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Electrogenerated chemiluminescence aptasensor for ultrasensitive detection of thrombin incorporating an auxiliary probe

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ABSTRACT

A novel electrogenerated chemiluminescence (ECL) aptasensor for ultrasensitive detection of thrombin incorporating an auxiliary probe was designed by employing specific anti-thrombin aptamer as a capture probe and a ruthenium(II) complex-tagged reporter probe as an ECL probe and an auxiliary probe to assist the ECL probe close to the surface of the electrode. The ECL aptasensor was fabricated by self-assembling a thiolated capture probe on the surface of gold electrode and then hybridizing the ECL probe with the capture probe, and further self-assembling the auxiliary probe. When analyte thrombin was bound with the capture probe, the part of the dehybridized ECL probe was hybridized with the neighboring auxiliary probe, led to the tagged ruthenium(II) complex close to the electrode surface, resulted in great increase in the ECL intensity. The results showed that the increased ECL intensity was directly related to the logarithm of thrombin concentrations in the range from 5.0×10^{-15} M to 5.0×10^{-12} M with a detection limit of 2.0×10^{-15} M. This work demonstrates that employing an auxiliary probe which exists nearby the capture probe can enhance the sensitivity of the ECL aptasensor. This promising strategy will be extended to the design of other biosensors for detection of other proteins and genes.

1. Introduction

The detection and quantification of extremely low concentrations of specific proteins play pivotal roles in basic research and clinical applications. Aptamers are artificial oligonucleotides (DNA or RNA) that can bind to a wide variety of entities (e.g., metal ions, small organic molecules, proteins, and cells) with high affinity, equal to or often superior to that of antibodies [1–4]. In recent years, aptamers taken as molecular recognition elements have been received considerable attention in protein analysis due to their advantages such as simple synthesis, easy labeling, good stability, and wide applicability [5]. Aptasensors (aptamer-based biosensors) which fuse the exquisite sensitivity and specificity of the probes with the suitable transducers, are simple and inexpensive analytical devices that may be able to provide escalating quantities of protein information [6–9]. A variety of aptasensors including optical [10], piezoelectric [11], voltammetric [12–13], electrochemical impedimetric [14,15], and electrogenerated chemiluminescence (ECL) [16–18] aptasensor have been reported for the detection of small molecules and proteins. Among them, ECL aptasensor is a promising one owing to the combination of advantages of both electrochemical aptasensor and chemiluminescent aptasensor, such as high sensitivity and easy controllability [19–21]. However, these reported aptasensors have common detection limits in the nM range. Therefore, design of ultrasensitive aptasensors for the detection of protein biomarkers at an ultralow level in the early stage of diseases is required.

Many reports have been devoted to improve the sensitivity of ECL aptasensors for the determination of proteins. These include employment of nanomaterials, such as electrode modified materials [22], probe carriers [23–25], and signal materials [26] to enhance and to amplify signal; investigation of transduction strategies, such as target-induced strand displacement [27–29], target-binding induced conformational changes of surface confined aptamers in which the distance of ECL signal is altered [30–32]; exploration of the smart assist approaching, such as supersandwhich approachs [33], rolling circle amplification (RCA) [34], strand displacement amplification (SDA) [35] and enzyme label [36]. These signal amplification methods bear the advantage of high sensitivity, yet most of the previously reported ECL aptasensors involve complicated structural design of the capture probes, complex pretreatment of electrodes, and frequent interference from the detection environment [37]. Mao's group first reported an electrochemical aptasensor with ferrocene-tagged aptamer-complementary DNA oligonucleotides as probe [38]. Upon target (thrombin) binding, the aptamers confined onto electrode surface

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dissociate from their respective cDNA(aptamer-complementary DNA) oligonucleotides into the solution and the single-stranded cDNA could thus tend to form a hairpin structure through the hybridization of the complementary sequences at both its ends. Such a conformational change of the cDNA resulting from the target binding-induced dissociation of the aptamers essentially leads to the change in the voltammetric signal of the redox moiety labeled onto the cDNA and thus constitutes the mechanism for the electrochemical aptasensors for specific target sensing. Zhang's group demonstrated a new approach to improve the sensitivity of the electrochemical aptasensor fabricated by self-assembling a thiol functionalized capture probe containing aptamer and an auxiliary probe on the surface of gold electrode and then complementarily hybridizing a methyl blue-tagged reporter probe with the capture probe [13]. The auxiliary adjunct probe can increase the chance of the dissociative reporter probe to collide with the electrode surface and facilitating the electron transfer. The biosensor with an adjunct probe exhibits improved sensitivity and a large dynamic range for DNA and the thrombin assay. To the best of our knowledge, an ECL aptasensor was designed by employing an auxiliary probe to assist the ECL probe close to the surface of the electrode that has not been reported.

The aim of the present work is to explore the development of a highly sensitive ECL aptasensor for the detection of proteins by simply introducing an auxiliary probe. As a model system, thrombin was chosen as a model analyte since it is a common protein that catalyzes many coagulation related reactions responsible for blood clotting [39]. The fabrication of the ECL aptasensor and ECL detection of thrombin is showed in Scheme 1. The ECL aptasensor was fabricated by self-assembling a thiolated capture probe on the surface of gold electrode and then hybridizing the ECL probe with the capture probe, and further self-assembling the auxiliary probe. When the analyte thrombin was bound with the capture probe, the part of the dehybridized ECL probe was hybridized with the neighboring auxiliary probe, which led to the tagged ruthenium(II) complex becoming close to the electrode surface, and resulted in a great increase in the ECL intensity. In this paper, the function of the designed auxiliary probe is discussed and the fabrication and analytical performance of the ECL aptasensor are presented.

2. Experimental

2.1. Reagents and apparatus

All oligonucleotides used were purchased from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). Their sequences are listed as follows.

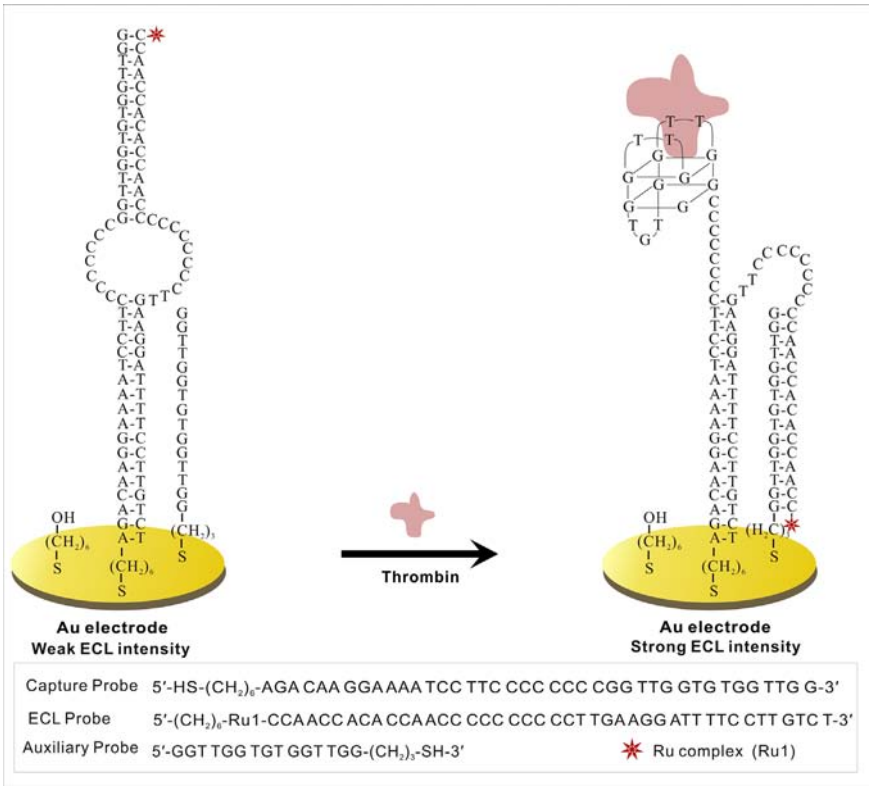
Capture probe, 5′-HS-$(CH_2)_6$-AGA CAA GGA AAA TCC TTC CCC CCC CGG TTG GTG TGG TTG G-3′ (Italic bases are the sequence of anti-thrombin aptamer).

Auxiliary probe, 5′-GGT TGG TGT GGT TGG-$(CH_2)_3$-SH-3′ (It is partly matched with the Italic part of the capture probe).

One base mismatched auxiliary probe, 5′-GGT TGG ***A***GT GGT TGG-$(CH_2)_3$-SH-3′

Reporter probe, 5′-NH_2-$(CH_2)_6$-*CCA ACC ACA CCA ACC* CCC CCC CCT TGA AGG ATT TTC CTT GTC T-3′ (Italic bases can be hybridized with the Italic part of the capture probe. The reporter probe was tagged with ruthenium(II) complex as ECL probe.).

$Ru(bpy)_2(mcbpy\text{-}O\text{-}Su\text{-}ester)(PF_6)_2$ (abbreviated as Ru1, ruthenium (II) complex), human alpha-thrombin, bovine plasma albumin (BSA), hemoglobin (Hb) and immunoglobulin G (IgG, Goat Anti-rabbit IgG) were purchased from Sigma-Aldrich, Inc. (U.S.A.) and used as received without further purification. 6-mercaptohexanol (MCH), Tri-*n*-propylamine (TPrA) and N,N-dimethylformamide (DMF) were obtained from Xi'an Chemical Reagent Factory (China). All chemical reagents were analytical grade and used without further purification. Millipore Milli-Q water (18.2 MΩ cm) was used throughout. 10 mM phosphate buffers saline (PBS, pH 7.4, 0.10 M NaCl + 10 mM NaH_2PO_4/Na_2HPO_4) was used as incubation buffer and washing buffer.



Scheme 1. Scheme for ECL detection of thrombin.

ECL measurements were performed with a MPI-E electrogenerated chemiluminescence analyzer (Xi'an Remex Analyze Instrument Co., Ltd, Xi'an, China). A commercial cylindroid glass cell was used as an ECL cell. A three-electrode system was composed of a gold electrode or a fabricated aptasensor as working electrode, a platinum plate as counter electrode, and a Ag/AgCl (saturated KCl) as reference electrode. All potentials were referred to this reference electrode.

2.2. Synthesis of the ECL probe

The ECL probe, Ru1–tagged reporter probe, was synthesized according to references [20,24] with some modification. Briefly, 2.0 OD (OD="optical density"; 1 OD at 260 nm ≈ 33 μg/mL ssDNA) of the reporter probe was dissolved in 1.00 mL of 10 mM PBS (pH 7.4). 100 μL of 9.9×10^{-3} M (1 mg/100 μL) Ru1 prepared by DMF was added to the reporter probe solution prepared above, allowed to shake at low speed at room temperature for 12 h. The Ru1–reporter probe was subsequently separated from free Ru1 complex via centrifugation using procedures described in reference [23]. 100 μL of 3.0 M sodium acetate and 2.0 mL of cold ethanol were added to the above mixture. The resulting solution was chilled for 24 h at −16 °C and then centrifuged for 30 min at 12,000 rpm. This mixture was centrifuged in a micro-centrifuge at 12,000 r/min for 30 min. The supernatant was carefully removed and the precipitate was rinsed with cold 70% ethanol for three times and dried in air. The dried precipitate was redissolved in 200 μL of the PBS. The resulting solution was used as a stock solution of the ECL probe and the working solution was diluted with PBS (pH 7.4).

2.3. Fabrication of ECL aptasensor

The gold disk electrode (2.0 mm diameter, CHI Co. Ltd., Shanghai, China) was first mechanically polished with 0.3 and 0.05 μm alumina slurry (Beuhler), followed by thorough rinsing with water for 3 min and then, this gold electrode was electrochemically cleaned using cyclic voltammetry between −0.2 and +1.5 V vs Ag/AgCl at 100 mV/s in 0.10 M H_2SO_4 until the CV characteristic of a clean Au electrode was obtained. The cleaned electrode was thoroughly rinsed with water.

The pretreated gold electrode was first immersed in 100 μL of 0.5 μM capture probe for 4 h at room temperature and then, the self-assembled electrode was immersed in 100 μL of 10 mM PBS containing 0.5 μM capture probe and 0.08 μM auxiliary probe for 2 h. The capture probe/auxiliary probe-assembled electrode was treated with 1 μM MCH at room temperature for 1 h to block the uncovered gold electrode surface. The above electrode was further immersed into 100 μL of 0.5 μM ECL probe for 1 h to obtain the ECL aptasensor. After each incubation, the electrode was washed with the washing buffer.

For control, ECL aptasensor without an auxiliary probe was fabricated following the protocol described above except without an auxiliary probe; ECL aptasensor with a 1-base mismatched auxiliary probe was fabricated following the protocol described above except that 0.08 μM auxiliary probe was replaced by 1-base mismatched auxiliary probe.

2.4. ECL measurements

The ECL aptasensor fabricated was immersed in 200 μL of a fixed concentration of thrombin for 30 min, followed by thoroughly washing with the washing buffer to remove unbound thrombin. The ECL measurement was performed with a constant potential of +0.85 V in 3.0 mL of 10 mM PBS containing 0.10 M TPrA. The cell was placed directly in front of a photomultiplier (PMT operated at −800 V) and the PMT window was only opened toward the working electrode to eliminate the blank ECL from the counter electrode. The concentration of thrombin was quantified by increased ECL intensity ($\Delta I = I_s - I_0$), where I_0 and I_s are the integrated ECL intensity in the absence and presence of thrombin, respectively. The integrated time was 30 s after the potential was applied.

3. Results and discussion

3.1. Characterization of the ECL probe

The synthesized ECL probe was characterized by UV–vis absorption spectrophotometry and the ECL technique. Fig. 1A shows UV–vis spectra of the reporter probe (line a), Ru1 (line b) and ECL probe (line c). A characteristic absorption peak of the reporter probe at ~260 nm is observed, whose concentration can be calculated with the Beer's law as ~17 μM on the basis of $\varepsilon^{260\ nm}=3.0\times10^5\ M^{-1}\ cm^{-1}$ [40], where ε is the molar extinction coefficient of reporter probe. Ru1 in PBS shows three absorption peaks at ~230, 290, and 458 nm (line b), and has a value of $\varepsilon^{458\ nm}=1.4\times10^4\ M^{-1}\ cm^{-1}$ in aqueous solution [41]. Line c of

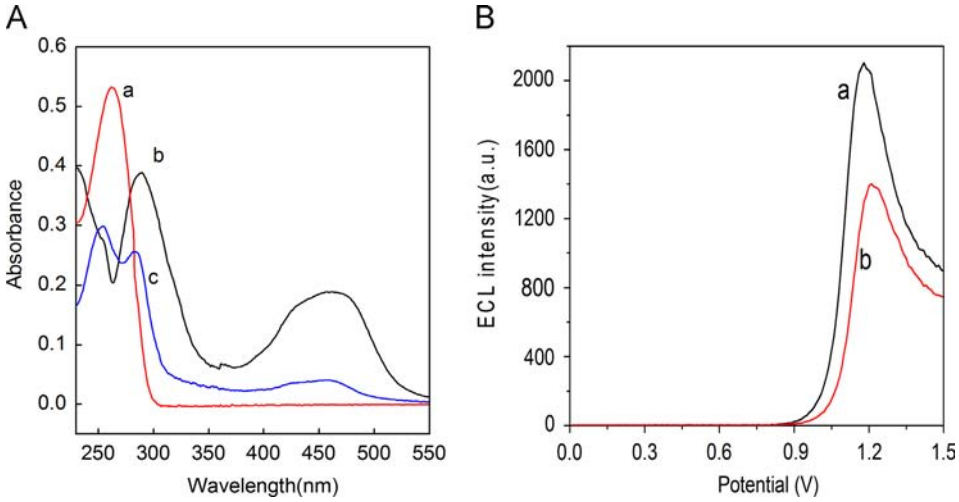


Fig. 1. (A) UV–vis absorption spectra of the reporter probe, Ru1 and ECL probe in PBS. (a) 17 μM reporter probe, (b) 130 μM Ru1, and (c) 27 μM ECL probe. A 1-mm quartz cuvette was used for all measurements. (B) ECL intensity–potential profiles of Ru1 and ECL probe. (a) 1.5×10^{-7} M Ru1; (b) 1.5×10^{-7} M ECL probe. The ECL measurements were carried out in 10 mM PBS (pH 7.4) containing 0.10 M TPrA with a scan rate of 100 mV/s.

Fig. 1A shows both the reporter probe and the Ru1 characteristic absorption peaks. This indicates that Ru1 is tagged to the reporter probe. The mole ratio of the Ru1 to the reporter probe in the ECL probe was calculated to be approximately 1:1 on the basis of the data shown in Fig. 1A (c) with the absorbance values at λ_{max}=260 nm for reporter probe and at λ_{max}=458 nm for Ru1 as well as their respective ε values. The concentration of the ECL probe was estimated to be 27 μM according to the Beer's law [40].

The ECL probe was also characterized by the ECL method at a gold electrode using a triangular potential waveform. Fig. 1B shows ECL intensity–potential profiles of Ru1 (a), and Ru1-tagged reporter probe (b) in 10 mM PBS containing 0.10 M TPrA. Their peak height is 2104 and 1401, respectively. Both ECL profiles display the characteristic ECL behavior of the aqueous $Ru(bpy)_3^{2+}$/TPrA system [42]. The ECL intensity obtained from Ru1 solution at 1.18 V is 1.5 times stronger than that of Ru1-tagged reporter probe at 1.21 V, although the two tested solutions have the same Ru1 concentration of 10 μM. From Fig. 1B, it can be calculated that the ECL peak intensity of ECL probe is 67% than that of Ru1, and the peak potential shift is 0.03 V from +1.18 V (Ru1) to +1.21 V.

3.2. Function of the auxiliary probe in the ECL aptasensor

To improve the detection sensitivity, an auxiliary probe nearby the capture probe (Scheme 1) is introduced. A ruthenium(II) complex-tagged reporter probe is also introduced to hybridize with the capture probe. The formation of rigid, duplex DNA prevents the ruthenium(II) complex tag from approaching the electrode surface, decreasing the chance of the dissociative element of the ECL probe to collide with the electrode surface. After incubation with thrombin, the anti-thrombin aptamer sequence bound thrombin by forming G-quadruplex and led to conformational change of the anti-thrombin aptamer. The auxiliary probe attached nearby the capture probe functions as a fixer to immobilize the flexible ECL probe which is displaced by the hybridization of target thrombin with the capture probe, consequently increasing the chance of the dissociative unwinding part of the ECL probe to collide with the electrode surface and producing a readily detectable ECL signal.

To check the effect of auxiliary probe on the ECL intensity of ECL aptasensors, both ECL aptasensors with an auxiliary probe and without an auxiliary probe were fabricated. As shown in Fig. 2A, B, after interaction with 5.0×10^{-14} M thrombin, the integrated ECL intensity of the aptasensor with an auxiliary probe increased from 76,593 to 269,256 (Fig. 2A), while the integrated ECL intensity of aptasensor without an auxiliary probe increased from 76,593 to 104,116 (Fig. 2B). In other words, ΔI of the ECL aptasensor with an auxiliary probe was approximately 7 times higher than ΔI of ECL aptasensors without an auxiliary probe, indicating that an auxiliary probe can greatly increase the ECL response. However, the signal increase was improved by as much as 3.2 times in the electrochemical biosensor with an adjunct probe compared to that without an adjunct probe [13]. The increase in ECL response could be attributed to the immobilization of flexible ECL probe onto the electrode surface by the hybridization of the auxiliary probe with the unwinding part of the ECL probe, which greatly shortened the distance between Ru1 and the electrode and facilitated the electron transfer and resulted in great increase in the ECL intensity.

To further understand the reason of improved sensitivity by the introduction of an auxiliary probe, the effect of both sequence and concentration of auxiliary probes upon the detection sensitivity was checked. Both 1-base mismatched and perfect matched auxiliary probes for the ECL probe were employed. ECL aptasensors with 1-base mismatched auxiliary probe were fabricated according to the protocol described in Section 2.3. After ECL aptasensors interacted with 1.0×10^{-13} M target thrombin, the ECL signal from the aptasensor with a 1-base mismatched auxiliary probe increased by as much as 10% as compared to that without an auxiliary probe. In contrast, the ECL signal from the aptasensor with a perfect matched auxiliary probe improved by as much as 53% as compared to that without an auxiliary probe. These results demonstrated that the sequence of the auxiliary probes played an important role in the detection sensitivity of the ECL aptasensor. The slight increase of ECL signals in the ECL aptasensor with a 1-base mismatched auxiliary probe is attributed to the fact that its bonding force with the ECL probe is weaker than that of matched auxiliary probe with the ECL probe.

To study the effect of the concentration of the auxiliary probe upon the detection sensitivity, we modified the electrode surface with different concentrations (0.01, 0.02, 0.05, 0.08, 0.1, and 0.12 μM) of perfect matched auxiliary probes. Fig. 3 shows the variance of ECL signal with the concentration of the auxiliary probes in response to 1.0×10^{-13} M thrombin. The ECL intensity of the ECL aptasensor before (curve *a*) and after (curve *b*) interaction with 1.0×10^{-13} M thrombin was examined. The nearly steady state ECL intensity of ECL aptasensor (curve *a*) is observed from the aptasensor in the range from 0.02 to 0.12 μM, while ECL intensity of ECL aptasensor (curve *b*) increases with increasing concentrations of auxiliary probe from 0.02 to 0.08 μM, but beyond the concentration of 0.08 μM, the ECL intensity slowly decreased with the increase of auxiliary probe concentration. The decrease of ECL intensity with the auxiliary probe concentration beyond 0.08 μM might result from that the high-concentration

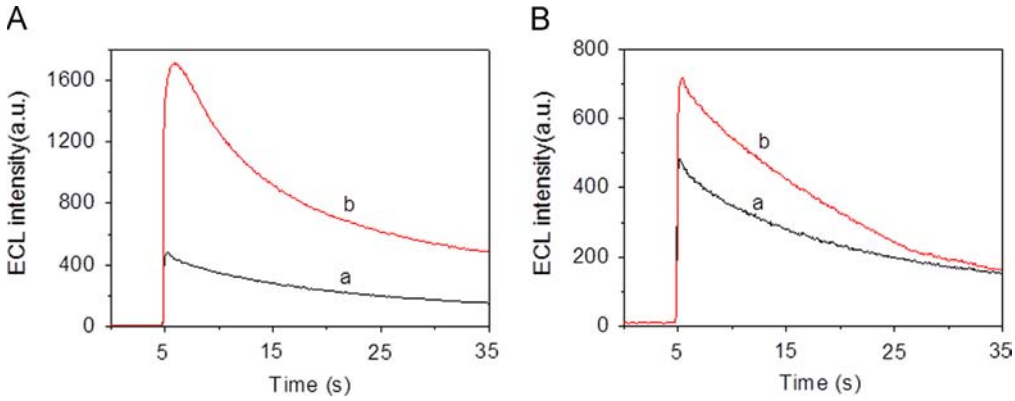


Fig. 2. The ECL signals obtained (a) before and (b) after treated with 5.0×10^{-14} M target thrombin. (A) The ECL signals from the ECL aptasensor with an auxiliary probe. (B) The ECL signals from the ECL aptasensor without an auxiliary probe. ECL measurements are performed in 10 mM PBS containing 0.10 M TPrA. Applied potential, +0.85 V. Integration time 30 s from 5 s to 35 s.

auxiliary probes might weaken the density of the capture probes on the electrode surface and increase the steric hindrance of the microenvironment, adversely preventing the dissociative ECL probe from colliding with the electrode surface [43]. The improvement of ECL signal with the increase of auxiliary probe concentration could be easily explained by the function of the auxiliary probes to capture more of the dissociative ECL probe onto the electrode surface and to facilitate the electron transfer. These results demonstrated that the concentration of the auxiliary probes played a critical role in detecting sensitivity of the ECL aptasensor. Therefore, 0.08 μM of auxiliary probe was used in the following experiments.

3.3. Optimization of hybridization time and binding time

The ECL measurement for the ECL aptasensor was performed with a constant potential of +0.85 V. The potential has the benefit of reusability of the probe because the thiol–gold bond is easily broken at potentials higher than +1.0 V [30].

Important experimental parameters including hybridization time and binding time were optimized to obtain a high sensitivity of the fabricated ECL aptasensors. The hybridization time between the capture probe and the ECL probe was checked since it can influence the hybridization efficiency and the succeeding response to thrombin (Fig. 4A). The result showed that the ECL intensity increases with an increase of hybridization time from 5 to 60 min and reached equilibrium more than 60 min, which is in accordance with recent reports ranging from 60 min to 70 min [44,45]. To ensure the completeness of hybridization between the capture probe and the ECL probe, 70 min was set as the hybridization time.

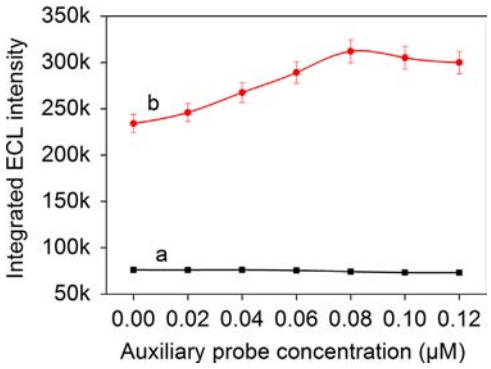


Fig. 3. Dependence of the ECL intensity on the concentration of perfect matched auxiliary probes. (a) before and (b) after treated with 1.0×10^{-13} M target thrombin. ECL measurement conditions were the same as in Fig. 2. The error bars show the standard deviation of three replicate determinations.

Beside the effect of hybridization time between the capture probe and the ECL probe, it was found that a different binding time of thrombin caused a visible difference in the increase of ECL intensity. Fig. 4B shows the dependence of the ECL intensity on binding time of the ECL aptasensor with the target thrombin. The ECL intensity of the ECL aptasensor after interaction with 1.0×10^{-14} M thrombin was examined. ECL intensity greatly increased from 0 to 25 min and then tended to stabilize from 25 to 40 min. Therefore, 30 min was chosen as the binding time of ECL aptasensors with thrombin.

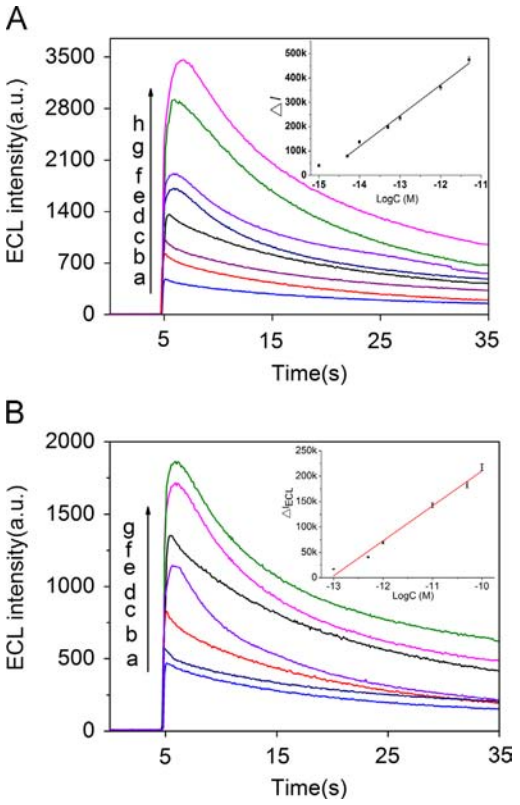


Fig. 5. (A) ECL response of the ECL aptasensor after interaction with different concentrations of thrombin. (a) blank, (b) 1.0×10^{-15} M, (c) 5.0×10^{-15} M, (d) 1.0×10^{-14} M, (e) 5.0×10^{-14} M, (f) 1.0×10^{-13} M, (g) 1.0×10^{-12} M, and (h) 5.0×10^{-12} M. (B) ECL response of the ECL aptasensor after interaction with different concentrations of thrombin. (a) blank, (b) 1.0×10^{-13} M, (c) 5.0×10^{-13} M, (d) 1.0×10^{-12} M, (e) 1.0×10^{-11} M, (f) 5.0×10^{-11} M, and (g) 1.0×10^{-10} M. Inset: calibration curve for thrombin. ECL measurement conditions were the same as in Fig. 2.

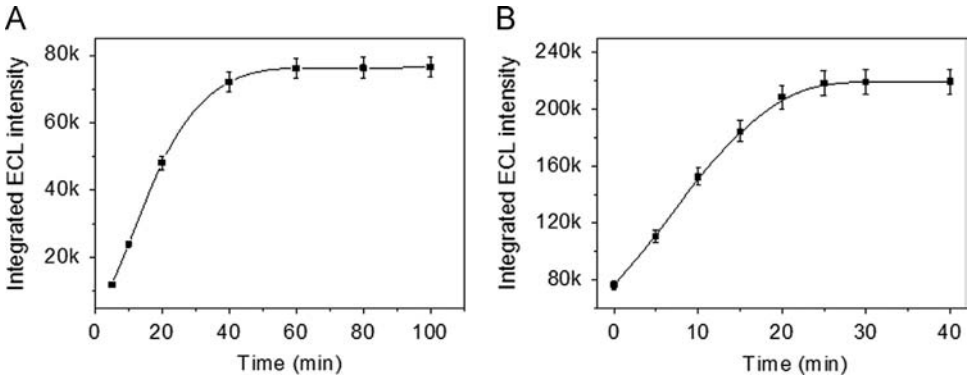


Fig. 4. (A) Dependence of the ECL intensity on hybridization time between capture probe and ECL probe. 0.5 μM capture probe, 0.5 μM ECL probe, binding time 30 min, hybridization temperature 37 °C. (B) Dependence of the ECL intensity on binding time between thrombin and aptasensor after the ECL aptasensor interaction with 1.0×10^{-14} M thrombin. ECL measurement conditions were the same as in Fig. 2.

Table 1
Detection limits for thrombin using different aptasensors.

Methods	Amplification	Rang of Linear	Limit of detection	References
ECL	No	5.0 fM–5.0 pM	2.0 fM	This work
ECL	No	0.05–50 pM	0.02 pM	[17]
ECL	Gold nanoparticle	56–900 nM	10 nM	[16]
ECL	Gold nanoparticle Carbon nanotube	2.7 pM–2.7 nM 10 fM–10 pM	0.80 pM 3.0 fM	[44]
ECL	Gold nanoparticle	100 aM–100 fM	26 aM	[46]
EIS	Gold nanoparticle	0.12–30 nM	0.06 nM	[14]
EIS	No	1.0–20 ng/mL	0.30 ng/mL	[15]
Voltammetric	No	3.0–80 nM	3.0 nM	[12]
SWV	No	0.01–10 nM.	20 pM	[13]

Note: ECL, electrogenerated chemiluminescence; EIS, electrochemical impedance spectroscopy; SWV, square wave voltammetry.

Table 2
Analytical results for different serum samples.

Samples	Added thrombin concentration/M	Concentration found/M	Recovery/ %	RSD/%, $n=3$
1	1.0×10^{-14}	0.93×10^{-14}	93.0	6.2
2	1.0×10^{-13}	1.06×10^{-13}	106	5.6
3	1.0×10^{-12}	1.08×10^{-12}	108	4.3

3.4. Performance of the ECL aptasensor

Fig. 5A shows the ECL responses of the ECL aptasensor after interaction with different concentrations of thrombin measured by the aptasensor with an auxiliary probe. From inset of Fig. 5A, it can be seen that the ECL signal increase with increasing concentration of thrombin and the increased integrated ECL intensity was directly related to the logarithm of thrombin concentration in the range from 5.0×10^{-15} M to 5.0×10^{-12} M. The regression equation was $\Delta I=1.9\times10^{6}+1.3\times10^{5}\log C$ (M) with a correlation coefficient 0.9949. The detection limit was 2.0×10^{-15} M using $3s/S$, where s was the standard deviation of the blank solution with 11 parallel measurements, and S (1.9×10^{6}) was the slope of the calibration curve.

Fig. 5B shows the ECL profiles of the ECL aptasensor without an auxiliary probe after interaction with different concentrations of thrombin. The results showed that the increased integrated ECL intensity of aptasensor had a linear relationship with the increasing concentration of thrombin in the range from 1.0×10^{-13} M to 1.0×10^{-10} M. The regression equation was $\Delta I=9.0\times10^{5}+6.9\times10^{4}\log C$ (M) with a correlation coefficient 0.9909. The detection limit was 3.3×10^{-14} M, which was 17 fold higher than that at the ECL aptasensor with an auxiliary probe. Therefore, a high sensitivity of the ECL aptasensor developed with an auxiliary probe was obtained. The low detection limit was attributed to the introduction of an auxiliary probe in the ECL aptasensor, which made more ECL signal compound Ru1 immobilized onto the electrode surface and facilitated the electron-transfer from the Ru1 to the electrode.

As shown in Table 1, the detection limit of the ECL aptasensor in this work was lower than those of the previous methods for thrombin detection except Wang's work [46]. The precision was estimated for 1.0×10^{-13} M thrombin with 7 ECL aptasensors and yielded reproducible results with a relative standard deviation (RSD) of 3.7%.

The potential application of the ECL aptasensor with an auxiliary probe was also checked using a standard addition method [17]. A series of samples were prepared by adding thrombin of different concentrations to human blood serum (obtained from Hospital of Shaanxi Normal University, China). Table 2 shows the acceptable relative standard deviation and quantitative recoveries, indicating that the present aptasensor can be potentially used in biological samples.

The specificity of the as-fabricated ECL aptasensor was examined by detecting sample solution containing 1 nM BSA, 1 nM IgG, 1 nM Hb and 1 pM thrombin under the same experimental conditions. Only thrombin gave an ECL response even though the thrombin concentration was 1000 times less than the concentration of the negative control proteins. The ΔI for BSA, IgG and Hb were 4%, 7%, and 8% of that for thrombin, respectively. The cross sensitivity of the aptasensor in a mixture of four different proteins containing thrombin was also examined. The signal obtained from the mixture was similar to that obtained from pure thrombin solution. These tests indicated that the developed strategy could be used to identify thrombin with high specificity. The result was in agreement with that the previously reported unique G-quartet conformation formed by the thrombin-specific aptamer sequence which is quite specific to thrombin [47]. This may be explained by the fact that the ruthenium complex label has not initiated the biodegradation and denaturation of anti-thrombin aptamer and the splitting of the aptamer into two segments as well as an additional folded structure was eliminated in the present strategy. Therefore, the recognition activities of the aptamer was maintained.

4. Conclusions

A highly sensitive ECL aptasensor for the detection of thrombin is developed by employing ruthenium complex as an ECL label and an auxiliary probe as a fixer to immobilize the dissociative Ru1 onto the electrode surface. The ruthenium complex is not labeled on the anti-thrombin aptamer so that the recognition activities of the aptamer are not altered. The auxiliary probe can capture the ECL probe which is released when the target protein binds with the anti-thrombin aptamer, increasing the chance of ECL reagent to collide with the electrode surface and facilitating the electron transfer. The aptasensors with an auxiliary probe have exhibited a sensitive detection of thrombin with lower detection limit of 2.0×10^{-15} M and a high specificity. Importantly, the use of this aptasensor is not limited to such application to proteins detection and could provide a promising platform for aptamer-based small molecule detection.

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