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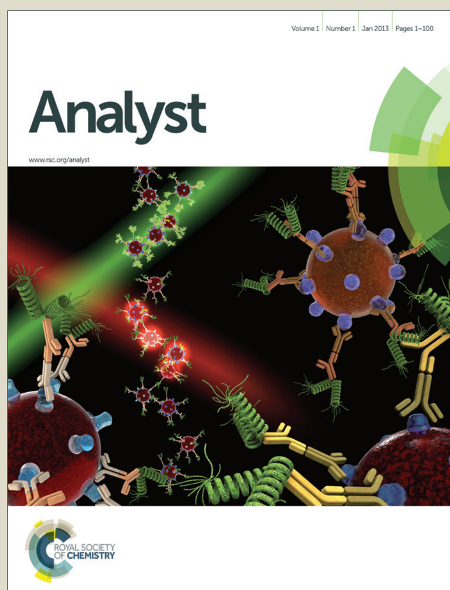

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ARTICLE

A simple approach to study the conformational switching of i-motif DNA by fluorescence anisotropy

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Fluorescence anisotropy, dictated by the rotational diffusion of the fluorophore associated entity, is sensitive to the volume and structural change, and the measurements can be performed in a homogeneous manner. In this work, a simple approach based on fluorescence anisotropy was proposed for the study of conformational switching of the i-motif structure. Factors influencing the stability of the i-motif structure, including the composition of buffer, the number of i-motif tetrads and coexistence of the complementary DNA, were interrogated in detail. This study elucidated the superiority of the fluorescence anisotropy measurement as a simple, cost-effective and sensitive method in the detection of DNA structural switching, opening a new avenue for the research of DNA structures and functions.

Introduction

The i-motif structure is a kind of quadruplex formed in cytosine-rich sequences as disclosed by Gehring et al. in 1993.[1-3] This tetrameric structure is composed of two parallel duplexes held together in an anti-parallel manner through intercalated base pairs, where the parallel duplex is built on the blocks of hemiprotonated cytosine-cytosine base pairs. Because hemiprotonation of the cytosine base is required for the formation of the quadruplex, i-motif structure is usually stable at acidic pH,[3-4] although recent researches have revealed that certain sequences can form a stable i-motif structure in the neutral pH with the assistance of molecular crowding.[5-7] The sensitive structural switching in a narrow pH range demonstrates the striking capability of the i-motif structure in pH sensing and the development of artificial molecular signaling networks with ultrahigh sensitivity, promoting the research in nanotechnology, bioengineering as well as the sensor development.[8-16] It is noteworthy that most of aforementioned applications of the i-motif structure are based on the DNA conformational switching. The stability of the i-motif structure greatly affects the sensitivity of structural switching, consequently the performance of nanomachines and sensors. Therefore, a systematic and in-depth understanding of the stability of i-motif structure is highly desirable for the extended application.

To date, a number of characterization techniques have been reported for the study of the i-motif structure, including fluorescence spectroscopy,[17-19] UV absorption,[20] circular dichroism (CD),[21] nuclear magnetic resonance (NMR),[2, 22] X-ray diffraction,[23] and laser tweezers.[24] Among these methods, fluorescence spectroscopy is one of the most important optical techniques in the research of the i-motif structure, because it is sensitive to structural changes and compatible to versatile detecting strategies, other than being cost-effective and user-friendly. With fluorescent tags and quenchers becoming commercially available, fluorescence based techniques have gained increasing popularity.[18, 25-26] Fluorescence resonance energy transfer (FRET)[26] can effectively monitor i-motif structural switching by appropriately labeling the DNA sequence with donor and acceptor fluorophores, since the FRET efficiency is highly sensitive to the distance between the donor and the acceptor. The similar strategy is applicable to fluorescence quenching, with which fluorescence intensity decreases when the fluorophore and the quencher are in close proximity.[18] Recently, fluorescence correlation spectroscopy (FCS)[25] has found the important application on the mechanism and dynamics of the i-motif structure formation at the single-molecule level, opening a new avenue for fluorescence-based techniques in the research of the DNA conformational change. However, the aforementioned methods suffer from some weakness. Specifically, two DNA fluorescence tags are usually necessary to facilitate FRET and fluorescence quenching; FCS measurements require sophisticated instrumentation and complicated data analysis. Therefore, the experimental expenses for these fluorescence-based techniques are still relatively high, and the development of a simple, rapid and more cost-effective method is highly desired.

Fluorescence anisotropy is a measure of emission depolarization when the fluorophore is excited with polarized light.[27] The anisotropy value could well report the rate of rotational diffusion of the interrogated molecule, thus is

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sensitive to the volume and structural changes of the fluorescent entity.[28-30] In our previous work,[31] we demonstrated that fluorescence anisotropy signal of terminally labeled double-stranded DNA (dsDNA) could be modulated by tuning the length and end structure of dsDNA, indicating the potential of fluorescence anisotropy in the research of DNA conformational changes. Besides, fluorescence anisotropy is a ratiometric measurement, just like signal normalization with an inner reference, thus is independent of fluctuations of light intensity and pH, facilitating simple and high-throughput measurements of systems containing i-motif DNA structures. FRET, quenching and other intensity-based measurements are more or less vulnerable to the pH change, thus are less advantageous in monitoring i-motif structure switching. Moreover, only one tag is needed for DNA modification, and a typical fluorescence anisotropy set-up simply requires a pair of polarizers, reducing substantially reagent and instrumental costs.

In this work, we demonstrate the application of fluorescence anisotropy in the study of the i-motif structure by labeling DNA at the end of the strand. Factors influencing the stability of the i-motif structure are systematically investigated, including the composition of buffer, the number of layers of i-motif tetrads and the competition between formation of i-motif and the double helical structures. Fluorescence anisotropy signal exhibits fast response to the event associated with the i-motif structural change, suggesting the potential of fluorescence anisotropy as a simple, rapid and effective method, in the research involving DNA structural changes.

Experimental

Materials and instrumentation

All the single-stranded (ssDNA) were synthesized and HPLC-purified by Sangon Biotech (Shanghai) Co., Ltd with detailed sequence information provided in the supporting information. All fluorophores used in this study, 6-carboxy-x-rhodamine (ROX) and 5-carboxytetramethylrhodamine (TAMRA), were attached to the 5′-end of the ssDNA. Glycine (AR) and trizma base (AR) were purchased from Beijing Xinjingke Biotechnology Co., Ltd. HCl (AR) and other reagents were purchased from Beijing Chemical Works. Wahaha® purified water was used throughout the study. Circular dichroism spectra were obtained with a J-810 spectropolarimeter (JASCO).

Fluorescence anisotropy measurements

Steady-state fluorescence anisotropy was measured using FLS 920 spectrofluorometer (Edinburgh Instruments Ltd.) with 587.4 nm excitation for ROX and 553 nm for TAMRA. All fluorescence measurements were carried out with the temperature of the sample compartment maintained by a circulating water bath at 25 ± 0.1 °C unless indicated. Briefly, components of the solution to be tested were added to a 1.5-mL Eppendorf tube to a final volume of 400 μL and mixed by vortex for 10 s. For dsDNA system, two DNA strands were first annealed by heating to 85 °C and slowly cooling down to room temperature. Then, hydrochloric acid with the quantity as indicated was added to the annealed dsDNA solution to form the i-motif structure. For ssDNA system, hydrochloric acid with the quantity as indicated was added to the DNA solution without the annealing step.

The anisotropy, r, of the test solution was calculated by
$r = (I_{VV}I_{HH} - I_{VH}I_{HV}) / (I_{VV}I_{HH} + 2I_{VH}I_{HV})$
where I represents the intensity of the fluorescence signal and the subscript defines the orientation (H for horizontal and V for vertical) of the excitation and emission polarizers, respectively.

Results and discussion

For fluorophore-labeled short dsDNA, fluorescence anisotropy is generally affected by the segmental motion fraction (α) of the fluorophore and the average rotational correlation time of the dsDNA, which could be described by the harmonic mean correlation time (θ_H) for non-spherical system (Equation 1).[27]

$$r = \left(1\text{-}\alpha\right) r_0 / (1 + \tau / \theta_{\mathrm{H}}) \quad (1)$$

where r_0 is the fundamental anisotropy of the fluorophore and τ is the lifetime of the fluorophore.

To obtain a high fluorescence anisotropy signal, a low segmental motion fraction is desired. In this study, ROX and TAMRA, both having positively charged centers, have been used as the fluorescent label for DNA, providing the strong electrostatic interaction with the negatively charged DNA backbone to yield a low α value. On the other hand, DNA structural transformation leads to the alteration of rotational correlation time (θ_H), which could be detected by the change of fluorescence anisotropy signal, laying the foundation for fluorescence anisotropy measurements in the application of researches related to DNA structural switching.

Effects of buffer concentration and the ionic strength on the formation of the i-motif structure

Since the hemiprotonated cytosine-cytosine base pair ($C{\cdot}C^+$) is the building block of i-motif structure, pH is the key factor for the i-motif structural formation. With the given DNA sequence, the composition of buffer, especially the concentration dictates the pH sensitivity to proton concentration, hence the structural switching of i-motif DNA.

A signal-off strategy was utilized to investigate the effect of buffer concentration on the formation of the i-motif structure (Figure 1a). Initially, i-motif DNA was hybridized with its fluorescently labeled complementary DNA, forming a rigid duplex. Our previous study proved that the double helix has a more significant contribution to the rotational correlation time than flexible ssDNA, and an overhang on the non-fluorescent strand can significantly enhance the fluorescence anisotropy signal.[30] Therefore, a high fluorescence anisotropy value was observed (Figure 1b). With the introduction of protons, solution pH gradually decreased, leading to the folding of the

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tetrameric i-motif structure and the release of the fluorescent strand. Hence, the fluorescence anisotropy signal dropped significantly. With a high buffer concentration, a less sensitive pH response to the proton was observed, and a higher concentration of proton was required to accomplish the formation of the i-motif structure. Therefore, a least buffer concentration to maintain the necessary buffer capacity was preferred to achieve the sensitive response to the structural switching.

The ionic strength is important to stabilize the double-helical structure and an insufficient ionic strength could result in the dissociation of dsDNA.[32-33] As illustrated in Figure 1c, higher fluorescence anisotropy values were observed for buffer with higher ionic strength, indicating the enhanced stability of the duplex structure. In addition, less fluctuated signals were obtained with higher ionic strength, suggesting that a high ionic strength was also favorable for stabilizing the i-motif structure.

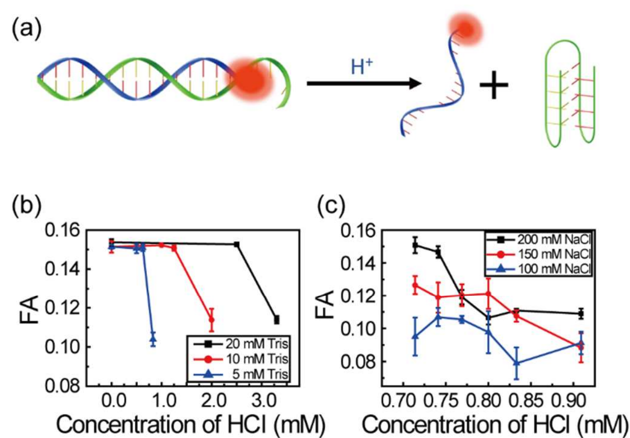

Figure 1 The schematic illustration of the signal-off mode for evaluation of the buffer and ionic strength effects (a); the effect of buffer concentration (b) and the ionic strength (c) on the formation of the i-motif structure.

Effect of the number of i-motif tetrads on the structural stability

The hemiprotonated cytosine-cytosine base pair ($C{\cdot}C^+$) is formed by three hydrogen bonds. Theoretical calculation revealed that the base-pairing energy for canonical Watson-Crick G·C is 96.6 kJ/mol, while for neutral C·C homodimer the energy is only 68.0 kJ/mol.[34] Therefore, it is difficult to form the i-motif structure at neutral or alkaline pH. However, once cytosine is protonated, the base-pairing energy for $C{\cdot}C^+$ increased to 169.7 kJ/mol,[34] even higher than that for G·C pairs. Such a stronger base-pairing interaction in proton-bound $C{\cdot}C^+$ dimer is the major driving force for the formation of the non-canonical i-motif structure in acidic pH, and a larger number of $C{\cdot}C^+$ dimers could make a more remarkable contribution to the thermodynamic stability of the i-motif structure.

As illustrated in Figure 2a, a signal-on mode was utilized to investigate the effect of the number of tetrads on the formation of the i-motif structure. The original weak fluorescence signal was from the flexible ROX-labeled ssDNA (Figure 2b). With the ascending concentration of the acid, anisotropy signal gradually increased due to the higher value of the rotational correlation time (θ_H) attributed to the folded i-motif structure. The i-motif structure with larger numbers of tetrads was more sensitive to the introduction of protons because of the higher thermodynamic stability originated from more $C{\cdot}C^+$ dimers. Besides, a larger layer number also led to a bigger rotational volume; hence, the final structure exhibited a higher anisotropy value. Same phenomena were also observed with TAMRA as the fluorescent label (Figure 2c), confirming the effect of the number of tetrads on the stability of the i-motif structure and also indicating that the fluorescence anisotropy measurement was a reliable and robust technique to study the DNA conformational switching (Figure S1). The signal change expand (~0.05) is comparable to most currently available fluorescence anisotropic methods, suggesting the sensitive response of fluorescence anisotropy measurements to the DNA structural change.

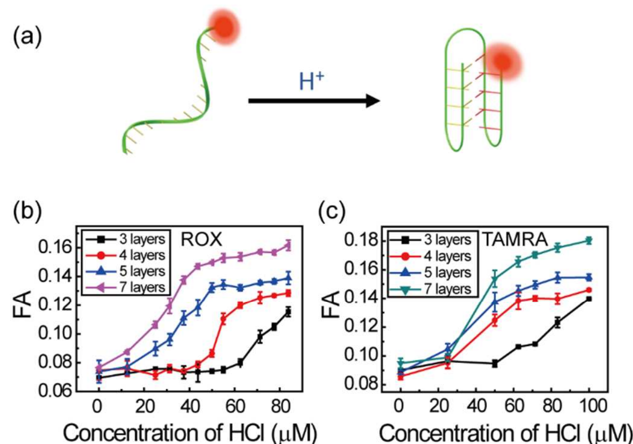

Figure 2 The schematic illustration of the signal-on mode for evaluation of the effect of the number of i-motif tetrads (a); the effect of the number of i-motif tetrads on the structural stability of ROX-labeled (b) and TAMRA-labeled (c) i-motif sequences.

The competition between i-motif structure and Watson-Crick duplex

When i-motif DNA coexists with its complementary DNA, there is competition between formation of the tetrameric i-motif structure and the canonical Watson-Crick duplex, which is dictated by temperature and pH. At a high temperature, for example higher than 60 °C, all folded structures dissociated to single strands; at room temperature, both dsDNA and i-motif structures could possibly be stable and the reaction equilibrium was mostly determined by solution pH.[35]

In alkaline condition, $C{\cdot}C^+$ base pairs are thermodynamically less favorable,[35] thus dsDNA is the major species (Figure 3a). With the descending of pH, DNA sequences were more apt to fold into i-motif structures due to the stronger $C{\cdot}C^+$ interaction than G·C base-pairing, leading to the increase of fluorescence anisotropy signal (Figure 3b). In the presence of mismatches, the initial double helix was destabilized and might dissociate to fold into the i-motif structure, resulting in the high initial fluorescence anisotropy value.

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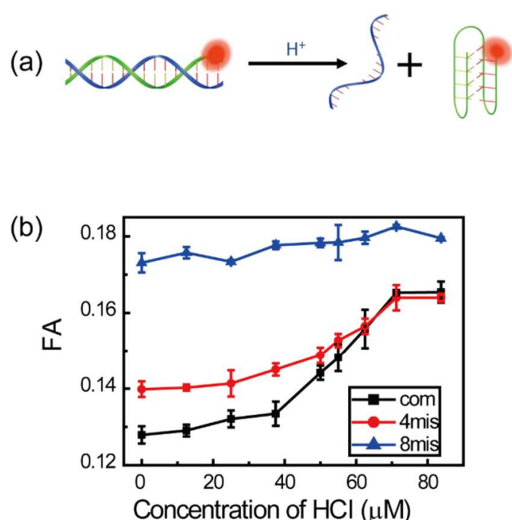

Figure 3 (a) The schematic illustration for evaluation of the competition between i-motif structure and Watson-Crick duplex; (b) The competition between i-motif structure and Watson-Crick duplex with no mismatched site (com), 4 (4mis) and 8 (8mis) mismatched sites.

Circular dichroism (CD) spectroscopy confirmed the conformational changes with pH alteration. Initially, the i-motif DNA was a flexibly coiled single strand with no characteristic peak of the i-motif structure, while its complementary sequences could fold into either a parallel (Figure 4a) or an anti-parallel (Figure 4c) G-quadruplex.[36] When the i-motif DNA was annealed in the presence of the complementary DNA, the CD spectrum was distinctive from the sum of the two separate spectra, suggesting formation of a new structure, i.e. dsDNA. However, if the i-motif strand was annealed with a strand with 8 mismatched sites, the spectrum was similar to the sum of the two separate spectra, indicating a compromised hybridization efficiency of the two sequences. With the introduction of sufficient amount of protons, the i-motif DNA could finally successfully fold into the tetrameric structure with a characteristic positive peak at 287 nm and a negative peak at 263 nm,[37] whereas G-quadruplex structures were not affected by proton (Figure 4b and 4d). For a dsDNA containing the i-motif sequence, the presence of proton caused remarkable structure transformation, with the rising of a new peak at 287 nm and the attenuation of the original peak at 263 nm. The resulted spectrum was distinctly different from the sum of the spectrum of two individual strands at its stable conformation, suggesting that only part of the initial dsDNA dissociated and then transformed to i-motif structure. Nevertheless, with mismatches in the complementary strand, the CD spectra showed a mixture of i-motif and G-quadruplex structures, indicating that the relative thermodynamic stability of i-motif structure to dsDNA was increased with the introduction of mismatched sites.

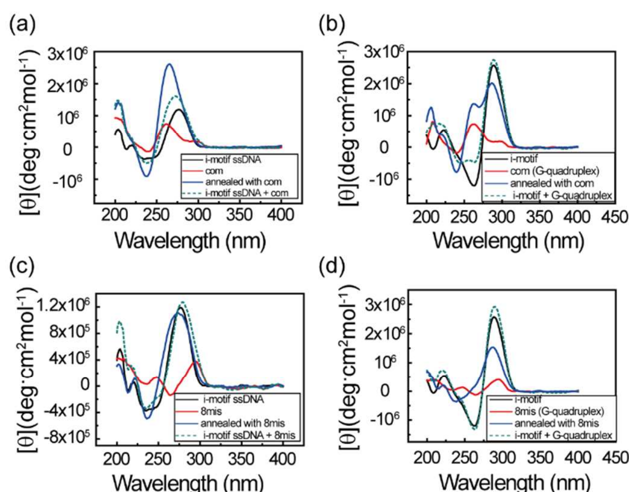

Figure 4 The CD spectra of i-motif DNA, complementary DNA and the annealed DNA mixture without introduction of HCl (a,c) and with sufficient HCl (b,d). The initial and final states of the complementary DNA with no mismatched site (a,b) and 8 mismatched sites (c,d).

Reducing the stability of the i-motif-containing dsDNA could effectively help the switch from double helix to the i-motif structure. Reducing the length of dsDNA was one approach to destabilize dsDNA for the promotion of structural transformation. As shown in Figure 5a, when dsDNA was shortened by five base pairs with the number of i-motif tetrads unchanged, no remarkable differences were observed with the coexistence of complementary DNA or the strands with mismatched sites, indicating that the i-motif structure was more preferable than dsDNA and no mismatches were required to further reduce the thermodynamic stability of dsDNA. On the other hand, reducing the number of i-motif tetrads could also jeopardize the stability of dsDNA. Despite that it was also disadvantageous for the stability of i-motif structure, the conformational transformation was still encouraged in this case and no mismatches were further needed (Figure 5b).

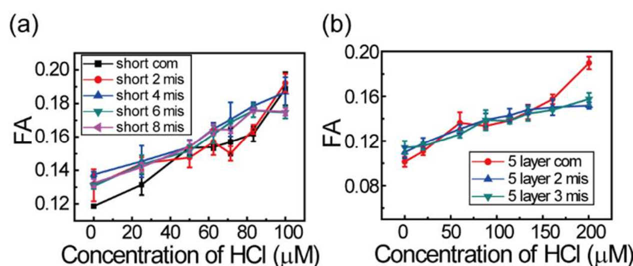

Figure 5 The promotion of structural switching from dsDNA to the i-motif structure by reducing the length of dsDNA (a) and reducing the number of i-motif tetrads (b).

Conclusions

Fluorescence anisotropy was for the first time used as an approach to study the structural switch of the i-motif structure. The factors influencing the stability of the i-motif structure, including the composition of buffer, the number of the i-motif

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tetrads and the coexistence of the complementary DNA, were systematically investigated, providing the foundation for future sensor development and nanomachine fabrication involving the i-motif structure. Moreover, this study demonstrated the superiority of the fluorescence anisotropy measurement as a facile, cost-effective and sensitive structural change responsive method in the research of DNA structures and functions.

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