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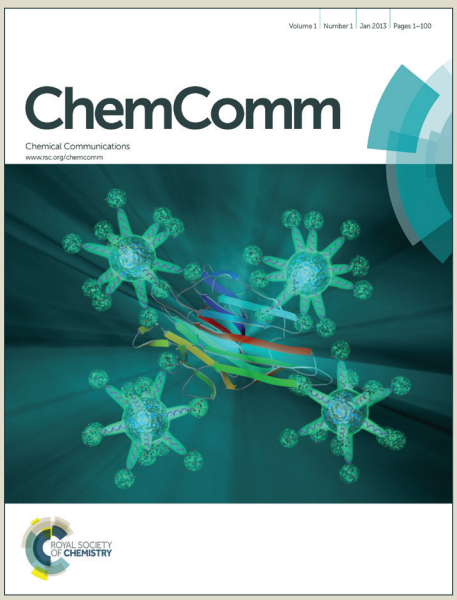

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Probing structural changes of self assembled i-motif DNA†

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We report an i-motif structural probing system based on Thioflavin T (ThT) as a fluorescent sensor. This probe can discriminate the structural changes of RET and Rb i-motif sequences according to pH change.

Molecular recognition is the basic module for discovering novel types of probing systems and developing diverse types of drugs.[1] Studying self-assembly of DNA and RNA has gained a lot of attention not only for designing bio-inspired nanomaterials,[2] but also as an attractive component of molecular recognition modules.[3] There are several types of natural self-assembled DNA and RNA structures, such as classical double helix, triplex, 4-way junction, hairpin, Z-DNA, G-quadruplex, and i-motif structures.[4]

Among these structures, G-quadruplex is composed of G (guanine)-rich sequence with a guanine quartet formed by Hoogsteen base pair and alkali cations in the center; whereas i-motif is induced by C (cytosine)-rich sequences and stabilized at low pH due to the formation of hemi-protonated C·C+ base pair.[5] G-quadruplex and i-motif have been discovered in the promoter regions of human genes, including proto-oncogene[6] (RET) and retinoblastoma[7] (Rb) gene. The RET gene encodes a tyrosine kinase which has been connected to the growth of human cancer,[6] while Rb gene encodes a tumor suppressor (retinoblastoma), which is a nuclear phosphoprotein related to the cell cycle.[7]

Name	Sequence (5' to 3')
i1 (RET)	CCCGCCCCGCCCCGCCC
i2 (Rb)	CCGCCCAAAACCCCCC

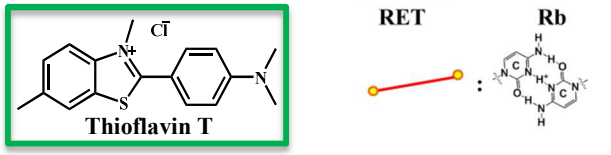

Fig. 1 Sequences, and structures of i-motif (RET, Rb) and Thioflavin T (ThT).

Thioflavin T (ThT), which is a benzothiazole moiety used to probe amyloid fibrils,[8] was recently reported for recognizing duplex,[9] and G-quadruplex DNA structures.[10] ThT is composed of dimethylaminobenzene and benzothiazole moieties which are linked by a single carbon bond.[11] This carbon bond tends to be in a non-fluorescent twisted state with a dihedral angle of 90°. ThT requires an interaction with a "host" molecule in order to prevent the benzothiazole and the benzene rings from twisting which renders it fluorescent. In this communication, we utilize Thioflavin T as a novel fluorescent sensor to detect the i-motif DNA in RET proto-oncogene and Rb retinoblastoma genes (Fig. 1). Fluorescence and CD spectroscopy were employed to study the interactions between i-motif DNA structures (RET and Rb) and ThT moiety at different pH values.

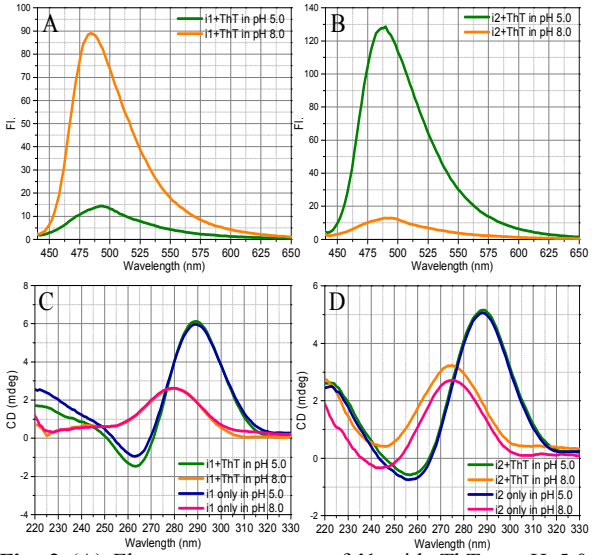

Fig. 2 (A) Fluorescence spectra of **i1** with ThT at pH 5.0 (i-motif structure) and pH 8.0 (random coil), (B) Fluorescence spectra of **i2** with ThT at pH 5.0 (i-motif structure) and pH 8.0 (random coil), (C) CD spectra of **i1** with ThT at pH 5.0 (i-motif structure) and pH 8.0 (random coil), (D) CD spectra of **i2** with ThT at pH 5.0 (i-motif structure) and pH 8.0 (random coil). All samples were prepared by 1.0 μM DNA and 6.0 μM ThT in 50 mM Tris-HCl buffer at 25 °C, and the fluorescence spectra were measured after excitation at 425 nm.

A dramatic change occurred in the fluorescence emission spectra of the ThT with **i1** and **i2** upon its structural transition from random coil to i-motif structure (Fig. 2A and B). Interestingly, the patterns of the fluorescence are opposite. In the case of **i1**, the emission was decreased around 6.3 times according to the pH change from pH 8 to pH 5. In the case of **i2**, the emission was increased around 10 times according to the pH change from pH 8 to pH 5. Such increases in fluorescence intensity of ThT (**i1** at pH 8.0 and **i2** at pH 5) mainly occur when i-motif sequences intercalate with hosts or cavity structures.[9]

We also recorded circular dichroism (CD) spectra to study the conformational transitions in the i-motif sequences (Fig. 2C and D). The characteristic features of the i-motif structures (pH 5.0) were represented by a strong positive band near 290 nm and a negative band around 260 nm.[6, 7] The i-motif spectra were compared (**i1** and **i2** individually) with and without ThT. The obtained results suggest that addition of ThT did not interrupt the conformation of the i-motif structure.

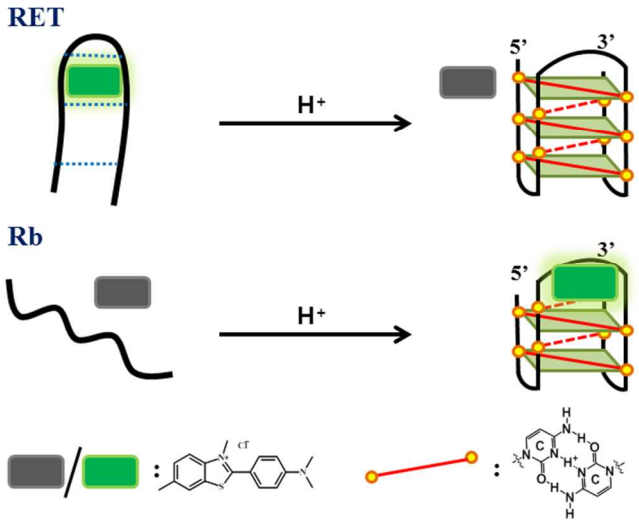

Fig. 3 Schematic diagrams for the intercalation of ThT into the i-motif sequences (RET and Rb).

For the RET sequence, a fitting cavity is available in a flexible hairpin structure[12] at a neutral pH value (Fig. 3). On the other hand, a satisfactory cavity is located in the i-motif structure of Rb due to the presence of the big adenosine loop (-AAAA-). The interaction of the intercalated ThT with the cavities of the sequences is the stabilizing force for ThT binding (Fig. 3). These interactions limit the torsional rotation between the dimethylaminobenzene and benzothiazole moieties, decreasing the nonradiative twisted internal charge-transfer (TICT) state.[13] Because of these cavities, these stacking interactions can occur in the i-motif DNA, and increase the fluorescence intensity.

We then investigated the fluorescence and CD spectra of **i1** with ThT at various pH values. Decreasing the pH value from 8.0 to 5.0 resulted in a decrease in the fluorescence intensity and a minor bathochromic shift (~10 nm) of the fluorescence maximum (Fig. 4A). Plotting fluorescence depending on pH revealed that the change was sigmoidal with a transition midpoint at pH 6.4 (Fig. 4C). The changes in fluorescence intensity were consistent with the structural changes observed in the CD spectra (Fig. S1). The most significant changes in the fluorescence and CD spectra occurred at a pH range 6.0-7.0, thus we believe that this fluorescent probe will have good sensitivity when used in biological systems.

We also checked fluorescence and CD spectra of **i2** with ThT at various pH values. Decreasing the pH value from 8.0 to 4.0 resulted in a fluorescence intensity increase in the range of 8.0 to 6.3 followed by a slight decrease in the range of 6.3 to 4 (Fig. 4B). Plotting the spectra depending on pH showed two sigmoidal transitions in the fluorescence with midpoints at pH 6.9 and 5.9 (Fig.4D). These changes in fluorescence intensity were consistent with the structural changes observed in the CD spectra (Fig. S2). The most significant changes in the fluorescence and CD spectra occurred in the pH range 6.0–7.0. These are also consistent with the CD results without the ThT (Fig. S2). This phenomena is not similar with the results of the natural Rb i-motif,[7] but agrees with the data of the modified Rb sequences.[14]

The obtained fluorescence of complexation process of the ThT with **i1** and **i2** was correlated with a Job's plot that indicates a 1:1 stoichiometry (Fig. S3). The association constant for the complexation process of **i1** with ThT was estimated to be 2.516 x 10^5 M^{-1} (error < 10%) and for **i2** with ThT is found to be 1.332 x 10^5 M^{-1} (error < 10%) from Benesi–Hildebrand equation using fluorescence data at pH 8 and 5 respectively (Fig. S4).

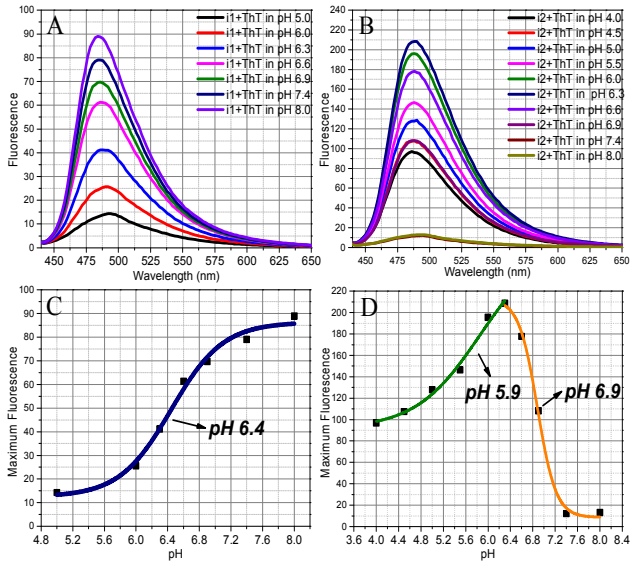

Fig. 4 (A) Fluorescence spectra of **i1** with ThT at various pH values from pH 5.0 (black line, i-motif structure) to pH 8.0 (purple line, random coil), (B) Fluorescence spectra of **i2** with ThT at various pH values from pH 4.0 (black line, i-motif structure) to pH 8.0 (dark yellow line, random coil), (C) Maximum fluorescence intensity of **i1** depending on pH values, (D) Maximum fluorescence intensity of **i2** depending on pH values. All samples were prepared by 1.0 μM DNA and 6.0 μM ThT in 50 mM Tris-HCl buffer at 25 °C, and the fluorescence spectra were measured after excitation at 425 nm.

We finally measured the melting temperatures using CD to determine the stability of the structures at low pH values (Table 1; Fig. S5–S7). There are no critical differences between the values obtained with and without ThT (0.4-0.7 ^{o}C). In the case of **i2**, however, there are significant differences between pH 6.3 and pH 4.0 (4.4-5.3 ^{o}C). This implies that the structure at pH 6.3 is more stable than that at pH 4.0.

	i1, pH 5.0	**i2**, pH 6.3	**i2**, pH 4.0
With ThT	60.5 °C	57.8 °C	53.4 °C
Without ThT	61.2 °C	58.2 °C	52.9 °C

Table 1 Melting temperatures measured by CD for **i1** and **i2** with and without ThT. All samples were prepared by 1.0 μM DNA and 6.0 μM ThT in 50 mM Tris-HCl buffer. Melting of i-motif structure was monitored at 290 nm.

The reason can be due to the acid sensitivity of the –AAAA- loop,[2] which can make different sized cavities according to the pH value.[15] This ThT probing system can detect these slight changes better than the typical CD measurement.

Conclusions

We have discovered an interesting relation between ThT and i-motif sequences (RET and Rb) for probing conformational changes. More importantly, there are dramatic changes measured in the fluorescence emission spectra of the ThT with **i1** (RET) and **i2** (Rb) upon its structural transition from random coil to i-motif structure. The fluorescence patterns are opposite because of the cavity but follow the patterns of the CD spectra exactly. More experimentation is currently underway to expand the scope of the work by testing different structural mutations to better explain the mechanism of interaction. This simple system is useful for probing i-motif structures and should assist in the development of stable DNA-based nanostructures.

Notes and references

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† Electronic Supplementary Information (ESI) available: Experimental details, CD spectra and CD melting curves. See DOI: 10.1039/c000000x/

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