RSC Advances

Scheme 1 Schematic illustration of the RLS aptasensor for selective detection of human thrombin.

Combining a Loop-Stem Aptamer Sequence with Methylene Blue: A Simple Assay for Thrombin Detection by Resonance Light Scattering Technique

Xiaopeng Huang,[a†] Yuqin Li,[b†] Xiang Huang,[a] Yaowen Chen,[c] and Wenhua Gao,[ac]*

[a] Department of Chemistry, Shantou University, Shantou, Guangdong 515063, P. R. China.

[b] Department of Pharmacy, Taishan Medicine College, Taian, Shandong 271016, P. R. China.

[c] Analysis & Testing Center, Shantou University, Shantou, Guangdong 515063, P. R. China.

Corresponding author. Tel: +86-22-86502774; Fax: +86-22-82903941
E-mail address: whgao@stu.edu.cn

[†]Both the authors contributed equally to the paper.

Abstract

An ingenious sensing strategy for detecting thrombin in human serum has been developed on the basis of a hairpin DNA sequence and resonance light scattering (RLS) technique. A thrombin aptamer sequence was embedded inside the hairpin DNA strand (H-eTBA), which was designed to be the loop-stem structure. Moreover, methylene blue (MB) was utilized as the RLS signal indicator according to its different affinity to single or double stranded DNA. Upon the addition of thrombin, the thrombin aptamer inside H-eTBA interacted specifically with thrombin. Thus the conformation of H-eTBA would change. After the introduction of the DNA strand (CTBA), which was complementary to H-eTBA, the amount of double stranded DNA would decrease as a consequence. Later when MB solution was added, the RLS signal would present various response value based on different amount of thrombin. The determination of thrombin in human serum could be obtained with a detection limit of 0.32 nM and this specific sensor could be applied to detect thrombin practically. Furthermore, this aptasensor showed quite good selectivity and simplicity toward thrombin. Finally, the proposed sensing method showed its superiority with selectivity and practicability, which could be used as a simple platform for thrombin detection.

1. Introduction

Thrombin occupied an essential position undoubtedly in many physiological and pathological processed, such as blood coagulation, thrombosis, inflammation, angiogenesis and metastasis[1]. Thrombin could also be utilized as a therapeutic and a biomarker[2,3] for diagnosis of coagulation abnormalities. Obviously, the development of a new strategy that is selective and simple is necessary and crucial for the detection of thrombin.

Aptamers, which are single stranded DNA molecules or RNA, could selectively bind to different target molecules[4,5] with high affinity and gain broader concerns because of their simplicity of synthesis. Moreover, the properties of excellent stability, wide applicability and superior sensitivity make aptamer a suitable analytical agent in many medical diagnosis[6-9]. Additionally, hairpin DNA sequence shows high hybridization specificity because of its loop-stem structure. It can easily discriminate the complementary strand from single-mutation target or mismatched DNA probe. Namely, hairpin DNA probe could be used to get a better selectivity in a DNA based probe.

Methylene blue (MB) is one of the most popular phenothiazine dye. It has demonstrated its different affinities towards single (ss) and double stranded (ds) DNA[10]. Positively charged MB molecule could be accumulated on the surface of double helix structure of negative charged dsDNA through electrostatic attraction. This unique affinity of MB with ss and ds DNA allowed the application of MB as an indicator in DNA based protein assays[11]. Resonance light scattering (RLS) is a kind of

elastic light scattering, which is produced while the incident beam is close to its molecular absorption band[12]. Pasternack initially established the RLS method, which was developed for analytical application by Huang *et al*[13]. Over the next two decades, RLS was applied to study widely in the aspect of detecting nucleic acids[14], anti-cancer drugs[15,16] and proteins[17], *etc*. An increasing number of studies indicated that RLS technique was becoming a most popular testing method in daily application.

Different methods such as optical[18,19], electrochemical[20,21], surface enhanced resonance Raman scattering[22], surface plasmon resonance[23] and so on have been developed to detect thrombin. However, not only the conventional techniques mentioned above but also some limited conditions[24] have a negative impact on the detection of thrombin. In this paper, a combination of aptamer with hairpin DNA structure revealed significantly improved analytical performance towards thrombin detecting and this combination accompanied with RLS technique has not been reported. Particularly, the adding of thrombin could lead to the structural change of hairpin sequence, after which different amount of dsDNA or ssDNA would form when the complementary strand of hairpin sequence was added to interact with hairpin DNA. Subsequently, the adding of MB solution expressed distinct affinity toward dsDNA and ssDNA and the system demonstrated different RLS signal that could be used to make quantitatively analysis. Therefore, an aptamer sensor could be attained, which could be used for thrombin detection in human serum sample successfully.

2. Experimental

2.1 Regents and apparatus

Oligonucleotides were synthesized by Sangon Biotechnology Co., Ltd. (Shanghai, China) and their sequences are shown in Table 1. (Bolded bases are aptamer segments of thrombin except the single-base mismatched DNA. Italic bases are the stem segments of the hairpin DNA. Underlined bases are totally complementary to the DNA sequence added subsequently.)

[Table 1]

Methylene blue (MB) was obtained from Aladdin Chemical Reagent Co., Ltd. (Shanghai, China). Human α-thrombin, bovine serum albumin (BSA), trypsin, α-chymotrypsin were purchased from Bomei Biotechnology Co., Ltd. (Hefei, China). Tris-HCl buffer solution (20 mM) containing 50 mM NaCl and 100 mM $MgCl_2$ was utilized to prepare all solutions. The fresh human serum samples were obtained from the infirmary of Shantou University. Millipore Milli-Q water (18 ΩM cm) supplied by a Millipore Milli-Q water purification system (Bedford, MA. USA) was applied in whole process of the experiment. All chemicals used for investigations were of analytical grade purity.

The RLS spectra were measured on an F-7000 fluorescence spectrophotometer (Hitachi, Japan) equipped with a 1cm×1cm quartz cuvette. Absorption spectra was recorded on a Lambda-950 UV-Vis spectrophotometer (Perkin-Elmer, USA). All pH measurements were made with a PHS-3CA precision acidity meter (Dapu, China).

2.2 Measurement procedure

First, 898 μL of thrombin solution with various concentrations were respectively added into a 1.5 mL centrifugal tube containing 1 μL H-eTBA solution (10 μM) (denatured at 95 °C for 10 min, then cooling to room temperature) in Tris-HCl buffer solution (pH7.40). After mixing for 25 min at 37 °C, 1 μL CTBA solution (10 μM) was added and the mixture was reacted for another one hour at 37 °C. Then 100 μL MB solution (50 μM) was added quickly into the tube and mixed thoroughly at room temperature. The resulting solution was transferred to a 1 cm quartz cuvette for spectral recording without any incubation time. RLS spectra were obtained by scanning synchronously from 225 to 700 nm with $\Delta\lambda=0$ nm. The excitation and emission slit widths were kept at 5 nm and 2.5 nm respectively. The decreased RLS intensity of the reaction system was presented as $\Delta I_{RLS}=I^0_{RLS}-I_{RLS}$, where I_{RLS} and I^0_{RLS} were the RLS intensities of the MB-DNA system with and without thrombin.

2.3 Validation test

Additionally, a validation test was proposed to verify that MB molecule could induce the RLS signal change when it interacted with dsDNA. The validated steps were shown as follow: 1 μL H-eTBA (10 μM) and 1 μL CTBA solution with different concentrations were mixed with 898 μL buffer solution thoroughly in a 1.5 mL centrifugal tube. After incubating at 37 °C for an hour and cooling it down to the room temperature, 100 μL of MB solution (50 μM) was added into the tube. Finally, this resulting solution was transferred to a 1 cm micro quartz cuvette for UV-vis

spectra recording.

3. Results and discussion

3.1 Characteristics and comparison of RLS spectra

Contrast experiment was made to identify and characterize the RLS spectra as indicated in Fig. 1. It could be seen that the spectrogram shape maintained nearly the same from curve a to h. The light scattering peak at 370 nm could be regarded as the RLS peak of the reaction system. As shown in Fig. 1, the RLS intensity of MB + H-eTBA + CTBA (curve a) was strong. On the contrary, the RLS intensities of MB + H-eTBA, MB + CTBA, MB + thrombin, thrombin, MB and H-eTBA + CTBA (inset: curve c to h) were rather weak in the whole scanning wavelength range, which could be ignored in this reaction system.

[Fig. 1]

However, when thrombin was introduced (12.28 nM, curve b), a decreased RLS intensity was clearly observed. Based on RLS theory[13,25], light scattering is caused by the presence of particles with proper diameter and the RLS intensity is proportional to the number of particles. As above, when the thrombin was added to the system, the amount of MB-dsDNA complexes decreased, which could be easily recognized from curve b in Fig. 1. Moreover, from the inset in Fig. 1, it was noted that the RLS intensity of curve c was a little stronger than curve d. This phenomenon demonstrated that MB had reacted with dsDNA to some extent due to the loop-stem structure of H-eTBA.

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3.2 Sensing mechanism

A schematic representation of the mechanism of the sensing thrombin was illustrated in scheme 1. Based on the RLS theory mentioned above, the reaction system showed two quite different RLS intensity results when the target thrombin was present or not.

[Scheme 1]

To be detailed, H-eTBA was in random in the form of loop-stem structure (hairpin) in the beginning. On one hand, when there was no target thrombin, the complementary sequence towards H-eTBA (CTBA) could be highly complementary to H-eTBA. Therefore, MB molecular added afterwards could interact with a large number of dsDNA. Thus MB-dsDNA complexes with large size were formed in the reaction system, which could induce a strong RLS signal.

On the other hand, while thrombin was added, H-eTBA could bind with thrombin and thus reduced the RLS intensity. With the addition of thrombin, H-eTBA could react with thrombin through its thrombin aptamer sequence embedded inside the hairpin DNA. Next, CTBA towards H-eTBA was introduced. Thus there would be less dsDNA existing in the reaction system because the target thrombin had interacted with some of the H-eTBA. As MB solution was added subsequently, weak RLS signal was produced because MB molecular interacted with a small number of dsDNA. By monitoring the change in RLS intensity, thrombin target could be detected with selectivity and speediness.

We then studied the hypochromicity effect of the reaction system through

UV-vis spectra. It would present a much more indicative view of the sensing mechanism. As shown in Fig. 2, the absorbance of MB-dsDNA at 666 nm decreased with the reduction of the concentration of CTBA. The hypochromicity demonstrated a fact that MB molecule could accumulate on the surface of the double helix structure, which was produced because of the combination of H-eTBA and CTBA, by electrostatic attraction[26]. Meanwhile, the maximum absorption wavelength did not change and this also strongly indicated that MB molecules combined to dsDNA externally.

[Fig. 2]

3.3 Optimization of experimental conditions

To optimize the sensing conditions, the experimental concentration of H-eTBA and CTBA should be explored in the beginning. As shown in Fig. S1, we tested the RLS value upon different concentration of H-eTBA in the absence of CTBA. It showed that the 10 μM H-eTBA was the optimized condition. In Fig. S2, it could reach a RLS maximum after 10 μM CTBA reacted with H-eTBA. So 1 μL, 10 μM were chosen for the detection of thrombin in this work. In addition, we also tested the incubation time presented in Fig. S3. We found that the RLS intensity hardly changed within 30 min. In order to shorten the time under the experimental premise, we considered it possible to present our work without any incubation time.

Certainly, the pH value played a very important role in the interaction between MB molecule and DNA. As shown in Fig. 3, the effect of pH value was investigated

by comparing the results in different pH conditions. In the range of 5.45-9.50, it was

apparent that the RLS intensity reached a maximum at pH7.40. Therefore, pH7.40

was selected to be the optimal pH value for the detection system.

[Fig. 3]

[Fig. 4]

The effect of MB concentration was tested by carrying through the H-eTBA (1

μL, 10 μM) and CTBA (1 μL, 10 μM) and thrombin (4.91 nM) at pH 7.40. In Fig. 4,

the experimental results indicated that the RLS intensity reached maximum when the

MB concentration was at 50 μM. It was obvious that the increasing concentration of

MB would result in the increasing of the RLS signal because of more MB molecules

interacted with dsDNA. However, the RLS intensity would decrease along with the

increasing concentration of MB solution. The reason probably was that MB molecules

would aggregate into dimers and thus it was not conducive to the combination of MB

with dsDNA. As a result, the RLS intensity would decrease obviously. So 50 μM was

chosen to be the optimal concentration of MB in this work.

3.4 quantitative detection of thrombin

On the basis of the above standard procedures and optimal conditions, various

concentrations of thrombin were introduced to evaluate the performance of the sensor.

As shown in Fig. 5, higher concentration of thrombin resulted in more RLS intensity

decrease (curve a to f). This result was in accordance with the inference as above.

Thus, the RLS peak of the reaction system at 370 nm could be used to give a

quantitative detection of thrombin.

[Fig. 5]

Furthermore, the proposed sensing strategy had linear relationship in the range from 0 to 17.18 nM. The equations for the resulting calibration plot was ΔI_{RLS}=261.96$C_{thrombin}$ +65.38 (R^2=0.9951). The detection limit was 0.32 nM, which was estimated on the concentration corresponding to the mean blank value plus 3 times the standard deviation of the blank value[27].

3.5 Selectivity, stability and reproducibility of the assay

In our proposal, it would be crucial for H-eTBA to recognize the other variable form of DNA strands because the system is quantified by the amount of dsDNA. Herein, we used single-base mutation DNA sequence (Mis-DNA) or non-specific aptamer sequence (Non-specific) for the comparison. The ability to sensitively discriminate that two kinds of DNA sequence was crucial for diagnosis at early stage. With the purpose of testing the selectivity of the loop-stem aptamer sensor, further investigation was made using the sequences as mentioned above.

As presented in Fig. 6, in a fixed concentration of thrombin at 4.91 nM, two contrast experiments were made. In the first comparison, a single-base mismatched strand was adopted to replace CTBA. In the second testing, a total non-thrombin specific DNA sensor (non-specific) was used to substitute H-eTBA. Simultaneously, a new complementary strand towards non-specific was utilized to replace CTBA. The procedure of these two independent experiments was the same as the process of

detecting thrombin as above.

It was clear that when H-eTBA was utilized, the RLS intensity was much stronger than the signal of Mis-DNA. The weaker RLS single of Mis-DNA implied that the hairpin sensor could discriminate the mutation sequence effectively. The Mis-DNA could not interact with H-eTBA thoroughly and a much weaker RLS signal were presented because MB reacted with fewer dsDNA strands (Fig. 6). This result demonstrated that even with the presence of a single base mutation complementary strand, H-eTBA could still recognize the difference because of its hairpin structure.

Additionally shown in Fig. 6, the RLS intensity of Non-specific sequence was similar to the signal of H-eTBA and even a little stronger than it. Because there was no thrombin aptamer embedded inside the non-specific hairpin strand, non-specific strand and its own complementary could highly combine with each other, which induced a quite strong RLS signal after MB solution was added.

[Fig. 6]

For the sake of a better illustration of the selectivity, we then used BSA, Trypsin and α-Chymotrypsin (all at 27.25 nM, except for thrombin at 17.18 nM) for interference measuring. As indicated in Fig. S4, significant change in RLS intensity was only observed for the target thrombin and not for other nontargeted proteins. The compared result indicated that our featured loop-stem aptamer structure could provide a good selectivity for thrombin detection.

The stability of the presented sensor was examined by detecting the RLS response with time variation. We studied the RLS signal of the reaction system after

the solution was stored at 4°C for 5 and 10 days. We found that the RLS response retained 92.4% of the initial RLS signal for 4.91 nM thrombin, demonstrating the good stability. Additionally, the reproducibility of this sensor was studied by analysis of the same concentration of thrombin (4.91 nM) using 3 sensors under the same experiment conditions. Closely RLS intensity was obtained with a relative standard deviation (RSD) of 5.3%. The results revealed that our proposal had an acceptable reproducibility.

3.6 Practical application

The performance of the thrombin probe in human serum sample was further investigated. Human serum samples were diluted 3-fold with Tris-HCl buffer solution in the beginning. As given in Table 2, satisfactory recoveries could be achieved using our method. The recovery of those measurements were in the range from 95.5% to 101.7% under the optimal condition, indicating that the designed thrombin sensor still worked well and have a more reliable result in real human serum sample application.

[Table 2]

Different methods for detecting thrombin were then compared and the results were presented in Table 3. It could be concluded that with the measurements of SERS (Surface-enhanced Raman Scattering), electrochemical, colorimetric or fluorescence methods, each one had either a narrow linear range or a high detection limit.

[Table 3]

Compared with those reported methods, our method presented a comparable linear range and detection limit, which were presented in Table 3 apparently. Additionally, our method was simple because it did not require any complicated experimental procedures and conditions.

4. Conclusion

To draw a conclusion, a sensing strategy using well-designed loop-stem structured DNA sequence was presented to demonstrate the feasibility for a simple detection of thrombin. More detailed, we tactfully combined the loop-stem structure with methylene blue and RLS detecting technique. Because of the thrombin aptamer embedded inside the hairpin DNA and the interaction between methylene blue molecule and dsDNA, the target thrombin could be detected effectively using RLS technique. In addition, the limit of detection was as low as 0.32 nM, which was practical for the detection of thrombin. Moreover, complicated and tedious experimental procedures were unnecessary for thrombin detection by this sensing strategy. Finally, because of its favorable selectivity and practicability, the authors believed that our sensing proposal could give a new promising method for the clinical application of detecting thrombin.

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Figure and table captions:

Fig. 1 The RLS intensity spectra of the MB-DNA reaction systems with and without thrombin. (a) MB + H-eTBA + CTBA; (b) MB + H-eTBA + CTBA + thrombin (12.28 nM); (c) MB + H-eTBA; (d) MB + CTBA; (e) MB + thrombin; (f) thrombin; (g) MB; (h) H-eTBA + CTBA. Conditions: MB: 50 μM; H-eTBA: 1 μL, 10 μM; CTBA: 1 μL, 10 μM; pH=7.40.

Scheme 1 Schematic illustration of the RLS aptasensor for selective detection of human thrombin.

Fig. 2 The hypochromicity effect of the reaction system. Conditions: MB: 50 μM; H-eTBA: 1 μL, 10 μM; CTBA: 1 μL, 0.1, 1, 2, 4, 8 and 10 μM; pH=7.40.

Fig. 3 The effect of pH value on the RLS intensity. Conditions: MB: 50 μM; H-eTBA: 1 μL, 10 μM; CTBA: 1 μL, 10 μM; thrombin: 4.91 nM; pH value: 5.45, 6.50, 7.40, 8.40 and 9.50. Error bars were the standard deviation of three repetitive measurements.

Fig. 4 The effect of the concentration of MB solution on the RLS intensity. Conditions: H-eTBA: 1 μL, 10 μM; CTBA: 1 μL, 10 μM; thrombin: 4.91 nM; MB: 10, 30, 50, 70 and 90 μM; pH=7.40. Error bars were the standard deviation of three
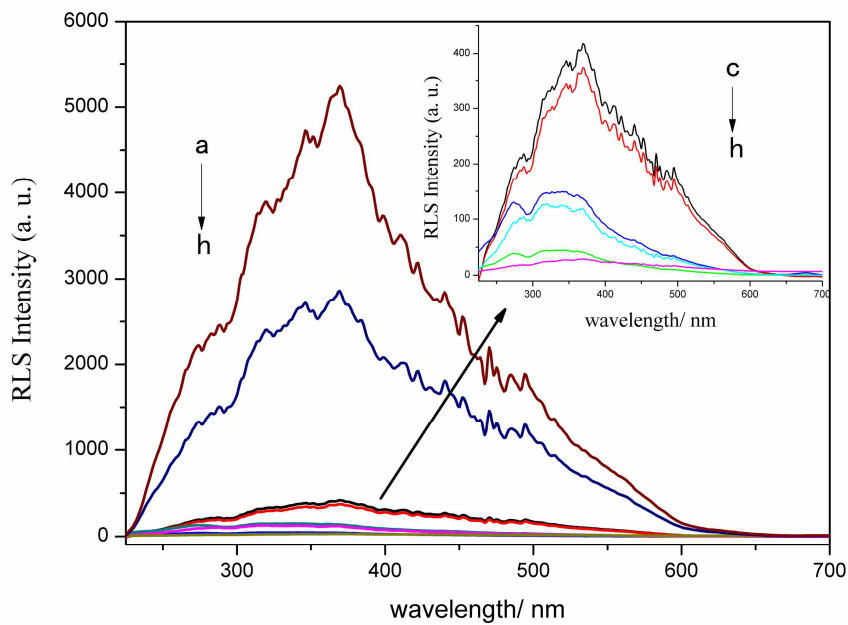
repetitive measurements.

Fig. 5 The RLS spectra of the reaction system upon the addition of human thrombin at different concentrations. a-f: 0, 4.91, 9.82, 12.28, 14.73 and 17.18 nM. Inset: the RLS peak absorbance change is linear with the thrombin concentration in the range from 0 to 17.18 nM. Conditions: MB: 50 μM; H-eTBA: 1 μL, 10 μM; CTBA: 1 μL, 10 μM; pH7.40. Error bars were the standard deviation of three repetitive measurements.

Fig. 6 Comparison of the aptasnesor selectivity using single-base mismatched strand (Mis-DNA) and non-specific aptamer sequence (non-specific). Conditions: thrombin: 4.91 nM; H-eTBA: 1 μL, 10 μM; CTBA: 1 μL, 10 μM; Mis-DNA: 1 μL, 10 μM; both Non-specific DNA and its complementary strand: 1 μL, 10 μM; MB: 50 μM; pH7.40. Error bars were the standard deviation of three repetitive measurements
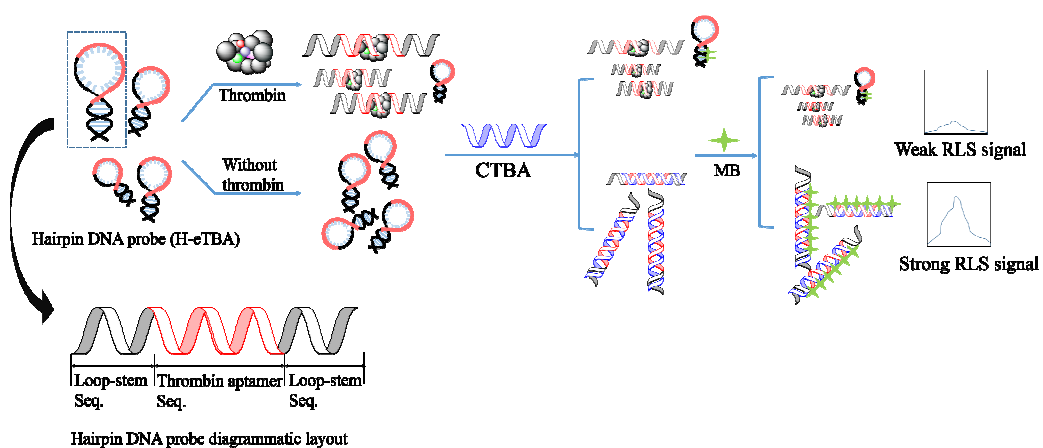
Table 1 Sequences of oligonucleotides used in this work.

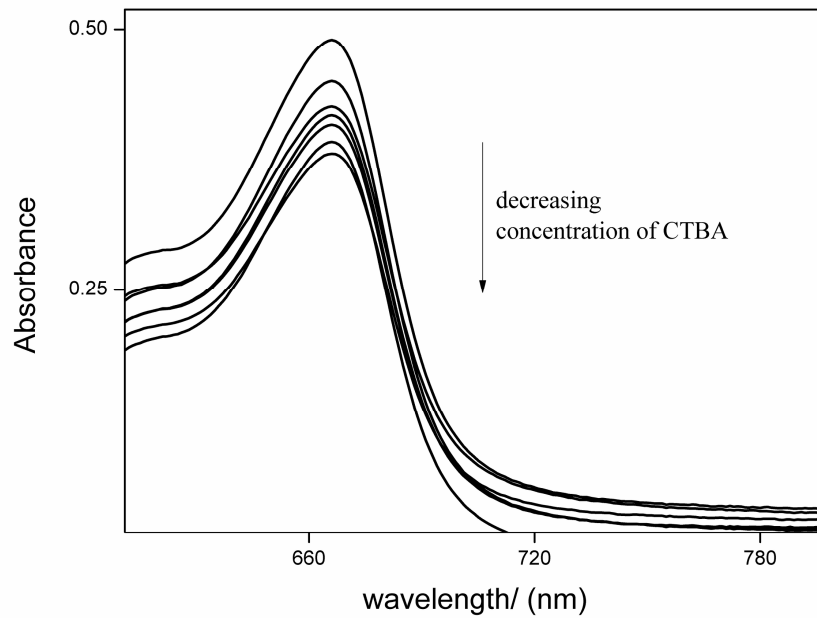Table 2 Determination results of thrombin in human serum.

Table 3 Comparison of different methods for the detection of thrombin

Fig. 1 The RLS intensity spectra of the MB-DNA reaction systems with and without thrombin. (a) MB + H-eTBA + CTBA; (b) MB + H-eTBA + CTBA + thrombin (12.28 nM); (c) MB + H-eTBA; (d) MB + CTBA; (e) MB + thrombin; (f) thrombin; (g) MB; (h) H-eTBA + CTBA. Conditions: MB: 50 μM; H-eTBA: 1 μL, 10 μM; CTBA: 1 μL, 10 μM; pH=7.40.
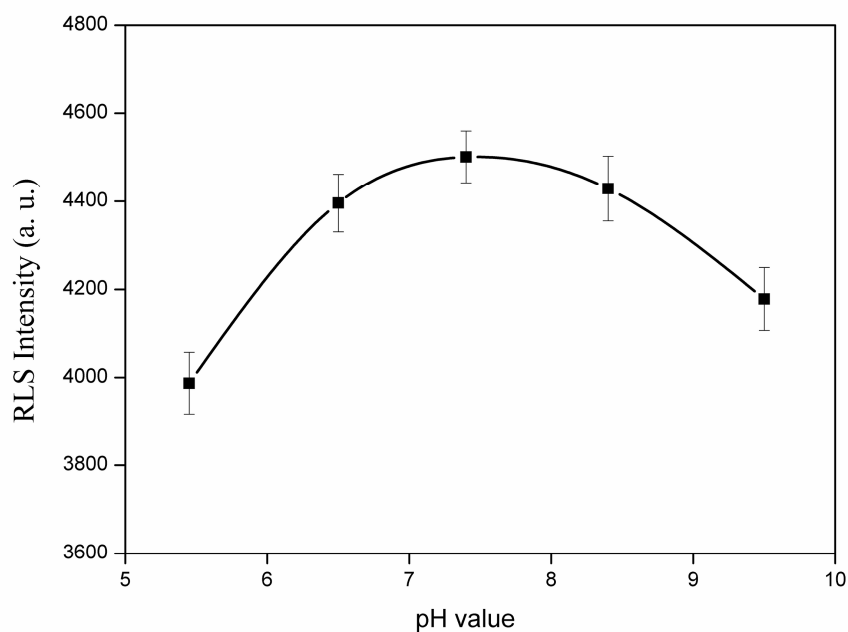
Scheme 1 Schematic illustration of the RLS aptasensor for selective detection of human thrombin.

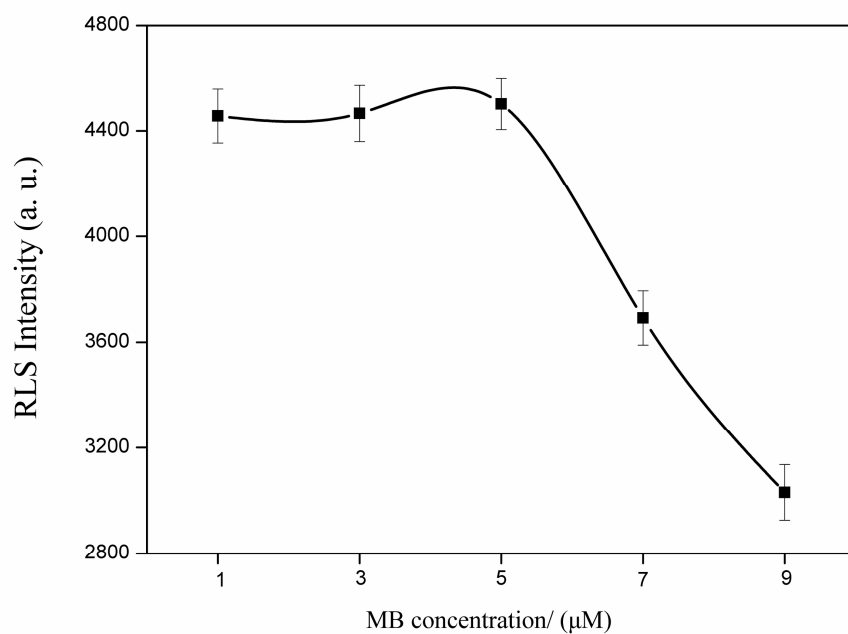**Fig. 2** The hypochromicity effect of the reaction system. Conditions: MB: 50 μM; H-eTBA: 1 μL, 10 μM; CTBA: 1 μL, 0.1, 1, 2, 4, 8 and 10 μM; pH=7.40.
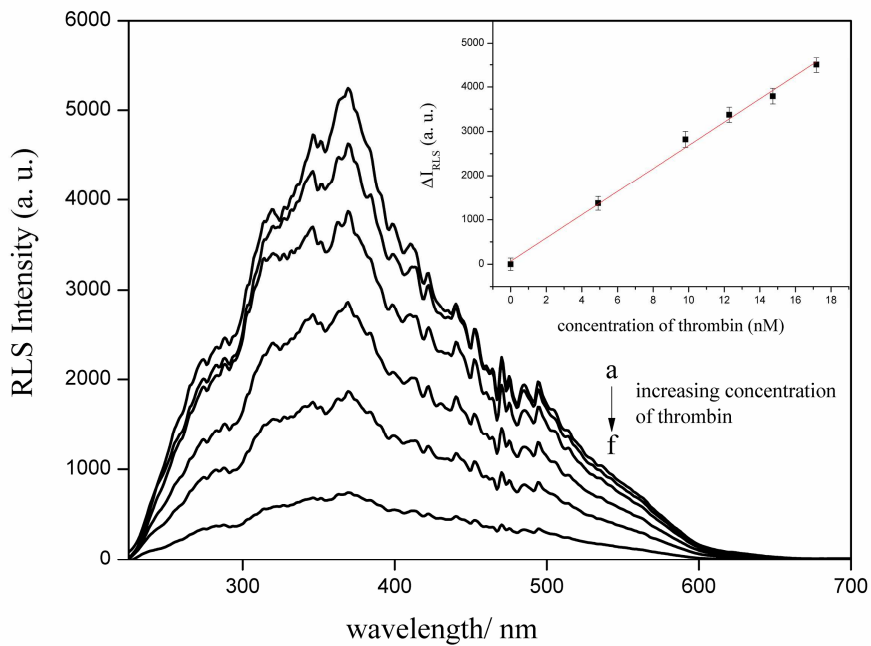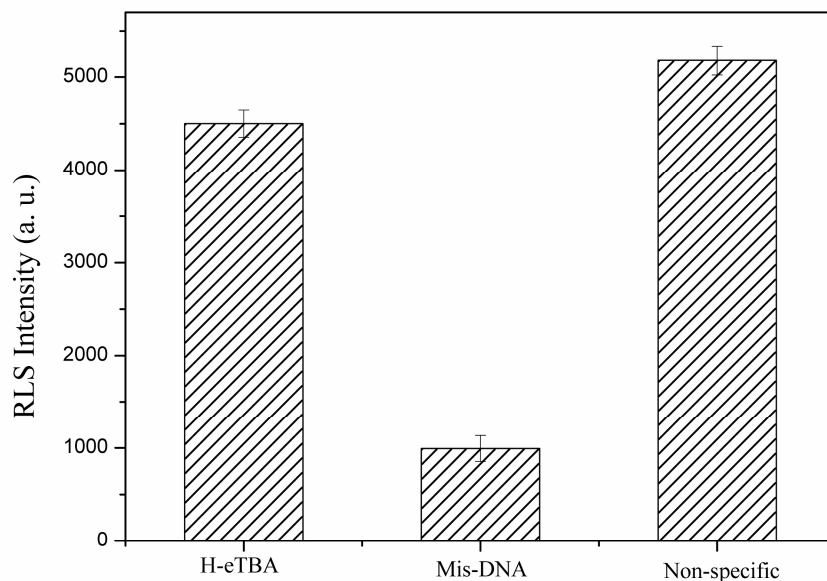
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Table 1 Sequences of oligonucleotides used in this work.

Name [a]	Sequences
H-eTBA	5'-*GAATTC* TTAAA **GGTTGGTGTGGTTG** *GAATTC*-3'
CTBA	5'-CCAACCACACCAACCTTTAAGAATT-3'
Mis-DNA	5'- CCAACCACAC**G**AACCTTTAAGAATT-3'
Non-specific	5'-*GAATTC* TTTTTTCCCCCCTTTTTT *GAATTC*-3'
Complementary strand towards non-specific	5'-CAAAAAAGGGGGGGAAAAAAGAATT-3'

[a] H-eTBA: Hairpin DNA embedded a thrombin binding aptamer; CTBA: Complementary strand to H-eTBA; Mis-DNA: Single-base mismatched DNA (double underline and in bold); Non-specific: totally non-specific DNA sequence.

Table 2 Determination results of thrombin in human serum. [a]

Sample Number	Thrombin Added (nM)	Thrombin Found (nM)	Recovery (%)	RSD (%, n=3)
1	4.08	3.90	95.6	3.8
2	6.81	6.69	98.2	5.2
3	9.54	9.69	101.6	4.2
4	12.26	12.35	100.7	7.3
5	14.98	14.80	98.8	6.1

[a] Human serum samples were diluted 3-fold with buffer solution (pH7.40) before detection. Each data was given as the average value obtained from three independent experiments.

Table 3 Comparison of different methods for the detection of thrombin

Method	Sensor	Linear range (nM)	Detection limit (nM)	Ref.
SERS	Au/SH-TBA/TB/TBA/AuNPs/Raman Probe/AgNPs	Not given	0.5	28
SERS	Si substrase/AuNPs/SH-TBA(-FITC) /TB	1×10^{-4}-0.01	0.02	29
Electrochemical	Magnetic bat/eletrode/AuMNP/SH-Apt1/TB/SH-Apt2-CS-AuNPs-HRP	1.0×10^{-5}-0.01	5.4×10^{-6}	30
Electrochemical	Ferrocene-graphene nanosheets/Ru(bpy)$_3^{2+}$	0.5-25	0.21	31
Electrochemical	Enzyme-free and non-label fluorescent G-quadruplex DNA	0.01-1	0.005	32
Photoelectrochemical	Cationic macromolecules PEI and MPA modified G-CdS nanocomposites	0.002-0.6	0.001	33
Colorimetric	Nanorose/SH-Apt15/TB/SH-Apt29/nanorose	1.6-30.4	1.0	34
Fluorescence	GO-FAM-peptide complex	2.2-10	2.0	35
RLS	**Loop-stem thrombin aptamer DNA sequence**	**0-17.18**	**0.32**	**This assay**