



A solid-state electrochemiluminescence biosensing switch for detection of thrombin based on ferrocene-labeled molecular beacon aptamer

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ARTICLE INFO

Article history:

Received 4 February 2009

Received in revised form 12 April 2009

Accepted 14 April 2009

Available online 22 April 2009

Keywords:

Solid-state electrochemiluminescence

biosensing switch

Molecular beacon aptamer

Thrombin

Ferrocene

Quenching effect

ABSTRACT

A solid-state electrochemiluminescence (ECL) biosensing switch system based on special ferrocene-labeled molecular beacon aptamer (Fc-MBA) has been developed successfully for thrombin detections. Such special switch system includes two main parts, an ECL substrate and an ECL intensity switch. The ECL substrate was made by modifying the complex of Au nanoparticle and Ruthenium (II) tris-(bipyridine) ($\text{Ru}(\text{bpy})_3^{2+}$ -AuNPs) onto Au electrode. A molecular beacon aptamer labeled by ferrocene acted as the ECL intensity switch. The loop bases of the ECL intensity switch are designed with special anti-thrombin aptamer sequence which could be combined with its target protein *via* the reaction between aptamer and thrombin. During the reactions, the molecular beacon aptamer opened its stem-loop, and the labeled Fc was consequently kept away from the ECL substrate. Such structural change resulted in an obvious ECL intensity increment due to the decreased quenching effect of Fc to the ECL substrate. The analytic results are sensitive and specific.

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1. Introduction

Among various biosensing technologies, electrochemiluminescence (ECL) including solution-state and solid-state ECL systems has the inherent advantages of versatility, sensitivity, selectivity, simplified optical setup, good temporal and spatial control, and wide linear range for clinical tests and biomolecules detection (Fähnrich et al., 2001; Richter, 2004; Wang et al., 2006; Zhan and Bard, 2007). Compared with solution-state ECL system (Jirka and Nieman, 1994; White and Bard, 1982), solid-state ECL can reduce the consumption of expensive reagent, enhance the ECL signal, simplify experimental design and create regenerable sensors (Choi et al., 2006; Ding et al., 2005; Gao et al., 2007; Guo et al., 2004; Lin and Chen, 2006; Sun et al., 2007; Yi et al., 2005; Zhang and Dong, 2006; Wei and Wang, 2008; Qi et al., 2009). Therefore, solid-state ECL system is more suitable for biomolecular sensing (Wei et al., 2007). However, the development of solid-state ECL biosensor is restricted by the fact that a significant loss of the biomolecular activity would result from mixing biomolecules with Ruthenium (II) tris-(bipyridine) ($\text{Ru}(\text{bpy})_3^{2+}$), the most used as ECL substrate (Wei et al., 2007; Zhang et al., 2007). Thus, the combination of the

solid-state $\text{Ru}(\text{bpy})_3^{2+}$ -ECL system with biomolecules together for making a biosensor has come to significant attraction.

Molecular beacon (MB), a hairpin-shaped oligonucleotide with a fluorophore and a quencher linked to each end of its stem, was initially described in 1996 (Tyagi and Kramer, 1996) as a switch able to undergo spontaneous conformational change following hybridization with the complementary nucleic acid target to turn fluorescence “off” or “on” (Tyagi et al., 1998). It is emerging as a novel probe for real time detection of nucleic acids and proteins with advantages of high sensitivity, excellent reproducibility, fast speed and convenience (Bourdoncle et al., 2006; Jenkins et al., 2006; Li et al., 2008a,b; Miranda-Castro et al., 2007; Riccelli et al., 2001; Wabuyele et al., 2003). However, the lack of sequence specificity in protein binding is one apparent and critical disadvantage of MBs in protein recognition (Li et al., 2002).

Aptamers are single-stranded DNA or RNA molecules, generally 25–60 nucleotides in length. They have been selected by SELEX technology from a combinatorial library by their capability to bind a specific target (Hermann and Patel, 2000; Luzzi et al., 2003). Theoretically, it is possible to obtain aptamers which can recognize virtually any target molecules with high affinity and specificity. Aptamers used for specific protein binding studies have drawn much interest recently (Balamurugan et al., 2006; Bowser, 2005; Chou et al., 2005; Le Floch et al., 2006; Zayats et al., 2006; Li et al., 2008a,b). Though aptamers have the advantage of binding specificity and generality, they lack the built-in mechanism for signal transduction, especially the capability of “detection without separation” which is

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right the characteristic of molecular beacons (MBs) (Li et al., 2002). MB shows high sensitivity for fast protein monitoring, the lack of selectivity limited the assay to a few nonspecific ssDNA binding proteins. Thus, in order to develop a novel protein-reporting probe for quantitation in homogeneous solutions and real time protein monitoring, it is desirable to combine the specific protein-binding capability of aptamers with the excellent signal transduction capability of MB together, and the formed probe was called molecular beacon aptamer (MBA). Actually, studies shown that the MBA can be functioned as capture agents and signaling probes at the same time by building an intramolecular signal transduction mechanism into the aptamers, thus the need for labeling the target proteins or a second signaling probe was eliminated (Cao et al., 2005). The MBA used as intracellular protein recognition agents has functions of probing proteins in different environments and monitoring protein–DNA/RNA interactions, and some studies have described that the novel MBA was used to detect the existence of the Tat protein of HIV-1 and the interaction between thrombin and aptamer (Bang et al., 2005; Hamaguchi et al., 2001; Li et al., 2002; Radi et al., 2006; Yamamoto et al., 2000).

The ferrocene-labeled molecular beacon (Fc-MB) was designed to be acted as a molecular recognition elements as well as a signal transduction elements with consideration of its high ECL quenching efficiency of Fc to Ru(bpy)₃²⁺ (Cao et al., 2006). We have developed a controllable solid-state Ru(bpy)₃²⁺-ECL film, and the solid-state ECL and the biosensing system was successfully combined (Wang et al., 2008). The system utilized Ru(bpy)₃²⁺-AuNPs modified Au electrode to emit ECL, and ferrocene-labeled molecular beacon (Fc-MB) to control the ECL intensity. Herein, we further utilize the special solid-state Ru(bpy)₃²⁺-ECL film to develop a novel solid-state ECL biosensing switch by designing the molecular beacon with anti-thrombin aptamer sequences, which could react with thrombin. Due to the combination of molecular beacon aptamer with the target thrombin, the consequent conformation adjustment of the molecular beacon aptamer results in the Fc departing away from the electrode surface. It is found that the increased ECL intensity can quantify the thrombin with sensitivity and selectivity.

2. Experimental

2.1. Reagents and apparatus

Oligonucleotides were purchased from Shenggong Bioengineering Ltd. Company (Shanghai, China). Molecular beacons aptamer (MBA): 5'-NH₂-(CH₂)₆-CCCGTGGTGGTGGATTGATCGTAG-GTACAACC-(CH₂)₆-SH-3' (underlined nucleotides are self-complementary (stem) segments of MBA, italic nucleotides are aptamer of thrombin). Purified thrombin, bovine serum albumin (BSA) and bovine hemoglobin (BHb) were purchased from Dingguo Biological Technology Corporation of Shanghai. Ru(bpy)₃²⁺ (99.95%), HAuCl₄, 6-mercapto-1-hexanol (SH-(CH₂)₆-OH, >97.0%), cysteamine (SH-(CH₂)₂-NH₂) and

1-ethyl-3-[(3-dimethylamino)propyl]carbodiimide (EDC) were purchased from Sigma (USA). Ferrocenecarboxylic acid (FCA) was purchased from Maoji Bioengineering Ltd. Company (Shanghai, China). 10 mM PBS containing 2 mM Mg²⁺, 10 mM K⁺ and 100 mM LiClO₄ (pH 7.0) was used in all the experimental processes. 20 mM PBS containing 1.0 mM tri-*n*-propylamine (TPrA) and 5.0 mM LiClO₄ (pH 8.7) was used as the detecting solution. Other reagents were of analytical reagent grade. All of the solutions were prepared with ultrapure water from a Millipore Milli-Q system.

ECL was recorded with MPI-E electrogenerated chemiluminescence analyzer (Xi'an Remax Electronic Science Tech. Co. Ltd.), a CHI 660A electrochemical analyzer (CHI instruments Inc., USA) was used to carry out impedance and cyclic voltammogram (CV) measurements in a 10 mL analytical cell.

2.2. Preparation of Fc-MBA-Ru(bpy)₃²⁺-AuNPs electrode

The Fc-MBA-Ru(bpy)₃²⁺-AuNPs electrode was prepared according to the reported protocols (Wang et al., 2008). As shown in Fig. 1, a self-assembled monolayer of cysteamine is prepared onto Au electrode firstly, then, the Ru(bpy)₃²⁺-AuNPs composite is assembled onto the cysteamine-derivated Au electrode to form the luminescent substrate (Fig. 1a). To obtain a solid-state ECL biosensing switch of Fc-MBA-Ru(bpy)₃²⁺-AuNPs on Au electrode (Fig. 1b), the Fc-MBA is attached onto the electrode *via* Au-S interactions, and SH-(CH₂)₆-OH solution is then dropped onto the Fc-MBA-Ru(bpy)₃²⁺-AuNPs electrode to occupy the unassembled surface of Ru(bpy)₃²⁺-AuNPs as well as adjust the Fc-MBA distribution on the electrode surface. The following is the detailed preparation processes.

2.2.1. Formation of luminescent substrate of Ru(bpy)₃²⁺-AuNPs on Au electrode

The surface of Au electrode was polished with alumina slurry, rinsed with water and ethanol in an ultrasonic bath briefly. The Au electrode was further treated electrochemically in 0.5 M H₂SO₄ by scanning the potential from -0.2 to 1.7 V at a scan rate of 10 V s⁻¹ for 15 min until an ideal voltammogram was observed. It was soaked in 0.1 M cysteamine aqueous solution for 2 h at room temperature to form the cysteamine monolayer (cysteamine-derivated Au electrode). After that the electrode was thoroughly rinsed with water to remove physically adsorbed cysteamine. 2 μL aliquot of the suspension of Ru(bpy)₃²⁺-AuNPs composite was placed on the cysteamine-derivated Au electrode surface. The as-prepared electrode was air-dried at room temperature. After rinsed with water thoroughly, the luminescent substrate of Ru(bpy)₃²⁺-AuNPs was immobilized on the Au electrode surface.

2.2.2. Formation of solid-state ECL biosensing switch of Fc-MBA-Ru(bpy)₃²⁺-AuNPs on Au electrode

2 μL aliquot of 10 μM Fc-MBA was pipetted onto the surface of Ru(bpy)₃²⁺-AuNPs immobilized Au electrode as uniformly as

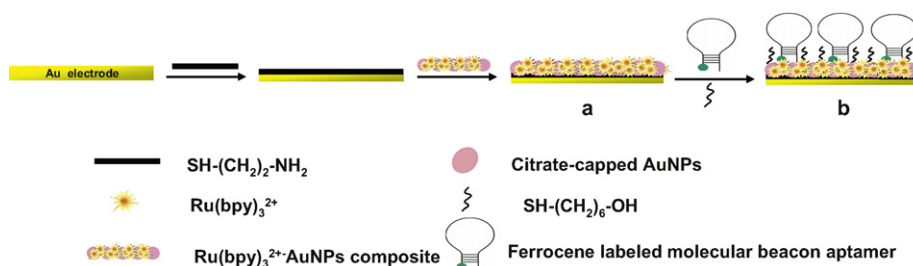


Fig. 1. Schematic representation of the preparation of Fc-MBA-Ru(bpy)₃²⁺-AuNPs electrode. (a) Luminescent substrate of Ru(bpy)₃²⁺-AuNPs on Au electrode and (b) solid-state ECL biosensing switch of Fc-MBA-Ru(bpy)₃²⁺-AuNPs on Au electrode.

possible and self-assembled for 12 h at room temperature, then the electrode was washed with 10 mM PBS containing 2 mM Mg^{2+} , 10 mM K^+ and 100 mM LiClO_4 (pH 7.0). The electrode was treated with 1.0 mM $\text{SH}-(\text{CH}_2)_6-\text{OH}$ solution containing 100 mM LiClO_4 for 2 h to hold the unassembled surface by the Fc-MBA. After rinsed thoroughly, solid-state ECL biosensing switch of Fc-MBA- $\text{Ru}(\text{bpy})_3^{2+}$ -AuNPs was formed on the Au electrode surface. The electrode was sealed to avoid evaporation during the whole assembling procedure.

2.3. The specific recognition of thrombin onto Fc-MBA- $\text{Ru}(\text{bpy})_3^{2+}$ -AuNPs electrode

The Fc-MBA- $\text{Ru}(\text{bpy})_3^{2+}$ -AuNPs electrode was incubated with thrombin in 10 mM PBS (pH 7.0) containing 2 mM Mg^{2+} , 10 mM K^+ and 100 mM LiClO_4 at room temperature for 20 min. Then the electrode was washed with the same buffer to remove the unreacted thrombin analyte. The aptamer-thrombin complex was formed onto Au electrode.

2.4. Electrogenerated chemiluminescence detection

The ECL determinations were performed at room temperature in a 10 mL homemade quartz cell. A three-electrode system was used with the modified Au electrode (2 mm in diameter) as the working electrode, an Ag/AgCl (sat.) as the reference electrode and a platinum wire as the counter electrode. Cyclic voltammetry mode with continuous potential scanning from 0.0 to 1.2 V and scanning rate of 0.1 V s^{-1} was applied to achieve ECL signal in 20 mM PBS containing 1.0 mM TPrA and 5.0 mM LiClO_4 (pH 8.7). A high voltage of -800 V was supplied to the photomultiplier for luminescence intensity determination. The ECL and CV curves were recorded simultaneously.

3. Results and discussion

3.1. The characterization of the Fc-MBA- $\text{Ru}(\text{bpy})_3^{2+}$ -AuNPs electrode

Electrochemical impedance spectroscopy (EIS) and ECL method were used to monitor the preparation of the solid-state ECL biosensing electrode. For EIS measurements, $[\text{Fe}(\text{CN})_6]^{3-}/[\text{Fe}(\text{CN})_6]^{4-}$ was utilized as the redox probe and Nyquist plots were used to calculate the R_{et} for modified electrodes. As shown in Fig. 2, the R_{et} for bare Au electrode was 246.4Ω , and then increased to 327.9Ω for the $\text{Ru}(\text{bpy})_3^{2+}$ -AuNPs electrode and 737.6Ω for the Fc-MBA- $\text{Ru}(\text{bpy})_3^{2+}$ -AuNPs electrode. The corresponding cyclic voltammogram (CV) curves of the electrodes using $[\text{Fe}(\text{CN})_6]^{3-}/[\text{Fe}(\text{CN})_6]^{4-}$ as the redox probe were shown in the inset of Fig. 2. The successive increase in the R_{et} and the CV curves of the electrodes illustrate the successful modification of the biosensing components onto Au electrode.

The corresponding ECL intensity-potential curves of the electrodes are presented in Fig. 3. As shown in the figure, no ECL signal can be found for the bare Au electrode (curve a), while the immobilization of $\text{Ru}(\text{bpy})_3^{2+}$ -AuNPs onto Au electrode results in a strong ECL signal (curve b). A clear decrease of ECL signal is found for the Fc-MBA- $\text{Ru}(\text{bpy})_3^{2+}$ -AuNPs electrode (curve c), which is due to the efficient quench of Fc-MBA film to the ECL of $\text{Ru}(\text{bpy})_3^{2+}$ -AuNPs. The mechanism for such Fc-quenching ECL of $\text{Ru}(\text{bpy})_3^{2+}$ has been reported as the bimolecular energy and electron transfer between $\text{Ru}(\text{bpy})_3^{2+}$ and the oxidized species of Fc, ferrocenium (Fc^+), along with suppression of radical reactions (Cao et al., 2006). Herein, all buffer solutions used in the experiments contain 100 mM LiClO_4 to inhibit the decomposition of Fc^+ (Han et al., 2000; Jenkins et al., 2006). Moreover, it can be found that

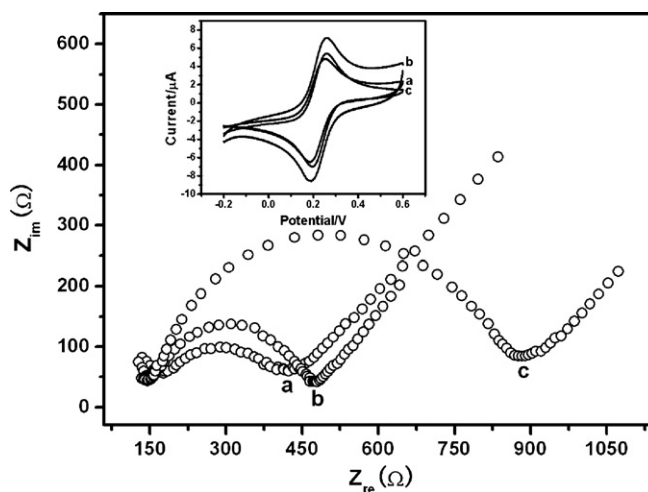


Fig. 2. Nyquist plots for the impedance measurement in 10 mM $[\text{Fe}(\text{CN})_6]^{3-}/[\text{Fe}(\text{CN})_6]^{4-}$ solution for the bare Au electrode (a), $\text{Ru}(\text{bpy})_3^{2+}$ -AuNPs electrode (b) and Fc-MBA- $\text{Ru}(\text{bpy})_3^{2+}$ -AuNPs electrode (c).

the $\text{Ru}(\text{bpy})_3^{2+}$ -AuNPs composite immobilized on the cysteamine-derived Au electrode surface *via* Au-S interactions is very stable, and as shown in the inset of Fig. 3, even after continuous potential scanning the electrodes can give an unchanged ECL intensity without the obvious loss of $\text{Ru}(\text{bpy})_3^{2+}$ from the electrode surface.

3.2. Recognition of thrombin by using the solid-state ECL biosensing switch

Thrombin as one important physiological protease in blood (Centi et al., 2007; Stubbs and Bode, 1993) has been chosen as the target for investigating the application of this solid-state ECL biosensing system to special protein. Herein, the loop bases of the Fc-MBA are designed with the specific anti-thrombin aptamer sequence, which is expected to transform into G-quartet structure to combine with thrombin as displayed in Fig. 4. Consequently, the Fc was far away the electrode surface and the ECL intensity was increased significantly. The enhanced ECL signal is expected to quantify the thrombin.

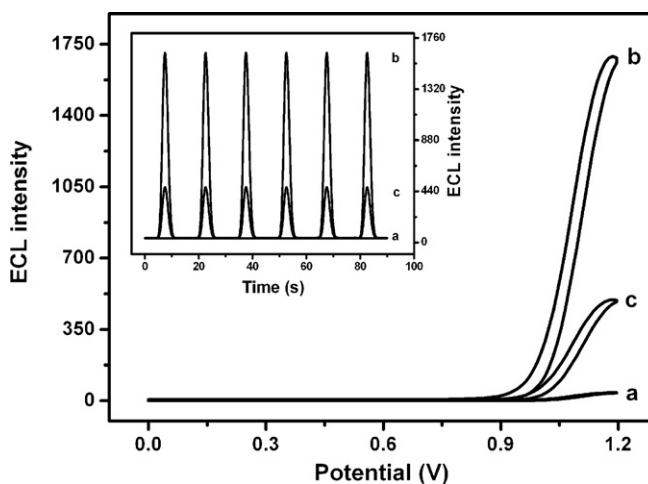


Fig. 3. ECL intensity vs. potential curves for the bare Au electrode (a), $\text{Ru}(\text{bpy})_3^{2+}$ -AuNPs electrode (b) and Fc-MBA- $\text{Ru}(\text{bpy})_3^{2+}$ -AuNPs electrode (c). Inset: ECL intensity vs. time curves for the electrodes under continuous CVs for six cycles. ECL curves were measured in 20 mM PBS containing 1.0 mM TPrA and 5.0 mM LiClO_4 (pH 8.7). Scan rate: 0.1 V s^{-1} , scan range: 0.0–1.2 V.

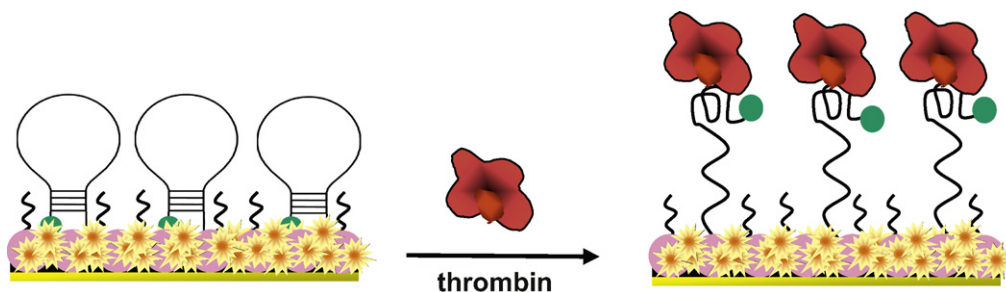


Fig. 4. The scheme of the specific recognition of thrombin by using the Fc-MBA-Ru(bpy)₃²⁺-AuNPs electrode.

3.3. The incubation time of Fc-MBA with thrombin

The incubation time of the Fc-MBA-Ru(bpy)₃²⁺-AuNPs electrode with thrombin is a vital parameter for aptamer-thrombin complex formation. With the incubation time increased, the ΔI_{ECL} increased rapidly and reached a plateau after 25 min. It meant that an almost complete binding reached after 25 min. So, we employed 25 min as the incubation time.

3.4. Control experiment

The evaluation of the selectivity of solid-state ECL biosensing switch was performed by examining thrombin, BSA, BHB or mixed samples. As shown in Fig. 5, the change of the ECL intensity for the Fc-MBA-Ru(bpy)₃²⁺-AuNPs electrode reacting with different concentration of BSA (curves b and c) or BHB (curves d and e) can be neglected when compared with the Fc-MBA-Ru(bpy)₃²⁺-AuNPs electrode (curve a). Only the samples containing thrombin can result in a remarkable enhancement of the ECL intensity (curves f–h). Moreover, even the concentrations of BSA or BHB were three log units higher than that of the thrombin analyte in the mixed sample of BSA, BHB and thrombin, the ECL signal had no apparent difference. Therefore, it can be concluded that the ECL intensity increase is caused by the thrombin combining with the molecule beacon aptamer as showed in Fig. 4. This also indicates that the solid-state ECL biosensing switch

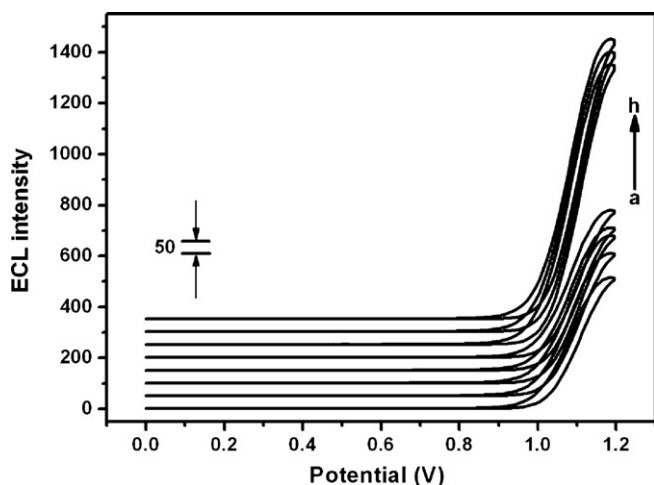


Fig. 5. ECL intensity vs. potential curves were measured in 20 mM PBS containing 1.0 mM TPrA and 5.0 mM LiClO₄ (pH 8.7) for the Fc-MBA-Ru(bpy)₃²⁺-AuNPs electrode (a), the Fc-MBA-Ru(bpy)₃²⁺-AuNPs electrode incubated with 10 nM BSA (b), 10 μ M BSA (c), 10 nM BHB (d), 10 μ M BHB (e), 10 nM thrombin (f), mixed sample containing 10 nM thrombin, 10 nM BSA and 10 nM BHB (g), mixed sample containing 10 nM thrombin, 10 μ M BSA and 10 μ M BHB (h), respectively. Scan rate was 0.1 V s⁻¹, scan range was 0.0–1.2 V.

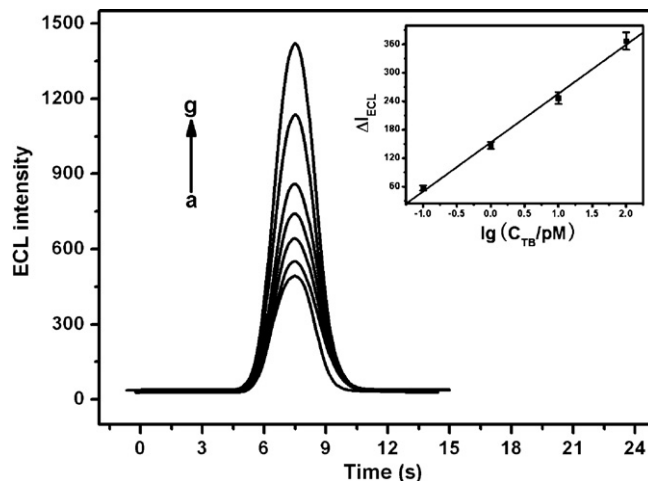


Fig. 6. ECL intensity–time curves for various MBA–thrombin complex electrodes. The concentrations of thrombin were 0.0 M (a), 0.1 pM (b), 1 pM (c), 10 pM (d), 100 pM (e), 10 nM (f) and 10 μ M (g), respectively. Inset: the calibration curve of thrombin detection. ECL curves were measured in 20 mM PBS containing 1.0 mM TPrA and 5.0 mM LiClO₄ (pH 8.7). Scan rate: 0.1 V s⁻¹, scan range: 0.0–1.2 V.

has a good selectivity for discriminating thrombin from other proteins.

3.5. The calibration curve of thrombin detection

The sensitivity of the solid-state ECL biosensing switch was investigated. Fig. 6 shows the ECL signals of the Fc-MBA-Ru(bpy)₃²⁺-AuNPs electrode under the condition that different concentration thrombin interacted with the Fc-MBA. The ECL intensity was enhanced when the thrombin concentration increased, and the change of the ECL intensity, ΔI_{ECL} , was found to be linear with the logarithm of thrombin concentration in the range from 0.1 pM to 100 pM (as shown in the inset). The equation for the resulting calibration plot was $y = 103 \lg x + 153$ (x is the concentration of thrombin divide pM and y is the ΔI_{ECL}) with correlation coefficient of 0.9978 and detection limit of 50 fM.

4. Conclusions

A solid-state ECL biosensing switch for detection of thrombin has been fabricated based on ferrocene-labeled molecular beacon aptamer. The ECL intensity is controlled by the distance from the Fc to the Ru(bpy)₃²⁺-modified electrode due to the Fc-MBA structure conformation. The results are specific and sensitive. Therefore, such novel biomolecular sensing switch is promising for real sample analysis in the field of biomedicine. It is also expected to be involved into bio-chip fabrication.

Acknowledgments

We would like to express our gratitude to the National Nature Science Foundation of China (Grant No. 20675031), and Shanghai Science and Technology Committee (Grant No. 06PJ14032) for financial support of this work.

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