles at 295 nm of MIX at pH 5.8 and 7.4 in the presence and absence of $K^+$ (50 mM).

The CD spectrum of MIX showed a positive peak around 286 nm at pH 5.8 in the absence of $K^+$ (Figure 2a, □). This spectrum is similar to that of the control sequence ConC (see Figure S2a) under the same conditions, which indicates that the i-motif structure can be formed with one long loop. Control sequences confirmed that the mutation of 2 or 6 cytosine residues partially or completely abolishes i-motif formation, respectively (see Figures S2b and S2c). The long G-rich loop formed trace amounts of a G-quadruplex under these conditions, as demonstrated by NMR spectroscopy (see below). In the presence of $K^+$ (50 mM) at pH 5.8, the intensity of the peak at 286 nm in the spectrum of MIX decreased, and the intensity of the peak at 260 nm increased (Figure 2a, ○). The sequence ConG had a spectrum characteristic of a parallel G-quadruplex structure (Figure S2b, blue). Interestingly, when we mutated two guanine residues, the peak at 260 nm disappeared (see Figure S2b). Therefore, in the spectrum of MIX with $K^+$ at pH 5.8, the two positive peaks around 286 and 260 nm demonstrate that both a G-quadruplex and an i-motif were formed.

For MIX at pH 7.4 in the absence of $K^+$ (Figure 2a), we observed a broad positive band from 250 to 290 nm. It was difficult to assign this spectrum, because the peak was very broad as compared to those in typical CD spectra of duplexes.[11] We hypothesize that under these conditions, MIX formed a mixture of unstructured single-strand and/or partially formed duplexes; this hypothesis was supported by UV and NMR spectroscopy, as discussed below. Interestingly, the CD spectrum of MIX at pH 7.4 in the presence of $K^+$ (50 mM; Figure 2a) had a higher-intensity peak at 260 nm and a lower-intensity peak at 286 nm as compared to the corresponding peaks in the spectrum recorded in the absence of $K^+$, whereas the peak at 260 nm disappeared in the spectrum of MutG2 under the same conditions (see Figure S2c). Therefore, we attribute the increase in intensity of the peak at 260 nm and the decrease in intensity of the peak at 286 nm in the CD spectrum of MIX in the presence of $K^+$ at pH 7.4 to the formation of a parallel G-quadruplex and unstructured C-rich strands. NMR spectroscopic analysis showed that MIX forms G-quadruplex structures and a trace amount of a duplex under these conditions (see below).

We used UV melting profiles and TDS to further demonstrate double-quadruplex formation.[14,15] For G-quadruplex and i-motif structures, but not for Watson–Crick duplexes, melting profiles show a hypochromic sigmoid transition at 295 nm.[14] The parallel G-quadruplex formed by the GGGTGGGTTGGGTGGG core should be very stable, whereas the stability of the i-motif depends on the pH value. At pH 7.4 in the absence of $K^+$, the UV melting profile of MIX at 295 nm did not show a sigmoid transition; we could therefore conclude that neither a G-quadruplex nor an i-motif is formed under these conditions (Figure 2b, △). In contrast, melting transitions were observed at pH 5.8 in the absence of $K^+$ ($T_m = 52\,°C$; Figure 2b, □) and at pH 7.4 in the presence of $K^+$ (50 mM; $T_m = 73\,°C$; Figure 2b, ▽). These two melting profiles were similar to those of the control sequences under the same conditions (see Figure S3). There was about a 3 °C difference in the $T_m$ values of ConC and MIX at pH 5.8. This difference may result from different contributions of the long guanine-rich or thymine-rich loops. In fact, the melting temperature of MutG, which has two guanine mutations in its long loop, is 51 °C at pH 5.8 (see Figure S3b). The monophasic transitions observed for MIX at pH 5.8 in the absence of $K^+$ and at pH 7.4 in the presence of $K^+$ (50 mM) are probably due to temperature-dependent unfolding of the i-motif and G-quadruplex, respectively. Interestingly, MIX exhibits two sigmoid transitions at pH 5.8 in the presence of $K^+$ (50 mM; Figure 2b, ○). The $T_m$ value of the first transition (50 °C) is similar (2 °C lower, as expected[16]) to that of MIX at pH 5.8 in the absence of $K^+$. The second transition has a $T_m$ value of 73 °C, which is similar to the $T_m$ value of MIX at pH 7.4 in the presence of $K^+$. All mutant sequences showed monophasic transitions at pH 5.8 in the presence of $K^+$ (50 mM), except MutC (see Figures S3b and S3c). Therefore, we attribute the first transition to i-motif unfolding and the second to G-quadruplex melting.

To confirm the results of UV melting analysis, we also performed CD melting experiments (see Figure S4). Overall, the CD results were in excellent agreement with UV-absorbance results and provided complementary information. Thus, these melting experiments support the hypothesis that MIX forms a double quadruplex at pH 5.8 in the presence of $K^+$.

Under certain solution conditions, the $T_m$ values of G-quadruplex and i-motif structures are significantly different (by about 20 °C). We were therefore able to obtain TDS individually by subtracting the absorbance at a mid-range temperature ("M", about 60 °C) from the absorbance at a high temperature ("H", > 90 °C) or the absorbance at a low temperature ("L", about 20 °C) from that at the mid-range

  

temperature. The TDS of both G-quadruplex and i-motif structures exhibit a negative peak around 295 nm; furthermore, two positive peaks are observed for G-quadruplexes at around 275 and 243 nm, and one positive peak is observed for i-motifs at around 239 nm.[15] For duplexes that have a high GC-base-pair content, the major positive peak is located around 276 nm, and other peaks may be observed around 237 nm.[15] At pH 5.8 in the presence of $K^+$ (Figure 3b), the TDS for "H−M" and "M−L" are typical of the G-quadruplex and i-motif, respectively, and thus demonstrate that a double quadruplex is formed.

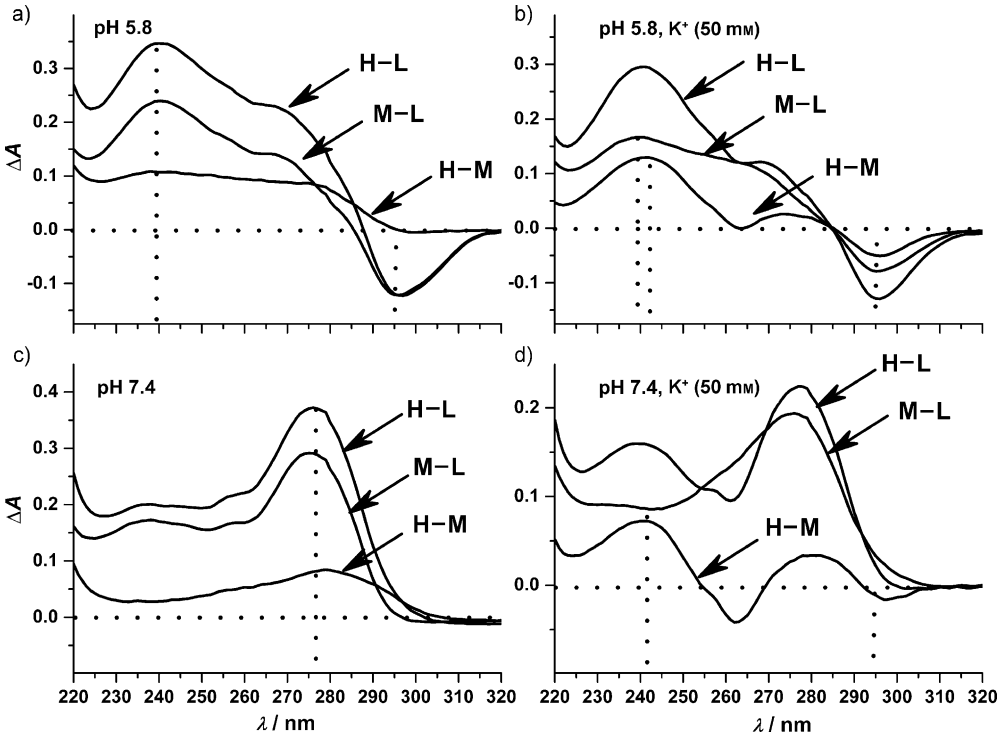


***Figure 3.*** TDS of MIX at a,b) pH 5.8 and c,d) pH 7.4 in the presence (b,d) and absence (a,c) of $K^+$ (50 mM). "H", "M", and "L" correspond to the absorbance at high, medium, and low temperature, respectively.

To confirm that MIX forms an intramolecular structure, we performed experiments at various strand concentrations. Melting temperatures for transition 1 (due to the i-motif) and transition 2 (due to the G-quadruplex) were concentration-independent (see Figure S5). These results demonstrate that intramolecular structures were formed.

It is possible that these two transitions correspond to an i-motif and a G-quadruplex formed on different strands rather than within the same strand. In support of the formation of a double quadruplex by a single oligonucleotide, the amplitudes of the transitions do not support partial formation of an i-motif or G4 for the MIX sequence; rather, the amplitudes are similar to those of the controls. If 50% of MIX is involved in G-quadruplex formation, the amplitude of that transition would be expected to be roughly half that of the transition observed for ConG, which is not the case. To further exclude this possibility, we analyzed the oligonucleotides by NMR spectroscopy.

Imino hydrogen atoms in different types of hydrogen bonds have various chemical shifts (Figure 4; see also Figure S6). For example, imino hydrogen atoms involved in a G-quartet have shifts between 10 and 12 ppm, those in $C{\cdot}C^+$ base pairs have shifts around 15–16 ppm, and those in Watson–Crick base pairs have shifts between 12 and 14 ppm.[4,17] At pH 5.8 in the presence of 50 mM $K^+$, peaks indicative of imino hydrogen atoms in both i-motif and G-quadruplex domains were observed (Figure 4a), in agreement with the previous CD and UV results. Interestingly, the sharpness of the peaks from both domains of the fully folded mixed-quadruplex structure ($H^+$ and $K^+$) is unusual for a long 38 nt sequence, especially at low pH values. This sharpness reveals a high level of compactness of the structure. In contrast, for the same double-quadruplex sequence, when one of the two domains is unfolded (in the absence of $H^+$ and $K^+$), we observed a significant broadening of the peaks from the remaining i-motif or G4 domains. The same broadening was also observed for the control and mutated sequences (Figure 4; see also Figure S6), in which only one of the two domains exists. The disruption of the G4 or i-motif domains located on the same strand induces more dynamic behavior and lowers the compactness of these half-folded structures. Therefore, the sharpness of the peaks from the mixed-quadruplex structure clearly shows that the i-motif and the G4 form simultaneously on the same strand. The spectrum of MIX at pH 7.4 in the absence of $K^+$ provides evidence for the formation of a duplex (peak around 13 ppm; Figure 4d); this signal disappeared at temperatures above 40 °C, which indicates that the duplex is not very stable in the absence of $K^+$ (Figure 4e). In general, all these results demonstrate that the mixed quadruplex is formed under acidic conditions in the presence of $K^+$; the main structures of MIX under various conditions are shown in Figure 1.

To demonstrate the application of double-quadruplex structures, we examined the fluorescence response of crystal violet (CV) under various buffer conditions. The fluorescence intensity of CV excited at 550 nm was enhanced in the presence of MIX relative to that of free CV under the same conditions (see Figure S7a). Previous studies have shown that CV discriminates the parallel G-quadruplex from the antiparallel G-quadruplex. The end loop of the antiparallel structure binds CV and thus isolates it from the solvent and

  

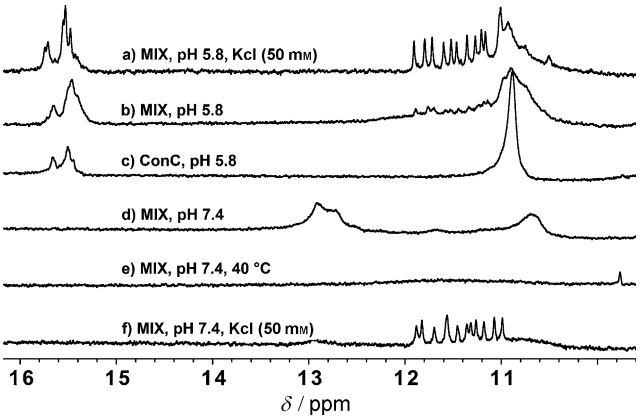


***Figure 4.*** Signals for the imino hydrogen atoms in the $^1$H NMR spectra of MIX and a control C-rich sequence under various conditions. All spectra were recorded at 25 °C, except (e), which was obtained at 40 °C for comparison (MIX, pH 7.4, 40 °C).

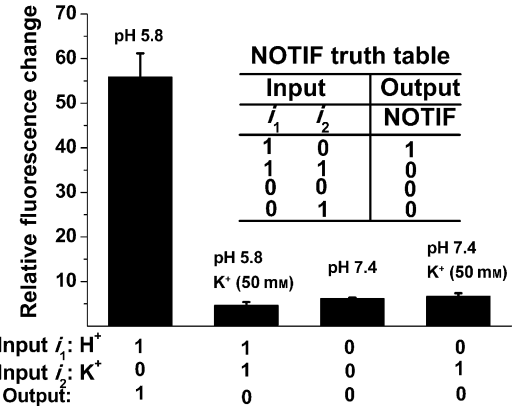


***Figure 5.*** Fluorescence enhancement of CV in the presence of MIX, as compared to the fluorescence of free CV under the same conditions. Error bars indicate the range of results obtained from triplicate experiments. The two input signals are $H^+$ ($i_1$) and $K^+$ ($i_2$), and the output signal is the fluorescence emission (0 or 1). The inset describes the NOTIF logic gate obtained.

causes the fluorescence intensity of CV to increase to a high level, whereas the side loops of the parallel structure do not.[18] In our system, the fluorescence intensity of CV under conditions in which MIX adopts the i-motif was higher (55.9 ± 5.3-fold) than that under conditions in which a mixed quadruplex, G-quadruplex, or duplex/single-strand form is formed (see Figure S7a). The high fluorescence intensity of CV bound to MIX in the i-motif form may result from the presence of the long guanine-rich loop, which may bind CV and induce the high level of fluorescence intensity of CV.[18] To test this hypothesis, we used control sequences MutG, MutG2, and ConC, which can all form the i-motif but have a different guanine content (see Figure S8). In all cases, at pH 5.8, the addition of $K^+$ led to a decrease in fluorescence emission. These results imply that the fluorescence increase is related to the presence of the long single-stranded guanine-rich region of MIX. Interestingly, with MIX, the fluorescence intensity of CV in the presence of $K^+$ was lower than with the control sequences MutG, MutG2, and ConC. This result further demonstrates that the G4 and i-motif structures are formed in the same strand.[18]

The double-quadruplex structure can be modulated to serve as a NOTIF logic gate (Figure 5). $H^+$ (pH 5.8; $i_1$) and $K^+$ (50 mM; $i_2$) are used as input signals, and the intensity of CV in the presence of MIX is used as the output signal. The fluorescence intensity at pH 5.8 in the absence of $K^+$ ($i_1 = 1$, $i_2 = 0$) is considerably enhanced (10-fold or more) relative to the intensities at pH 5.8 in the presence of $K^+$ ($i_1 = 1, i_2 = 1$), at pH 7.4 in the absence of $K^+$ ($i_1 = 0, i_2 = 0$), and at pH 7.4 in the presence of $K^+$ ($i_1 = 0$, $i_2 = 1$), confirming NOTIF gate behavior (Figure 5). Switching between the different states occurs within a few minutes or less, and may be repeated at least 10 times (see Figure S9).

In summary, we have demonstrated the coexistence of a G-quadruplex and an i-motif in a single strand for the first time. This structure was built on the basis of the principle that G-quadruplex formation requires the presence of a G-quadruplex-compatible cation, whereas i-motif formation demands acidic conditions. The constructed nanodevice is very simple and can be rapidly converted into other structures by varying the stimulus, such as the pH value or cation. Furthermore, this straightforward device can be used as a NOTIF logic gate. As compared to previous strategies, which have required labeling of the nucleic acid with a fluorescent dye, this device is simpler, cost-effective, and faster to respond. Other applications may be envisioned for this double quadruplex. First, C runs may sometimes be found adjacent to G runs in natural sequences, as shown by Johnson and co-workers.[19] Whereas G runs on both strands of duplex DNA could contribute to G-quadruplex structures, we offer an alternative possibility involving multiple structures on the same strand. Furthermore, since hydrogels can be formed on the basis of i-motif or G4 structures individually,[20,21] a double quadruplex has more triggers to induce the formation and dissociation of the hydrogel than a single structure. Therefore, the double quadruplex has unique features and may have applications in biology and nanotechnology.

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