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A label-free electrochemiluminescence aptasensor for thrombin detection based on host–guest recognition between tris(bipyridine) ruthenium(II)-β-cyclodextrin and aptamer

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ABSTRACT

An ultrasensitive label-free electrochemiluminescence (ECL) aptasensor for the detection of thrombin was developed based on the specific recognition between tris(bipyridine)ruthenium(II)-β-cyclodextrin (tris(bpyRu)-β-CD) and the anti-thrombin aptamer (aptamer). The NH_2-aptamer was first immobilized on the activated glassy carbon electrode (GCE) by coupling interaction. By use of the specific recognition between tris(bpyRu)-β-CD and aptamer, tris(bpyRu)-β-CD was then attached on the surface of GCE. Resulting from the outstanding photoactive properties of tris(bpyRu)-β-CD, the fabricated GCE performed strong ECL signal with the coreactant of 2-(dibutylamino)ethanol (DBAE). However, in the presence of thrombin, aptamer–thrombin bioaffinity complexes were formed, which restricted the recognition activities between aptamer and tris(bpyRu)-β-CD. Thus, fewer tris(bpyRu)-β-CD could be attached on the surface of GCE and led to an obvious decrease of ECL signal. Fortunately, the difference of ECL intensity before and after combination with thrombin was logarithmically linear with the concentration of thrombin in a wide range of 10 nM–1pM. Meantime, a detection limit of 0.1 pM without any other signal labeling or amplifying procedures indicated that the biosensor performed excellent sensitivity, operability and simplicity.

1. Introduction

In recent years, considerable attentions have been given to the applications of electrochemiluminescence(ECL) owing to its fundamental advantages like low background, high detection sensitivity and good controllability (Dennany et al., 2003; Miao et al., 2002). Hereinto, ECL-based biosensors have already been of widespread used in biomolecular detection or disease diagnosis (Gatto-Menking et al., 1995; Hu and Xu, 2010). One of the popular ECL system—Ru$(bpy)_3^{2+}$ (or its derivatives)/coreactant has been developed successfully (Leland and Powell, 1990; Richter, 2004) since Noffsinger and Danielson first reported ECL of Ru$(bpy)_3^{2+}$/aliphatic amines system in 1987 (James and Neil, 1987). In consideration of raising ECL efficiency and reducing environmental pollution, 2-(dibutylamino) ethanol (DBAE) was then considered as a new co-reactant in ECL system by Liu's group (Liu et al., 2007). After that, an increasing number of ECL-based sensors have been constructed upon the Ru$(bpy)_3^{2+}$/DBAE systems and performed good sensitivity and selectivity (Crespo et al., 2012; Sun et al., 2009; Xue et al., 2009).

Thrombin is involved in some physiological and pathological process, such as metastasis, wound pathological process, blood solidification and inflammation (Centi et al., 2007; Nierodzik and Karpatkin, 2006). In addition, the determination of thrombin is crucial in fundamental research and clinical practice so that a mass of works has been done through various methods, such as surface-enhanced Raman spectroscopy (SRS; Hu et al., 2009), electrochemiluminescence (ECL; Wang et al., 2009), surface plasmon resonance (SPR; Bai et al., 2013; Mani et al., 2011), fluorescence (Wang et al., 2011) and electrochemistry (EC; Fan et al., 2012). The concentration of thrombin in blood could be low-μM during the coagulation process under abnormal conditions, while the concentration close to low-nM could also be detected in hemostatic process (Shuman and Majerus, 1976). For the patient with coagulation abnormalities, thrombin circulates at pM level (Bichler et al., 1996), which needs to be detected by an ultrasensitive aptasensor within a detection limit of pM level or lower.

Aptamers, obtained from the technique named "systematic evolution of ligands by exponential enrichment (SELEX)", is a new class of single-stranded DNA (ss-DNA) or RNA oligonucleotides (Ellington and Szostak, 1990;Robertson and Joyce, 1990; Tuerk and Gold, 1990). The target compounds of aptamers could be small organic molecules (Liu et al., 2012), metal ions (Chan et al., 2013; Mei et al., 2013) and proteins (Goda and Miyahara, 2013; Jalit et al., 2013). Compared to conventional methods, aptamer-based sensors for thrombin detection

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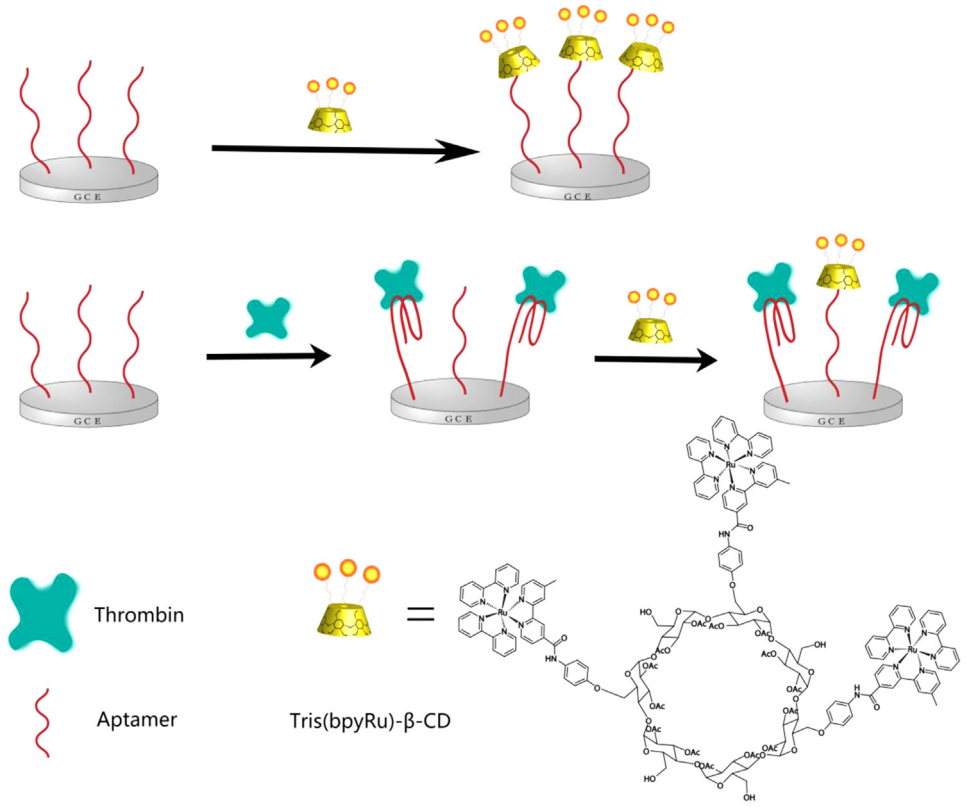
exhibit good specificity, low cost, simple preparation and easy storage (Kankia and Marky, 2001). Inspired by the advance of ECL measurements, ECL-based aptasensors for thrombin detection have gradually been employed in the pursuit of a lower detection limit (Li and Cui, 2013; Gan et al., 2012). To further improve the ECL sensitivity, polymers (Wang and Yu, 2000), nanoparticles (Gui et al., 2013) or chemical labeling on the biomolecules (Zhou et al., 2003) have been applied in most instances. With the acquaintance of polynuclear metallocyclodextrins, the effective signal molecules with excellent electronic and photoactive characteristics definitely have aroused the interests of researchers (Haider and Pikramenou, 2005). On the other hand, based on the specific properties of cyclodextrin, polynuclear metallocyclodextrins also possess of the recognition capability with guest molecules (Del Valle, 2004). The above features could be fully utilized to construct a novel biosensor with signal amplification. For this reason, as a typical example of polynuclear metallocyclodextrins, tris(bpyRu)-β-CD was successfully synthesized in our group and played great ECL performance as well as recognition capability. Based on the host–guest recognition between dabcyl and tris(bpyRu)-β-CD, a sensitive ECL-based DNA sensor was once proposed by employing a dabcyl-labeled hairpin DNA (Chen et al., 2013).

Bases or ss-DNA, as a new class of guests for cyclodextrins, have been gradually applied into bioprocess and DNA-sensors (Abdolkarim and Abolhassan, 2008, 2012; Kondo and Nishikawa, 2007; Spies and Schowen, 2002). Based on above facts, the recognition between cyclodextrin and aptamer is supposed to be feasible and is expected to open up a new route in the determination of proteins, small organic molecules or metal ions. Herein, by use of the host–guest recognition between tris(bpyRu)-β-CD and anti-thrombin aptamer (aptamer) as well as the excellent photoactive properties of tris(bpyRu)-β-CD, an ultrasensitive label-free ECL-based aptasensor was successfully designed as Scheme 1. The NH_2-terminated aptamer was previously immobilized on the glassy carbon electrode (GCE) by coupling interaction. After effective recognition with tris(bpyRu)-β-CD, the fabricated GCE performed high ECL intensity. In the presence of thrombin, aptamer–thrombin bioaffinity complexes were formed due to its higher stability, and then interferenced the host–guest recognition of tris(bpyRu)-β-CD and aptamer. As a result, fewer tris(bpyRu)-β-CD could be attached on the surface of electrode and led to an obvious decrease of ECL signal. Thus, the concentration of thrombin could be monitored by the decrease of ECL intensity. Beyond that, it is worthwhile to mention that the specific recognition capability of tris(bpyRu)-β-CD contributes to the direct combination of aptamer and tris(bpyRu)-β-CD, which could cut out the signal labeling procedures in construction of aptasensor. Besides, the multi-(bpyRu) component of tris(bpyRu)-β-CD would greatly enhance the sensitivity in ECL determination of thrombin. By taking full advantages of tris(bpyRu)-β-CD above, the proposed ECL-based aptasensor not only exhibited excellent sensitivity but also performed high efficiency and simplicity without any other signal labeling or amplification procedures. Therefore, this approach offered great potentials for the ultrasensitive, simple and efficient detections of diverse aptamer-specific binding targets.

2. Experimental

2.1. Chemicals and materials

Anthranilic acid (ABA), 2-(dibutylamino) ethanol (DBAE), N-hydroxysuccinimide (NHS), Tween-20, N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), lysozyme and thrombin were purchased from Sigma-Aldrich (Shanghai, China). Tris-(hydroxymethyl)aminomethane (Tris) was obtained from Sangon Biotech Co., Ltd (Shanghai, China). Human IgG was purchased from Dingguo Biotechnology Inc (Beijing, China). NH_2-anti-thrombin aptamer (NH_2-aptamer) was prepared by Sangon Biotech Co.,



Scheme 1. Schematic diagram for the proposed ECL-based aptasensor for thrombin detection.

Ltd (Shanghai, China), as following: 5′-NH_2-$(CH2)_6$-GGTTGGTGTG GTTGG-3′; tris(bipyridine)ruthenium(II)-β-cyclodextrin was synthesized and characterized as previously reported by our group (Chen et al., 2013). All of the solutions were prepared with ultrapure water from a Millipore Milli-Q water purification system.

2.2. Apparatus

All electrochemical measurements were performed on a CHI 660C electrochemical analyzer (CH Instruments Co., Shanghai). The electrochemical system was the conventional three-electrode system: a glassy carbon electrode as working electrode, a platinum wire as counter electrode and an Ag/AgCl electrode as reference electrode. All ECL measurements were performed on an LK5100 electrochemiluminescence analyzer (Lanlike Electronic Science Tech. Co., Ltd, China).

2.3. The immobilization of the aptamer on the electrode

Prior to use, glassy carbon electrode (GCE) was polished sequentially with 1.0, 0.3 and 0.05 μm alumina slurry, followed by ultrasonic cleaning with ethanol and ultrapure water and dried under nitrogen gas. Then, the ABA was electropolymerized on GCE by immersing the electrode in a solution of 0.05 M ABA/1 M H_2SO_4 as reported (0–1 V, 8 cycles; Li et al., 2004). After cleaned with ultrapure water for three times, the poly(ABA) coated electrode was activated with 4 mM EDC/1 mM NHS solution for 30 min. Then, the electrode was immediately covered with 50 nM NH_2-aptamer solution of TE buffer (10 mM Tris–HCl, 1 M NaCl, pH 7.4) for another 3 h. Followed by complete washing, the aptasensor for thrombin detection was finally fabricated for further use.

2.4. ECL measurement for thrombin

As shown in Scheme 1, the electrode immobilized with the aptamer was incubated in various concentrations of thrombin for 1 h at 37 °C. After rinsing with washing buffer (25 mM Tris–HCl, 0.1 M NaCl, 5 mM $MgCl_2$, 1.0% (v/v) Tween-20, pH 7.4) and ultrapure water respectively, the electrode was immersed in 10 μM tris(bpyRu)-β-CD for 1.5 h. Afterwards, the ECL behavior was finally monitored over a scan range of 0.4–1.25 V *vs.* Ag/AgCl in 0.1 M phosphate buffer solution (PBS) containing 20 mM DBAE, with a scan rate of 100 mV/S.

In addition, to show the binding specificity of the proposed aptasensor to thrombin, comparative experiments between thrombin and other interfering proteins were carried out under the same conditions. Here, 1 μM IgG, 1 μM hemin and 1 μM lysozyme were selected to operate the aptasensor, respectively.

3. Results and discussion

3.1. ECL property of tris(bpyRu)-β-CD

Tris(bpyRu)-β-CD, consisting of multiple ruthenium centers and cyclodextrin (Scheme 1), was expected to play great ECL performance and recognition capability. First, to verify the excellent photoactive properties of tris(bpyRu)-β-CD, ECL measurement of 1 μM tris(bpyRu)-β-CD in 0.1 M PBS buffer with 20 mM DBAE as a coreactant was carried out by employing a bare GCE as a working electrode. Compared to the ECL intensity of $Ru(bpy)_3Cl_2$ detected under the same condition (curve b in Fig. 1), the ECL intensity of tris(bpyRu)-β-CD (curve a in Fig. 1) was nearly tripled, which accorded with the particular "tris(bpyRu)"-structure of tris (bpyRu)-β-CD. Now that tris(bpyRu)-β-CD was endowed with much greater ECL performance, it was advisedly applied into the construction of biosensors for further improvement of sensitivity.

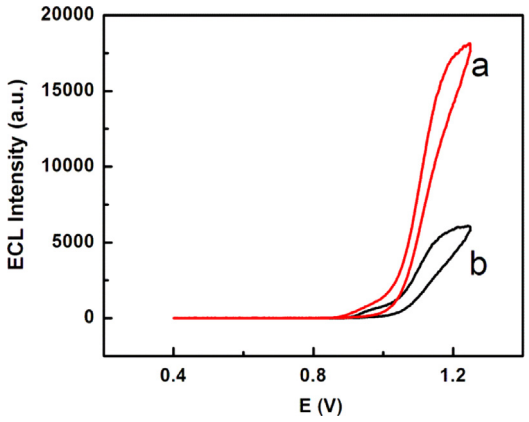


Fig. 1. ECL spectra of 1 μM (a) tris(bpyRu)-β-CD and (b) $Ru(bpy)_3Cl_2$ in 0.1 M PBS buffer (pH 7.5) containing 20 mM DBAE. (ECL experiments were performed on a bare GCE at a scan rate of 100 mV/S ranging from 0.4 V to 1.25 V (*vs.* Ag/AgCl).)

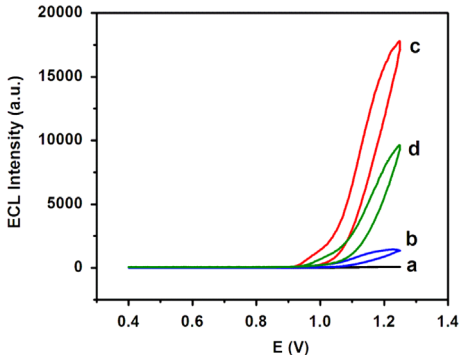


Fig. 2. ECL spectra of (a) aptamer/GCE, (b) $Ru(bpy)_3Cl_2$/aptamer/GCE, (c) tris (bpyRu)-β-CD/aptamer/GCE, and (d) tris(bpyRu)-β-CD/thrombin(10 nM)/aptamer/GCE in 0.1 M PBS containing 20 mM DBAE at a scan rate of 100 mV/S ranging from 0.4 V to 1.25 V (*vs.* Ag/AgCl).

3.2. The host–guest recognition between tris(bpyRu)-β-CD and the DNA probe (aptamer) immobilized on the electrode

The effective host–guest recognition between β-cyclodextrin and ss-DNA or bases has been already reported in the past few years (Abdolkarim and Abolhassan, 2008, 2012; Kondo and Nishikawa, 2007; Spies and Schowen, 2002). Here, for the purpose of demonstrating the host–guest recognition between tris(bpyRu)-β-CD and the DNA probe (aptamer), ECL behaviors of tris(bpyRu)-β-CD and $Ru(bpy)_3Cl_2$ on the electrodes immobilized with aptamer were investigated respectively in our approach. First of all, the NH_2-aptamer was immobilized onto the activated GCE in the presence of EDC/NHS, which performed no ECL signal (curve a in Fig. 2). After aptamer/GCE was immersed in 10 μM tris(bpyRu)-β-CD or $Ru(bpy)_3Cl_2$ for 1.5 h, tris(bpyRu)-β-CD/aptamer/GCE or $Ru(bpy)_3Cl_2$/aptamer/GCE was then obtained correspondingly. As shown in Fig. 2, the ECL signal of tris(bpyRu)-β-CD/aptamer/GCE (curve c) was extremely stronger than that of $Ru(bpy)_3Cl_2$/aptamer/GCE (curve b), indicating that the recogniton between "β-cylcodextrin component" and aptamer indeed played a main role in the attachment of tris(bpyRu)-β-CD onto aptamer/GCE instead of the electrostatic interaction between "(bpy)Ru(II) component" and aptamer. Thus, such specific recognition capability could be further utilized into the construction of label-free aptasensors.

3.3. ECL behavior of the proposed ECL-based aptasensor

The strategy of the proposed aptasensor was illustrated as Scheme 1, which contained just several simple steps in the construction and determination process. The NH_2-aptamer was previouly immobilized on the activated GCE via coupling reaction. By use of the speicific host–guest recognition between aptamer and tris(bpyRu)-β-CD, tris(bpyRu)-β-CD was successfully attached onto the surface of aptamer-modified GCE and was then monitored by ECL measurements. Resulting from the remarkable photoactive properties of tris(bpyRu)-β-CD (multi-(bpyRu) component), the fabricated electrode would apparently perform strong ECL signal with DBAE as a coreactant (curve c in Fig. 2). However, in the presence of thrombin, aptamer–thrombin bioaffinity complexes were easily formed to restrict the recognition between aptamer and tris(bpyRu)-β-CD due to the higher stability of aptamer–thrombin complexes. Therefore, fewer tris(bpyRu)-β-CD was captured by the thrombin (10 nM)/aptamer/GCE, which led to a sharp decrease in the ECL performance (curve d in Fig. 2) in comparision of tris(bpyRu)-β-CD/aptamer/GCE (curve c in Fig. 2). As mentioned above, the aptasensor exhibited obvious difference of ECL intensity between the absence and presence of thrombin without any other additional signal labeling or amplification procedures. In other words, the decrease of ECL intensity of the proposed aptasensor was supposed to be the key parameter for thrombin detection. Consequently, such a simple and distinctive aptasensor was proved to be well operated in the detection of thrombin.

3.4. Effect of the aptamer concentration onto the surface of GCE for the aptasensor

Based on the quenching mechanism of the proposed aptasensor, the competition capability of thrombin against tris(bpyRu)-β-CD was preferred to be the strongest under optimized condition. In this work, ECL responses of tris(bpyRu)-β-CD/thrombin–aptamer/GCE and tris(bpyRu)-β-CD/aptamer/GCE were correspondingly regarded as signal and control. To achieve the minimum signal-to-control ratio, different initial concentration of aptamer onto the surface of GCE was investigated. More precisely, different concentrations of NH_2-aptamer (from 0.5pM to 0.5 μM) were employed to cover the suface of activated GCE for 3 h, then the GCEs with different concentrations of aptamer were immersed in Tris-buffer or 1 μM thrombin for 1 h respectively. After reacting with 10 μM tris(bpyRu)-β-CD for another 1.5 h, all the fabricated GCE were respectively monitored by ECL measurents in 0.1 M PBS containing 20 mM DBAE at a scan rate of 100 mV/S, ranging from 0.4 V to 1.25 V (*vs*. Ag/AgCl). That being the case, ECL signals with different concentrations of aptamer were detected from Tris-buffer as control(curve a in Fig. 3) and thrombin at 1 μM as signal (curve b in Fig. 3) in parallel.

Fig. 3 showed the ECL responses of tris(bpyRu)-β-CD/aptamer/GCE within different aptamer concentrations (0.5 μM–0.5pM), and the trendline of ECL intensity was represented with regularity. With the increase of the concentration of aptamer from 0.5pM to 50 nM, the ECL intensity of tris(bpyRu)-β-CD/aptamer/GCE gradually increased and reached a maximum at 50 nM. It could be undoubtedly explained that the greater amount of aptamers onto the surface of GCE are able to combine more tris(bpyRu)-β-CD complexes, which was in favor of the enhancement of ECL intensity. However, with the further increase of the concentration of aptamer from 50 nM to 5 μM, the ECL intensity decreased instead, which probably resulted from the high concentration of aptamer on surface of GCE being unfavorable for the recognition with tris(bpyRu)-β-CD due to the steric-hindrance effects.

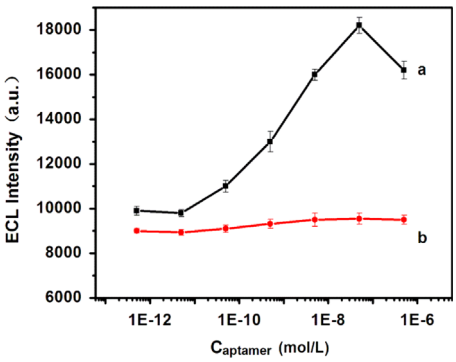


Fig. 3. ECL signal responses of (a) tris(bpyRu)-β-CD(10 μM)/aptamer/GCE and (b) tris(bpyRu)-β-CD(10 μM)/thrombin–aptamer/GCE with different concentrations of aptamer ranging from 0.5pM to 0.5 μM in 0.1 M PBS containing 20 mM DBAE at a scan rate of 100 mV/S ranging from 0.4 V to 1.25 V (*vs*. Ag/AgCl).

On the other hand, curve b in Fig. 3 showed the ECL responses of tris(bpyRu)-β-CD/thrombin–aptamer/GCE with corresponding concentrations of aptamer. It experienced a slight upward trend from 0.5pM to 0.5 μM as a whole. After all, the concentration of aptamer exerted weaker effect on the ECL behaviors of tris(bpyRu)-β-CD/thrombin–aptamer/GCE due to the excessive concentration of thrombin. Besides, the ECL intensity of tris(bpyRu)-β-CD/thrombin–aptamer/GCE probably resulted from: (1) the electrostatic interaction between phosphate backbone and tris(bpyRu)-β-CD. (2) The inevitable aptamers that do not combine with thrombin related to the binding efficiency.

As can be seen in Fig. 3, a minimum signal-to-control ratio was found when the concentration of aptamer was at 50 nM. Therefore, 50 nM should be chosen as the optimal in the following experiments.

3.5. Determination of thrombin

Under optimal conditions, the feasible ECL-based aptasensor was employed for the determination of thrombin. Fig. 4A showed the ECL responses of the aptasensor after immersing in different concentrations of thrombin, where the curves from a to g corresponded to the thrombin concentrations from 0 to 10 nM, respectively. In the absensce of thrombin, the ECL signal was extremely larger in comparision with that of others (curve a). In addition of thrombin, the ECL signal correspondingly decreased with the increasing thrombin concentrations (curves from b to f), demonstrating that thrombin was quantitatively measured by the ECL aptasensor.

Fig. 4B displayed the relationship between ΔI and thrombin concentrations, which was found to be logarithmically related to the thrombin concentrations in a range from 1pM to 10 nM (R=0.995). In this work, three parallel samples of each concentration were investigated to confirm the accuracy of the experiments. Besides, the detection limit of 0.1 pM was close to or lower than the previously reported detection of thrombin (Hu et al., 2009; Lee et al., 2012; Yoon et al., 2013; Zhao et al., 2013), indicating that the sensitivity of the proposed aptasensor was satisfatory.

3.6. Selectivity for the aptasensor

Control experiments were conducted to investigate the specificity of the ECL-based aptasensor. In this work, human IgG, lysozyme and hemin were chosen to operate the proposed aptasensor instead of thrombin under the same experimental conditions. Fig. 5 showed that none of the above proteins caused obvious ECL signal decrease even with a concentration as high as 1 μM, while 10 nM of thrombin led to a significant quenching of

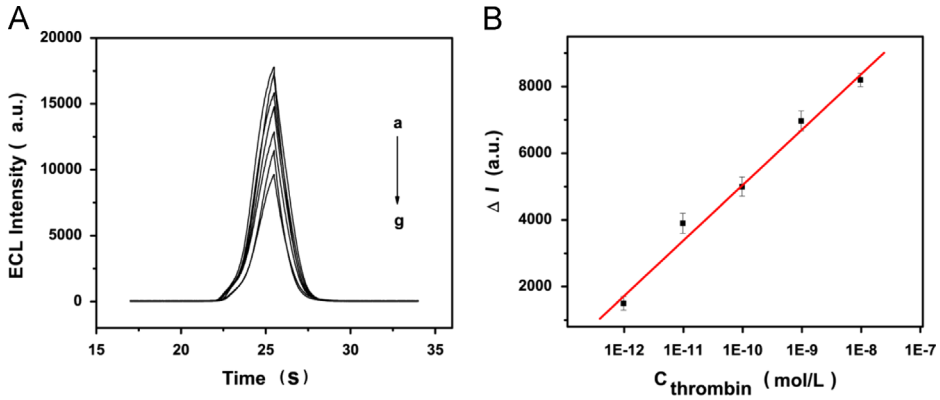


Fig. 4. (A) ECL responses of the aptasensor incubated with different concentrations of thrombin (a) 0 M, (b) 0.1 pM, (c) 1 pM, (d) 10 pM, (e) 100 pM, (f) 1 nM, and (g) 10 nM. (B) The resulting calibration curve for the absolute difference of the ECL intensity as a function of the logarithm with different thrombin concentrations from 1 pM to 10 nM. ($\Delta I = I_0 - I$, I_0 and I were the ECL intensity of the aptasensor in the absence and presence of thrombin, respectively.)

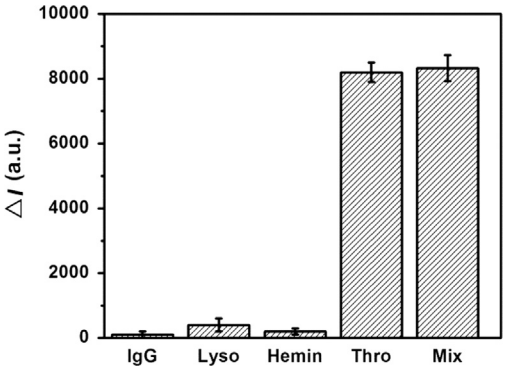


Fig. 5. Selectivity of the ECL aptasensor to thrombin (10 nM) by comparing it to the interfering proteins at 1 μM level (IgG, lysozyme, and hemin).

ECL signal. Furthermore, a mixed solution (containing all the proteins above) performed approximate ECL intensity as thrombin itself did, which demonstrated that the aptasensor could resist the interference from other proteins in determination of thrombin. Additionally, three parallel experiments were carried out to demonstrate the specificity for the aptasensor. Therefore, the results proved that the proposed aptasensor performed excellent specificity for thrombin determination.

3.7. *Application of ECL-based aptasensor in human serum sample*

The feasibility of the aptasensor for the clinical applications was further investigated in human serum. A series of samples were prepared by adding certain concentrations of thrombin in human serum. The results in Table S1 described the acceptable quantitative recoveries of the aptasensor in a range of 95.2–102.0%, which indicated that the developed aptasensor was applicable for the determination of thrombin in complex biological samples.

4. Conclusion

This work described an ultrasensitive label-free ECL-based aptasensor for the detection of thrombin by use of tris(bpyRu)-β-CD. Thanks to the remarkable advantages of tris(bpyRu)-β-CD, a wider range of thrombin detection with a detection limit of 0.1 pM was successfully obtained based on the excellent photoactive properties of multi-(bipyridine)ruthenium component as well as the specific host–guest recognition between cyclodextrin component and ss-DNA. Furthermore, the aptasensor was simply constructed and operated well in thrombin detection without any other complicated signal labeling or amplification procedures. Such ECL-based aptasensor created new opportunities for detection of aptamer-specific protein targets and was expected to be widely applied in protein validation and diagnostics.

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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.2013.11.028.

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