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Label-free triple-helix aptamer as sensing platform for "signal-on" fluorescent detection of thrombin

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ABSTRACT

The design of a label-free aptamer for separation of recognition sequence from signal reporter is significant to ensure the high-efficiency affinity between aptamer and target. This work develops a label-free triple-helix aptamer (THA) as sensing platform for "signal-on" fluorescent detection of thrombin. THA was composed of aptamer sequence and help DNA 1 (H1), which contained the complementary sequence of hexachloro-fluorescein (HEX) labeled help DNA 2 (H2). The specific recognition event between aptamer and thrombin triggered the dismission of THA to release H1. The released H1 then reacted with the signal probe of H2/graphene oxide (GO) nanocomposite to form H1-H2 duplex, leading to the fluorescence recovery of H2 due to the detachment of H1-H2 duplex from the surface of GO. With employment of THA as a signal transducer and GO as a "superquencher", this method shows a sensitive response to thrombin with a wide concentration range from 5 to 1200 nM. The limit of detection is 1.8 nM (S/N=3) with excellent selectivity. Considering the universality of THA, the proposed aptasensor would provide a platform for homogeneous fluorescent detection of a wide range of analytes.

1. Introduction

Triple-helix DNA (THD) is one of nontraditional nucleic acid structures, which involves a third strand in the major groove of a Watson-Crick duplex via Hoogsteen hydrogen bonding [1–4]. Based on groove recognition of THD towards biomolecules, the THD has been extensively employed as therapeutic agents in biomedical and biotechnological applications [5–6]. Recently, THD has been used as template for preparation of site-specific silver nanoclusters with high-stability [7]. By modifications of its nuclear acid structure, THD as signaling probe has been applied in detection of double-stranded DNA (dsDNA) [8,9], microRNA [10], DNA [11], Hg^{2+} [12], and adenosine triphosphate [13], in combination with the techniques of fluorescence and chemiluminescence. The above methods are on the basis of spatial change of THD structure after target recognition for fluorescent signal readout with THD labeling. However, such structural alteration may not induce the enhanced signal for sensitive detection. Therefore, the further functionalization of THD is a promising way in the design of new molecular biology tools, especially for integrating with aptamer as a recognition element.

Aptamers are single-stranded DNA or RNA nucleic acids isolated from random-sequence nucleic acids libraries by SELEX [14,15]. By reason of their high affinity, aptamers are considered to be promising recognition elements for biosensor applications in a wide range of targets including small molecules, ions, and proteins [16–30]. For example, a silver nanocluster functionalized aptamer as sensing platform was designed for multiplexed analysis of pathogenic DNAs via fluorescence resonance energy transfer (FRET) between silver nanocluster and graphene oxide [31]. A sensitive aptasensor was constructed by using two-photon dye labeling aptamer as molecular probe for quantitative detection of adenosine triphosphate [32]. However, due to the requirement of labeling fluorophores or quenchers on the aptamers, these methods are expensive, time-consuming and inconvenient. Meanwhile, another significant problem arises that modifications of dyes on aptamer will influence aptamer's affinity and specificity to target [33]. In order to solve these problems, a triple-helix molecular switch was developed by using a hairpin-shaped aptamer sequence and a dual-labeled oligonucleotide serving as a signal transduction probe [34]. In this work, we design a label-free triple-helix aptamer (THA) as sensing platform for "signal-on" fluorescent detection of thrombin.

Thrombin plays a key role in regulation of tumor growth, metastasis, and angiogenesis [35] and thus it is worth to develop a sensitive and specific method for detection of thrombin in biological samples. Here, by integrating with the advantages of graphene oxide (GO) as efficient supporter and quencher [36], a

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label-free THA was developed as sensing platform for convenient and homogeneous fluorescent detection of thrombin (Fig. 1). First, the label-free THA and signal probe were conveniently synthesized by the mixing of Apt29 and help DNA 1 (H1), and the hexachloro-fluorescein labeled help DNA 2 (H2) and GO, respectively. After aptamer-thrombin recognition event, the released H1 could react with signal probe of GO/H2 to form H1-H2 duplex, leading to the detachment of H2 from the surface of GO, and thus an enhanced fluorescence was realized for readout. Based on the distinguishment of GO towards single-stranded DNA against double-stranded DNA [37,38] and high affinity between aptamer and target, a fluorescent strategy is achieved for specific detection of thrombin. The label-free THA provides a convenient way for constructing the powerful sensing platform towards the versatility of target in bioanalysis and clinical diagnosis.

2. Experimental

2.1. Materials and reagents

The oligonucleotides along with hexachloro-fluorescein (HEX) labeled DNA were synthesized and HPLC-purified by Sangon Biological Engineering Technology & Co. Ltd. (Shanghai, China). The oligonucleotide sequences were shown in Table 1. Thrombin (Tmb) and horseradish peroxidase (HRP) were purchased from Sigma-Aldrich (Shanghai) Trading Co. Ltd. Bovine serum albumin (BSA) was obtained from Nanjing Sunshine Biotechnology Co. LTD. Human immunoglobulin G antibody (IgG) was obtained from Sangon Biological Engineering Technology & Co. Ltd. (Shanghai, China). Graphene oxide (GO) was obtained from XFNano Materials Tech Co. Ltd. (Nanjing, China). Inorganic metal salts were obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). All other reagents were of analytical reagent grade and used as purchased without further purification. Phosphate buffer solution (PBS) (10 mM, containing 20 mM KCl and 2.5 mM $MgCl_2$, pH 6.2) was used for homogeneous fluorescence detection of thrombin. Tris–HCl buffer (20 mM, containing 100 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 8.5% glycerol (v/v), pH 7.4) was used to dissolve thrombin. Human blood serum samples were obtained from Jiangsu Province Tumor Hospital (Nanjing, China). Ultrapure water obtained from a Millipore water purification system ($\geq$ 18 MΩcm, Milli-Q, Millipore) was used in all experiments.

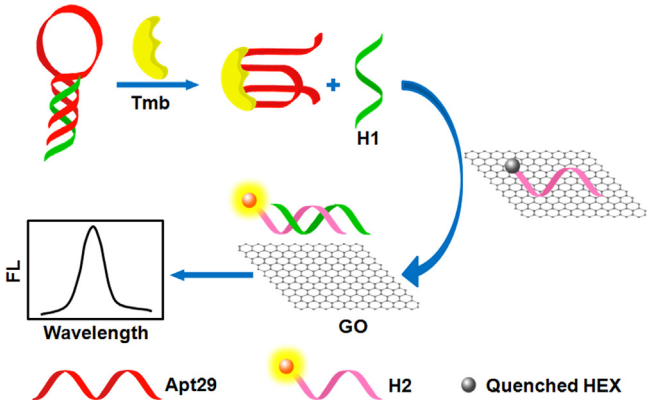

Fig. 1. Schematic illustration of label-free triple-helix aptamer as sensing platform for fluorescent detection of thrombin.

2.2. Apparatus

The synchronous scanning fluorescence spectra were recorded from 500 to 620 nm with a step of 1 nm at the excitation wavelength of 482 nm on a RF-5301PC spectrofluorometer (Shimadzu Co., Japan) equipped with a xenon lamp. The slit width was 5 nm, and the stokes shift ($\Delta\lambda$) was 18 nm for HEX.

2.3. Preparation of triple-helix aptamer and signal probe

The DNA stock solutions of Apt29 (1 μM) and help DNA 1 (H1, 1 μM) were first heated at 90 °C for 10 min, and gradually cooled down to room temperature. Subsequently, THA (0.5 μM) was prepared by mixing the same volume of Apt29 (1 μM) and H1 (1 μM) in 10 mM PBS, and then incubated at room temperature for 30 min.

To obtain the optimal performance, a series of GO dispersions were involved in the preparation of signal probe (GO/H2). The optimal ratio of GO (150 μg mL^{-1}), H2 (1 μM) and PBS was 5:2:43 in volume. The final concentration of the signal probe of GO/H2 was 15.2 μg mL^{-1}.

2.4. Homogenous fluorescence detection of thrombin

4 μL THA (0.5 μM) were mixed with different concentrations of thrombin in a certain volume of PBS to form 50 μL of reaction mixture. After the mixture was incubated at 37 °C for 40 min, 50 μL GO/H2 and 100 μL PBS were added to the reaction mixture at room temperature for 50 min. Subsequently, the final solution was used for fluorescent detection.

3. Results and discussion

3.1. Principle of sensing platform

The mechanism of label-free THA as fluorescent sensing platform for detection of thrombin based on GO is shown as Fig. 1. The work employed THA as a signal trigger which was composed of Apt29 and H1 by Watson-Crick and Hoogsteen base pairings [5]. In the absence of Tmb, H2 was absorbed on GO surface by π-π stacking, and its fluorescence was quenched though FRET [39], that is, the fluorescence of GO/H2 was turned off. Upon the introduction of Tmb, THA structure was dismissed and thus released H1 due to the specific recognition between Apt29 and Tmb. Afterwards, the released H1 reacted with signal probe of GO/H2 to form H1-H2 duplex that was desorbed from surface of GO, leading to the recovery of the fluorescence of signal probe. Therefore, the "signal-on" fluorescence strategy was simply achieved for the sensitive detection of Thrombin with high specificity.

Table 1
Oligonucleotide sequences used in this work.

Oligonucleotide	Sequence
Apt29	5'–CTCTCTTT***AGTCCGTGGTAGGGCAGGTTGGGGTGACT***TTTCTCTC-3'
help DNA 1 (H1)	5'–AAAGAGAGCGTA-3'
help DNA 2 (H2)	5'–TACGCTCTCTTT-HEX-3'

HEX is hexachloro-fluorescein. The aptamer sequence is bold in italic type.

3.2. Feasibility of strategy

The feasibility of the proposed method was demonstrated by fluorescence emission spectra of signal probe under different conditions (Fig. 2). The fluorescence spectrum of GO/H2 exhibited extremely weak fluorescence intensity owing to the strong adsorption of H2 on the surface of GO and the high fluorescence quenching efficiency of GO by FRET (curve a). The introduction of THA brought about no change of fluorescence intensity, because THA was bond up by Watson-Crick and Hoogsteen base pairings (curve b). Once THA was mixed with Tmb followed by GO/H2, the fluorescence intensity increased 37.4 times than that of GO/H2 (curve c). When mixing GO/H2 with free H1, an obvious fluorescence restoring of HEX labeled to H2 was obtained (curve d). These results indentified that THA could release H1 to trigger the fluorescence recovery of HEX in the presence of Tmb, leading to a "signal-on" fluorescence of sensing platform for detection of the target.

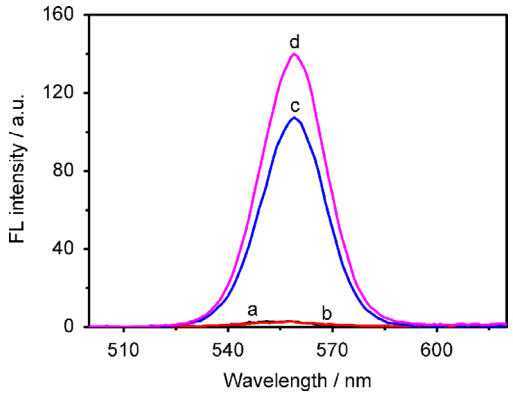

Fig. 2. Fluorescence emission spectra of GO/H2 (a), THA+GO/H2 (b), THA+Tmb+GO/H2 (c), and GO/H2+H1 (d). The concentrations of THA, Tmb, H1 and GO/H2 were 10, 1000, 100 nM and 3.8 μg mL^{-1}, respectively.

3.3. Optimization of conditions

In order to achieve the optimal performance, the concentration of GO as a critical factor for detection efficiency was optimized. As shown in Fig. 3A, the fluorescence intensity ratio of sensing platform, F/F_0, first increased and subsequently decreased as the concentration of GO increased. The results demonstrated that small amount of GO was insufficient to quench the HEX labeled on H2, and by contrast excess GO provided spare area to absorb THA or released H1 resulting in that low fluorescence intensity was recovered. Therefore, the corresponding concentration of GO (3.75 μg mL^{-1}) was chosen as the optimal value for the next experiments.

The effects of reaction time between Apt29 and H1, THA and Tmb, as well as THA/Tmb and signal probe on fluorescence intensity ratio of F/F_0 were investigated step by step. After mixing Apt29 with H1 at the same molar, 30 min was identified as the optimal time to ensure that THA was completely formed (Fig. 3B). For incubation time of THA and Tmb, the value of F/F_0 increased and reached a plateau at 40 min (Fig. 3C), which indicated the THA fully reacted with Tmb and liberated H1. When addition of GO/H2 into the mixture, the F/F_0 ratio first increased and subsequently reduced (Fig. 3D). The phenomena could be explained that the liberated H1 from THA couldn't completely react with signal probe within a short time. But a long reaction time could result in fluorescence enhancement of background due to

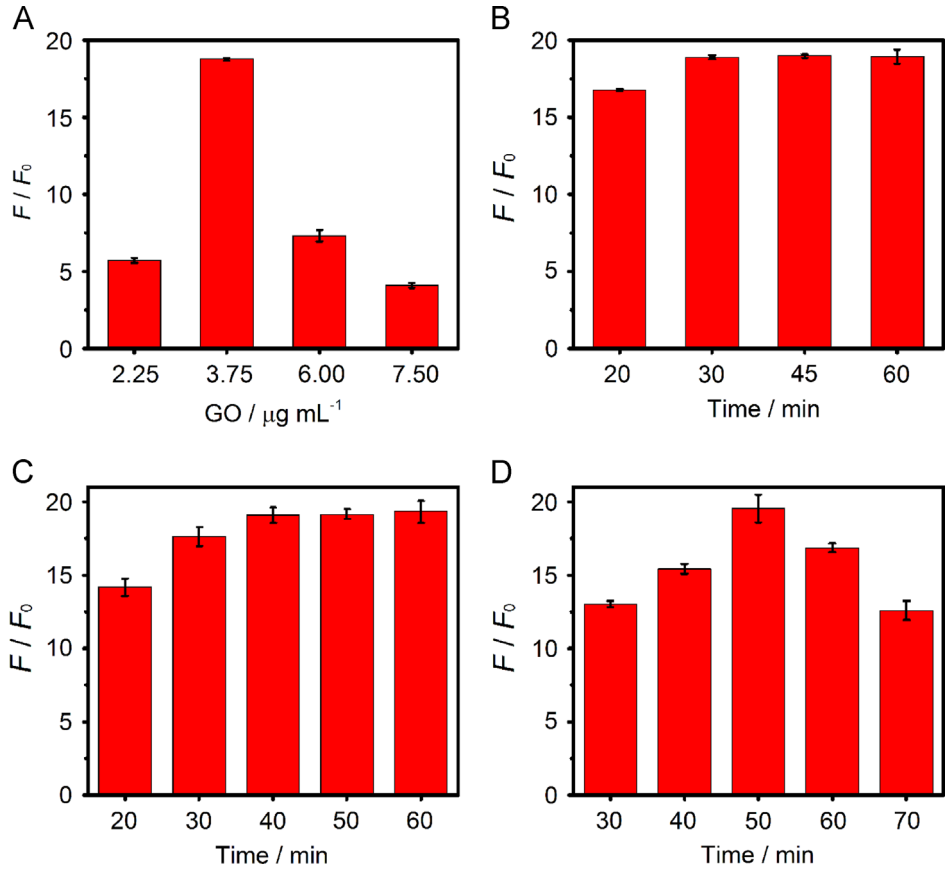

Fig. 3. Effects of (A) GO concentration, incubation time for (B) Apt29+H1, (C) THA+Tmb, and (D) THA+Tmb+GO/H2 on the fluorescence ratio of F/F_0. F and F_0 are the fluorescence intensities of sensing system in the presence and absence of Tmb, respectively. All assays were carried out in 10 mM PBS with 500 nM Tmb, 10 nM THA, while the other parameters were under their optimal conditions.

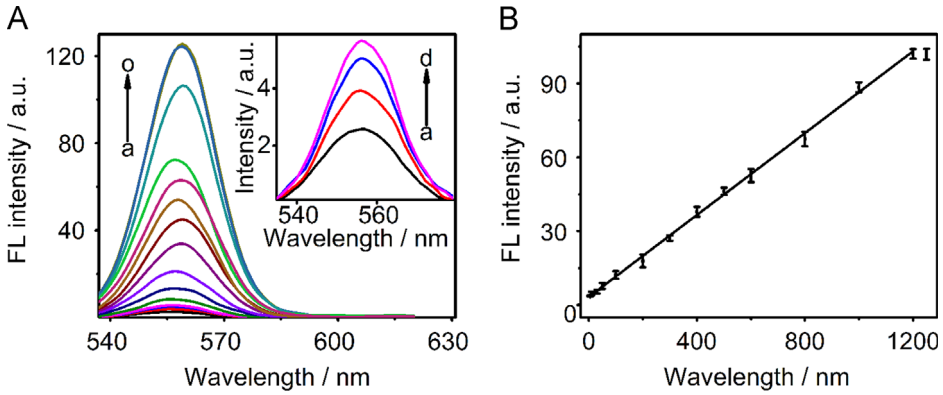

Fig. 4. (A) Fluorescence emission spectra of sensing platform in response of Tmb at 0, 5, 10, 30, 50, 100, 200, 300, 400, 500, 600, 800, 1000, 1200 and 1250 nM (from a to o). Inset: Amplified fluorescence emission spectra in response of Tmb at 0, 5, 10, 30 nM (from a to d). (B) Calibration curve for Tmb detection.

Table 2
Comparison of the fluorescent label-free aptamer-based biosensors for thrombin.

Detection method	LOD (nM)	Linear range (nM)	Ref
Fluorescent detection using triple-helix aptamer	1.8	5–1200	This work
Fluorescent aptasensor based on multi-branched pyrazine derivative	50	5–2500	41
Fluorescent detection based on H_2O_2-catalyzed oxidation via hemin/G-quadruplex DNAzyme	10	0–100	42
Fluorescent detection based on QDs/Ru complex photoinduced charge transfer	50	200–1600	43
Fluorescent detection based on inhibition of polymerase reaction	62.5	0–500	44

the competition of THA with the absorbed H2 onto GO, and thus the F/F_0 ratio reduced [40]. Hence 50 min was chosen as the optimal reaction time for fluorescence detection.

3.4. Tmb detection

Under the optimal concentration of GO and reaction time, the fluorescence intensity of sensing platform enhanced dramatically with the increasing concentration of Tmb in the wide range from 5 to 1200 nM (Fig. 4). The linear regression equation was $I=3.30+0.08\times c$ ($R^2=0.9989$), where I is the fluorescence intensity and c is the Tmb concentration. The detection limit was estimated to be 1.8 nM at 3σ. As shown in Table 2, the detection limit of the proposed aptasensor for thrombin was lower than those of the previous fluorescent label-free aptamer-based methods [41–44]. The high sensitivity and the wide detection range of our method are attributed to the high-efficiency affinity between label-free THA and Tmb, and the strong fluorescence quenching effect of GO on fluorophore labeled on single-stranded DNA. In addition, a relative standard deviation was calculated to be 4.2% for 300 nM thrombin with 6 different aptasensors, indicating acceptable reproducibility.

3.5. Selectivity of sensing platform

To evaluate the selectivity of our strategy for Tmb, the fluorescence responses of sensing platform to Tmb and other proteins containing HRP, BSA and IgG were individually investigated at the same concentration (500 nM). As shown in Fig. 5, the fluorescence intensity of Tmb was approximately 5.9 times higher than that of IgG. For BSA and HRP, the fluorescence intensity of target was 3.4 times and 3.8 times higher than negative controls, respectively. The results could be attributed to that nonspecific adsorption of interference proteins onto GO caused a fraction of H2 to be discharged from surface of GO. The selectivity of the sensing platform resulted from the specific recognition of aptamer to Tmb and the specific hybridization of H1 with H2. It could be summarized that THA as fluorescent sensing platform is promising

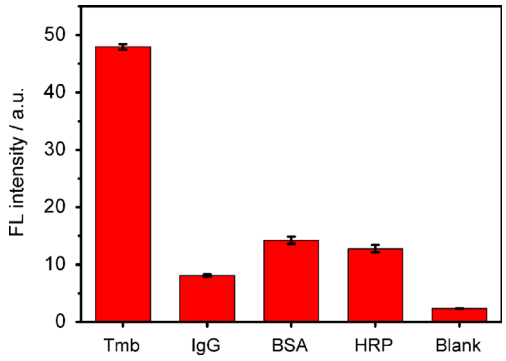

Fig. 5. Fluorescence intensity changes of the aptasensor toward Tmb and other proteins under the same condition. The concentrations of Tmb, IgG, BSA and HRP were 500 nM.

to be applied in homogeneous fluorescent detection of Tmb and related analytes.

3.6. Tmb detection in serum samples

Considering sensitive detection of thrombin in real sample is of great importance for disease diagnosis and drug discovery, thrombin in serum samples was detected by the proposed aptasensor. A series of samples were prepared by spiking the standard solution of 500 nM to different human blood serum samples. All of recoveries were from 97.7% to 92.9%, indicating the proposed strategy could be promising for thrombin detection in real biological samples.

4. Conclusions

The novel label-free triple-helix aptamer was successfully constructed as sensing platform for homogeneous fluorescent detection of target. THA is composed of aptamer sequence and help DNA 1, which functions as the recognition element for specific recognition of Tmb, and the trigger for fluorescence recovery of signal probe,

respectively. Taking advantages of the specific recognition of aptamer to Tmb and the specific hybridization of H1 with H2, the THA-based sensing platform could selectively detect thrombin with a wide linear range and a low detection limit. Moreover, the separation of aptamer from signal reporter ensures the high affinity between aptamer sequence and target. Besides, this method possesses some merits such as convenient operation and low cost. The label-free triple-helix aptamer as a universal signal transducer can provide a simple, sensitive, selective homogeneous protocol in bioanalysis.

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