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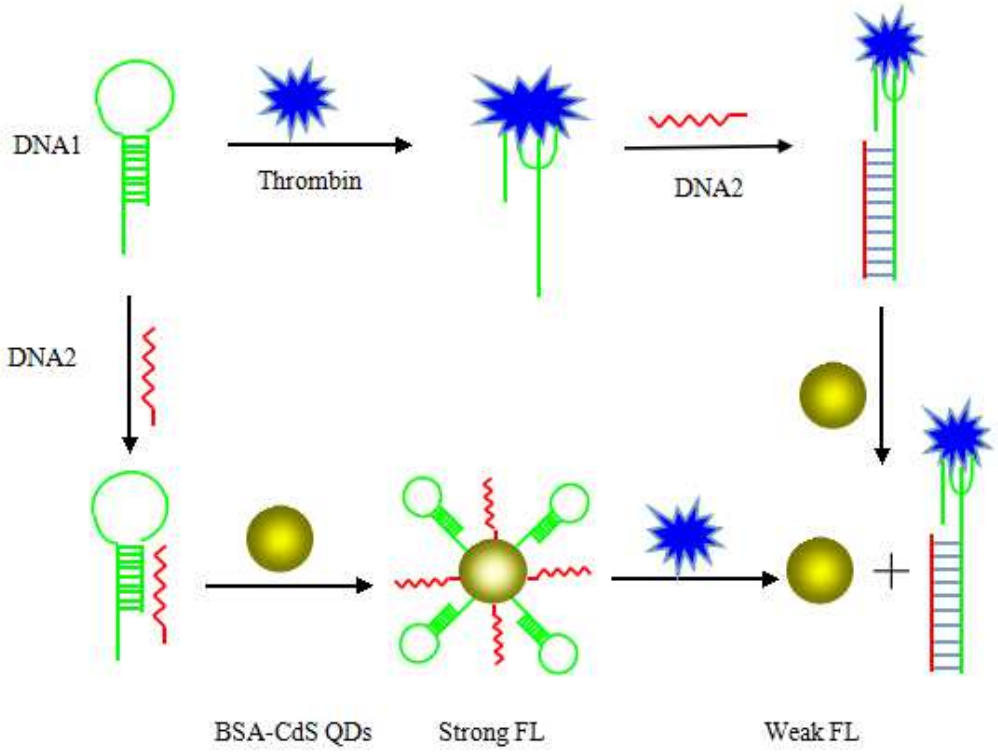
Graphic Abstract



Label-free aptamer biosensor for selective detection of thrombin

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ABSTRACT

We fabricated a novel fluorescence biosensor for the selective detection of thrombin by using bovine serum albumin-capped CdS quantum dots (BSA-CdS QDs). Two kinds of designed DNA (DNA1 and DNA2) could bind to CdS QDs through the electrostatic interaction between DNA and Cd^{2+} on the surface of CdS QDs. The obtained DNA/BSA-CdS QDs kept stable in the solution with the fluorescence intensity obviously enhanced. Hairpin structure of DNA1contained two domains, one is the aptamer sequence of thrombin and the other is the complementary sequence of DNA2. When thrombin was added, it would bind to DNA1 and induce the hairpin structure of DNA1 changed into G-quadplex structure. Meanwhile, DNA2 would transfer from the surface of CdS QDs to DNA1 via hybridization, which resulted in the removal of DNA1 and DNA2 from the surface of CdS QDs, and led to the fluorescence intensity of CdS QDs reduced. Thus, the determination of thrombin could be achieved by monitoring the change of the fluorescence intensity of CdS QDs. The present method is simple and fast, and exhibits good selectivity for thrombin over other proteins. We have successfully detected thrombin in human serum samples with satisfactory results.

Key word: Thrombin, thrombin aptamer, fluorescence, CdS quantum dots

1. Introduction

Thrombin, a kind of serine protease, plays a significant role in blood coagulation. It can directly transform soluble fibrinogen into insoluble fibrin that subsequently forms the fibrin gel for clots [1-3]. Thrombin is not present in the blood of healthy individuals. However, during the coagulation process, the concentration in blood varies considerable even from nM to mM [4,5]. The high or low concentration of thrombin in blood is dangerous to patients. Excessive thrombin will induce thrombosis while low content of thrombin will induce an excessive bleeding. Moreover, it is considered as a useful tumor marker in the regulation of tumor growth, apoptosis, metastasis [6-8]. Therefore, it is significant to detect thrombin by a sensitive and selective method.

An aptamer, a single-stranded DNA or RNA, can especially recognize and bind to the targets with high affinity, good stability and storage properties [9-11]. Two well-known thrombin-binding aptamers for thrombin with 15 (Apt15) and 29 (Apt 29) bases have been specifically selected that bind to its two electropositive exosites, namely fibrinogen and heparin sites, situated on the sides of the active site [12,13]. Since the thrombin-binding aptamer was selected, numerous aptasensors have been fabricated based on the conformational changes induced by target binding. The key issue in the development of aptasensors is how to convert target recognition and binding into measurable signals. Up to now, many methods have been used for this purpose, including colormetric [14], fluorescence [15,16], electrochemiluminescence [17,18], surface plasmon resonance [19,20] and so on. For example, Li. et al. developed a colorimetric assay for thrombin. Apt 15 labeled paramagnetic particles and Apt 15 labeled Ag nanoparticals, can recognize the fibrinogen and heparin sites of thrombin, would be hold together by thrombin to form a sandwich-type complex. The sandwich-type complex has the catalytic ability. The colorimetric

detection was achieved through the reduction of dye catalyzed by the sandwich-type complex [21]. He. et al. developed a novel thrombin detection strategy based on dual-hairpin DNA structure. First, DNA S1 was stabilized on the gold electrode and formed a hairpin DNA structure due to the designed five pairs of base matches in the stem region. DNA S2, modified with methylene blue (MB), who could be captured by S1 via forming Dual-Hairpin structure. Upon the addition of thrombin, the dual-hairpin structure was changed, and the S2 would divorce from the S1 modified electrode. Because the S2 that contained the signal molecules MB was far away from the electrode, the current would drop sharply [22]. These sensor platforms exhibited a broad dynamic range, high sensitivity. Among these methods, fluorescence methods have attracted significant interest due to its rapidness, cost-effectiveness, simplicity, and nondestructive. In fluorescence methods, QDs always be used for fabricating the sensing system owing to their ideal optical properties. A series of QDs-based fluorescence sensors have been designed for various protein detection, such as lysozyme detection [23,24]. However, only few sensors were designed for the assay of thrombin.

In this work, we constructed a new label-free biosensor for thrombin detection by employing a conformation change-based hybridization reaction (Scheme 1). We designed two kinds of DNA. The first one is a hairpin structured DNA, defined as DNA1, contained two domains termed as I and II according to their different functions. Region I is the sequence of Apt 15 and region II is the complementary sequence of DNA2. The other one is a 27-mer DNA, defined as DNA2, could hybridize with region II of DNA1. In the absence of thrombin, DNA1 and DNA2 could combine with the BSA-CdS QDs to passivate its surface defects, and the fluorescence of the CdS QDs would be enhanced. In the presence of thrombin, Region I of DNA1 would recognize and combine

with the thrombin, the hairpin structure will be opened upon the interaction between DNA1 and thrombin to form a stable G-quadruplex structure, then the region II sequence of DNA1 and DNA2 would bind together via complementary base pairing. The fluorescence intensity of CdS QDs would be reduced since the removal of DNA1 and DNA2 from the surface of CdS QDs. The fluorescence intensity of CdS QDs would gradually decrease with the increasing of the thrombin concentration.

2. Experimental section

2.1. Materials

All reagents were of at least analytical grade. The water used in all experiments had a resistivity higher than 18 MΩ cm^{-1}. Cadmium (II) chloride ($CdCl_2$), sodium hydroxide (NaOH), Sodiumsulfide nonahydrate ($Na_2S•9H_2O$), trihydroxymethyl aminomethane (Tris) and hydrochloric acid were purchased from Shanghai Qingxi Technology Co., Ltd. Thrombin and Bovine serum albumin were purchased from Sigma-Aldrich Corporation. The oligonucleotides with the following sequences were obtained from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. DNA1 (5'-GGT TGG TGT GGT TGG CAT CAA AAA AAT CCT CAG ATG CCA ACC-3') , DNA2 (5'-GGT TGG CAT CAG AGG ATT TTT TTG ATG-3'). The 0.1 mol/L Tris-HCl buffered solution (pH 7.4) was used as the medium for detection process.

2.2 Apparatus

The fluorescence spectra were obtained by using a Shimadzu RF-5301 PC spectrofluorophotometer equipped with a xenon lamp using right-angle geometry. UV-vis absorption spectra were obtained by a Varian GBC Cintra 10e UV-vis spectrometer. In both experiments, a 1 cm path-length quartz cuvette was used. All pH measurements were made with a

PHS-3C pH meter (Tuopu Co., Hangzhou, China). Transmission electron microscopy (TEM) experiments were performed on a Philips Tecnai F20 TEM operating at 200 KV acceleration voltage.

2.3 Preparation of BSA-CdS QDs

BSA-CdS QDs were synthesized in aqueous solution. 400 μL 0.5 mmol/L BSA solution, 120 μL 50 mmol/L $CdCl_2$ solution and 150 μL 0.1 mol/L Tris-HCl buffer (pH 7.0, 150 μL) were added into a 2 mL calibrated centrifuge tube and shaken thoroughly for 10 minutes. After that, 90 μL 30 mmol/L Na_2S solution was added into the centrifuge tube and diluted to 1500 μL with deionized water followed by the thoroughly shaking and equilibrated for 15 minutes. 50 μL BSA-CdS QDs was diluted to 1.5mL with deionized water. The fluorescence spectra were recorded from 405 nm to 650 nm with the excitation wavelength of 340 nm. The slit widths of excitation and emission were both 10 nm. The fluorescence quantum yield of BSA-CdS QDs was 23.46%.

2.4 Preparation of DNA/BSA-CdS QDs

The BSA-CdS QDs solution (50 μL), 150 μL 0.1mol/L Tris-HCl buffer (pH 7.0, 150 μL), 12 nmol/L DNA1 and 12 nmol/L DNA2 was added into a 2.0 mL calibrated centrifuge tube. Then, the solution was diluted to 1.5 mL with deionized water followed by the thoroughly shaking and equilibrated for 10 minutes until the solution was fully mixed. The fluorescence spectra were recorded from 405 nm to 650 nm with the excitation wavelength of 340 nm. The slit widths of excitation and emission were both 10 nm.

2.5 The detection of thrombin

The BSA-CdS QDs solution (50 μL), 0.1mol/L Tris-HCl buffer (pH 7.0, 150 μL), 12 nmol/L DNA1 and 12 nmol/L DNA2 was added into a 2.0 mL calibrated centrifuge tube. Different

concentrations of thrombin were successively added into the calibrated centrifuge tube and diluted to the 1.5 mL mark with deionized water followed by the thoroughly shaking and equilibrated for 30 minutes. The fluorescence intensity of the maximum emission peak was used for the quantitative analysis of the target molecules.

2.6 Detection of thrombin in human blood serum

For serum samples detection, drug-free human blood samples were collected from healthy volunteer at the Hospital of Changchun China–Japan Union Hospital. All experiments were performed in compliance with the relevant laws and institutional guidelines, and the writing of informed consent for all samples was obtained from human subjects. All the blood samples were obtained through venipuncture and centrifuged at 10000 rpm for 10 min after standing for 2 h at room temperature. The serum samples were diluted 10 times with Tris-HCl solution before detection.

3. Results and discussion

3.1 The strategy for the detection of thrombin

Single-stranded DNA (ssDNA) bear a negatively charged phosphate groups that will control the synthesis of CdS QDs by binding Cd^{2+}, and these ssDNA also display functionalities such as endocyclic and exocyclic amines that can passivate the surfaces of CdS QDs [25], render them water-soluble stable and retain the ssDNA secondary structure for the recognition of targets [26]. As shown in Fig. 1(A), the black dash line is represented the fluorescence spectra of BSA-CdS QDs. It can be seen that the fluorescence intensity of CdS QDs increased obviously after the addition of DNA1 and DNA2, since they could serve as the co-ligands to passivate the surface deficiency (orange dash line). Upon the addition of thrombin, DNA1 would change its secondary

structure from hairpin structure to fold into a G-quadruplex structure by a Hoogsteen hydrogen bond between four G-bases, then bound with DNA2 via complementary base pairing, resulting in the removal of DNA1 and DNA2 from the surface of CdS QDs and the fluorescence intensity of CdS QDs decreased (red dash line). Moreover, the introducing of thrombin to BSA-CdS QDs had little influence on the fluorescence intensity of CdS QDs (blue dash line). Such phenomena might meaningfully confirm the evidence aforementioned in the representation of Scheme 1.

As shown in Fig. 1(B), both DNA1 and DNA2 could acted as the co-ligands for CdS QDs, but the fluorescence intensity of DNA2/BSA-CdS QDs is stronger than that of DNA1/BSA-CdS QDs. This phenomenon should be attributed to the different number in the base sequence that was not involved in the formation of DNA secondary structure. As we known, double-stranded DNA (dsDNA) could not stabilize the surface of QDs, only the single-stranded DNA (ssDNA) could serve as the ligand for the synthesizing of QDs [27]. After the addition of thrombin to DNA1/BSA-CdS QDs, the fluorescence intensity of CdS QDs would be enhanced. It is because the structural change of DNA1 from hairpin structure to G-quadruplex structure induced by thrombin would increase the number of base sequence that was not involved in the formation of secondary structure. All the results demonstrated that only the base sequence without secondary structure or hybridization could be acted as the ligand of QDs.

3.2 Characterization of the DNA/BSA-CdS QDs

The transmission Electron Microscopy (TEM) image in Fig. 2(A) revealed the shape of BSA-CdS QDs was nearly spherical with the average diameter of 6 nm. As shown in Fig. 2(B), the black dash line and the black solid line represented the absorption and fluorescence emission spectra of BSA-CdS QDs, respectively. There was an increased absorption below 300 nm and a shoulder

177 around 275 nm that was the result of $1S_h$–$1S_e$ excitonic transition characteristic of CdS QDs [28].
178 The fluorescence emission spectra of the BSA-CdS QDs showed a fluorescent peak with
179 maximum emission wavelength of 535 nm which arised from excitonic emission of CdS QDs [29].
180 After the introduction of DNA to the above solution, both the absorption intensity (red dash line)
181 and the fluorescence intensity (red solid line) of BSA-CdS QDs became enhanced.

182 *3.3 Optimization of CdS QDs for thrombin detection*

183 We firstly studied the effect of DNA concentration on the fluorescence intensity of CdS QDs.
184 As shown in Fig. S1, the fluorescence intensity of CdS QDs increased sharply when the total
185 addition level of DNA1 and DNA2 ranged from 0 to 0.024 μmol/L, and then keep almost
186 unchanged with increasing the concentration of DNA, which indicated 0.024 μmol/L DNA was
187 the optical concentration for the passivation of CdS QDs surface deficiency. The concentration
188 proportion of DNA1 to DNA2 was 1:1 (0.012 μmol/L DNA1 and 0.012 μmol/L DNA2) for the
189 sake of the fully removal of DNA1 and DNA2 after the hybridization in the presence of thrombin.
190 In addition this ratio could ensure the wider linear range for the thrombin analysis.

191 As shown in Fig.S2, we systematically investigated the influence of different pH on the
192 fluorescence intensity of DNA/BSA-CdS QDs before and after the addition of thrombin. It could
193 be seen that the fluorescence intensity of both DNA/BSA-CdS QDs (red line) and DNA/BSA-CdS
194 QDs-thrombin system (black line) increased with the increasing of the pH value from 1.0 to 9.0
195 and decreased sharply when the pH value further increased from 9.0 to 14.0 . The fluorescence
196 quenching induced by thrombin reached the maximum value when the pH value was at 7.0.
197 Therefore, pH 7.0 was selected to be the optimum pH value for the thrombin detection.

198 The optimum reaction temperature of DNA and thrombin in the detection process was
199 investigated. As shown in Fig. S3, the quenching effect of thrombin on the fluorescence of
200 DNA/BSA-CdS QDs at 37 □ was the highest, which was consistent with the optimum
201 temperature of the catalytic behavior of thrombin. Therefore, we chose 37 □ as the optimal
202 reaction temperature in the further experiments. The effect of incubation time on the fluorescence
203 quenching of DNA/BSA-CdS QDs by thrombin was studied. The result in Fig. S4 showed that the
204 quenching effect was completed within 20 minutes and kept almost constant until 30 minutes. To
205 ensure the completeness of fluorescence quenching of DNA-CdS QDs, we chose the 30 minutes as
206 the optimum reaction time.

207 *3.4 The detection of thrombin*

208 Under the optimal conditions, the developed DNA/BSA-CdS QDs-based biosensor was
209 applied for the thrombin detection. Fig. 3 showed the evolution of fluorescence spectra of
210 DNA/BSA-CdS QDs with thrombin in different concentrations. It can be observed that the
211 fluorescence intensity of DNA/BSA-CdS QDs decreased gradually with the increasing of
212 thrombin concentration. Fig. 3 Inset showed that there was a good linear relationship between the
213 relative fluorescence intensity ratio F/F_0 (F_0 and F is the fluorescence intensity of DNA/BSA-CdS
214 QDs before and after the addition of thrombin, respectively) and the thrombin concentration in the
215 range from 0.5 nmol/L to 10.5 nmol/L. The regression equation is

216 $$F/F_0 = 0.9947-0.03637[\text{thrombin}], \text{nmol/L}$$

217 The corresponding regression coefficient (R^2) is 0.9954, and the detection limit for thrombin is
218 0.15 nmol/L. Some analytical parameters in other methods for the detection of thrombin were
219 listed in Table 1. [30-34] Compared with those reports about thrombin assay, our present method

offered a comparable detection limit and dynamic range. Although many strategies have been designed for the sensitive detection of thrombin, very few detection were in combination with QDs. In this work, the functionalization of BSA capped CdS QDs with two kinds of specially designed DNA was selective and specific for the detection of thrombin. With the satisfactory results, our present method based on the two ligand stabilized CdS QDs offered an alternative for thrombin analysis.

3.5 Interference study

Under optimized experimental conditions, the selectivity of the developed method was evaluated by using thrombin (5 nM) and other 9 common proteins (50 nM), including lysozyme, pepsin, BSA, trypsin, urase, HRP, ALP, IgG and BHB (Fig. 4). The results showed that only thrombin could cause the significant fluorescence quenching of DNA/BSA-CdS QDs, while the presence of other proteins have a neglect influence on the fluorescence intensity compared to the blank test (in the absence of proteins). This comparison clearly shows the high selectivity of the method. Therefore, this biosensor had the specific ability of sensing thrombin in the presence of other common proteins.

3.6 Detection of thrombin in human serum samples

To assess the application of this biosensor in complex biological systems, the analysis of thrombin in human serum samples were carried out. Serum is what remains from whole blood after coagulation, the chemical composition is similar to plasma but does not contain coagulation protein. We performed detection of thrombin in 10-fold-diluted serum samples under the optimal conditions. The results obtained by standard addition method were listed in Table 2. From Table 2, we can see that the RSD was lower than 3.4% and the average recoveries of thrombin in the real

samples were in the range of 101 – 104%, indicating that the accuracy and precision of the proposed method were satisfactory.

4. Conclusion

In this study, a simple, sensitive, and label-free fluorescence method for the determination of thrombin have been developed based on the DNA conformational changes induced fluorescence quenching of the CdS QDs. The BSA-CdS QDs was synthesized, and two kinds of designed DNA (DNA1 and DNA2) were used as co-ligands to obtain the strong fluorescence DNA/BSA-CdS QDs. With the addition of thrombin, the hairpin structure of DNA1 was changed into G-quadruplex structure then trigger the hybridization between DNA1 and DNA2, resulting in the removal of DNA1 and DNA2 from the surface of CdS QDs, and the fluorescence intensity of CdS QDs was decreased. Thus, thrombin detection was achieved via the fluorescence change of CdS QDs. The present method is simple, fast and exhibited excellent selectivity for thrombin over other proteins and was successfully applied in real sample. The sensitive and selective detection ability for thrombin holds great potential in the diagnosis of diseases associated with coagulation abnormalities and cancers.

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362

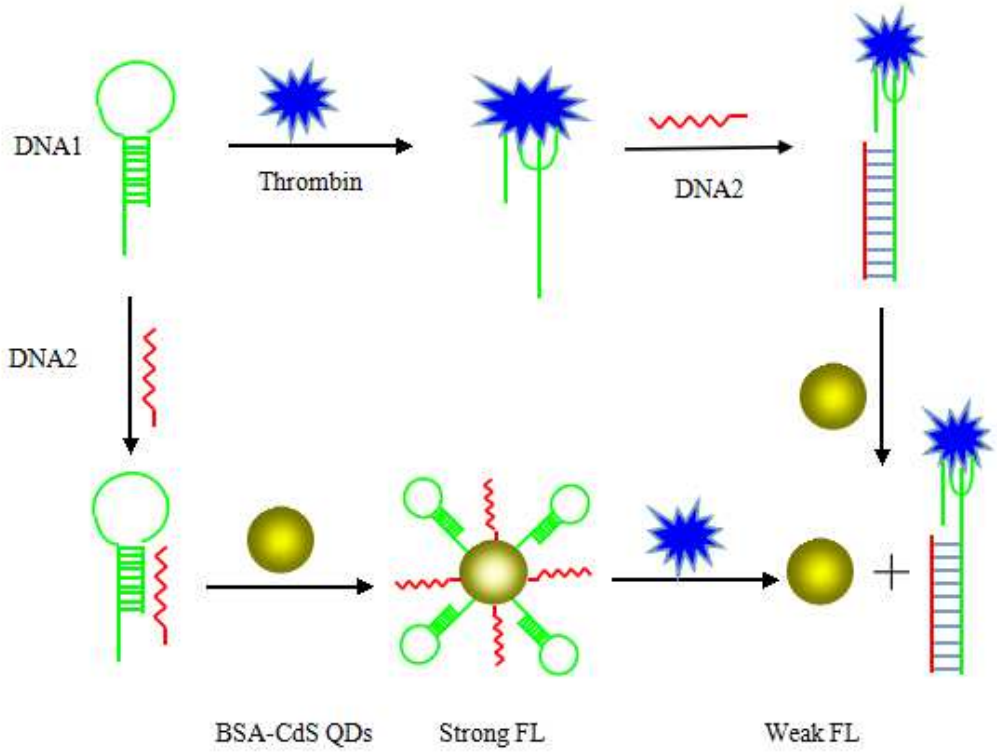
363

364 **Captions:**

365 Scheme 1. Schematic representation the fluorescent detection of thrombin based on BSA-CdS
366 QDs.

367 Fig. 1(A) The fluorescence emission spectra of the BSA-CdS QDs (black line), BSA-CdS
368 QDs-Thrombin (blue line), BSA-CdS QDs-DNA1-DNA2 (orange line) and BSA-CdS

369 QDs-DNA1-DNA2-Thrombin (red line). (B) The fluorescence intensity of BSA-CdS QDs,
370 DNA1/BSA-CdS QDs, DNA1/BSA-CdS QDs-Thrombin and DNA2/BSA-CdS QDs.
371 Fig. 2(A) TEM image of BSA-CdS QDs. (B)The UV-Vis absorption (Black dash line) and
372 fluorescence emission spectra (Black solid line) of the BSA-CdS QDs. The UV-Vis absorption
373 (Red dash line) and fluorescence emission spectra (Red solid line) of the DNA/BSA-CdS QDs.
374 Fig. 3 The fluorescence spectra of DNA/BSA-CdS QDs (0.05 μmol/L) in the presence of different
375 concentrations of thrombin (0, 0.5, 1.5, 3, 4.5, 6, 7.5, 9, 10.5, 12, 15, 18 nmol/L). Inset shows the
376 linear relationship between F/F_0 and the concentration of thrombin (from 0-10.5 nmol/L). F_0 is the
377 fluorescence intensity of DNA/BSA-CdS QDs (0.05 μmol/L) and F is the fluorescence intensity of
378 DNA/BSA-CdS QDs (0.05 μmol/L) after the addition of different concentrations of thrombin.
379 Reaction conditions: 10 mmol/L Tris–HCl buffer solution (pH 7.0), incubated for 30 minutes.
380 Fig. 4 The fluorescence intensity of DNA/BSA-CdS QDs (0.05 μmol/L) in the presence of
381 7.5nmol/L thrombin and 75 nmol/L other 9 common proteins, including lysozyme, pepsin, bovine
382 serum abumin (BSA), trypsin, urase, horseradish peroxidase (HRP), alkaline phosphatase (ALP),
383 IgG and bovine hemoglobin (BHB).
384 Table 1 The comparison of different method for the detection of thrombin.
385 Table 2 The detection of thrombin in human serum samples.



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387 Scheme 1

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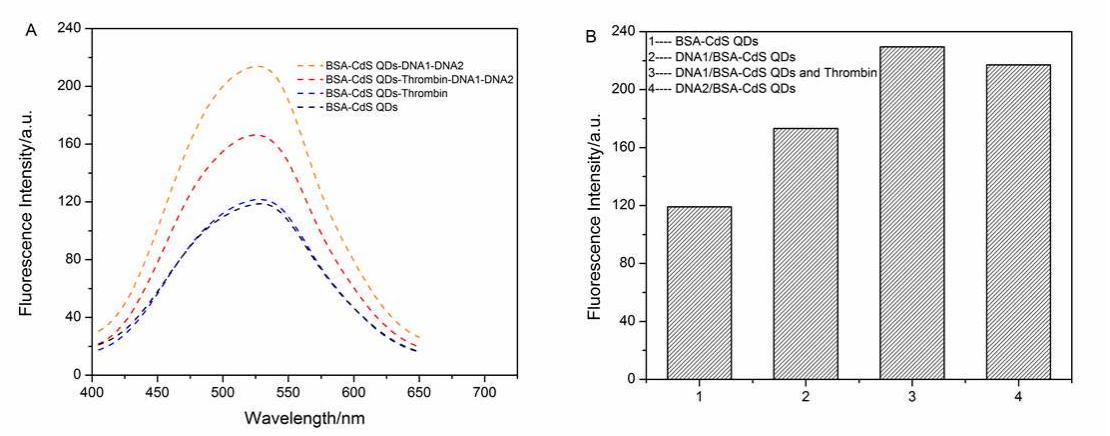

Fig. 1

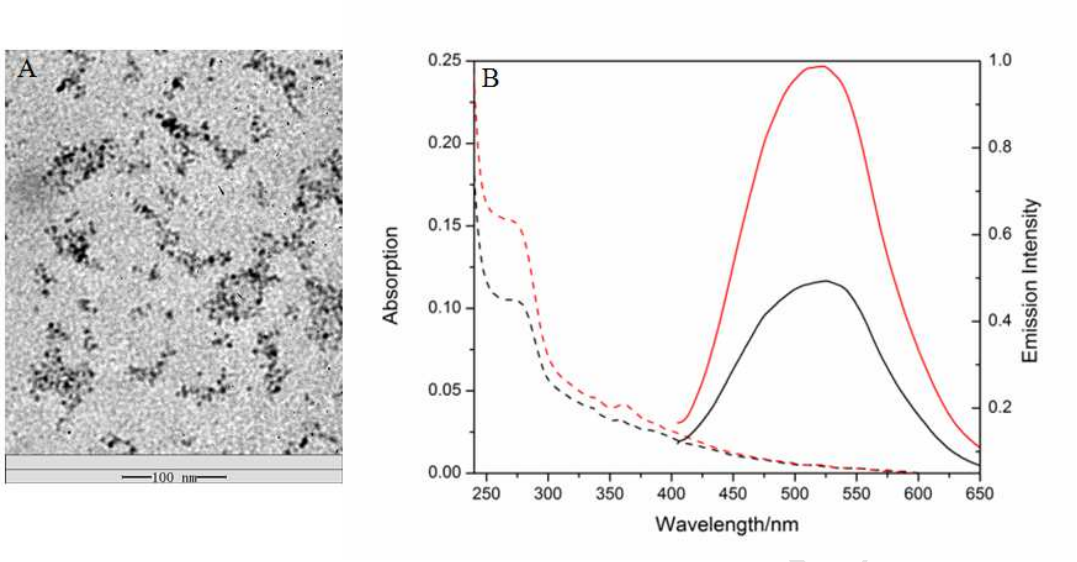

Fig. 2

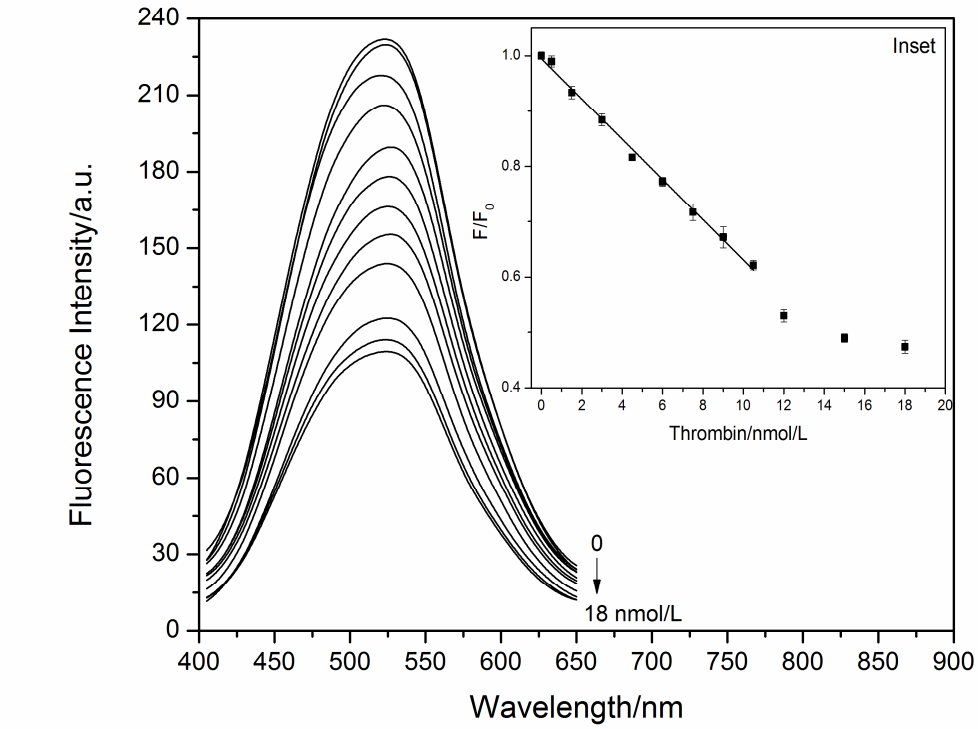

Fig. 3

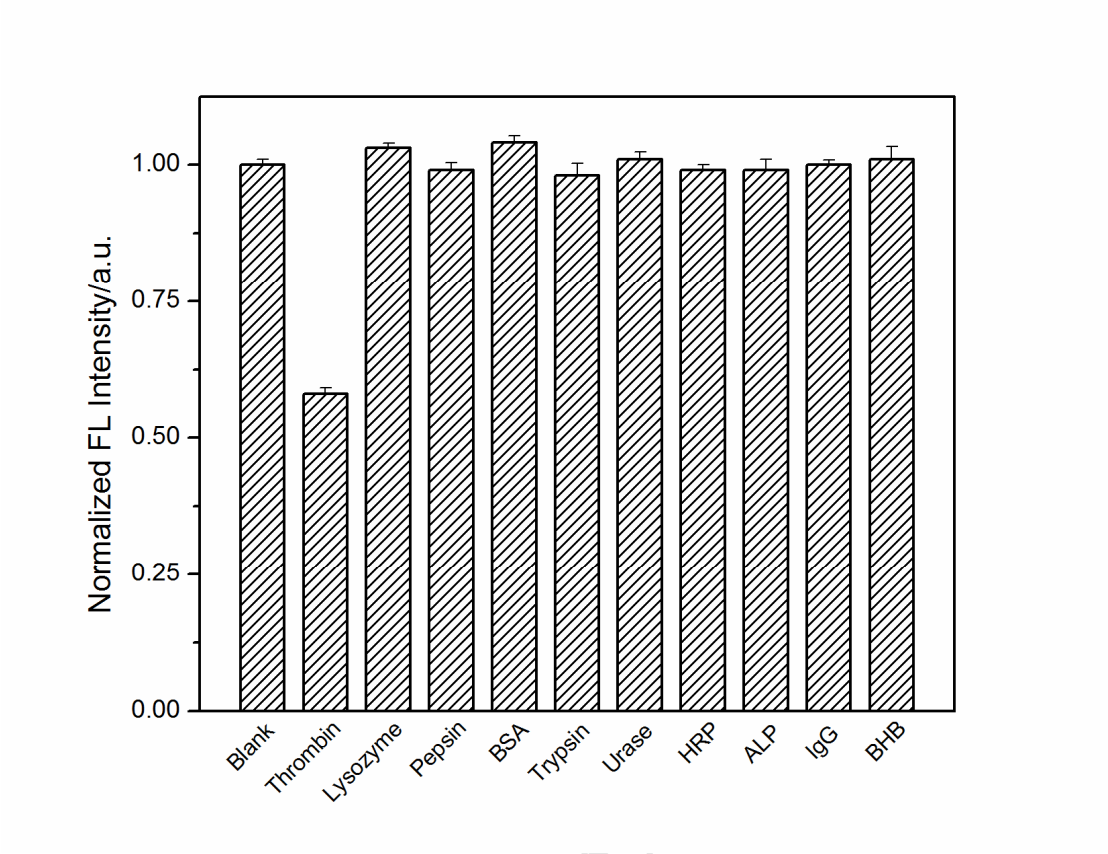

Fig. 4

448 Table 1

Detection method	LOD (nM)	Linear range(nM)	Ref
Dual polarization interferometry	0.1	2.5-100	30
Localized surface plasmon resonance	0.2	0-10	31
Colorimetric Detection	0.05	0-2	32
Electrochemiluminescence detection	0.12	0.3-30	33
Liquid chromatography/tandem mass spectrometry	0.30	0.5-50	34
Fluorescence detection	0.15	0-10.5	This work

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461 Table 2

Serum samples	Added (nmol/L)	Founded (nmol/L)	Recovery (%)	RSD (%, n=3)
1	1.5	1.56±0.08	1.04	3.4
2	3.0	3.09±0.05	1.03	3.0
3	4.5	4.54±0.06	1.01	2.3

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Highlights：

- A novel strategy for the detection of thrombin was established based on BSA-CdS QDs.
- DNA could serve as as the co-ligands to stabilize CdS QDs and enhance the fluorescence intensity.
- Thrombin could change the structure of DNA1 and quench the fluorescence of CdS QDs.
- Thrombin in real sample was detected with satisfactory results.

Supporting information

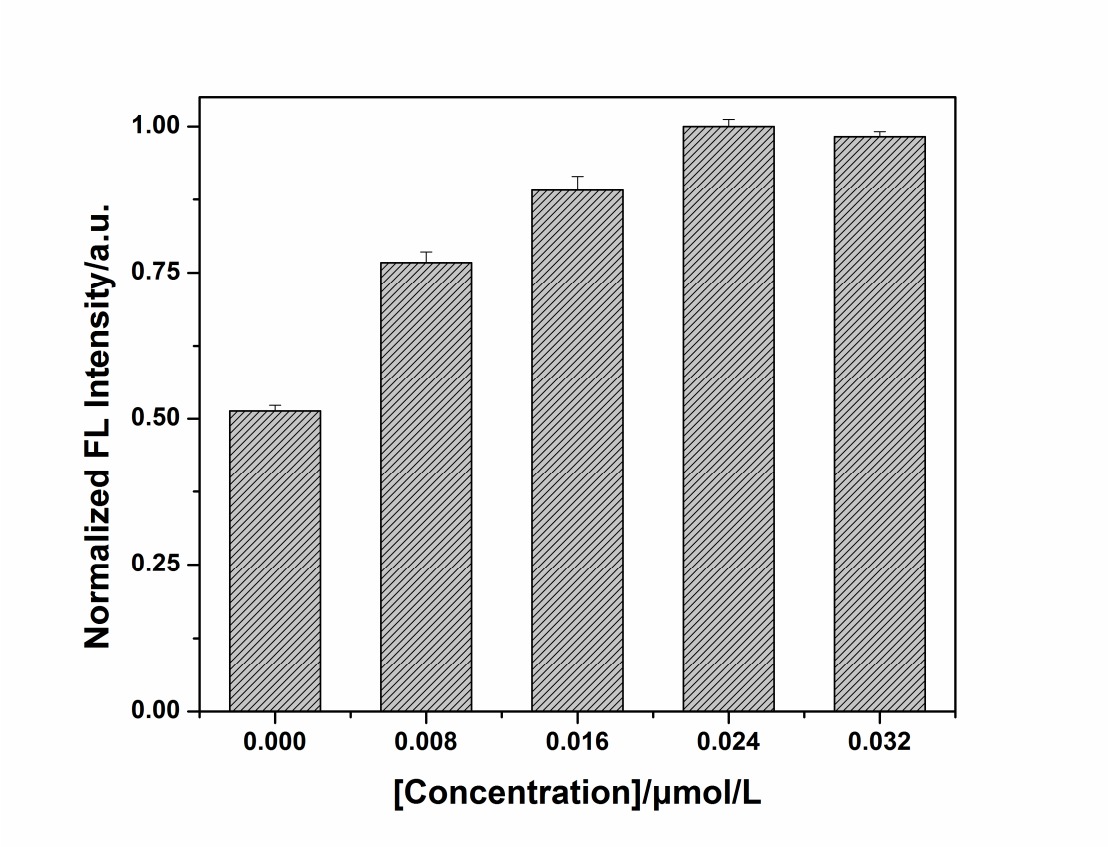

Fig. S1 The effect of DNA concentration (0, 0.008 , 0.016, 0.024, 0.032 μmol/L) on the fluorescence intensity of CdS QDs.

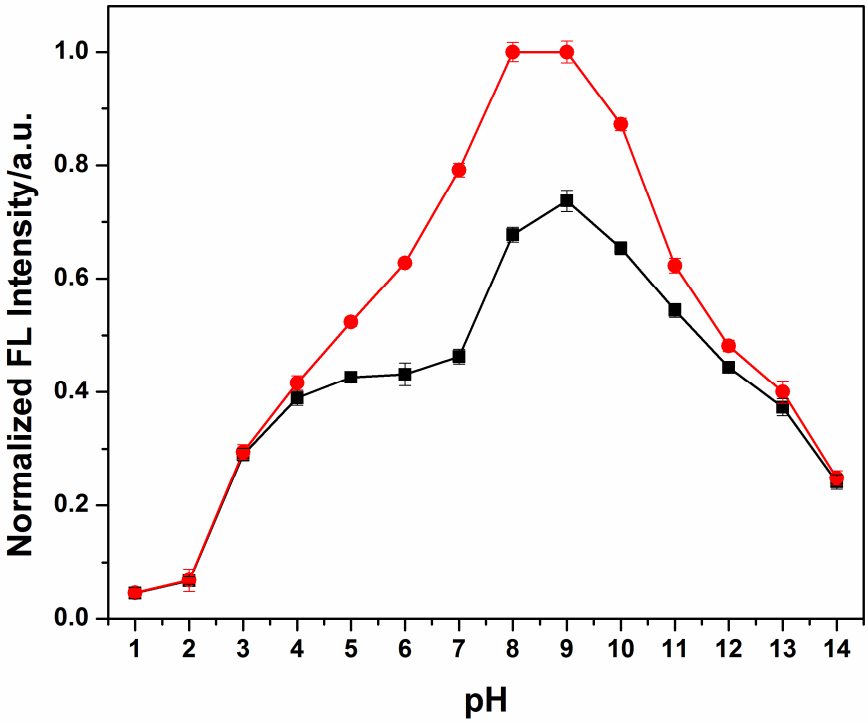

Fig. S2 FL intensity of DNA/BSA-CdS QDs (0.05 μmol/L) before (red line) and after (black line) addition of 9 nmol/L thrombin in different pH environments (pH 1-14).

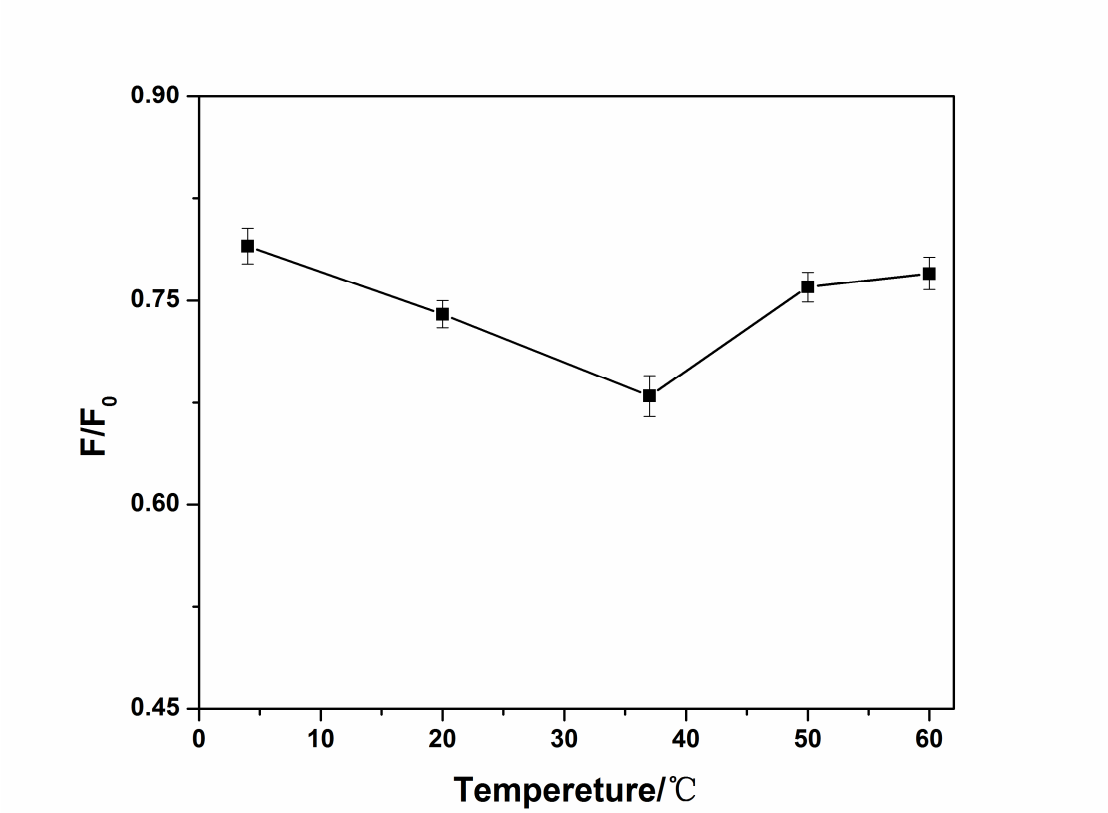

Fig. S3 The effect of reaction temperature on the fluorescence quenching of DNA/BSA-CdS QDs (0.05 μmol/L) by thrombin. F_0 and F is the fluorescence intensity of DNA/BSA-CdS QDs before and after addition of 9 nmol/L thrombin.

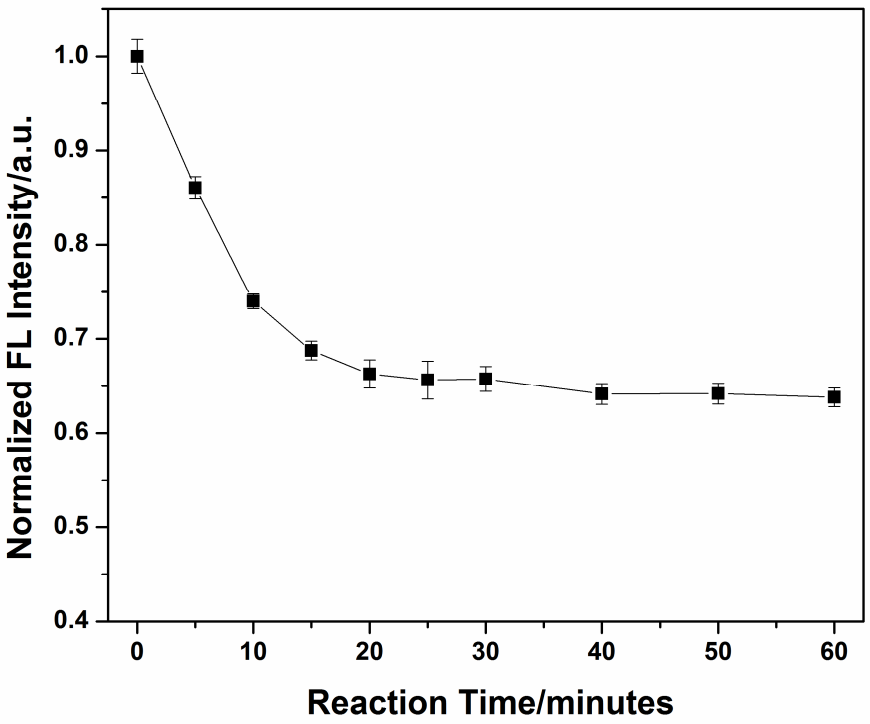

Fig. S4 The effect of incubation time (0, 5, 10, 15, 20, 25, 30, 40, 50, 60 minutes) on the fluorescence quenching of DNA/BSA-CdS QDs (0.05 μmol/L) by thrombin.