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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.

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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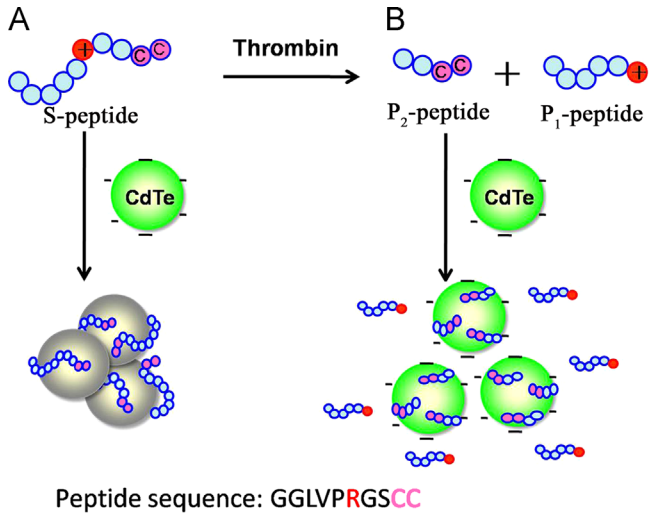
(Medintz et al., 2006). Very recently, one kind of QD as simultaneous donors and acceptors in a time-gated FRET, and an assembly of a concentric FRET relay on a QDs scaffold, has been reported for the multiplexed detection of protease activity (Mulvihill et al., 2010; Tang et al., 2006). However, most 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