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Label-free fluorescence assay for thrombin based on unmodified quantum dots

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ABSTRACT

Rapid and sensitive assay of thrombin and its inhibition in a high-throughput manner is of great significance in the diagnostic and pharmaceutical fields. In this article, we developed a novel biosensor for the detection of thrombin and its inhibition based on the aggregation behavior of the unmodified CdTe QDs. A cationic substrate peptide of thrombin (GGLVPRGSCC-NH_2, S-peptide) can attach to the surface of CdTe QDs, partly balance their surface negative charge, and induce the aggregation of QDs, which results in the fluorescence quenching of QDs. After hydrolysis of S-peptide by thrombin, two kinds of shorter peptides (P_1-peptide, GGLVPR, and P_2-peptide, GSCC) are produced. The uncharged P_2-peptide rather than the cationic P_1-peptide would bind to QDs. Hence, the CdTe QDs were kept stable in the solution with the fluorescence being maintained. The change of fluorescence intensity would sensitively respond to thrombin activity and its inhibition. Fluorescence spectroscopy, transmission electron microscopy and dynamic light scattering were performed to discuss the quenching mechanism. Under optimized conditions, this method enables measurement of thrombin in the range of 10–100 μU/mL with the detection limit of 1.5 μU/mL. Not only in buffer, but also in blood serum, such sensor exhibited extraordinarily high sensitivity and excellent specificity. In addition, the typical inhibitor of thrombin, hirudin, was also successfully assayed by this method (from 2 μU/mL to 30 μU/mL with the LOD of 0.21 μU/mL). Furthermore, the present approach could also be potentially extended to other proteases and their inhibitors detection with unmodified CdTe QDs.

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1. Introduction

Thrombin is a serine protease which plays a pivotal role in hemostasis and blood clotting through selectively cleaving Arg–Gly bonds in fibrinogen to form fibrin and activating platelet (Davie et al., 1991). Over-expression or activity imbalance of thrombin is associated with many diseases such as thrombosis, hemophilia, atherosclerosis, and inflammation (Maragoudakis et al., 2000). Therefore, accurate detection of thrombin activity is valuable for disease diagnosis and drug discovery. Compared with several thrombin assays based on electrochemical method (Du et al., 2010), spectrophotometry (Bing et al., 2010; Chen et al., 2010), chemiluminescence (Huang and Zhu, 2009), or surface enhanced Raman scattering spectroscopy (Bizzarri and Cannistraro, 2009), fluorescence assays are very attractive due to their short detection time, high sensitivity, and simple instrumentation. In these existing fluorescence-based thrombin assays, most of them relied on the use of fluorescent labeling techniques or thrombin specific recognition biomolecules (e.g., antibodies or aptamers; Wang et al., 2009; Zheng et al., 2010). Such assays are effective but require sophisticated labeling procedures or expensive recognition proteins; furthermore it is not thrombin activity but thrombin molecule that is detected by aptamer-based methods. Therefore, it is still a challenge to develop label-free, simple and cost-effective fluorescence assays for thrombin activity.

Quantum dots (QDs) are nanometer-size luminescent semiconductor crystals that exhibit unique optical and electronic properties (Medintz et al., 2005). Because of the outstanding optics properties (bright, narrow, and size-tunable photoluminescence) and easy modification, QDs, as novel fluorescent probes, have attracted substantial interest in biological imaging and bioanalysis (Jiang et al., 2009), including biocatalytic processes sensing. A series of functional-QDs have been designed as donors/receptors of energy or electrons in fluorescence resonance energy transfer (FRET) or electron transfer (ET) processes to achieve sensitive detection of enzyme catalyzed events, such as QDs–peptide conjugates labeled with organic fluorescent dyes/quencher to detect proteases activity by FRET (Medintz et al., 2006; Shi et al., 2006), QDs–aptamer beacon for the detection of thrombin based on ET (Wu et al., 2010), and gold nanoparticles–peptide–QDs complex to probe for collagenase sensing

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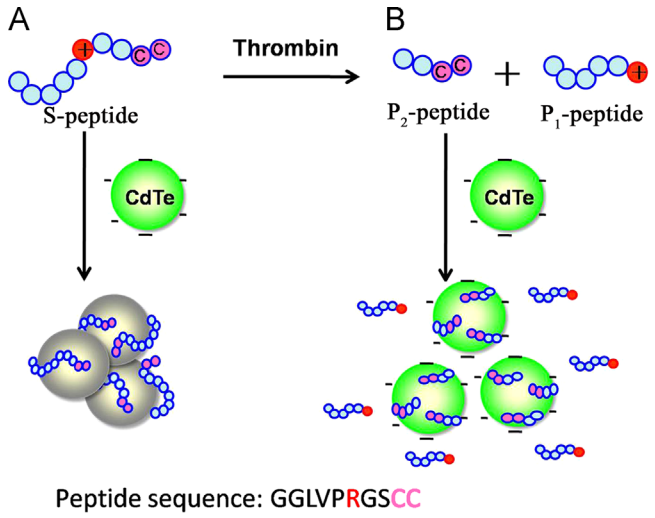
(Medintz et al., 2006). Very recently, one kind of QD as simultaneous donors and acceptors in a time-gated FRET, and an assembly of a concentric FRET relay on a QDs scaffold, has been reported for the multiplexed detection of protease activity (Mulvihill et al., 2010; Tang et al., 2006). However, most of the above mentioned QD-based methods, especially the methods based on FRET, require complicated and expensive labeled peptide or antibody and a modification process of QDs. Hence, we expect to develop a label-free fluorescent sensor for protease activity detection with unmodified QDs.

Over a wound site, fibrin, formed from fibrinogen, is polymerized to form a "mesh" for a hemostatic plug or clot formation (in conjunction with platelets; Hantgan et al., 1985). The triggering event for the transformation of fibrinogen to fibrin is the thrombin-catalyzed release of two small acidic peptides from the fibrinogen molecule. The resulting surface charge changed molecules spontaneously polymerize into a fibrin network (Maragoudakis et al., 2000). Unmodified QDs, like fibrinogen, are also very sensitive to charge change on their surface, which in turn can easily affect the stability of QDs suspension, resulting in the aggregation (Mulvihill et al., 2010) and the significant fluorescence change of QDs (Tang et al., 2006). Taking advantage of these properties, we develop a new bionic assay for thrombin activity detection based on peptides modulated CdTe QDs aggregation, where the surface charge of CdTe QDs is regulated by the hydrolysis of a thrombin substrate peptide (Scheme 1). Compared with the existing thrombin activity assays, the present assay does not require peptide labeling and the use of expensive antibodies or binding proteins. Additionally, as a mix-and-readout detection system, common steps in above-mentioned bioanalysis, such as modification and purification of QDs composites probe and several wash steps, are avoided in this method. Herein, we present a simple and novel method for profiling thrombin activity using unmodified CdTe QDs as a fluorescent probe. This method provides a new platform for the detection of thrombin activity.



Scheme 1. Concept of the fluorescence thrombin activity assay based on peptides modulated QDs aggregation. (A) S-peptide (a cationic substrate peptide of thrombin) can attach to the surface of CdTe QDs through thiol group-mediated chemisorptions, partly balance their surface negative charge, and induce the aggregation of QDs, which results in the fluorescence quenching of QDs. (B) After hydrolysis of S-peptide by thrombin, two kinds of shorter peptides (P_1-peptide and P_2-peptide,) are produced. The uncharged P_2-peptide rather than the cationic P_1-peptide would bind to CdTe QDs, and the QDs are stable in the solution with the fluorescence being maintained. Hence, the fluorescence of QDs responds sensitively to the activity of thrombin.

2. Experimental

2.1. Materials and measurements

Thrombin was purchased from Sigma (USA). Substrate peptide (S-peptide, GGLVPRGSCC-NH_2) for thrombin was purchased from GL Biochem Ltd. (Shanghai, China). Water-soluble CdTe QDs (carboxyl coated) were purchased from Zhong DS (Shenzhen, China). Tris was purchased from Bio Basic Inc. (Ontario, Canada). Serum sample was obtained from Hunan University Hospital. All solutions were prepared using ultrapure water (18.3 MΩ cm) from a Millipore Milli-Q system.

Fluorescence measurements were performed on Synergy Mx multimode microplate reader (BioTek, USA). All samples were illuminated at an excitation wavelength of 365 nm, and the fluorescence emission was scanned from 400 to 700 nm at room temperature. The fluorescence measurements were performed three times for each sample ($n=3$). Transmission electron microscopy (TEM) measurements were conducted on a JEOL electron microscope (JEM-2100F, JEOL, Japan). Zeta potential and dynamic light scattering (DLS) measurements were used to monitor the surface potential and the hydrated diameter of the QDs in different solutions, respectively, with a Nano Zetasizer ZS90 (Malvern, U.K.).

2.2. Peptide-induced QDs aggregation

Product peptides (P-peptides) were produced after hydrolysis of S-peptide by 1 mU/mL thrombin in 10 mM Tris–HCl buffer (pH 7.5) at 37 °C for 30 min. Then S-peptide or P-peptides (3.5 μM) were mixed with 20 μL of CdTe QDs suspension, and the fluorescence intensity was measured immediately. All concentrations presented in the manuscript were the final concentrations. The microwell plate loaded with samples was placed into a Synergy Mx multimode microplate reader and was shaked for 1 min with medium intensity; then the fluorescence emission spectra from 400 to 700 nm were measured at room temperature (excitation at 365 nm).

2.3. Detection of the activity and inhibition of thrombin

The thrombin-catalyzed reaction solutions were composed of thrombin (0–500 μU/mL) and S-peptide in 10 mM Tris–HCl buffer (pH 7.5). After incubation at 37 °C, the resulting solution containing P-peptides was obtained, and the QDs dispersion was added. S-peptide, instead of P-peptide solution, was added as a control. To optimize the detection, different concentrations of QDs and S-peptide, and different enzymatic hydrolysis times, were tested. The fluorescence intensity was measured as mentioned above. For hirudin (the inhibitor of thrombin) assay, hirudin with different concentrations was mixed with thrombin (100 μU/mL) and S-peptide (3.5 mM) in 10 mM Tris–HCl buffer (pH 7.5). After incubation at 37 °C for 30 min, the resulting solution was mixed with the QDs dispersion, and then the fluorescence was measured in the same way as that for the detection of thrombin activity.

2.4. Determination of thrombin activity in real serum sample

Serum sample from a healthy volunteer was centrifuged at 12,000 rpm for 15 min, and then the supernate filtered through a paper filter was collected as the prepared serum. Thrombin with different concentrations was spiked in the prepared serum separately. 2 μL of the resulting sample containing different concentrations of thrombin was mixed with S-peptide solution and incubated at 37 °C for 30 min. 35 μL of the resulting solution (3.5 μM final peptide concentration) was added into the QDs dispersion (20 μL, 4 μg/mL final concentration). S-peptide, instead

of P-peptide, was used as a negative control. The fluorescence measurement protocol was set as the above mentioned procedure.

3. Results and discussion

3.1. Effect of S-peptide or P-peptides on the unmodified CdTe QDs

Thrombin, a serine protease with multiple functions, is the key enzyme participating in the whole process of blood coagulation. It was reported that the thrombin-catalyzed hydrolysis of small acidic peptides from fibrinogen molecule makes the resulting fibrin spontaneously to aggregate, followed by insoluble gel (clot) formation in conjunction with platelets (Xu et al., 2010). Therefore, we speculate whether thrombin can modulate the aggregation of unmodified QDs, since the stability of unmodified QDs can be easily affected by additional charges either on their surface or in the surrounding environment (Mulvihill et al., 2010). Here, a substrate peptide of thrombin (S-peptide, GGLVPRGSCC), containing a positive charge (+), was designed to interact with carboxyl coated CdTe QDs. The –SH groups in cysteine residues at the carboxy-terminal end of S-peptide make it convenient for the peptide to attach to the surface of CdTe QDs through thiol group-mediated chemisorptions. When attaching to the particles, the positively charged S-peptide can partly balance the negative charge covered on the surface of CdTe QDs, and induce the aggregation of the latter, which will result in the fluorescence quenching of CdTe QDs. After hydrolysis of S-peptide by thrombin, two kinds of shorter peptides (P-peptides), GGLVPR (P_1-peptide) and GSCC (P_2-peptide), are produced. Although P_1-peptide has a positive charge, two cysteine residues enable the uncharged P_2-peptide much easier to anchor the QDs. Hence, the net charge of the CdTe QDs is not changed, and the particles are stable in the solution and maintain their fluorescence. Therefore, the thrombin hydrolysis process can be monitored by a fluorescence spectrometer. Such a process is described in Scheme 1.

The proof-of-concept experiment was carried out to investigate the effect of S-peptide or P-peptides on the fluorescence of unmodified CdTe QDs. Fig. 1 shows fluorescence emission spectra of QDs dispersions and that in the presence of S-peptide or P-peptides. The addition of S-peptide induces a significant decrease of QDs fluorescence as well as a red-shift in emission from 535 to 560 nm. Correspondingly, as shown in the inset of Fig. 1, the bright green fluorescence of QDs turns to be very weak when QDs are mixed with S-peptide under ultraviolet irradiation (365 nm).

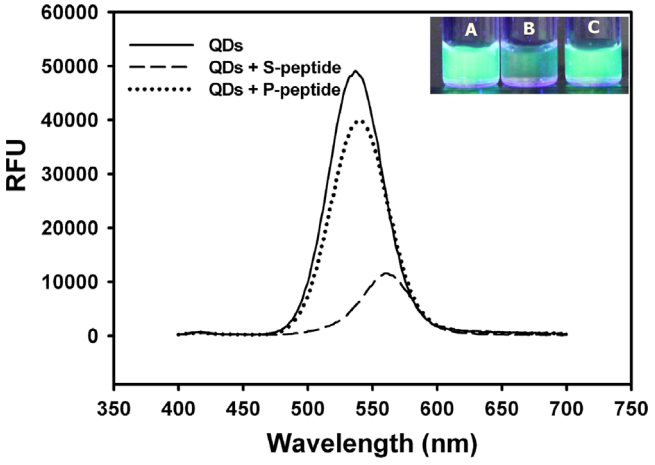


Fig. 1. Fluorescence emission spectra of CdTe QDs and the mixture of CdTe QDs with S-peptide or P-peptides in 10 mM Tris–HCl buffer, pH 7.5. Inset: fluorescence photograph of the control CdTe QDs (A) and QDs mixed with S-peptide (B) or P-peptide (C).

However, a relatively high fluorescence intensity of QDs is retained (above 80%) after the addition of P-peptides. To further explore the peptide-induced fluorescence quenching of QDs, several experiments were performed. First of all, the Zeta potential measurement was used to monitor the surface charge of the QDs (Fig. S1). It was found that Zeta potential of the initial negatively charged CdTe QDs was about −30.0 mV. Addition of the positively charged S-peptide caused a significant change of Zeta potential from −30.0 to −10.8 mV. Nevertheless, a negligible change in Zeta potential was found in the case of mixing CdTe QDs with P-peptides (−27.1 mV). Since the higher the Zeta potential (positive or negative), the better the particle stability, such low Zeta potential of CdTe QDs/S-peptide mixture indicates that the existence of S-peptide would induce QDs aggregation. Secondly, the particle size of QDs before and after mixing with peptides was investigated by TEM. Fig. 2(A) displays that the original QDs disperse very well, with an average diameter of 4.3 ± 0.7 nm. In the presence of S-peptide, the aggregation of QDs was observed and the diameter of aggregated particles was more than 80 nm (Fig. 2(B)). Comparatively, few CdTe QDs were aggregated upon addition of P-peptides (Fig. 2(C), the average diameter of the aggregate grew up to a size on the order of 8.2 ± 1.0 nm), and most CdTe QDs were well dispersed and maintained a similar size as that of initial CdTe QDs. Thirdly, the change of CdTe QDs dispersion in the presence of S-peptide or P-peptides was also detected through DLS. Fig. S2 shows that the hydrodynamic diameter of QDs/S-peptide sample is about 85 nm. However, the hydrodynamic diameter of both CdTe QDs and QDs/P-peptides sample was too small to be detected under the Nano Zetasizer. Such results of DLS measurements are well in accord with TEM image. These above-mentioned results prove that the binding of cationic S-peptide on QDs surface partly neutralizes the surface negative charge of QDs, reduces interparticle electrostatic repulsion, and further induces the formation of QDs aggregates. As reported previously (Koole et al., 2006), the aggregation of QDs could cause the red-shift and drastic quenching of the QDs fluorescence by electronic energy transfer between neighboring dots in the QDs aggregate. Therefore, on the basis of all these facts, unmodified CdTe QDs have the potential to be a fluorescent probe for selective detection of thrombin activity.

3.2. Detection of the activity and inhibition of thrombin

To improve the sensing performance, the concentrations of QDs and S-peptide and enzyme catalysis time were optimized. With a fixed concentration of S-peptide or P-peptides (4 μM), 4 μg/mL QDs can best distinguish S-peptide and P-peptides (Fig. S3). Fig. S4 shows that the fluorescence intensity of QDs drastically decreases with the increase of S-peptide concentration from 0 to 3.5 μM, while it increases when the S-peptide concentration is higher than 3.5 μM. The increase of fluorescence signal may be attributed to the increased stability of CdTe QDs when net charge on the surface of QDs becomes positive with more S-peptide. Therefore, 3.5 μM is chosen as the optimal S-peptide concentration among the tested concentrations to quench the fluorescence of CdTe QDs. Thrombin hydrolysis time was also optimized, and the result is shown in Fig. S5. The fluorescence intensity increased slowly when the enzyme reaction time was longer than 30 min (thrombin, 1 mU/mL). Considering improving the reaction efficiency and reducing the time, 30 min was employed in the subsequent assay of enzyme activity.

Detection of thrombin activity was further studied under the preoptimized conditions (4 μg/mL CdTe QDs, 3.5 μM S-peptide) with different amounts of thrombin (0–500 μU/mL). The fluorescence intensity was measured after mixing the enzyme-treated solution with CdTe QDs suspension (Fig. 3A). The fluorescence intensity increased with the increasing concentration of thrombin,

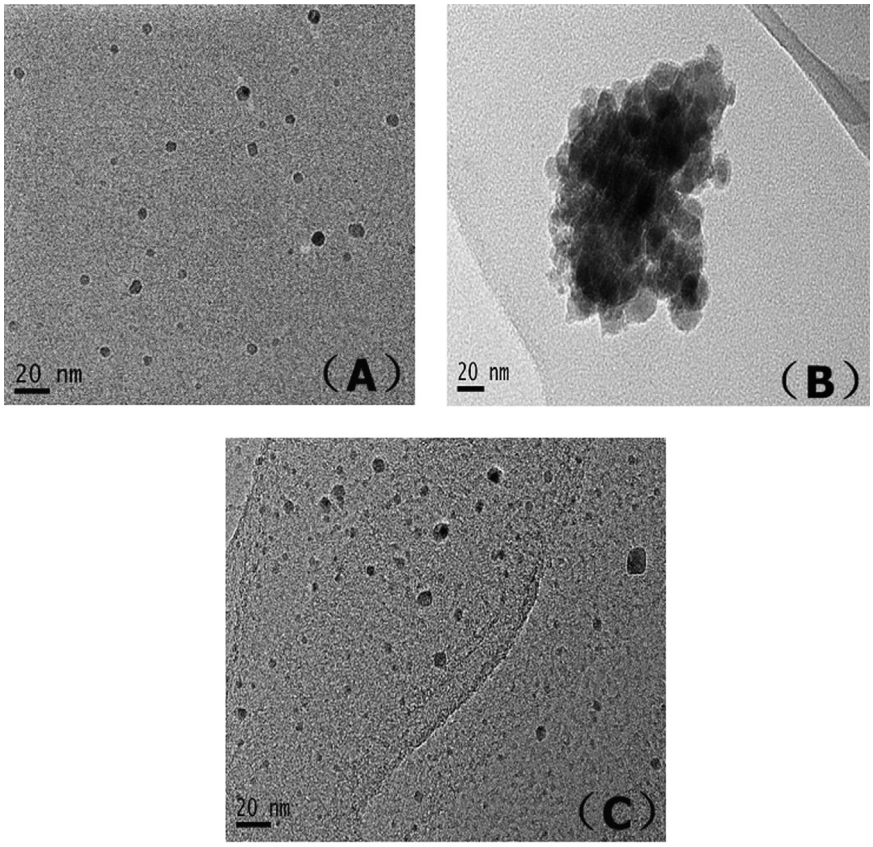


Fig. 2. TEM images of the control CdTe QDs (A) and the CdTe QDs mixed with S-peptide (B), or P-peptide (C).

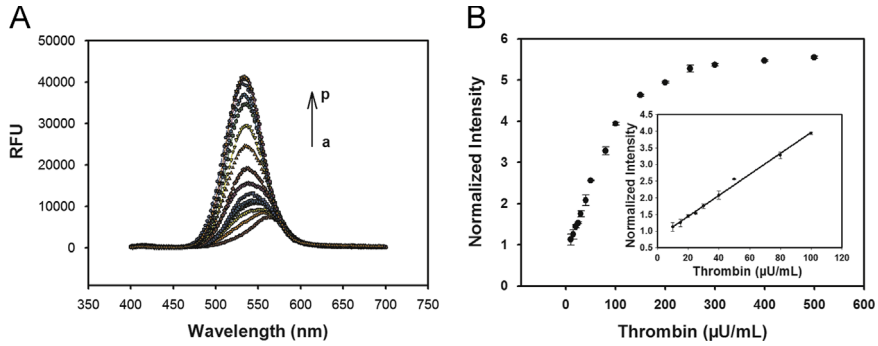


Fig. 3. (A) Fluorescence emission spectra of CdTe QDs (4 μg/mL) response to the thrombin concentration: (a) 0; (b) 10; (c) 15; (d) 20; (e) 25; (f) 30; (g) 40; (h) 50; (i) 80; (j) 100; (k) 150; (l) 200; (m) 250; (n) 300; (o) 400; and (p) 500 μU/mL. (B) Plot of the normalized fluorescence intensity of CdTe QDs versus the concentration of thrombin. The normalized fluorescence intensity of CdTe QDs is defined as F/F_0, where F and F_0 are the fluorescence intensities in the presence of P-peptide and S-peptide, respectively. Inset: linear plot of the normalized intensity versus the concentration of thrombin in the range of 10–100 μU/mL.

indicating that a higher degree of hydrolysis induced a smaller quantity of aggregation. As shown in Fig. 3(B), the normalized fluorescence intensity (F/F_0, where F and F_0 are the QDs fluorescence intensity in the presence of P-peptides and S-peptide, respectively) is linear with the concentration of thrombin from 10 μU/mL to 100 μU/mL ($R^2=0.9948$; Fig. 3B, inset) with the LOD of 1.5 μU/mL (equal to 0.20 nM; $S/N=3$). The LOD is above 13 fold lower than that of electrochemiluminescence biosensor (2.72 nM) and five fold lower than that of fluorescence method (1 nM; Bing et al., 2010; Huang and Zhu, 2009). The relative standard deviation (RSD) of 1.1% was obtained from three replicate detections of 50 μU/mL thrombin, which revealed a good reproducibility of our method. Comparatively, when CdTe QDs were mixed with the other protease treated solutions, the fluorescence intensity gave no significant recovery (Fig. 4A). These results confirmed that the fluorescence increasing was caused by specific digestion of S-peptide by thrombin. Moreover, the coexistence of other proteases had a negligible effect on the fluorescence response of QDs to thrombin, indicating that the detection of thrombin was not disturbed by other proteases (Fig. 4B). Besides low cost, low detection limit and good precision, our method uses unmodified QDs as a fluorescent probe and a mix-and-readout detection system, so that the complex process of QDs modification and purification, and several wash steps, is avoided in this method. Therefore, simplicity of operation and stability of the sensor are better than those of other fluorescence-based thrombin assays.

Detection and screening of protease inhibitors play significant roles in new drug research and development. Here, hirudin, one of the most powerful thrombin inhibitors, is used to test whether our method has the potential to be extended to thrombin inhibitor detection. Hirudin is a natural peptide in salivary glands of medicinal leeches, which has a blood anticoagulant property

through inhibition of thrombin activity (Nowak and Schrör, 2007). Hence, coexistence of hirudin can protect S-peptide from being digested by thrombin. It was found that with the fixed concentrations of thrombin (100 μU/mL) and S-peptide (3.5 μM), the fluorescence intensity remarkably decreased upon the increase of hirudin concentration (Fig. 5A), indicating that hirudin can intensively inhibit the activity of thrombin. The inhibition percentage of thrombin was calculated from the following equation: inhibition percentage $=\Delta F_i/\Delta F_0$, where ΔF_i is the fluorescence change of inhibition reaction system (QDs, hirudin, S-peptide, and 100 μU/mL thrombin) and ΔF_0 is the change of the fluorescence intensity in the absence of hirudin. When the inhibition percentage of thrombin versus the hirudin concentration is plotted (Fig. 5B), it is clearly seen that inhibition percentage rises with increasing concentration of hirudin and reaches plateau over 30 μU/mL hirudin. Inhibition percentage is linear with the concentration of hirudin from 2 μU/mL to 30 μU/mL ($R=0.9857$; Fig. 5B, inset) with the LOD of 1.5 μU/mL ($S/N=3$). Moreover, IC_{50} (half maximal inhibitory concentration) was 13 μU/mL (equal to 1.3 ng/mL), which is similar to that reported previously (3 ng/mL; Gast et al., 1994; Grutter et al., 1990; Rydel et al., 1991). Such data, in return, show that the QD-based method is viable to detect thrombin inhibitor and apply to inhibitor drug screening.

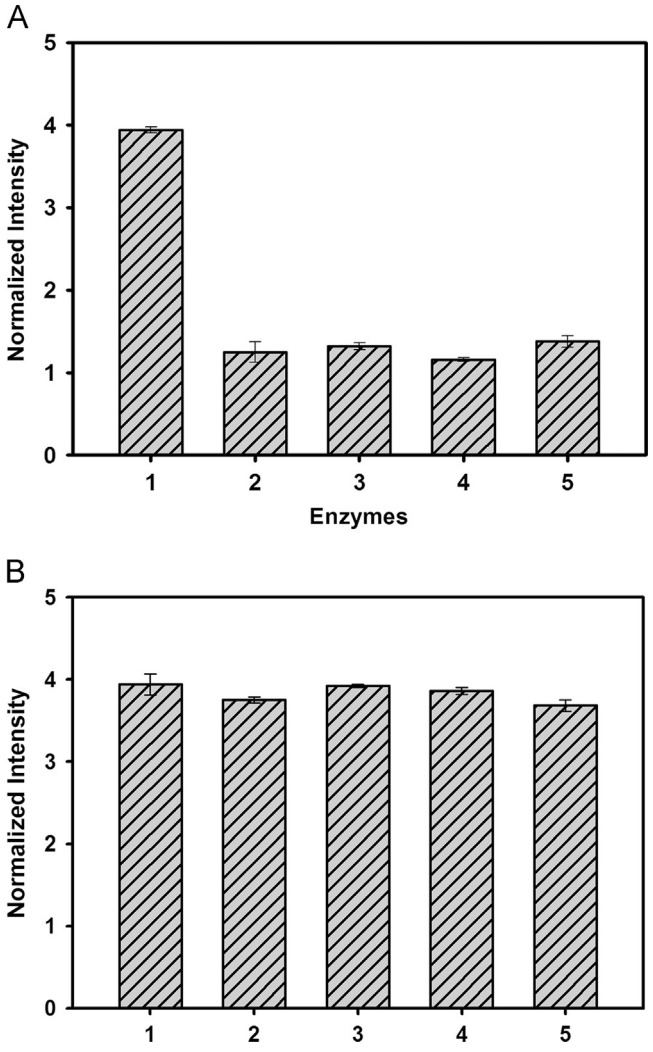


Fig. 4. (A) Normalized fluorescence intensity of CdTe QDs/S-peptide mixture, where S-peptide was treated with different proteases before mixing: (1) thrombin; (2) trypsin; (3) chymotrypsin; (4) pepsin; and (5) papain. (B) Normalized fluorescence intensity of CdTe QDs/S-peptide mixture, where S-peptide was hydrolyzed by thrombin and other proteases: (1) thrombin; (2) thrombin+trypsin; (3) thrombin+chymotrypsin; (4) thrombin+pepsin; and (5) thrombin+papain. Thrombin: 100 μU/mL (equal to 0.5 μg/mL). Trypsin, chymotrypsin, pepsin, and papain: 10 μg/mL.

3.3. Determination of thrombin in serum sample

Sensitive and rapid determination of thrombin in real sample is of great significance for disease diagnosis and drug discovery. Considering that thrombin is a key enzyme in blood clotting process, the thrombin activity in real serum samples was detected with our method. Standard addition method was performed since there is no thrombin but prothrombin in serum. Table 1 shows that this method presents satisfactory accuracy (recovery between 94.3% and 104.7%) and precision (RSD between 2.3% and 6.4%) in serum sample analysis. Hence, our method based on the charge-induced aggregation behavior of the unmodified CdTe QDs has the potential to be applied for thrombin detection in complex matrix, even in real biological samples, and further for disease diagnosis and drug discovery (Wu et al., 2007).

Table 1
Measurement of thrombin activity in serum by this method (repeated five times).

Thrombin added (μU/mL)	Thrombin found (μU/mL)	Recovery (%)	RSD (%)
10.0	9.7	97.0	6.4
30.0	28.3	94.3	3.4
60.0	62.8	104.7	3.0
90.0	87.9	97.7	2.3

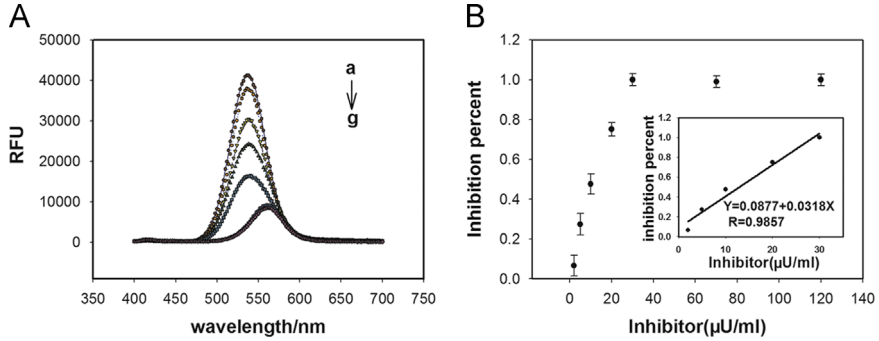


Fig. 5. (A) Fluorescence emission spectra of CdTe QDs versus the hirudin concentration: (a) 2; (b) 5; (c) 10; (d) 20; (e) 30; (f) 70; and (g) 120 μU/mL. The spectra were obtained from the mixtures of CdTe QDs (4 μg/mL), thrombin (100 μU/mL), S-peptide (3.5 μM), and different concentrations of hirudin (0–120 μU/mL). (B) Plot of the inhibition percentage of thrombin versus the concentration of hirudin. The inhibition percentage of thrombin was calculated from the following equation: inhibition percentage $=\Delta F_i/\Delta F_0$, where ΔF_i is the fluorescence change of inhibition reaction system (QDs, hirudin, S-peptide, and 100 μU/mL thrombin) and ΔF_0 is the change of fluorescence intensity in the absence of hirudin. Inset: linear plot of the normalized intensity versus the concentration of thrombin in the range of 2–30 μU/mL.

4. Conclusions

In this study, a simple, sensitive, and label-free fluorescence method for the determination of thrombin activity has been developed based on the charge-induced aggregation behavior of unmodified CdTe QDs, which simulates the physiological process of blood clotting catalyzed by thrombin. The activity of thrombin was achieved by detecting the fluorescence intensity change of CdTe QDs. Under the optimized conditions, the unmodified CdTe QDs based nanosensor enables sensing thrombin activity in the range of 10–100 μU/mL with LOD of 1.5 μU/mL, and further successfully performing thrombin inhibitor detection and real serum sample analysis. According to previous work (Jasmina et al., 2005), the concentration of CdTe QDs (4 μg/mL final concentration) we used in the assay would not induce cytotoxicity, indicating the safety of this sensor. Compared to other thrombin fluorescence assays, our sensor exhibits several practical advantages, including no need for labor-intensive QDs modification, simple experimental process, and fast response. Such advantages enable our method to be conducted in a multiwell plate system and present a promising technique for high-throughput screening in thrombin-related drug discovery. Furthermore, by substituting the S-peptide with other peptides, this strategy could be conveniently extended for other acid proteases or alkaline proteases detection, such as trypsin, hyaluronidase, and *Staphylococcus* alkaline protease.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.10.021.

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