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A novel fluorescent reagent for recognition of triplex DNA with high specificity and selectivity

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Abstract: A fluorescent agent (DMT) was screened for recognizing triplex DNA with the specific and selective characteristic, which was embedded into the triplex DNA structure. The triplex DNA was firstly formed by a complementary target sequence through two distinct and sequential events. The conditions including pH and hybridization time, fluorescent agent concentration and embedding time were optimized in the experiment. Under the optimum condition, the fluorescent signal was enhanced up to 9-fold in comparing with the DMT embedding into the ssDNA, dsDNA and G-quadruplex. Under the same fluorescence conditions, the changes of fluorescence signal were also investigated by several kinds of base mismatched DNA in the experiment. The results showed that our biosensor provided excellent discrimination efficiency toward the perfectly mismatched target DNA with no formation of triplex DNA. We preliminarily deduced the mechanism of the fluorescent reagent for recognizing triplex DNA with high specificity and selectivity.

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Introduction

DNA structures can be divided into single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), triplex DNA and G-quadruplexe.[1, 2] Triplex DNA has been greatly developed since the first description of triple-hehical nucleic acid structures in 1957, [3,4] which is formed through the interaction between a double-helical DNA target and a target DNA. Stable triplex formation occurs essentially through DNA recognition of three-two-base motifs in the third strand, TC (pyrimidine), GA (purine) and GT motifs. [5, 6] The formation of triplex DNA can be regulated by external triggers such as salt concentration, pH and temperature.[7-9]Triplex DNA has attracted intense interest as a target of small molecule therapeutic agents, and triplex DNA has been recognized as a potentially powerful tool for numerous applications in medicine and molecular biology.[10-14] Triplex DNA has also been one of the most attractive recognition motifs in the design of strategies for sequence-specific labeling, regulating gene expression and constructing DNA-based nanostructures.[15-19] Therefore, it is significant to develop new selective and specific methods for the recognition and detection of triplex DNA.[20]

Nowadays, a few typical methods for screening and detecting triplex DNA have been reported, such as competitive dialysis,[21] mass spectroscopy,[22] gold nanoparticle -based sensors,[23] gold nanoparticle-based microarrays[24] and electrophoretic mobility with UV vis melting experiment.[25-27] Most of these methods have high equipment cost, complex synthesis procedures, time-consuming sample preparation and low-throughput screening. Due to their high sensitivity, low costs and miniaturization

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possibilities, electrochemical methods also provide interesting perspectives in the field of nucleic acid analysis.[28,29] However, electrochemical methods have poor stability and reproducibility. Fluorescence is a favored signaling technology for molecular diagnoses on account of its simplicity and high sensitivity, and fluorescence-based strategies have been exploited for the screening purposes.[30,31] A few fluorescent dyes have been reported to present a more intense fluorescence emission in the presence of G-quadruplexes than dsDNA.[32-34] Naphthylquinoline compounds and derivative compounds have been reported for binding to triplex DNA with an excellent affinity.[35,36] Few fluorescence reagents which are selective and specific recognition for triplex DNA have been reported.[37] Therefore, it is necessary to developed a novel fluorescence agent to recognize the triplex DNA with high selectivity and specificity.

In our present work, a pyridine derivative named methyl-2, 6-[2-(4-methyl-sulfanyl-phenyl)-vinyl]-pyridine (DMT) was screened to recognize the triplex DNA. The structure of DMT was shown in Figure 1. When DMT was embedded in ssDNA, dsDNA, triplex DNA and G-quadruplex, the obvious different fluorescence was observed in the experiment. Here we investigated the condition of the triplex DNA formation and difference of fluorescence between DMT and these DNA by a fluorescence spectroscopy. The interaction conditions were systematically optimized and the interaction mechanism was deduced between triplex DNA and DMT. At the optimized condition, the fluorescence characteristics were investigated by several kinds base mismatched DNA in comparing with a target DNA for the formation of

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triplex DNA.

Experimental section

Materials and apparatus

Tris [hydroxymethyl- aminomethane] hydrochloride, hydrochloric acid and sodium chloride were of analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). They were used directly without further purification. The oligonucleotides were synthesized and purified through HPLC by Sangon Biotechn. Co., Ltd. (Shanghai, China). Their sequences are listed as following: H_2: 5'- CC TTTTCTTTTAT-3', H_1:5'-TATTTTCTTTTCCCCCCAGTATTATTCCCCCTTTTC TTTTAT-3', Target DNA: 5'-GGAAAAGAAAATA-3', d(TTAGGG)$_4$, single base mismatched target: 5'-GGATAAGAAAATA-3', two base mismatched target: 5'-GGA TAAGAACATA-3', a perfectly mismatched target: 5'-CCATTACATTATA-3'. All oligonucleotides were dissolved in ultrapure water and stored at -4 □. The DMT reagent was supplied by Lu group (Guangdong University of technology) and dissolved in ultrapure water as a stock solution. The ^{1}H NMR and ^{13}C NMR spectra were recorded by AVANCE III 400 (Bruker, Switzerland). A high-resolution ESI-MS was performed by LCQ Fleet (Thermo Fisher Scientific, USA). CD spectra were measured at room temperature on an Aviv Model 410 (Aviv Biomedical, USA). IR spectra were measured by Nicolet 360 (Nicolet, USA). Ultrapure water obtained from a Millipore water purication system and puried by using a Milli-Q Academic purication set (Millipore, Bedford, MA, USA).

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Fluorescent measurements

The fluorescent spectroscopy of the system was measured by a Sanco 970CRT Spectrofluorophotometer (Shanghai Tianmei company), which was comprised of an xenon short arc lamp as an excitation source, a 200-900 nm double grating excitation and emission monochromators. When the DMT emission was measured, the excitation wavelength was set to 425 nm and the scanning range was 500～600 nm. The sensitivity was set to 5 and the slits widths were 10 nm for both excitation and emission with a quick scan. Three replicate samples were performed and calculated by an average in each experiment.

Procedure for triplex DNA formation

All DNAs were dissolved in the ultrapure water to obtain 1 μM. Then, 20 μL H_1 and 20 μL target DNA were firstly mixed and diluted to 200 μL by 100 mM NaCl/ Tris-HCl buffer solution (pH=7.4). The final solution was heated at 37 □ for the formation of the triplex DNA (Figure 1A).

Results and discussion

DMT characteristics

Methyl-2, 6-[2-(4-methyl-sulfanyl-phenyl)-vinyl]-pyridine (DMT) was supplied by the Lu group and the structure showed in Figure 1. The 1H NMR and 13C NMR spectra were recorded at 400 and 100 MHz, respectively. The results show as following: 1H NMR (400 MHz, DMSO) δ 8.41 (t, J = 8.1 Hz, 1H), 8.26 (d, J = 8.1 Hz, 2H), 7.82 (d, J = 8.5 Hz, 4H), 7.76 (d, J = 15.9 Hz, 2H), 7.64 (d, J = 15.9 Hz, 2H),

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7.37 (d, J = 8.5 Hz, 4H), 4.29 (s, 3H), 2.55 (s, 6H); 13C NMR (100 MHz, DMSO) δ 153.82 (s), 143.27 (s), 142.36 (s), 131.95 (s), 129.43 (s), 126.13 (s), 124.01 (s), 118.45 (s), 42.14 (s), 14.74 (s). The IR spectrum was recorded at the characteristic peak (Figure 2). A high-resolution ESI-MS was calcd for $C_{24}H_{24}NS_2$ (M^+) m/z 390.3913 and observed m/z 390.1217. These results confirmed that the structure of DMT was reasonable.

To confirm DMT with a selective and specific recognition for triplex DNA, DMT were embedded into the different DNA chains (ssDNA, dsDNA, triplex DNA and G-quadruplex) in comparing with these common fluorescent reagents (EB, Green1 and Green2), respectively. The fluorescent intensity of these common fluorescent reagents was steadily enhanced with increase of DNA chain numbers (table 1). The results indicated that three reagents had no selective and specific recognition for triplex DNA. However, we firstly observed that the fluorescent intensity of DMT in the triplex DNA had markedly improved to 9-fold than in the presence of ssDNA, dsNDA and G-quadruplex (Figure 1B). Therefore, the DMT reagent had different characteristic from other fluorescent reagents for the recognition of triplex DNA.

Optimal conditions and verification for triplex DNA formation

The influencing factors including pH and hybridization time were investigated to explore the chemical microenvironment for the triplex DNA formation. As a target DNA, oligonucleotides (TFOs) bind to double-stranded DNA (dsDNA) for the triplex DNA formation. Triplex DNA are formed by two Hoogsteen hydrogen bonds between T and C in the TFO with AT and GC base pairs in dsDNA, respectively, which is

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stable less than the Watson-Crick base pairing in the duplex DNA.[38] The Hoogsteen parallel interaction was almost insensitive to pH dependence.[39-41] The pH (6.0, 7.4, 8.8) was investigated in the experiment. The evidence of sensing mechanism for triplex DNA formation was also supported by the obtained results (Figure 3). The results indicated the fluorescent intensity was the strongest at pH 7.4 and the evidence of result also showed that Hoogsteen interactions were much stable at pH 7.4. Therefore, the pH 7.4 was selected for the triplex DNA formation.

Another factor, the hybridization time between H_1 DNA chain and target DNA was also important for the triplex DNA formation. The hybridization time could influence the degree of their combination between the H_1 DNA and the target DNA. Therefore, based on the change of the fluorescent intensity by the ability of DMT embedding into the triplex DNA, the hybridization time of H_1 DNA and target DNA was investigated for the triplex DNA formation. The results showed that the fluorescent intensity increased with the increasing of hybridization time between H_1 DNA chain and target DNA (Figure 4). However, when the hybridization time was up to 120 min, the fluorescent intensity was stable. Therefore, 120 min was selected as hybridization time.

The amount of DMT embedding into the triplex DNA could influence the fluorescent intensity. In order to investigate the amount of DMT embedding into the triplex DNA, the DMT concentration and embedding time were studied. Different DMT concentrations including 2 μM, 10 μM, 25 μM and 50 μM were performed to the experiment. The result showed that the fluorescent intensity increased with the

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increase of DMT concentrations (Figure 5). When the DMT concentration was up to 25 μM, the degree of improvement was slight, the phenomenon also indicated that 25 μM could mainly fill up the groove of triplex DNA. Hence, we chose 25 μM as the DMT concentration.

The time of embedding into triplex DNA could influence amount of DMT, which further affected the fluorescent intensity. Therefore, different embedding times were investigated at the room temperature. When the embedding time was up to 50 min, the loading capacity of triplex DNA reached equilibration (Figure 6).

To prove the formation of triplex DNA, CD spectra of H_1 DNA and triplex DNA have been recorded (Figure 7). Compared with the spectra of H_1 DNA (curve a), a new characteristic peak (285 nm) appears in the spectra of triplex DNA (curve b), which proves the formation of "triplex-stem" DNA.

Sequence selectivity

To explore the specificity and selectivity of target DNA for the formation of triplex DNA, a few base mismatched targets DNA were investigated to compare the perfectly matched target DNA. We were able to observe some significant signal changes in the presence of the base mismatched target DNA. Compared a perfect matched DNA, the single mismatched DNA gave a slight change. The fluorescent signal decreased gradually with the increasing of the mismatched degree. When a perfectly mismatched target DNA was employed in the experiment, it was observed that the fluorescent intensity was up to ssDNA or dsDNA signal. The results also confirmed that the formation of triplex DNA was significant to the fluorescent signal. It was a

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reasonable explanation that different mismatched target DNAs had different affinity constants (K_D) with H_1 DNA chain.

Preliminary mechanism

A new method was provided for discriminating the triplex DNA in the experiment. To further explore the excellent selectivity and specificity, we should recognize the structure of DMT. Firstly, the rotating of double bonds are unable to form a rigid plain structure, thus, DMT has weak fluorescence. When the DMT is embedded into the triplex DNA, the aromatic ring surface can stack well on the base triplets within the triplex structure. Compared to the ssDNA, dsDNA and G-quadruplex, the triplex structure can possibly prevent DMT molecules turning for the better rigid plane structure, therefore, the fluorescent intensity of DMT is improved. Secondly, the pyridine ring nitrogen atom and terminal sulfhydryl group of the side chain are protonated in the intercalation complex, yielding a dication with a favorable polyelectrolyte contribution to improve the binding free energy (ΔG), and the triplex binding free energies were relation to the binding constant (*Ka*).[36]

$$\Delta G = - RT\ln Ka \quad (1)$$

The negative value of ΔG indicates higher triplex binding affinity. Therefore, the structure of DMT has specificity and selectivity for triplex DNA. Thirdly, pyridine ring nitrogen atom shows preferential binding to the T·AT triplex structure.[42, 43] Lastly, it is possible due to the fact that the structure of triplex DNA can improve the solubility of DMT, which can increase the effective probe concentration. In summary, no matter from theory or experiment, DMT has excellent specificity and selectivity

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for triplex DNA with compared to ssDNA, dsDNA and G-quadruplex.

Conclusions

The H_1 DNA was bound a target DNA through hybridization technique, which could lead to the triplex DNA formation at the optimal hybridization conditions. Compared to DMT embedding into the ssDNA, dsDNA and G-quadruplex, the fluorescent intensity had obviously improved up to 9-fold at the same fluorescent determination condition. The max ability to embed DMT of the triplex DNA has been investigated and the mechanism of selectivity for triplex DNA has been discussed. The drawback of our approach may be represented by the fact that the sequences of the triplex DNA formation are usually limited to detect the target DNA with background interference of ssDNA or dsDNA. Therefore, as a result of the extraordinary fluorescent reagent, it can only provide an excellent and efficient discrimination for the triplex DNA.

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Figure 1

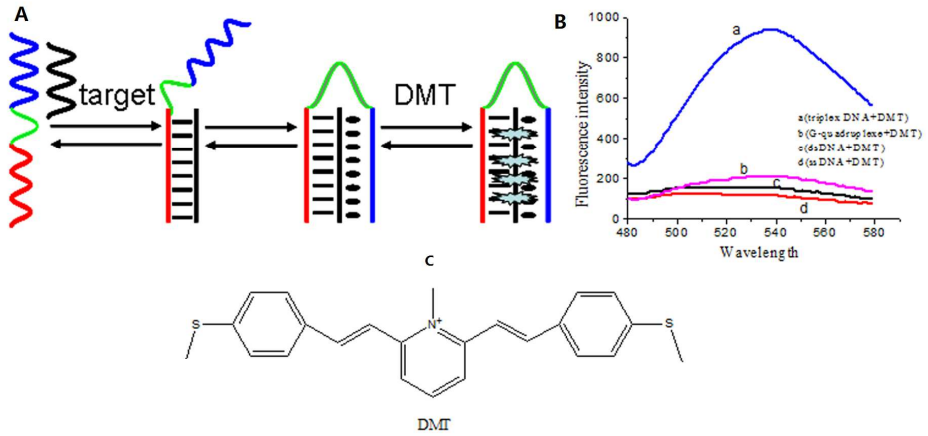

Figure 1 (A)The scheme of triplex DNA formation; (B)The fluorescence intensity curves of the DMT embedding into ssDNA, dsDNA, triplex DNA and G-quadruplex, respectively; (C) The DMT structure. Conditions: 100 mM NaCl/ Tris-HCl buffer solution (pH=7.4) at 37 □ for triplex DNA formation and 25μM DMT was added to the mixture solution; Excitation wavelength: 425 nm; Scanning range: 500～600 nm.

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Figure 2

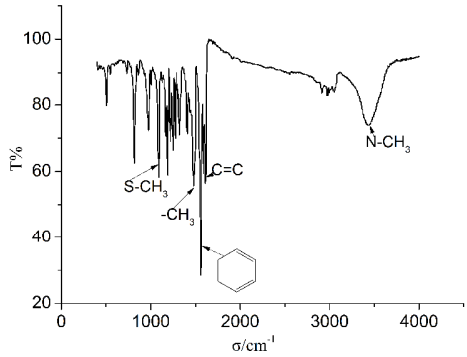

Figure 2 The FT-IR spectra of DMT.

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Figure 3

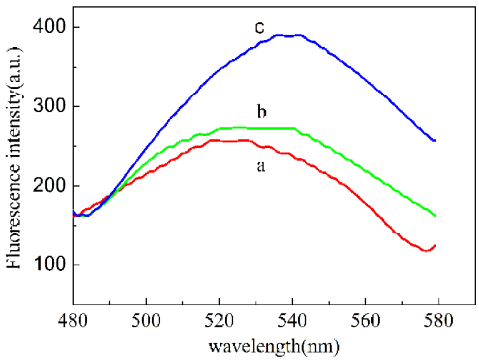

Figure 3 The effect of pH on the fluorescence intensity of triplex DNA formation (a) pH 8.8; (b) pH 6.0; (c) pH 7.4. Conditions: 100 mM NaCl/ Tris-HCl buffer solution at 37 □for triplex DNA formation with various pH; other conditions: see Fig.1

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Figure 4

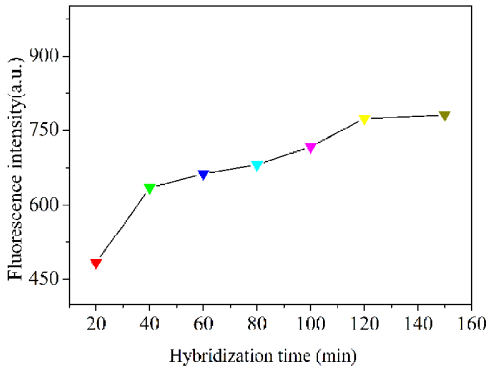

Figure 4 The effect of hybridization time on the fluorescence intensity of triplex DNA formation. Conditions: 100 mM NaCl/ Tris-HCl buffer solution at 37 □ for triplex DNA formation with various hybridization times (20, 40, 60, 80, 100, 120, 140, 160 min); other conditions: see Fig.1

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Figure 5

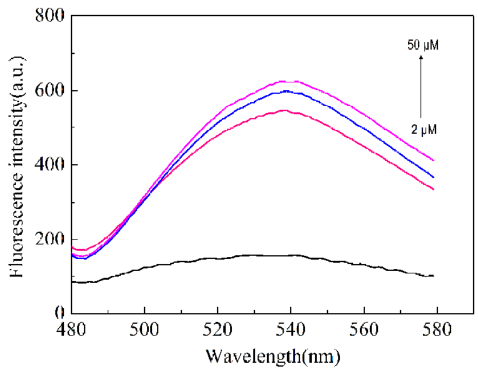

Figure 5 The fluorescence curves of different DMT concentrations embedding into triplex DNA. Conditions: 100 mM NaCl/ Tris-HCl buffer solution at 37 □ with different concentrations (2μM, 10 μM, 25 μM and 50 μM) embedding into triplex DNA; other conditions: see Fig.1

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Figure 6

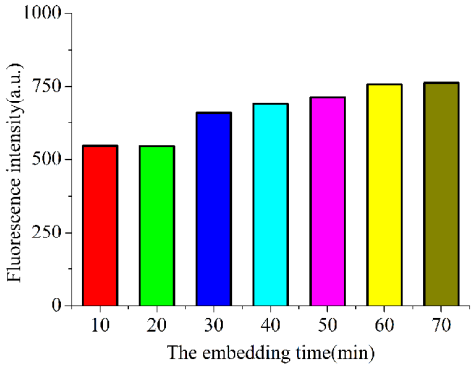

Figure 6 The effect of embedding time on the fluorescence intensity between DMT and triplex DNA. Conditions: 100 mM NaCl/ Tris-HCl buffer solution at 37 □ with different embedding times (10, 20, 30, 40, 50, 60, 70 min); other conditions: see Fig.1

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Figure 7

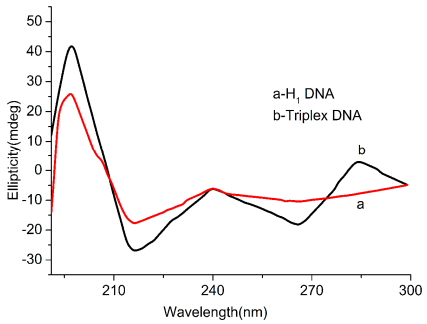

Figure 7 The CD spectra of H_1 DNA (a) and triplex DNA (b). Condition: 20 μM H_1 DNA, 20 μM target DNA for triplex DNA formation with 100 mM NaCl/ Tris-HCl buffer (pH7.4), 120 min for hybridization time at 37 □.

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Table 1 The fluorescent intensity between fluorescent reagents and different DNA

fluorescent reagents	fluorescent intensity(a.u)			
	ssDNA	dsDNA	G-quadruplex	Triplex DNA
EB	136.21	363.24	614.56	508.16
Green1	127.24	432.14	684.18	624.09
Green2	367.23	173.57	224.36	268.17
DMT	168.77	197.28	234.18	908.39

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Table 2 The comparison of different methods for recognizing triplex DNA

Characteristic / Methods	Recognition motif	recognized object	refers
competition dialysis	phenyl-furan-phenyl ring scaffold	triplex DNA	21
mass spectroscopy	agarose bead	triplex DNA	22
sensors	gold nanoparticle	triplex DNA	23,24
electrochemical	MB for signal	triplex DNA	20
fluorescent sensor	4-aminonaphthalimide	triplex DNA	31
our work	DMT	triplex DNA	