We report herein a label-free and sensitive fluorescent method for detection of thrombin using a G-quadruplex-based DNAzyme as the sensing platform. The thrombin-binding aptamer (TBA) is able to bind hemin to form the G-quadruplex-based DNAzyme, and thrombin can significantly enhance the activity of the G-quadruplex-based DNAzyme. The G-quadruplex-based DNAzyme is found to effectively catalyze the H$_2$O$_2$-mediated oxidation of thiamine, giving rise to fluorescence emission. This allows us to utilize the H$_2$O$_2$–thiamine fluorescent system for the quantitative analysis of thrombin. The assay shows a linear toward thrombin concentration in the range of 0.01–0.12 nM. The present limit of detection for thrombin is 1 pM, and the sensitivity for analyzing thrombin is improved by about 10 000-fold as compared with the reported colorimetric counterpart. The work also demonstrates that thiamine is an excellent substrate for the fluorescence assay using the G-quadruplex-based DNAzyme as the sensing platform.

**Introduction**

Nowadays, besides the traditional roles as a genetic material and the substrates of enzymes for DNA manipulation, the new functions of nucleic acids as recognition elements and enzyme-like biocatalysts have attracted much attention. The DNAzyme (also called DNA enzyme, deoxyribozyme or catalytic DNA) is a class of catalytically active nucleic acid molecules, which has found a promising methodology termed **in vitro** selection or SELEX (systematic evolution of the exponential enrichment). Compared with protein enzymes, DNAzymes are relatively less expensive to produce and self-replicate more readily. Furthermore, DNAzymes are impressively stable under ambient and even elevated temperatures, and can be stored at room temperature (ca. 20 °C). DNAzymes can recognize target analytes or catalyze specific chemical and biological reactions. Many cofactors, such as amino acids, nucleic acids, metal ions and small organic molecules, can affect the catalytic activity of DNAzymes, which makes it a novel platform for the development of highly selective sensors.

An important development in the field of DNAzymes is the discovery of the G-quadruplex-based DNAzyme formed by the G-quadruplex and hemin. The G-quadruplex-based DNAzyme displays peroxidase-like activity. To date, the G-quadruplex-based DNAzyme has been used as a peroxidase mimetic for the detection of many analytes including metal ions, small molecules, DNA and proteins. In these reported sensing platforms based on the G-quadruplex-based DNAzyme, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) has predominantly been used as the chromogenic substrate. The G-quadruplex-based DNAzyme is found to catalyze the H$_2$O$_2$-mediated oxidation of ABTS, and the oxidized product of ABTS has a maximal absorption at about 420 nm ($\varepsilon = 3.6 \times 10^3$ L mol$^{-1}$ cm$^{-1}$). Thus, the analytes can be detected via absorbance methods. Though the colorimetric method has some advantages (such as simplicity and rapidity), some inherent problems of these systems with UV-visible spectrophotometric measurements are presented: (1) the systems lack sufficient sensitivity; and (2) the linear range of the systems is narrow. For example, in the reported H$_2$O$_2$–ABTS system for thrombin, the net absorbance signal change in the range of 0.02–0.12 μM thrombin was only 0.07. In addition, ABTS can turn green after oxidation, but the green product is not stable and can quickly decay to a colorless product in aqueous media within 10 min. That is, ABTS is not an ideal substrate for the G-quadruplex-based DNAzyme sensing platform. Hence, exploration for other appropriate substrates is required.

Fluorescence detection is now used for a wide range of quantitative applications and offers the advantages of high sensitivity, rapidity, stability, simplicity and feasibility. In the presence of HRP, the oxidation of several fluorogenic substrates by H$_2$O$_2$ into fluorescent molecules has been well applied to detect H$_2$O$_2$ and other compounds. There is little doubt that the G-quadruplex-based DNAzyme, as the peroxidase-mimetic,
can also catalyze the \( \text{H}_2\text{O}_2 \)-mediated oxidation of fluorogenic substrates. However, to the best of our knowledge, few applications in the G-quadruplex-based DNAzyme fluorescence analysis have been reported.\(^2\) In this work, we use thiamine, one of the most attractive fluorogenic substrates,\(^19,20\) to replace ABTS (a chromogenic substrate), and its aim is to extend the G-quadruplex-based DNAzyme to fluorescence analysis. Thrombin serves as an ideal analyte for testing a new sensing system due to its biological significance. The thrombin-binding aptamer (TBA) is found to bind hemin to form a G-quadruplex-based DNAzyme, and the activity of the G-quadruplex-based DNAzyme is significantly promoted in the presence of thrombin. The G-quadruplex-based DNAzyme is found to bind hemin to form a G-quadruplex-based DNAzyme. Scheme 1 depicts the designed fluorescent method for thrombin with TBA as the sensing element. In the presence of thrombin, the fluorescent product, thiochrome, which exhibited strong fluorescence emission, which inspires us to pioneer a novel DNAzyme-based approach to sensing thrombin in a label-free fluorescent manner. The analysis conditions are optimized, and the performances of the proposed system are investigated. The present limit of detection of thrombin is about 1 pM, which is much lower than that of the reported detection of thrombin with the ABTS–\( \text{H}_2\text{O}_2 \) colorimetric system (the detection limit for analyzing thrombin was 20 nM).\(^17\)

**Experimental**

**Reagents**

Hemin, human thrombin (1710 U mg\(^{-1}\)) and HRP (type VI, 330 U mg\(^{-1}\)) were obtained from Sigma (St. Louis, MO, USA). Hemin stock solution (5 mM) was prepared in DMSO, stored in the dark at \(-20^\circ\text{C}\), and diluted to the required concentration with buffer solution (20 mM Tris-\( \text{HCl} \), 40 mM KCl, 200 mM NaCl, 0.06%(v/v) Triton X-100, pH 7.4). Thrombin was dissolved in the binding buffer (20 mM Tris-\( \text{HCl} \), 140 mM NaCl, 5 mM KCl, 1 mM CaCl\(_2\), 1 mM MgCl\(_2\), pH 7.4). The 15-mer DNA oligonucleotide (5’-GGT TGG TGT GGT TGG-3’) and 29-mer aptamer (5’-AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3’) were synthesized by Beijing Dinguo Biotechnology Co. Ltd. (Beijing, China). The purchased DNA was dissolved in Tris-\( \text{HCl} \) buffer solution (10 mM, pH 7.4) and stored at 4°C. The concentration of DNA was quantified by UV-visible absorption spectroscopy with the following extinction coefficients (\(\varepsilon_{260\text{nm}}, \text{M}^{-1}\text{cm}^{-1}\)): \(\varepsilon = 15400, G = 11500, C = 7400, T = 8700\), 30% \(\text{H}_2\text{O}_2\) was purchased from Xi’an Reagent Plant (Xi’an, China) and thiamine was purchased from Beijing Dinguo Biotechnology Co. Ltd. (Beijing, China). The used metal salts (KCl, NaCl, Pb(\(\text{Ac}\))\(_2\), etc.) were purchased from Sinopharm Group Chemical Reagent Co., Ltd. (Shanghai, China). Millipore Milli-Q (18 MΩ cm) water was used in all experiments.

**Apparatus**

All fluorescence measurements were made using Hitachi model F-4600 fluorescence spectrophotometer (Kyoto, Japan). UV-visible adsorption spectra were recorded on a Hitachi model U-3900H UV-vis spectrophotometer (Kyoto, Japan) at room temperature. A Chirascan Model Circular Dichroism Spectrometer (Leatherhead, Surrey, UK) was utilized to collect the circular dichroism spectra of DNA.

**Preparation of G-quadruplex-based DNAzyme**

The 50 \(\mu\)L of 20 \(\mu\)M TBA solution was heated at 88°C for 10 min to dissociate any intermolecular interactions, and gradually cooled to room temperature. Then, to this solution was added 50 \(\mu\)L thrombin with different concentrations. The mixture solution of TBA and thrombin was incubated at room temperature for 3 h to form the TBA–thrombin complex. Finally, 50 \(\mu\)L of 20 \(\mu\)M hemin and 850 \(\mu\)L Tris-\( \text{HCl} \) (25 mM, pH 7.4) buffer solution were added into the TBA–thrombin solutions, keeping the mixtures at room temperature for 2 h to form the thrombin–TBA–hemin complex.

**Fluorescence detection of thrombin**

The fluorescence detection of thrombin utilizing the G-quadruplex DNAzyme was performed in the thiamine–\( \text{H}_2\text{O}_2 \) reaction system at room temperature. Briefly, 50 \(\mu\)L of the above prepared G-quadruplex-based DNAzyme was added into the mixture of 100 \(\mu\)L of 206 \(\mu\)M thiamine and 550 \(\mu\)L of 80 \(\mu\)M \(\text{H}_2\text{O}_2\), and then 200 \(\mu\)L of 20 mM PBS (pH 12.0) was quickly added to the above mixture solution. The peroxidation reaction began to produce the fluorescent product, thiochrome, which exhibited strong fluorescence at 440 nm with an excitation wavelength of 370 nm. The fluorescent spectra of the reaction mixture were recorded within 5 min using a fluorescence spectrophotometer whose excitation and emission slits were set at 10 nm. The increased fluorescence intensity (\(\Delta F, \Delta F = F - F_0\), where \(F\) and \(F_0\) are the fluorescence intensity of the system in the presence and absence of thrombin, respectively) at 440 nm with an excitation wavelength of 370 nm was used for the quantitative analysis of thrombin.

**Results and discussion**

**Principle of sensing thrombin via G-quadruplex DNAzyme**

The 15-mer thrombin-binding aptamer (TBA) is widely used as a sensing element for constructing the thrombin aptasensor.\(^26\) The TBA (5’-GGT TGG TGT GGT TGG-3’) is a single-stranded guanine-rich nucleic acid. This aptamer is thought to adopt a loose random coil structure in the absence of K\(^+\) or thrombin. Upon addition of K\(^+\) or thrombin, TBA folds into a compact G-quadruplex. On the other hand, TBA can bind hemin in the G-quadruplex state to form a complex with peroxidase-like activity. Interestingly, the addition of thrombin can significantly promote the DNAzyme activity of the hemin–G-quadruplex complex,\(^17\) which provides a facile approach to sensing thrombin with high sensitivity.

Thiamine is a non-fluorescent substrate, but thiochrome (the oxidation product of thiamine with \(\text{H}_2\text{O}_2\)) is a strongly fluorescent compound.\(^23,25\) We find that the G-quadruplex-based DNAzyme can effectively catalyze the thiamine–\( \text{H}_2\text{O}_2 \) reaction at room temperature. Thus, we propose a new strategy for the fluorescent detection of thrombin using a G-quadruplex-based DNAzyme. Scheme 1 depicts the designed fluorescent method for thrombin with TBA as the sensing element. In the presence of thrombin, TBA folds into a compact G-quadruplex conformation, and then the folded TBA can bind hemin to form the G-quadruplex-based DNAzyme with high peroxidase-like activity.
activity, whereas the hemin–TBA complex shows very low activity in the absence of thrombin. The activity of the G-quadruplex-based DNAzyme can be analyzed in the thiamine–H₂O₂ reaction by fluorescence spectroscopy. As shown in Fig. 1, the fluorescence intensity of the thiamine–H₂O₂ reaction system is greatly enhanced upon the addition of thrombin.

UV-Vis absorption and circular dichroism (CD) spectroscopy are utilized to study the interactions of thrombin–TBA–hemin. Fig. 2A showed the UV-Vis absorption spectra of different concentrations of thrombin-binding hemin–G-quadruplex complexes. Hemin has a Soret absorption band centered at 397 nm, and in the presence of TBA, the shape and intensity of the Soret absorption is similar to that of hemin itself. After incubation with different concentrations of thrombin, a sharp hyperchromicity is always observed in the Soret band of hemin. The increasing absorbance of hemin has been considered as an indicator of the hydrophobic nature of the hemin-binding site.\(^{27}\) The interaction of TBA with hemin/thrombin could be also monitored by the absorption change of the TBA (G-rich DNA). When thrombin and hemin were added into the DNA solution, the absorption peak of the DNA at 257 nm increased significantly, which indicated the formation of the G-quadruplex structure.\(^{3,27}\) In a word, with the addition of thrombin, the increase of the absorbance at about 400 nm and 257 nm showed that the hemin–TBA complex was more and more compact; as a result, the DNAzyme’s activity would be enhanced. On the other hand, in the presence of hemin, TBA displays a major positive peak near 295 nm and a negative peak near 265 nm in the CD spectra (Fig. 2B), which is typical of an antiparallel quadruplex conformation. No appreciable change in their CD patterns is observed in the presence of thrombin, but the peak intensity largely increases at 295 nm in the presence of thrombin. It is obvious that the pattern of the G-quadruplex structure does not change, but the antiparallel structure becomes more compact. Therefore, the results of the UV-vis absorption and CD spectroscopy further confirm that thrombin can promote the activity of the G-quadruplex-based DNAzyme.

**Optimization of assay condition**

The performance of the developed sensing for thrombin is strongly influenced by the assay conditions. Different assays were investigated in our studies.

TBA has two different sequences: one is the 15-mer DNA (5’-GGT TGG TGT GGT TGG-3’), and the other is 29-mer aptamer (5’-AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3’). The two DNA sequences are both found to enhance the catalytic activity of hemin. After the addition of thrombin, there is a large improvement of the G-quadruplex-
based DNAzyme activity when the 15-mer TBA is used, whereas a smaller improvement is observed in the case of the 29-mer TBA. In the presence of hemin and thrombin, 15-nt TBA displays a major a positive peak near 295 nm and a negative peak near 265 nm in CD spectroscopy (Fig. 2B), which is typical of an anti-parallel quadruplex conformation. However, the structure of 29-nt TBA–thrombin–hemin may be a parallel quadruplex because the complex of the 29-nt TBA–thrombin–hemin displays a negative peak near 240 nm and a positive peak near 260 nm (Fig. S1†). So, we think, the different structure of the quadruplex would result in the difference in DNAzyme activities for the 15-nt TBA and 29-nt TBA. The dissociation constant \( (K_d) \) of the 29-mer TBA interaction with thrombin is smaller than that for 15-mer TBA. That is to say, the interaction between 29-nt TBA and thrombin is stronger, which is not consistent with the low catalytic activity of the 29-mer TBA–hemin–thrombin complex. The possible reason is that the DNAzyme catalytic activity mainly depends on the structure of the G-quadruplex DNAzyme. So, the 15-mer TBA is considered as the better sensing element, and is adopted in the following experiments.

In general, DNA G-quadruplexes need coordination cations, especially \( K^+ \), to remain stable. \( K^+ \) can be located at the center cavity between two stacked G-tetrads and coordinates with eight carbonyl oxygen atoms of the G residues, significantly promoting the proper folding of the G-quadruplex. In this work, the thrombin–TBA–hemin complex exhibited rather a low peroxidase-activity when the \( K^+ \) concentration is less than 5 mM. The possible reason is that the TBA does not form G-quadruplexes and does not bind hemin under salt-deficient conditions, and the presence of more than 5 mM \( K^+ \) sufficiently favors the formation of thrombin–TBA–hemin complexes. According to a previous report, the aptamer–hemin complexes possess the best DNAzyme function in 25 mM Tris-HCl buffer (pH 7.4, 20 mM KCl, 200 mM NaCl, 0.05% Triton X-100, 1% DMSO). Therefore, this buffer condition is used for the formation of the G-quadruplex-based DNAzyme.

Based on the principle of the G-quadruplex-based DNAzyme (thrombin–TBA–hemin supramolecular complex), there are two main interactions: thrombin–TBA binding and hemin–TBA binding. Three different analytical processes were designed. In strategy one, TBA and thrombin were first mixed and allowed to interact for a certain period, then hemin was added into the solution of TBA–thrombin to form the thrombin–TBA–hemin supramolecular complex. In strategy two, TBA and hemin were first mixed and allowed to interact for a certain period, then thrombin was added into the TBA–hemin solution to form the thrombin–TBA–hemin supramolecular complex. In strategy three, hemin and thrombin were first mixed, and the mixture of hemin and thrombin was added into the TBA solution to form the thrombin–TBA–hemin supramolecular complex. We compared the three different strategies. After 0.8 nM thrombin was added, the increased fluorescence intensities were 808(±32) (in strategy one), 376(±14) (in strategy two), and 354(±13) (in strategy three), respectively. It is obvious that the sensitivity of the assay in strategy one is higher than that in the other two strategies. For the TBA–thrombin–hemin equilibrium process, the order of adding the reagent should not matter with DNAzyme activity. In this work, the concentration of thrombin (<0.12 nM) was far lower than that of hemin (20 \( \mu \)M); the incubation time (2 h) was not enough for this equilibrium process. So, some kinetic or competitive effects may govern this system, and the different order of addition may result in the different fluorescence intensities. Thus, we choose strategy one (shown in Scheme 1) as the analytical strategy of the assay.

The fluorescence intensity of this system also depends on the conditions of the thiamine–\( H_2O_2 \) reaction. We optimized the reaction conditions. The optimal amount of thiamine was 100 \( \mu \)L of 2.06 × 10\(^{-4}\) M, and the optimal amount of \( H_2O_2 \) was 550 \( \mu \)L of 80 \( \mu \)M. The reaction media strongly affected the fluorescence intensity of this system. The three buffering solutions (carbonate, Tris-HAc and phosphate) were tested in the thiamine–\( H_2O_2 \) system. The results (Fig. 3) showed that the fluorescence intensity in \( \text{pH 12.0} \) phosphate buffer solution (PBS) was the largest. In view of the nature of the thiamine–\( H_2O_2 \) reaction, which is more favored under basic conditions, an alkaline medium can improve the sensitivity of the system. However, the strong alkaline media maybe destroy the structure of the G-quadruplex-based DNAzyme, which forms in \( \text{pH 7.4} \) Tris-HCl buffer solution. So, we used the CD spectrum to investigate the effect of \( \text{pH} \) on the structure of the G-quadruplex-based DNAzyme. The experimental results (Fig. S2†) showed that the structure of the G-quadruplex-based DNAzyme did not change in the strong alkaline medium under the experiment conditions (5 min of incubation time). It is obvious that the DNAzyme possesses high peroxidase-like activity in strong alkaline medium. So, PBS (\( \text{pH 12.0} \)) was used as the reaction medium. Taking into account operational convenience, room temperature (ca. 20 °C) was chosen as the operating temperature for all experiments. In addition, the influence of the reaction time on the final fluorescence intensity was investigated. The results indicated that the fluorescence intensity was maximal and constant after 5 min, and the fluorescence intensity of the product (thiochrome) was constant for at least 12 h. However, in the ABTS–\( H_2O_2 \) colorimetric system, the green product is not stable and can quickly decay to a colorless product in aqueous media within 10 min. So, for the G-quadruplex-based DNAzyme(or HRP)–\( H_2O_2 \) system, thiamine is an ideal substrate because it is easy to obtain and is very stable.
Analytical performance of G-quadruplex DNAzyme-based sensing for thrombin. The quantitative behavior of the fluorescent assay was assessed with different concentrations of thrombin under the optimized conditions. From Fig. 4, it can be seen that the fluorescence intensity increases with increasing thrombin concentration. A good linear relationship between the increased fluorescence intensity ($\Delta F$, $\Delta F = F - F_0$, where $F$ and $F_0$ are the fluorescence intensity of the system at 440 nm in the presence and absence of thrombin, respectively) and thrombin concentration is observed in the range of 0.01–0.12 nM (Fig. 4, inset). The absence of thrombin, respectively) and thrombin concentration is fluorescence intensity of the system at 440 nm in the presence and absence of thrombin, respectively) and thrombin concentration is observed in the range of 0.01–0.12 nM (Fig. 4, inset). The regression equation is $\Delta F = 8304.8c + 98.9$ (where $c$ is the concentration of thrombin, nM) with a correlation coefficient of 0.9993 ($n = 8$). The relative standard deviation (R.S.D.) for $1.0 \times 10^{-10}$ M thrombin measurement is 3.4% ($n = 11$). The detection limit that is taken to be three times the standard derivation in the blank solution was found to be 0.001 nM. The sensitivity was increased more than 4 orders of magnitude over that of the G-quadruplex-based DNAzyme-based colorimetric method.\(^{17}\) The selectivity of this method for thrombin was evaluated by testing the response of the assay to other common proteins (human serum albumin, bovine serum albumin, human IgG, and lysozyme) at a concentration of 0.1 nM. The experimental results (Fig. 5) showed that only thrombin could significantly enhance the activity of the DNAzyme, and that other proteins had little influence on the catalytic activity, indicating that the TBA–hemin complex specifically responds to thrombin. The results reveal that this facile fluorescent method for thrombin detection is sensitive and effective.

Conclusion

We have introduced a novel method for the fluorescent detection of thrombin with the 15-mer thrombin-binding aptamer (TBA) as the sensing element. This aptamer can specifically bind thrombin to form the G-quadruplex structure, thus allowing the formation of a G-quadruplex-based DNAzyme after incubation with hemin. Combined with the $\text{H}_2\text{O}_2$–thiamine fluorescent system, the G-quadruplex-based DNAzyme provides a facile approach to sensing thrombin with a detection limit of 1 pM. Furthermore, the DNAzyme-catalyzed $\text{H}_2\text{O}_2$-mediated oxidation of thiamine is tested, and the results show that stable and cheap thiamine can be used as a fluorescent substrate to replace the traditional chromogenic substrate ABTS. The findings in this work should be useful in emerging fluorescence detection technology with the G-quadruplex-based DNAzyme as a label-free sensing platform, and also for fluorometric assay to discover anticancer drugs that inhibit telomere or genomics G-quadruplexes.\(^{34,30}\)

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