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A signal-on electrochemiluminescence aptamer biosensor for the detection of ultratrace thrombin based on junction-probe

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ABSTRACT

A novel signal-on junction-probe electrogenerated chemiluminescence (ECL) aptamer biosensor has been developed for the detection of ultratrace thrombin based on a structure-switching ECL-quenching mechanism. The ECL aptamer biosensor comprises 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 ($Ru(bpy)_3^{2+}$–AuNPs) on the surface of gold electrode (GE), and the ECL intensity switch contains three probes designed according to the "junction-probe" strategy. The first probe is capture probe (Cp) which was functionalized with a thiol group at one end and covalently attached to $Ru(bpy)_3^{2+}$–AuNPs modified GE through S–Au bonding. The second probe is aptamer probe (Ap), which containing 15-base anti-thrombin DNA aptamer. The third one is ferrocene-labeled probe (Fp), which was functionalized with ferrocene tag at one end. We demonstrated that, in the absence of thrombin, Cp, Ap and Fp will hybridize to form a ternary "Y" junction structure and resulted in a quenching of ECL of $Ru(bpy)_3^{2+}$. Whereas, in the presence of thrombin, the Ap prefers to form the G-quadruplex aptamer–thrombin complex and lead to an obvious recovery of ECL of $Ru(bpy)_3^{2+}$, which provided a sensing platform for the detection of thrombin. Using this reusable sensing platform, a simple, rapid and selective signal-on ECL aptamer biosensor for the detection of thrombin with a detection limit of 8.0×10^{-15} M has been developed. The success in the present biosensor served as a significant step towards the development of monitoring ultratrace thrombin in clinical detection.

1. Introduction

Biosensor, which combines the exquisite sensitivity and specificity of biological active substances with the suitable transducers, plays essential roles not only in basic research but also in genetic screening, medical diagnostics, food quality determination and environmental monitoring. In recent years, a variety of signal transduction techniques have been incorporated in the biosensor design such as electrochemical (Shen and He, 2007), optical (Aizawa et al., 2007) and electrogenerated chemiluminescence (ECL) techniques etc. (Wilson et al., 2003; Zhang et al., 2007a,b). Due to the fact that ECL biosensor has obvious advantages such as versatility, better sensitivity and selectivity, low background signal, simplified optical setup, good temporal/spatial control and wide linear range etc. (Wang et al., 2006; Zhan and Bard, 2007), it was widely recognized to be a promising approach for clinical detection. Among all ECL strategies, solid-state ECL, which can reduce the consumption of expensive reagent, enhance the ECL signal, simplify experimental design and create re-generable biosensors (Wei and Wang, 2008; Qi et al., 2009), is more suitable for biomolecular sensing (Wei et al., 2007a,b). In ECL biosensors reported previously, antibody was widely utilized as molecular recognition element for the high-specificity detection of proteins. However, antibody has some drawbacks such as in vivo production, limited target analytes, limited shelf lives and temperature-sensitive to undergo denaturation. All above shortages of antibody prompted researchers to seek an alternative (O'Sullivan, 2002).

Aptamer is single-stranded oligonucleotide that has been designed through an in vitro selection process called SELEX (Systematic Evolution of Ligands by Exponential Enrichment) (Ellington and Szostak, 1990; Tuerk and Gold, 1990). Compared with anti-

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body, aptamer not only has similar high affinity and selectivity for proteins, but also has several clear advantages including simple production, easy storage, good reproducibility, target versatility, easy modification and convenient regeneration etc. (Jayasena, 1999). Therefore, aptamer has been extensively applied as recognition element for the design of biosensors (Willner and Zayats, 2007; Mok and Li, 2008), especially ECL biosensors (Bruno and Kiel, 1999; Bruno and Kiel, 2002).

To date, various sensing strategies were developed to improve the sensitivity and selectivity of ECL aptamer biosensor (Li et al., 2008; Wang et al., 2009). For example, modulate the distance of redox tags from the electrode and result in an altering redox current by inducing the conformational change of aptamer. However, these strategies required a large-scale conformational switch and generally suffered from the problem of substantial background current. Recently, a novel nucleic acid detection technique called as junction-probe has been reported by Nakayama et al. (2008). Junction-probe operates via a concept called template-enhanced hybridization processes (TeHyP). TeHyP encompasses a design strategy whereby two probes, which does not hybridize to each other at a specific temperature, can be made to anneal to each other in the presence of a template (target) via the formation of "Y" junction structure. This method can amplify DNA detection signals under isothermal conditions, has better specificity for the detection of single-base mismatch, and has been used to fabricate fluorescence biosensor (Nakayama et al., 2008) and electrochemical biosensor (Zhang et al., 2009). However, the methods previously reported were all focused on the DNA detection, and the study on the ECL aptamer biosensor for the protein detection based on junction-probe has never been reported.

We herein reported a novel signal-on junction-probe ECL aptamer biosensor for the thrombin detection based on a structure-switching ECL-quenching mechanism. Compared with the existing "signal-off" ECL aptamer biosensors, which often suffers from the problem of limited signal gain and "false positive" caused by the contaminants, the proposed signal-on ECL aptamer biosensor has much higher sensitivity and better selectivity. The success in the present biosensor provides a promising alternative to conventional thrombin detection in clinical detection.

2. Experimental

2.1. Reagents and apparatus

The labeled oligonucleotides were synthesized by TaKaRa biotechnology Co. Ltd. (Dalian, China). Their concentrations were quantified by OD260 based on their individual absorption coefficients. Their base sequences were as follows: capture probe (Cp): 5′-HS-GAT CAA AAC CGT GGA CTC TAA AA-3′; ferrocene-labeled probe (Fp): 5′-CCA CAC CAT TTG ATC-3′-Ferrocene; aptamer probe (Ap): 5′-AGA GTC CAC GGT TGG TGT GGT TGG-3′ (underlined nucleotides are aptamer of thrombin). Human thrombin, human serum albumin (HSA), immunoglobulin G (IgG) and immunoglobulin A (IgA) used in this study were purchased from Sigma–Aldrich, and were diluted to appropriate concentrations with sterile water. $Ru(bpy)_3^{2+}$ (99.95%), $HAuCl_4$, 6-mercapto-1-hexanol ($SH–(CH_2)_6–OH$, >97.0%) and cysteamine ($SH–(CH_2)_2–NH_2$) were purchased from TCI Inc. (Japan). DNA immobilization buffer is 10 mM TE solution (pH 8.0) containing 10 mM TCEP (tris (2-carboxyethyl) phosphine hydrochloride) and 1 M NaCl. The detection buffer is 20 mM PBS solution (pH 8.7) containing 1.0 mM tri-n-propylamine (TPA) and 5.0 mM $LiClO_4$. The 10 mM PBS solution (pH 7.0) containing 2 mM Mg^{2+}, 10 mM K^+ and 100 mM $LiClO_4$ was used in other experimental processes. All chemicals were analytical grade and sterilized Milli-Q water (18 MΩ/cm) was used in all experiment.

The ECL detection system consisted of a BPCL-GP15-TGC ultra-weak luminescence analyzer (Institute of Biophysics, Chinese Academy of Science, Beijing, China), collecting ECL signal system controlled by a personal computer with BPCL program (Institute of Biophysics, Chinese Academy of Science) and a CHI 660D electrochemical system (Shanghai Chenhua Equipments, China). The electrochemical system consisted of a working electrode (ECL biosensor), a platinum wire as the auxiliary electrode and a reference electrode (Ag/AgCl). A glass cell with optically flat bottom was used as detection cell. The electrochemical cell was placed directly on the photomultiplier (PMT operated at 1050 V) and the PMT window was only opened towards the working electrode for reducing the interference of ECL from the auxiliary electrode. The electrochemical impedance spectra (EIS) and cyclic voltammograms (CVs) measurements was also carried out with CHI 660D electrochemical analyzer.

2.2. Preparation of $Ru(bpy)_3^{2+}$–AuNPs composite

AuNPs with a diameter of 16 nm were prepared by citrate reduction of $HAuCl_4$ in aqueous solution according to the literature (Doron et al., 1995). In brief, 100 mL of 0.01% $HAuCl_4$ solution was brought to reflux, and then 3.5 mL of 1% sodium citrate solution was introduced with stirring. The whole solution was kept boiling for 30 min and cooled to room temperature. The product (AuNPs solution) was stored in a dark glass bottle at 4 °C for further use. The $Ru(bpy)_3^{2+}$–AuNPs composite was prepared according to the literature (Wang et al., 2008). In brief, 150 μL of 40 mM $Ru(bpy)_3^{2+}$ aqueous solution was added into 20 mL of above AuNPs solution under vigorous stirring at room temperature. Several minutes later, a large amount of black precipitate ($Ru(bpy)_3^{2+}$–AuNPs composite) was formed. The resulting $Ru(bpy)_3^{2+}$–AuNPs composite was collected by centrifugation, and then was washed several times with water and suspended in water again.

2.3. Preparation of the junction-probe ECL aptamer biosensor

2.3.1. Preparation of $Ru(bpy)_3^{2+}$–AuNPs modified gold electrode

A gold disk electrode (2 mm in diameter) was cleaned following the reported protocol (Zhang et al., 2007a,b). The gold electrode was firstly polished with 1.0, 0.3 and 0.05 μM alumina powder respectively, followed by sonication in ethanol and water for 5 min respectively. Then, the electrode was electrochemically cleaned in fresh 0.5 M H_2SO_4 solution to remove any remaining impurities (Fan et al., 2003). The pretreated electrode was rinsed with water again. After drying with nitrogen gas, the gold electrode (GE) was soaked in 0.1 M cysteamine aqueous solution for 2 h at room temperature to form the cysteamine monolayer (cysteamine-derivatized GE). After that, the electrode was thoroughly rinsed with water to remove the physically adsorbed cysteamine, and a 5 μL of the suspension of $Ru(bpy)_3^{2+}$–AuNPs composite was placed on the cysteamine-derivatized GE surface. The GE was then air-dried at room temperature. After rinsing with water thoroughly, the luminescent substrate of $Ru(bpy)_3^{2+}$–AuNPs was immobilized on the GE surface.

2.3.2. Formation of quenching monolayer of junction-probe-$Ru(bpy)_3^{2+}$–AuNPs on GE surface

5 μL of 2 μM capture probe (Cp) solution was dript onto the surface of $Ru(bpy)_3^{2+}$–AuNPs modified GE for 2 h at room temperature (Cp–$Ru(bpy)_3^{2+}$–AuNPs modified GE). Then, the electrode was washed with 10 mM PBS containing 100 mM $LiClO_4$ (pH 7.3). After that, the electrode was treated with 1 mM MCH for 1 h to obtain well aligned DNA monolayer. The electrode surface was then washed with water to remove the unbound oligonucleotides. Aptamer probe (Ap) was pre-annealed with ferrocene-labeled

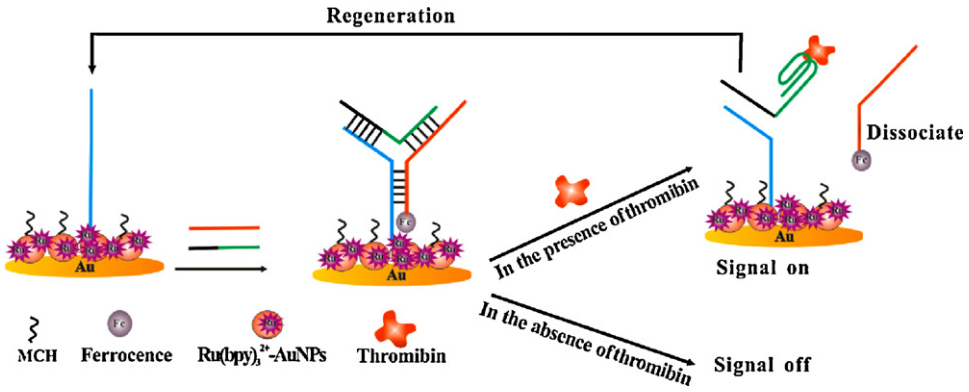

Fig. 1. Experimental principle of the junction-probe ECL aptamer biosensor for the detection of thrombin.

probe (Fp) at 30 °C for 30 min in hybridization buffer solution, and then 5 μL of the hybridization solution was placed on the surface of Cp–Ru(bpy)$_3^{2+}$–AuNPs modified GE for 1 h at room temperature. After rinsing thoroughly, a quenching monolayer of junction-probe–Ru(bpy)$_3^{2+}$–AuNPs was formed on the GE surface.

2.3.3. ECL measurement

The junction-probe–Ru(bpy)$_3^{2+}$–AuNPs modified GE (junction-probe ECL aptamer biosensor) 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 30 min. Then the electrode was washed with the same buffer to remove the un-reacted thrombin. The ECL determinations were performed at room temperature in an ECL cell with three-electrode system, which consists of above treated GE as the working electrode, an Ag/AgCl as the reference electrode and a platinum wire as auxiliary electrode. Cyclic voltammetry mode, which with a continuous potential scanning from 0.8 to 1.4 V and a scanning rate of 100 mV/s, was applied to achieve ECL signal in 20 mM PBS (pH 8.7) containing 1.0 mM TPA (a co-reactant for ECL) and 5.0 mM $LiClO_4$. The ECL and CVs curves were recorded simultaneously. The intensity of ECL signal was used for the quantitative detection of thrombin.

3. Results and discussion

3.1. The principle of the junction-probe ECL aptamer biosensor

The detailed principle of our work is illustrated in Fig. 1. As shown in Fig. 1, the ECL aptamer biosensor comprises two main parts: an ECL substrate and an ECL intensity switch. The ECL substrate was made by immobilizing a Ru(bpy)$_3^{2+}$–AuNPs composite on a cysteamine-derivatized GE. The controllable solid-state ECL film of Ru(bpy)$_3^{2+}$–AuNPs composite, which developed by Wang's group (Sun et al., 2005), has been demonstrated to have good stability, excellent ECL behavior and wonderful bio-conjunction. Moreover, the Ru(bpy)$_3^{2+}$–AuNPs film formed on the GE provide an advanced electrochemical functionality and an attractive surface for the immobilization of DNA probes. The ECL intensity switch contains three probes that were designed according to "junction-probe" strategy (Zhang et al., 2009). The first probe is capture probe (Cp, colored blue), which was functionalized with a thiol group at one end and then was covalently attached on Ru(bpy)$_3^{2+}$–AuNPs modified GE through S–Au bonding (Cp modified GE). The Cp modified GE was then blocked with 2-mercaptoethanol (MCH) to form a mixed monolayer in order to effectively prevent the nonspecific adsorption of thrombin on the electrode surface, optimize the orientation of the capture probes to make hybridization easier and displace the weaker adsorption contacts between probes and the substrates. The second probe is aptamer probe (Ap, colored green and black), which have a part of sequence of the 15-base anti-thrombin DNA aptamer (colored green). The third one is ferrocene-labeled probe (Fp, colored red), which was functionalized with ferrocene tag at one end. In the absence of thrombin, Cp, Ap and Fp will hybridize to form a ternary "Y" junction structure (junction-probe–Ru(bpy)$_3^{2+}$–AuNPs modified GE). In this case, the ferrocene was close to Ru(bpy)$_3^{2+}$–AuNPs film, which lead to a very week ECL intensity due to the high ECL quenching efficiency of ferrocene to Ru(bpy)$_3^{2+}$ (Cao et al., 2006). Whereas, in the presence of thrombin, the Ap prefers to form the G-quadruplex aptamer–thrombin complex (Macaya et al., 1993; Smirnov and Shafer, 2000) and the "Y" junction structure does not to be formed (Nakayama et al., 2008; Zhang et al., 2009). Thus, the Fp will be easily removed from the Ru(bpy)$_3^{2+}$–AuNPs modified GE by washing the GE with buffer solution and the ECL intensity will be increased greatly. The increasing ECL signal sensitively reflects the concentration of thrombin.

3.2. EIS characterization of the different stage of the junction-probe ECL aptamer biosensor preparation

In order to verify if indeed the ECL aptamer biosensor works as our expected, electrochemical impedance spectra (EIS) was investigated at different stages of the ECL aptamer biosensor preparation. EIS was measured in 10 mM PBS-10 mM $[Fe(CN)_6]^{4-/3-}$ solution (pH 7.0) in the frequency range of 0.1–10^5 Hz and Nyquist polts were used to calculate the electron-transfer resistance (*Ret*). The EIS at different GE surfaces was shown in Fig. 2. As shown in Fig. 2, the Cp–Ru(bpy)$_3^{2+}$–AuNPs modified GE showed a larger *Ret* (see Fig. 2c) in comparison with bare GE (Fig. 2a) and Ru(bpy)$_3^{2+}$–AuNPs modified GE (Fig. 2b), this is because the electrostatic repulsion between negative charges of the DNA backbone and the $Fe(CN)_6^{3-/4-}$ (Cheng et al., 2007; Jin et al., 2007).

When Cp–Ru(bpy)$_3^{2+}$–AuNPs modified GE was incubated in the solution containing Ap and Fp, Cp–Ap–Fp–Ru(bpy)$_3^{2+}$–AuNPs modified GE (junction-probe–Ru(bpy)$_3^{2+}$–AuNPs modified GE) showed a biggest *Ret* (Fig. 2e). This is because that Cp hybridized with Ap and Fp to form "Y" junction structure, and the formation of "Y" junction structure resulted in a much denser negative charges on the GE surface and generated a much stronger electrostatic repulsion between GE surface and $Fe(CN)_6^{3-/4-}$. Subsequently, we studied the change of the *Ret* in the present of thrombin. The curve d was the Nyquist plot of $Fe(CN)_6^{3-/4-}$ at the junction-probe–Ru(bpy)$_3^{2+}$–AuNPs modified GE after it reacted with thrombin, and it was clearly observed that the *Ret* of curve d was much smaller than that of curve e. This is because that in the present of thrombin, the Ap prefers to form the G-quadruplex aptamer–thrombin complex and the "Y" junction structure does not to be formed. Thus, the Ap and Fp dissociated from the elec-

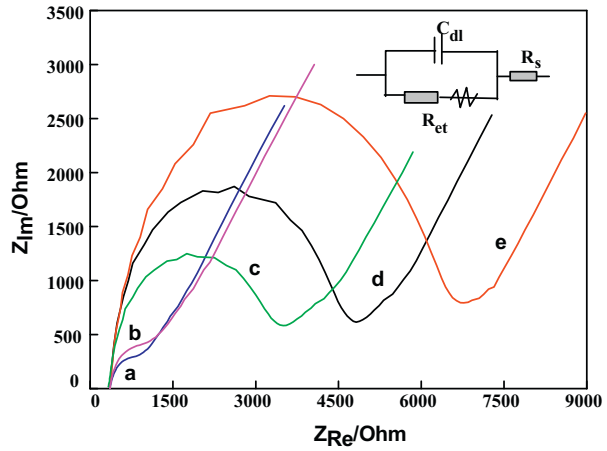

Fig. 2. (A) Impedance spectra (Nyquist plot) of bare GE (a), the $Ru(bpy)_3^{2+}$–AuNPs modified GE (b), the Cp–$Ru(bpy)_3^{2+}$–AuNPs modified GE (c), the junction-probe–$Ru(bpy)_3^{2+}$–AuNPs modified GE (e) and the junction-probe–$Ru(bpy)_3^{2+}$–AuNPs modified GE after it was incubated in the solution containing thrombin (d). The data were obtained in 10 mM $[Fe(CN)_6]^{4-/3-}$ and 10 mM PBS (pH 7.0) buffer solution. The biased potential was 0.18 V (versus Ag/AgCl) in the frequency range of 0.1–10^5 Hz and the amplitude was 5.0 mV.

trode and the negative charges on the GE surface decrease, and finally resulting in the decrease of *Ret*. The results of EIS indicated that the ECL aptamer biosensor indeed worked as our expected.

3.3. ECL characterization of the different stage of the junction-probe ECL aptamer biosensor preparation.

In this work, the junction-probe ECL aptamer biosensor was also characterized by ECL to demonstrate the modification process further. The corresponding ECL intensity vs time curves of the electrodes were presented in Fig. 3. As shown in Fig. 3, no ECL signal can be found for the bare GE (curve a), while the $Ru(bpy)_3^{2+}$–AuNPs modified GE showed a most strong ECL signal (curve e). The Cp–$Ru(bpy)_3^{2+}$–AuNPs modified GE showed a

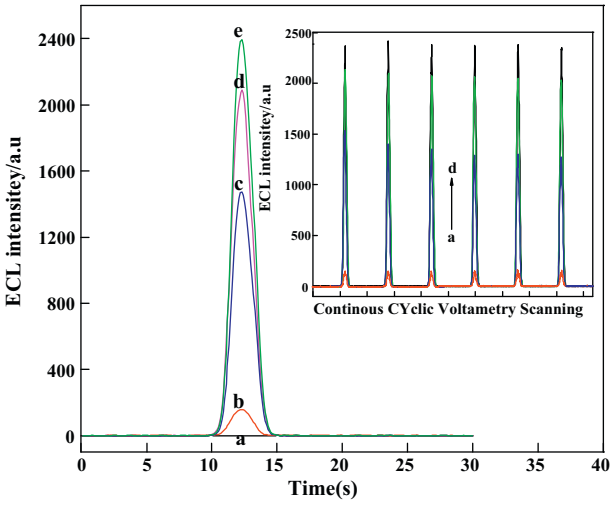

Fig. 3. ECL intensity vs time curves for the bare GE (a), $Ru(bpy)_3^{2+}$–AuNPs modified GE (e), Cp–$Ru(bpy)_3^{2+}$–AuNPs modified GE (d), junction-probe–$Ru(bpy)_3^{2+}$–AuNPs modified GE (b), and junction-probe–$Ru(bpy)_3^{2+}$–AuNPs modified GE after it was incubated in 100 pM thrombin (c). Inset: The ECL intensity of different GE under continuous CVs for six cycles. (a) Junction-probe–$Ru(bpy)_3^{2+}$–AuNPs modified GE, (b) Junction-probe–$Ru(bpy)_3^{2+}$–AuNPs modified GE after it was incubated in 100 pM thrombin, (c) Cp–$Ru(bpy)_3^{2+}$–AuNPs modified GE and (d) $Ru(bpy)_3^{2+}$–AuNPs modified GE. ECL curves were measured in 20 mM PBS containing 1.0 mM TPA and 5.0 mM $LiClO_4$ (pH 8.7). Scan rate: 0.1Vs 1, scan range: 0.0–1.4 V.

lower ECL signal (curve d) in comparison with $Ru(bpy)_3^{2+}$–AuNPs modified GE, this is because the binding of TPA (a co-reactant for ECL) to $Ru(bpy)_3^{2+}$ is hindered due to the spatial restriction of DNA self assembly monolayer. When the Cp–$Ru(bpy)_3^{2+}$–AuNPs modified GE was incubated in solutions containing Ap and Fp, the Cp brings the Ap and Fp to form "Y" junction conformation structure (junction-probe–$Ru(bpy)_3^{2+}$–AuNPs modified GE). Consequently, the Fc group was close to $Ru(bpy)_3^{2+}$–AuNPs film of GE and resulted in a significant decrease of ECL signal (curve b, compared curve b with curve d, ECL intensity decreases from 2385 to 157 and the ECL intensity was quenched by 93%.). This observation was consistent with the result obtained in the previous report (Wang et al., 2008). The mechanism for such Fc-quenching ECL of $Ru(bpy)_3^{2+}$ has been reported as the bimolecular energy and electron transfer between $Ru(bpy)_3^{2+}$ and the oxidized species of Fc, ferrocerium (Fc^+), along with suppression of radical reactions (Cao et al., 2006). It was also mentioned that the decrease in the ECL signal is also likely due to the steric/conformational effects. At such junction-probe–$Ru(bpy)_3^{2+}$–AuNPs modified electrode surface, we believe that the binding of TPA to $Ru(bpy)_3^{2+}$ is also seriously hindered due to spatial restriction of the"Y" junction configuration.

It was reported that human thrombin has two positively charged sites termed exosite I and II on the opposite sides of the protein (Cao and Tan, 2005). The 15-base anti-thrombin DNA aptamer can bind with the exosite I of thrombin with the dissociation constant (K_d) of 25 nM (Bock et al., 1992). As we mentioned above, a part of bases of the Ap are designed according to the anti-thrombin DNA aptamer sequence, which is expected to transform into G-quartet structure to combine with thrombin as displayed in Fig. 1. As shown in curve c of Fig. 3, when the junction-probe–$Ru(bpy)_3^{2+}$–AuNPs modified GE was incubated in thrombin, the ECL was significantly enhanced. Above results demonstrated that Ap prefers to form the G-quadruplex aptamer–thrombin complex in the presence of thrombin, and as a result, the "Y" junction structure does not to be formed. Thus, the Ap and Fp dissociated from the electrode and the ECL intensity was recovered. The recovery of ECL signal provided a sensing platform for the quantitative detection of thrombin. It is noteworthy that the $Ru(bpy)_3^{2+}$–AuNPs composite immobilized on the GE is very stable (see the inset of Fig. 3), the ECL intensity keeps no change even after continuous potential scanning. The results of ECL are consistent with that of EIS, indicated that our biosensor indeed works as our expected too.

3.4. The sensitivity and reproducibility of the junction-probe ECL aptamer biosensor for the thrombin detection

The surface density of capture probe seriously affects the sensitivity of aptamer biosensor by influencing the hybridization efficiency. In previous study, we have demonstrated that the surface density of 1.1×10^{12}–1.6×10^{12} molecules/cm^2 can give a stable hybridization efficiency and a highest sensitivity for junction-probe biosensor (Zhang et al., 2009). Under above surface density of Cp, the sensitivity of the ECL aptamer biosensor was investigated. The experimental results showed that ECL intensity increased with the increase of thrombin concentration. The relationship between ECL intensity and the concentrations of thrombin was shown in Fig. 4, a good linear relationship was observed between the ECL intensity and the logarithm of thrombin concentration in the range of 0.05 pM and 100 pM (as shown in the inset). The equation was

$$\Delta I_{ECL}\,(\text{a.u.}) = 368.5 \times \log C\,(\text{PM}) + 602.2$$

where I is ECL intensity and C is thrombin concentration. The regression coefficient R was 0.9965. The detection limit was calculated to be 8.0×10^{-15} M (defined as signal/noise = 3) and the relative

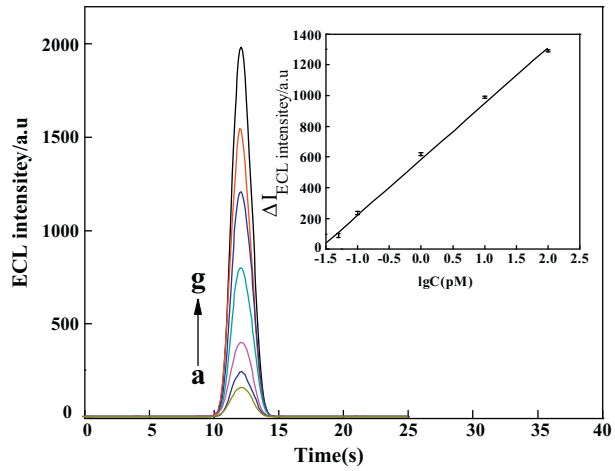

Fig. 4. The relationship between ECL intensity and thrombin concentration. The concentration of thrombin is: (a) 0.00 pM, (b) 0.05 pM, (c) 0. 1 pM, (d) 1 pM, (e) 10 pM, (f) 100 pM and (g) 1000 pM. Inset: the calibration curve of thrombin detection. Other experimental conditions are the same as those in Fig. 3.

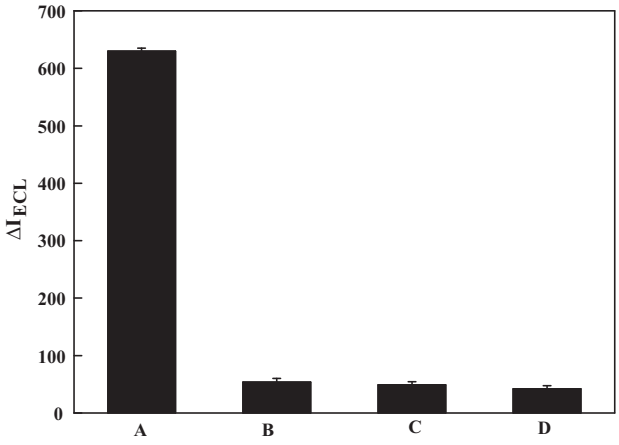

Fig. 5. ECL intensity on junction-probe–Ru(bpy)$_3^{2+}$–AuNPs modified GE after it reacted with thrombin and other three control proteins. Columns A is 1 pM thrombin, columns B is 1 nM human serum, columns C is 1 nM human IgG and columns D is 1 nM human IgA. Other experimental conditions are the same as those in Fig. 3.

standard deviation (RSD) was calculated to be 5.24% ($n = 5$) for the detection of 1 pM thrombin. The detection limit of 8.0×10^{-15} M is much lower than that of other methods such as optical (Wei et al., 2007a,b; Xiang et al., 2009) and electrochemical (Xiao et al., 2005) methods, is equal to that of the ECL aptasensor in which coupled with complicated nanomaterial-amplification technique (Li et al., 2010), indicating that the biosensor developed in this study has a high sensitivity and a good reproducibility.

So far, most ECL biosensors reported previously were developed based on the binding induced conformation change of ferrocene-labeled molecular beacon aptamer (Wang et al., 2009). In these ECL biosensors, the ECL probe (ruthenium complex-labeled aptamer) was directly immobilized on the surface of ECL substance and stayed in a relatively soft conformation, which led to a higher background and lower signal-to-noise ratio. Another shortage of these ECL biosensors is easily to give rise to "false positives", because the stem structure and internal secondary structure of the molecular beacon is unstable under certain conditions. The stem structure may gradually open to a chainlike strand, and as a result, the ferrocene molecule is far apart from the Ru(bpy)$_3^{2+}$ and the quenching of the ECL of Ru(bpy)$_3^{2+}$ becomes weak. Thus, a false ECL signal, which is not arisen from the binding of target analyte, will occur and affect the specificity of the biosensor. The proposed ECL aptamer biosensor offers the substantial advantage because all above problems were alleviated by the stable "Y" junction conformation. For this reason, the signal-on ECL strategy we described here has lower background signal and much higher sensitivity, this was demonstrated by the much lower detection limit of 8.0×10^{-15} M of our ECL biosensor in comparison with the existing ECL aptamer biosensors previously reported (Bruno and Kiel, 1999, 2002; Li et al., 2007, 2008; Wang et al., 2007).

3.5. Selectivity and regeneration of the junction-probe ECL aptamer biosensor

The specificity of the proposed ECL aptamer biosensor was also examined by detecting the ECL intensity change of the functionalized electrode in the presence of three control proteins. Fig. 5 exhibited the change of ECL signals (ΔI_{ECL}) of the biosensor after it reacted with 1 pM thrombin (A), 1 nM human serum albumin (B), 1 nM human IgG (C) and 1 nM human IgA (D), respectively. As shown in Fig. 5, only the samples containing thrombin can result in a remarkable enhancement of the ECL intensity (column A), thousand-folds of human serum albumin, human IgG and human IgA (in comparison with thrombin) only caused a neglectable enhancement on the ECL intensity (column B, C and D), indicating that the proposed ECL aptamer biosensor has a good selectivity for discriminating thrombin from other proteins.

After once determination, the ECL biosensor can be revived by rinsing it with buffer solution and de-ionized water several times, and then is used for the next cycle of hybridization and detection. We found that the ECL signal of second measurement only reduced 5.78% in comparison with first measurement, indicating that our ECL biosensor has good stability and regeneration. All about features makes our biosensor a promising alternative to conventional thrombin detection methods.

4. Conclusions

In summary, we have introduced a novel signal-on ECL aptamer biosensor for the detection of thrombin based on junction-probe. Compared with the exiting ECL aptamer sensor, which based on the binding induced conformation change of redox-labeled aptamers, the proposed ECL aptamer biosensor has the advantages of higher sensitivity, lower background current, better selectivity and reusability. These features, as well as its fabrication easiness and operation convenience, make it a promising alternative to conventional thrombin detection methods. Furthermore, such novel bio-molecular sensing switch is expected to be involved into biochip fabrication.

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