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A Thioflavin T-induced G-Quadruplex Fluorescent Biosensor for Target DNA Detection

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The detection of disease-related DNA is of great significance for early and accurate diagnosis and therapy. In this work, we successfully achieved the sensitive detection of target DNA based on a thioflavin T (ThT)-induced G-quadruplex fluorescent biosensor. ThT, a water-soluble fluorescent dye, can induce G-rich sequences to form G-quadruplexes and obtain an obviously enhanced fluorescence. In this work, it was employed to construct a biosensor for the detection of HIV. When the target HIV existed, the hairpin DNA probes would be opened in succession and release the completely exposed G-rich sequence to combine with ThT. The simple and rapid biosensor performed satisfactory selectivity; it also exhibited sensitivity with a detection limit of 2.4 nM. With good performance in human serum, we believe that this optical biosensor has the potential to be applied to the practical detection of target DNA.

Keywords Fluorescence, G-quadruplex, thioflavin T, human immunodeficiency virus

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Introduction

Deoxyribonucleic acid (DNA) contains many genes that are the carriers of genetic information, involving in regulating and controlling the growth and development of an organism. Moreover, since DNA oligonucleotide is closely related to human diseases,[1,2] the detection of disease-related DNA is of great significance for early and accurate diagnosis and therapy.[3] To achieve the accurate detection of target DNA, numerous methods have been reported, including colorimetric methods,[4-6] fluorometric methods,[7,8] electrochemical methods,[9-11] and so on. However, most cases are time-consuming and require expensive chemical modifications, bio-enzymes, or complicated immobilization.[12-14] It is therefore essential to develop a simple, rapid, and economical strategy for target DNA detection.

As a typical non-Watson–Crick DNA secondary structure, G-quadruplex comprises four strands due to the guanine-rich sequence.[15] In recent years, its property and applications have become a research hotspot.[16-19] A lot of colorimetric methods and electrochemical strategies have employed G-quadruplex systems for target detection because of the formation of a G-quadruplex/hemin complex, which is a horseradish peroxidase (HRP) mimicking DNAzyme.[9,20,21] Meanwhile, the G-quadruplex has also been widely applied in fluorescent biosensors[22-24] because it can specifically combine with some fluorescent dyes, causing significantly higher fluorescence intensities than those of dyes alone.[25,26]

Among these dyes for G-quadruplex, thioflavin T (ThT) has increasingly attracted the attention of researchers to become a new focus in the construction of G-quadruplex fluorescent biosensors, mainly due to its satisfactory water solubility and low cost.[27] Compared to conventional dyes, such as *N*-methylmesoporphyrin IX (NMM)[28] and protoporphyrin IX (PPIX),[29] the system adopting ThT as a fluorescent probe for G-quadruplex avoids using an organic solvent to dissolve the dye. Herein, we tactfully designed a simple biosensor for a target DNA assay by using ThT as a fluorescent probe for G-quadruplex. The performance of the designed biosensor was investigated based on a case study of the human immunodeficiency virus (HIV) in this study. Two metastable hairpin DNA (HP1 and HP2) structures, which had complementary regions, were employed. In the presence of target, hairpin structure of HP1 was opened, and a complementary region of it was released. Then, HP2 hybridized with unfolded HP1 based on the complementary base pairing, exposing the guanine-rich sequence. Finally, ThT was added to combine with G-quadruplex, causing an obvious enhancement of the fluorescence intensity. The employed biosensor obtained a detection limit of HIV as low as 2.4 nM, and a highly efficient and selective protocol was achieved. Moreover, the measuring process is simple and can be accomplished by using a single centrifugal tube, and thus avoiding complicated experimental steps. Importantly, this simple biosensor with the merits of rapidness and low cost can be applied to the detection of other disease-related DNA by adjusting the sequences of hairpins.

Experimental

Reagents

3,6-Dimethyl-2-(4-dimethylaminophenyl) benzo-thiazolium cation (ThT), potassium chloride (KCl), and magnesium chloride ($MgCl_2$) were purchased from Sangon Inc. (Shanghai, China). 2-Amino-2-(hydroxymethyl)-1,3-propanediol (Tris)

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Table 1 Sequences of the oligonucleotides used in this study

Name	Sequence (5′ to 3′) description
HIV	**AGTCAGTGTGGAAAATCT**CTAGC
HP1	***GTGTGGAAAATC****AGGGTA****GATTTTCCACAC****TGACT**
HP2	**AGGGTTAGGG**TTAGGGTTAGGGTCCCTAACCCTA-***ACCCTGATTTTCCACAC***
DNA1	AGTCAGTGTGGAAAA***A***CTCTAGC
DNA2	AGTCAGTGTGGAAAATCTC***A***AGC
DNA3	AGTCAGTGTGGAAAA***A***CTC***A***AGC

The bold letters in HIV and HP1 are the complementary bases. The bold italic letters in HP1 and HP2 are the complementary bases as well. Bold italic letters in DNA1, DNA2, and DNA3 are the mismatched bases. Underline regions in HP1 and HP2 are the stems of their hairpin structures.

was obtained from Aladdin Chemistry Co., Ltd. (Shanghai, China). Tris–HCl buffer (pH 7.48) was prepared with 10 mM $MgCl_2$, 20 mM Tris–HCl, and 50 mM KCl. A human serum sample was provided by the hospital of Southwest University (Chongqing, China) and diluted 10 times with Tris–HCl buffer (pH 7.48). The ultrapure water used throughout the whole experiment was 18.2 MΩ cm.

DNA oligonucleotides used in this study were synthesized and purified by Sangon Inc. (Shanghai, China). All oligonucleotides were dissolved in Tris–HCl buffer (pH 7.48) and stored at 4°C for preparation. The detailed sequences of the oligonucleotides are presented in Table 1.

Apparatus

All fluorescence spectra were performed on an F-2700 fluorescence spectrophotometer (Hitachi, Japan) at room temperature, which adopted a xenon lamp (150 W) as its excitation light sources. The spectra were recorded by exciting ThT at 425 nm and the wavelength range was from 450 to 600 nm. The PMT voltage of 400 V and the slits (ex/em) of 10/10 nm were set before the measurement.

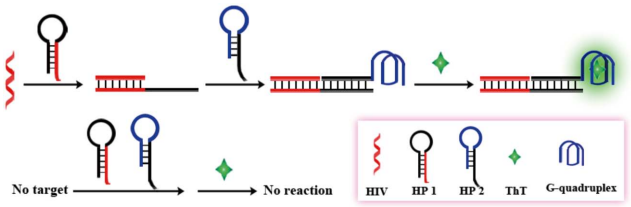
Fluorescence measurements

Before this study, HP1 and HP2 were annealed by heating at 90°C for 10 min and then cooling down slowly to room temperature for 3 – 4 h. Both of them were stored at 4°C for further experiments. First, HP1 (100 nM), HP2 (200 nM), and different concentrations of HIV were incubated at 37°C for 90 min, giving rise to the exposure of guanine-rich sequences. Subsequently, ThT (3 μM) was added to the above mixtures, and the final volume of 500 μL would be confirmed as well. Then, to promote the formation of the ThT/G-quadruplex complexes, the reaction of solutions lasted for 10 min at 37°C. Finally, the fluorescence intensities of the above-mentioned solutions were measured.

Results and Discussion

Detection mechanism

Scheme 1 exhibits the mechanism of the non-enzyme and label-free biosensor for detecting target HIV. We designed two hairpin DNA (HP1 and HP2), which were metastable structures with a particularly low rate of spontaneous hybridization in the absence of target. Once target HIV was added, HP1 hybridized with HIV partially, leading to unfolding of the HP1 hairpin structure and the release of a single-stranded region of HP1.



Scheme 1 Schematic representation of the non-enzyme and label-free fluorescent biosensor for target DNA detection.

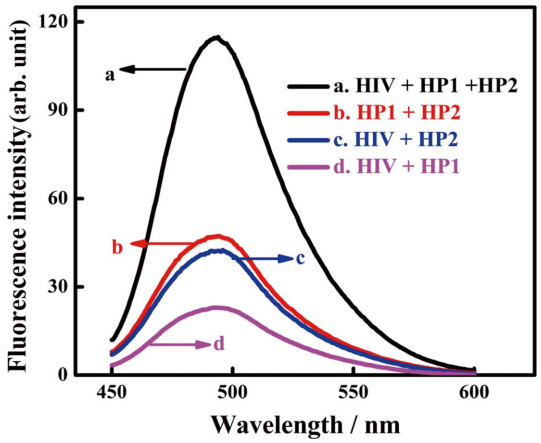


Fig. 1 Fluorescence spectra of the systems: a, HIV + HP1 + HP2; b, HP1 + HP2; c, HIV + HP2; d, HIV + HP1. Conditions: C_{HIV}, 100 nM; C_{HP1}, 100 nM; C_{HP2}, 200 nM; reaction time, 90 min; dyeing time, 10 min; concentration of ThT, 3 μM.

This region of HP1 destroyed the hairpin structure of HP2 based on the complementary base pairing, exposing the guanine-rich sequence. With the addition of ThT, whose fluorescence intensity was generally weak, itself, the G-quadruplex/ThT complex was formed, and an obvious enhancement of fluorescence intensity was obtained. Therefore, what we have designed achieved the detection of target HIV both rapidly and conveniently.

To ensure the feasibility of the proposed biosensor, we validated its principle through fluorescent spectrometry. As displayed in Fig. 1, the system containing HIV and HP1 (curve d) obtains a fairly low fluorescence response, indicating that the strong fluorescence intensity of ThT cannot be obtained without a G-rich sequence. The low signal response of the system with HIV and HP2 (curve c) confirmed that they cannot hybridize and cause the guanine-rich sequence to be released efficiently. When only HP1 and HP2 exist (curve b), the fluorescence intensity increases slightly compared to the above-mentioned system, manifesting that the interaction of HP1 and HP2 is weak without a target. The system including HIV, HP1, and HP2 inversely performs an obvious enhancement of the fluorescence intensity, and the maximum fluorescence peak is at 490 nm. These phenomena indicated that HIV can initiate unfolding of the hairpin structure. As stated above, the proposed simple biosensor was proved to be available for HIV detection.

Optimum conditions for the system

We investigated the following aspects to achieve the best performance of the biosensor. During a series of studies,

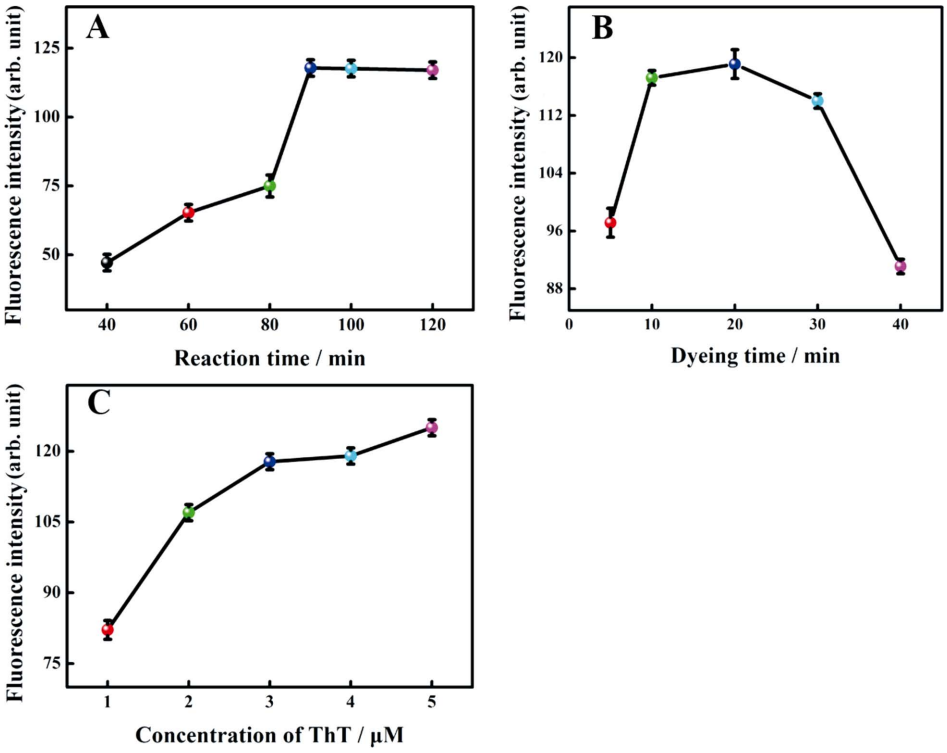


Fig. 2 (A) Effect of different reaction times on the fluorescence intensity. Conditions: C_{HIV} = 100 nM, C_{HP1} = 100 nM, C_{HP2} = 200 nM, dyeing time 10 min, concentration of ThT 3 μM. (B) Effect of different dyeing time on the fluorescence intensity. Conditions: C_{HIV} = 100 nM, C_{HP1} = 100 nM, C_{HP2} = 200 nM, reaction time 90 min, concentration of ThT 3 μM. (C) Effect of different concentrations of ThT on the fluorescence intensity. Conditions: C_{HIV}, 100 nM; C_{HP1}, 100 nM; C_{HP2}, 200 nM; reaction time, 90 min; dyeing time, 10 min. The error bars, standard deviation, n = 3.

the fluorescence intensity was a direct referenced index for determining the performance.

As described in Fig. 2A, the fluorescence intensity increases in the period of 40 to 90 min, and changes little in the period of 90 to 120 min, during which the value at 90 min reaches the maximum. This indicates that the substrate can react completely in 90 min. Thus, a reaction time of 90 min was chosen.

As shown in Fig. 2B, with the addition of ThT, the maximum fluorescence intensity of the dyeing time arose at 10 min, indicating that ThT had already combined with the G-quadruplex perfectly. Also, in the period of 10 to 30 min, the fluorescence intensity showed little change, indicating that the biosensor was relatively stable within the time range of 10 to 30 min. Therefore, the dyeing time was set as 10 min. Further, as can be seen in Fig. 2C, when the concentration of ThT increases to 3 μM, the fluorescence intensity is enhanced rapidly. Adding ThT sequentially cannot lead to a significant enhancement of the fluorescence intensity until the concentration increases to 5 μM. Thus, 3 μM ThT became the suitable concentration for the whole study.

Sensitivity and selectivity for HIV detection

We explored the fluorescence responses of different concentrations of HIV at 5, 10, 15, 30, 50, 70, 100, 150, and 200 nM under the optimized circumstance. As displayed in Fig. 3A, the fluorescence intensity continuously strengthens with increasing concentration of HIV from 0 to 200 nM. And, as shown in Fig. 3B, there is a linear relationship between the fluorescence intensity and the concentration of HIV from 5 to 200 nM with a regression equation of $F = 58.537 + 0.593 \times C_{HIV}$ ($R^2 = 0.9904$). In this equation, F and C_{HIV} represent the fluorescence intensity of the system and the concentration of the target HIV, respectively. The limit of detection was calculated to be as low as 2.4 nM in terms of the $3\sigma/s$ rule, where σ is the standard deviation of the blank sample and s is the slope of the standard curve. Compared to the previously reported methods for HIV detection, our fluorescent biosensor obtained a lower detection limit[30,31] and a wider linear range.[3] To evaluate the repeatability of our biosensor, we made five successive assays by analyzing the target DNA at 10, 100, and 200 nM. The relative standard deviations (RSDs) were calculated to be 4.04, 5.40, and 4.27%, demonstrating that the proposed biosensor possessed an acceptable repeatability.

Detecting mismatched DNA sequences is very important because it can supply information about single-nucleotide polymorphism (SNP), genetic mutation, and DNA damage. To confirm the selection of our proposed biosensor, we investigated three other mismatched DNA, which are listed in Table 1 and named as DNA 1, DNA 2, and DNA 3, respectively. As illustrated in Fig. 3C, the fluorescence intensity of the system containing DNA 1 is slightly lower than that with HIV. Compared with the system with DNA 2 or DNA 3, the system containing target HIV has a significant fluorescence response. These results showed that our strategy can successfully discriminate the target DNA and mismatched DNA, and that it has been potential for SNP analysis. To prove the feasibility of our established sensor in a complex environment, we detected the fluorescence responses of HIV in human serum samples. As can be seen from Fig. 3D, there are parallel fluorescence signals in both the buffer and the serum samples. These results confirm

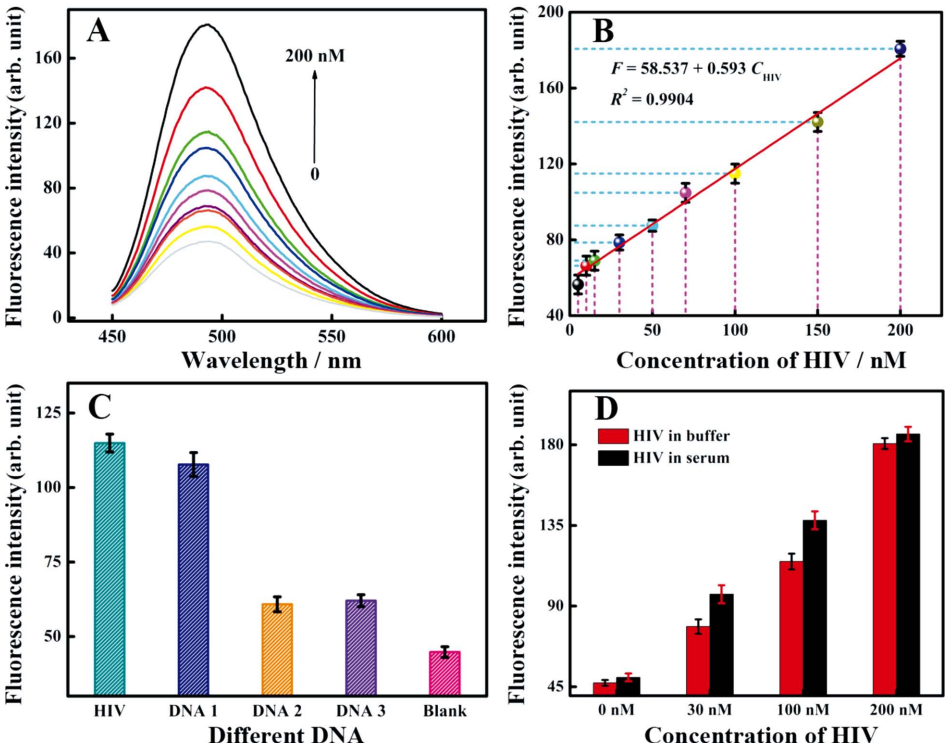


Fig. 3 (A) Fluorescence responses to the different concentrations of HIV (from bottom to top: 0, 5, 10, 15, 30, 50, 70, 100, 150, and 200 nM). (B) Linearity relationship between the fluorescence intensity and HIV concentration from 5 to 200 nM. (C) Fluorescence intensities of the systems in the presence of target HIV and mismatched DNA. The reaction mixture without target or mismatched DNA was as the blank sample. (D) Fluorescence responses to the systems with different concentrations of HIV in the buffer and diluted serum samples. Condition: C_{HP1}, 100 nM; C_{HP2}, 200 nM; reaction time, 90 min; dyeing time, 10 min; concentration of ThT, 3 μM. The error bars, standard deviation, $n = 3$.

that our method can also detect HIV accurately in a complex environment, and possess a potential application prospect to target DNA detection in clinical samples.

Conclusions

In the present work, we successfully developed a non-enzyme and label-free biosensor for target DNA detection based on a case study of HIV virus DNA. The method exhibited high sensitivity with a low detection limit, which was estimated to be 2.4 nM. It was also applied in the analysis of human serum sample where it presented a significant response. Meanwhile, it produced satisfactory selectivity against with other mismatched DNA. It is worth noting that this method is simple, rapid, and low-cost because of no requirements of any complex operation, expensive chemical modification, and bio-enzyme. The simple biosensor avoided the use of an organic reagent due to the good water solubility of ThT. In the case of changing the hairpin probe sequences, almost all of the target DNA detection and analysis could be realized. Thus, we believe that the proposed fluorescent biosensor would have greater applications in the future.

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