Analytica Chimica Acta 715 (2012) 86–92

Contents lists available at SciVerse ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Electrochemiluminescence biosensor for the assay of small molecule and protein based on bifunctional aptamer and chemiluminescent functionalized gold nanoparticles

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ARTICLE INFO

Article history:
Received 9 September 2011
Received in revised form
20 November 2011
Accepted 5 December 2011
Available online 14 December 2011

Keywords:
Electrochemiluminescence
N-(Aminobutyl)-*N*-(ethylisoluminol)
functionalized gold nanoparticles
Bifunctional aptamer
Adenosine
Thrombin
Biosensor

ABSTRACT

An electrochemiluminescence (ECL) biosensor for simultaneous detection of adenosine and thrombin in one sample based on bifunctional aptamer and *N*-(aminobutyl)-*N*-(ethylisoluminol) functionalized gold nanoparticles (ABEI-AuNPs) was developed. A streptavidin coated gold nanoparticles modified electrode was utilized to immobilize biotinylated bifunctional aptamer (ATA), which consisted of adenosine and thrombin aptamer. The ATA performed as recognition element of capture probe. For adenosine detection, ABEI-AuNPs labeled hybridization probe with a partial complementary sequence of ATA reacted with ATA, leading to a strong ECL response of *N*-(aminobutyl)-*N*-(ethylisoluminol) enriched on ABEI-AuNPs. After recognition of adenosine, the hybridization probe was displaced by adenosine and ECL signal declined. The decrease of ECL signal was in proportion to the concentration of adenosine over the range of 5.0×10^{-12}–5.0×10^{-9} M with a detection limit of 2.2×10^{-12} M. For thrombin detection, thrombin was assembled on ATA modified electrode via aptamer–target recognition, another aptamer of thrombin tagged with ABEI-AuNPs was bounded to another reactive site of thrombin, producing ECL signals. The ECL intensity was linearly with the concentration of thrombin from 5×10^{-14} M to 5×10^{-10} M with a detection limit of 1.2×10^{-14} M. In the ECL biosensor, adenosine and thrombin can be detected when they coexisted in one sample and a multi-analytes assay was established. The sensitivity of the present biosensor is superior to most available aptasensors for adenosine and thrombin. The biosensor also showed good selectivity towards the targets. Being challenged in real plasma sample, the biosensor was confirmed to be a good prospect for multi-analytes assay of small molecules and proteins in biological samples.

1. Introduction

The development of sensitive and selective high-throughput biosensors for simultaneous detection of two or more analytes within a single sample remains an extremely important goal in fields such as biomedical diagnostics, environmental monitoring, industrial process and quality control [1–3]. A variety of biosensors have been developed for multi-analytes detection of proteins, nucleic acids, tumors, toxins, and small molecules [4–6].

Aptamer based detection methods are attractive because of their high sensitivity, selectivity to the target molecule and good stability in complex physical and chemical environments. Many aptasensors for small molecules, proteins, even cells have been developed [7] and most efforts were devoted in single target detection [8,9]. The construction of multi-component sensing system that can give different signals in the presence of different molecules is highly desired [10,11] and two dominant modes of multi-analytes assay have been employed in aptasensors. For example, micro beads, microarray chip based aptamer arrays [12–14] for multiplexed protein measurements were developed, which relied on different aptamer recognition elements assembled on solid supports. Multi-label mode was also employed in aptamer based multi-analytes assay [15,16]. For example, aptamer-based chemiluminescence biosensing platform for cocaine and adenosine was reported by Yan et al. using alkaline phosphatase and horseradish peroxidase multi-labeling [17].

Recently, the combination of two aptamers for designing new molecule ligands or novel functionalities has been reported [18]. Two aptamers binding to different targets have been integrated into one sequence, which was used as recognition element for biosensor and provided a facile way for the detection of multiple analytes in one sample. The bifunctional aptamers have found growing interests in biosensing field and their multifunctional properties have been widely studied [19,20] due to its higher affinity, inhibitory activity than single aptamers [18] and simultaneous sensing ability of multiple targets. Up to now, several bifunctional

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doi:10.1016/j.aca.2011.12.006

aptamer involved biosensors have been fabricated, which mainly based on electrochemistry methods [21]. The parallel analysis of two analytes using a bifunctional aptamer has been demonstrated. Du et al. [22] reported a label-free electrochemical biosensor based on bifunctional aptamer for parallel detection of adenosine triphosphate and thrombin using electrochemical impedance spectroscopy. A sensitive biosensor for adenosine and lysozyme using $[Ru(NH_3)_6]^{3+}$ as signal transducer and gold nanoparticles (AuNPs) amplification was reported by Deng et al. [23]. Such sensing interfaces were not suitable for the simultaneous detection of multi-targets in one sample. More recently, a bifunctional aptamer probe was used for the determination of adenosine and thrombin in one spot by coupling the bio-bar-code based multi-labeling and anodic stripping voltammetry in Li's work [24]. Despite of the low detection limits in picomolar range and one-spot detection, such multi-labeling bears the disadvantages of complicated and cost procedure.

Owing the advantages of low back ground, being easily control and high sensitivity, electrochemiluminescence (ECL) has become an important and promising method for biosensors. However, to our knowledge, few papers have been published on ECL methodologies for the analysis of multiple targets using bifunctional aptamer. Recently, in our group, luminol functionalized AuNPs and *N*-(aminobutyl)-*N*-(ethylisoluminol) functionalized AuNPs (ABEI-AuNPs) with excellent ECL activity have been synthesized [25,26] and used as ECL labels to fabricate bio-probe for the determination of single target such as human immunoglobulin G (hIgG) [27], single-stranded DNA [28], human platelet-derived factor BB (PDGF-BB) [29] and cardiac troponin I [30]. Herein, we reported on a novel strategy to develop a sensitive ECL biosensor based on bifunctional aptamer probe and ABEI-AuNPs labeling for the detection of adenosine and thrombin, which play fundamental role in life related processes. The bifunctional aptamer for adenosine and thrombin (ATA) was used as recognition element of capture probe. ABEI-AuNPs were used as universal ECL label for the detection of both analytes. The hybridization probe with a partial complementary sequence of ATA and another thrombin aptamer (TA 2) were designed for the detection of adenosine and thrombin by virtue of competition reaction and aptamer–target recognition, respectively. The labeled ABEI-AuNPs on the hybridization probe and TA 2 served as ECL signal reporters. The conditions for the detection of adenosine and thrombin were optimized. The analytical performance and the selectivity of this sensor were studied. Finally, the applicability of the sensor in real plasma samples was explored.

2. Experimental

2.1. Materials and reagents

The 5′-biotinylated ATA (5′-GGTTGGGTGGTTGGACCTGG-GGGAGTATTGC GGAGGAAGGT-3′), 5′-biotinylated hybridization probe (5′-ACCTTCCTCCGC-3′), and 5′-biotinylated TA 2 (5′-AGTCCGTGGTAGGGCAGGTTGGGGTGACT-3′) were purchased from Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China) and purified by HPLC. ABEI was purchased from TCI Co. Ltd. (Tokyo, Japan). ABEI stock solution was prepared by dissolving ABEI in 0.1 M NaOH and kept at 4 °C. $HAuCl_4$ stock solution (2% $HAuCl_4$, w/w) was prepared by dissolving 1.0 g of $HAuCl_4{\cdot}4H_2O$ (Shanghai Reagent, China) in 412 mL of purified water and stored at 4 °C. Adenosine, uridine, guanine, thymine were purchased from Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China). Thrombin was purchased from Sigma (St. Louis, MO). PDGF-BB was purchased from Prospe-Tany Ltd. (York, UK). Streptavidin (SA), bovine serum albumin (BSA), Hemoglobin (Hb), glucose oxidase (GOx), hIgG were all obtained from Solarbio (Beijing, China). The human thrombin ELISA Kit was purchased from Y-Y Chemical Reagents (Shanghai, China). Different concentrations of adenosine and thrombin were prepared in the assemble buffer (20 mM pH 7.4 Tris–HCl containing 0.1 M NaCl and 5.0 mM $MgCl_2$). All other chemicals were of analytical grade. Ultrapure water was prepared by a Milli-Q (Millipore, France) system and used throughout.

2.2. Preparation of SA coated AuNPs modified electrode

The bare gold electrode (Ø 3.0 mm) was polished to mirror-like surface with chamois leather, rinsed with ethanol and ultra-pure water. The electrode was then cleaned by cycling on a CHI760B electrochemical workstation (Chenhua, China) between 0 and 1.5 V vs SCE in H_2SO_4 (0.5 M) at a scan rate of 100 mV s^{-1} until reproducible cyclic voltammograms were obtained. As-pretreated bare gold electrode was immersed in a 5 mM 1,3-propanedithiol ethanol solution and incubated at room temperature for 20 h. The thiol self-assembled monolayer (SAM) on the surface of the gold electrode was rinsed with ethanol and ultrapure water. 1,3-Propandithiol was chosen for the assembly of SAM due to its relatively shorter carbon chain and the SAM formed on the electrode would have smaller contact resistance [31]. Our previous work found that it took at least 14 h for 1,3-propandithiol to form SAM on the gold substrate. And in this experiment, 20 h were chosen to ensure formation of dense SAM on the electrode. After that, 60 μL aliquots of SA coated AuNPs colloid (Supplementary information) were dropped on the thiol self-assembled gold electrode for 4 h at 4 °C. After rinsing with ultrapure water, the SA coated AuNPs modified electrode was ready for further experiments.

2.3. Preparation of ABEI AuNPs labeled hybridization probe and TA 2 probe

The ABEI-AuNPs used in this work were synthesized according to Tian's work [26] with some modifications. ABEI-AuNPs with a diameter around 15 nm were prepared and the gold colloid was stored at 4 °C for future use. 1 mL pH-adjusted (pH 7.0) ABEI-AuNPs solution was added to 25 μL of SA (1.0 mg mL^{-1}), after incubated at room temperature for 0.5 h, the solution was further added with 5% BSA (w/w) solution to the final concentration of 1% BSA, then stirred for 5 min. The conjugate of ABEI-AuNPs with SA was centrifuged at 12,500 rpm (Universal 320, Hettich, Germany) for 20 min, and the red precipitates were dispersed with 200 μL 0.05 M Tris–HCl (pH 8.0) containing 0.3 M NaCl. 23 μL biotinylated hybridization probe and 23 μL biotinylated TA 2 (1×10^{-5} M, to a final concentration of 1×10^{-6} M) was added to the red solution and incubated at 37 °C for 1 h, respectively. The as-prepared mixture was centrifuged at 12,500 rpm for 10 min, and the red precipitates were suspended with assemble buffer to obtain ABEI-AuNPs labeled hybridization probe and TA 2 probe.

2.4. Preparation of the electrode modified with bifunctional aptamer probe

60 μL aliquots of the biotinylated ATA as capture probe (4×10^{-6} M) in assemble buffer were dropped with a pipette on the SA coated AuNPs modified electrode at 37 °C for 1 h and rinsed by washing buffer (10 mM pH 7.4 Tris–HCl containing 0.1 M NaCl) to remove the absorbed capture probe. Then 60 μL aliquots of 1% BSA in assemble buffer were dropped with a pipette on the modified electrode to block both the electrode and AuNPs, the electrode was incubated at 37 °C for 30 min and rinsed with washing buffer. The ATA modified electrode was ready for further experiments.

2.5. Detection of adenosine and thrombin using the ECL sensor

For adenosine detection, 60 μL aliquots of the ABEI-AuNPs labeled hybridization probe were dropped with a pipette on the ATA modified electrode at 37 °C for 1 h and rinsed with washing buffer. The ECL signal I_1 was detected. After that, 60 μL aliquots of adenosine contained solution or the mixed sample of adenosine and thrombin were dropped on the modified electrode at 37 °C for 1 h and rinsed with washing buffer. The hybridization probe labeled with ABEI-AuNPs was replaced by adenosine to form the adenosine–aptamer complex. A remarkable decrease of ECL signals on modified electrode was observed and ECL signal I_2 was detected. In this way, the ΔI ($\Delta I = I_1 - I_2$) of two events was obtained and can be used to quantify adenosine.

For thrombin detection, 60 μL aliquots of thrombin contained solution or the mixed sample of adenosine and thrombin were dropped on the ATA modified electrode at 37 °C for 1 h and rinsed with washing buffer. 60 μL aliquots of the ABEI-AuNPs labeled TA 2 probe were then dropped with a pipette on the modified electrode at 37 °C for 1 h and rinsed with washing buffer. The "aptamer/protein/aptamer conjugate" was obtained on the electrode and the ECL signal I_3 was recorded for the determination of thrombin.

2.6. ECL measurement

ECL measurements were carried out with a homemade ECL system consisted of a model CHI760B workstation, a model RFL-1 luminometer (Remax, Xi'an, China), an H-type electrochemical cell (self-designed) and a computer. A three-electrode system was used, being composed of a disk gold electrode modified with sensing interface as working electrode, a platinum wire as counter electrode, and a silver wire as the quasi-reference electrode (AgQRE). A 1.75 mM H_2O_2 solution containing 0.02 M carbonate buffer solution (CBS, pH 10.2) was used as working solution for the detection of adenosine and thrombin. During measurements, a 3.0 mL portion of the working solution and blank solution without H_2O_2 were added to the working compartment and the auxiliary compartment of the ECL cell, respectively. When a double-step potential (30 s pulse period, 0.1 s pulse time, 0.8 V pulse potential and 0 V initial potential) was applied to the working electrode, an ECL signal was generated and recorded.

3. Results and discussion

3.1. Strategy for ECL biosensor

Fig. 1 depicted schematically the assay protocol of the proposed ECL biosensor using bifunctional aptamer and ABEI-AuNPs labeling. First, 1,3-propanedithiol was self-assembled on the surface of gold electrode to form monolayer. The SA coated AuNPs were immobilized on 1,3-propanedithiol modified gold electrode, and then the biotinylated ATA probe was subsequently assembled on the modified electrode via biotin-SA bio-amplification system. The detection of adenosine and thrombin was carried out on separate electrode, respectively. For the detection of adenosine, as shown in Fig. 1 protocol A, the hybridization probe labeled with ABEI-AuNPs was first interacted with ATA assembled on the electrode. The ECL signal I_1 was detected. After adding adenosine, as aptamer can bind tightly to target molecules to form a tertiary complex with a binding constant greater than DNA duplex, the target–aptamer induced strand displacement of hybridization probe happened and the hybridization probe labeled with ABEI-AuNPs was replaced by the target to form the target–aptamer complex. A remarkable decrease of ECL signals on modified electrode was observed and ECL signal I_2 was detected. In this way, the ΔI ($\Delta I = I_1 - I_2$) of two events was obtained and can be used to quantify adenosine. For the detection of thrombin (Fig. 1 protocol B), after the binding of thrombin with ATA assembled on the electrode via target–aptamer recognition, ABEI-AuNPs labeled TA 2 probes were further combined with thrombin due to its two aptamer binding sites, the "aptamer/protein/aptamer conjugate" was eventually formed on the electrode and the ECL signal I_3 was recorded for the determination of thrombin.

3.2. Characterization of the modified electrode

Electrochemical impedance spectra (Fig. S1 of supplementary information) was used to monitor the modification procedures of ECL biosensor. The results showed that the electrodes modified with sensing interfaces (i.e. ECL biosensor) were fabricated as expected. The ECL behavior of the modified electrodes was studied with a double-step potential in 0.02 M CBS containing 1.75 mM H_2O_2 as shown in Fig. 2. For the detection of adenosine, as can been seen from Fig. 2A, no ECL responses were observed on bare gold electrode (curve a), 1,3-propanedithiol self-assembled electrode (curve b), SA coated AuNPs modified electrode (curve c) and ATA probe/SA coated AuNPs modified electrode (curve d). After the hybridization of ABEI-AuNPs labeled hybridization probe with ATA, strong ECL signals were observed (curve e), indicating that the ABEI-AuNPs labeled hybridization probe was successfully assembled on the electrode and the ECL signals were from ABEI-AuNPs. In the presence of adenosine, a decrease in ECL intensity (curve f) revealed that binding of adenosine to the duplex DNA resulted in the duplex dissociation and the reducing of ABEI-AuNPs assembled on the electrode. For the detection of thrombin, as shown in Fig. 2B, curve a–d was consistent with that of Fig. 2A. After the electrode was treated with 1×10^{-12} M thrombin, still no ECL response was observed (curve e). However, when ABEI-AuNPs labeled TA 2 probe was bounded to thrombin via aptamer–target recognition, ECL signals were detected on the electrode (curve f), demonstrating that the ECL signals were also from ABEI-AuNPs. These results revealed that the electrodes modified with sensing interfaces were fabricated as expected and the detection of adenosine and thrombin could be realized according to Fig. 1, protocol A and B. It can be seen that the pulse ECL signals remained stable in more than five pulses, thus the average intensity of five pulse ECL signal was used as an analytical signal for the analysis.

3.3. Detection of adenosine and thrombin coexisted in one sample

As mentioned before, a prominent advantage of the bifunctional aptamer based biosensor is its ability for flexible screening of adenosine and thrombin coexisted in one sample. As shown in the two protocols of Fig. 1, for adenosine detection in protocol A, when thrombin coexisted with adenosine, no ECL signal was generated from presence of thrombin because no ABEI-AuNPs labeled TA 2 was adopted in this protocol. The ECL signal change in protocol A arose only from adenosine. For the determination of thrombin, protocol B is employed. The ECL signal of protocol B was only from thrombin because ABEI-AuNPs labeled hybridization probe was not adopted and ECL signal change induced by adenosine would not occur. Therefore, the cross talk of two targets was evaluated by comparing the ECL responses in the presence of only one analyte with those in the presence of both analytes. As can be seen from Fig. 3A, when adenosine coexisted with 1×10^{-11} M thrombin in solution, compared with ΔI gained from pure adenosine solution, ΔI changed 1.7%, 2.2%, 0.6%, 0.2%, 0.7% for adenosine concentration of 1×10^{-11} M, 5×10^{-11} M, 1×10^{-10} M, 5×10^{-10} M, 1×10^{-9} M, respectively. In the presence of 1×10^{-10} M thrombin, 1.5%, 1.7%, 1.1%, 2.3%, 0.4% ΔI changes were observed for the adenosine concentration of 1×10^{-11} M, 5×10^{-11} M, 1×10^{-10} M, 5×10^{-10} M,

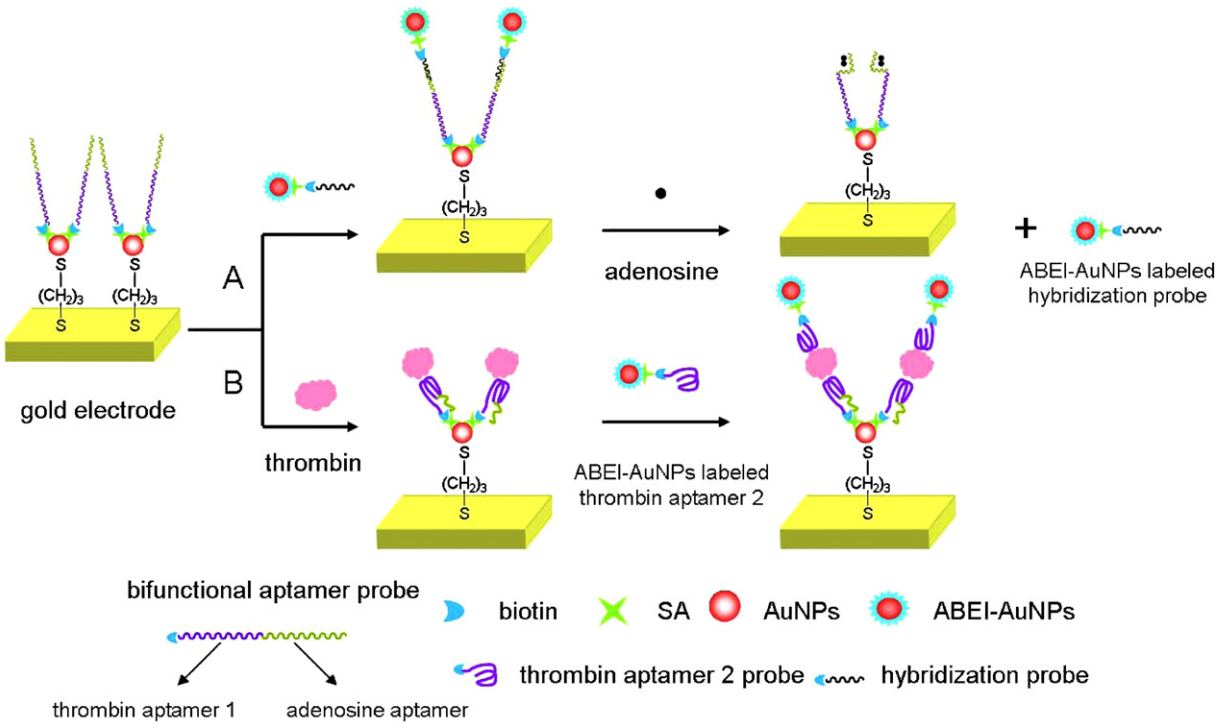

Fig. 1. Schematic diagram for the ECL biosensor fabrication based on ATA and ABEI-AuNPs labeling. (A) Protocol for the detection of adenosine. (B) Protocol for the detection of thrombin.

1×10^{-9} M, respectively. These results showed that the existence of thrombin in sample solution would not affect the detection of adenosine in protocol A. For the detection of thrombin, the similar results were observed (Fig. 3B). For the thrombin concentration of 5×10^{-14} M, 1×10^{-13} M, 1×10^{-12} M, 1×10^{-11} M, 1×10^{-10} M, only 0.3%, 0.9%, 0.1%, 0.9%, 0.2% of I_3 changes were observed in the presence of 1×10^{-10} M adenosine, respectively. And only 1.4%, 1.5%, 0.6%, 0.2%, 0.7% of I_3 changes were observed in the presence of 1×10^{-9} M adenosine, respectively. The negligible change of I_3 indicated that the existence of adenosine would not affect the detection of thrombin as well. These results revealed that the proposed ECL biosensor can be used for the detection of adenosine and thrombin coexisted in one sample solution.

3.4. Sensitivity of adenosine and thrombin detection

Important parameters of double-potential steps and CBS working solution were optimized (data not shown). The optimized experimental conditions were 0 V for initial potential, 30 s for pulse period, 0.1 s for pulse time, 0.8 V for pulse potential, pH 10.2 CBS and 1.75 mM H_2O_2 concentration. The quantitative behavior of the fabricated ECL biosensor for adenosine was assessed by measuring the dependence of ΔI upon the concentration of adenosine under the optimized experimental conditions (Fig. 4A). The ΔI was linear with the logarithm of concentration of adenosine over the range of 5.0×10^{-12}–5.0×10^{-9} M. The linear regression equation was $\Delta I = -258 + 810 \times \log C$ (where C was adenosine concentration

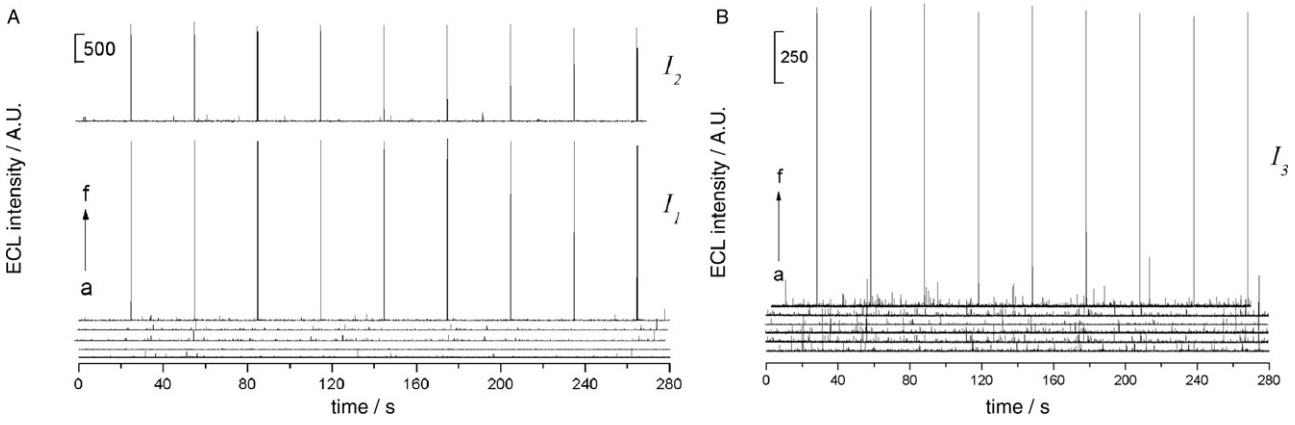

Fig. 2. ECL signals under pulse potential for the detection of adenosine and thrombin. (A) ECL signals obtained on (a) bare gold electrode, (b) 1,3-propanedithiol/gold electrode, (c) SA coated AuNPs/1,3-propanedithiol/gold electrode, (d) ATA probe/SA coated AuNPs/1,3-propanedithiol/gold electrode, (e) ABEI-AuNPs labeled hybridization probe/ATA probe/SA coated AuNPs/1,3-propanedithiol/gold electrode, and (f) adenosine (1×10^{-10} M) and ABEI-AuNPs labeled hybridization probe/ATA probe/SA coated AuNPs/1,3-propanedithiol/gold electrode. (B) ECL signals obtained on (a) bare gold electrode, (b) 1,3-propanedithiol/gold electrode, (c) SA coated AuNPs/1,3-propanedithiol/gold electrode, (d) ATA probe/SA coated AuNPs/1,3-propanedithiol/gold electrode, (e) thrombin (1×10^{-12} M)/ATA probe/SA coated AuNPs/1,3-propanedithiol/gold electrode, and (f) ABEI-AuNPs labeled TA 2 probe/thrombin (1×10^{-12} M)/ATA probe/SA coated AuNPs/1,3-propanedithiol/gold electrode. Initial potential, 0 V; pulse period, 30 s; pulse time, 0.1 s; pulse potential, 0.8 V. All ECL signals were measured in 0.02 M CBS (pH 10.2) solution containing 1.75 mM H_2O_2.

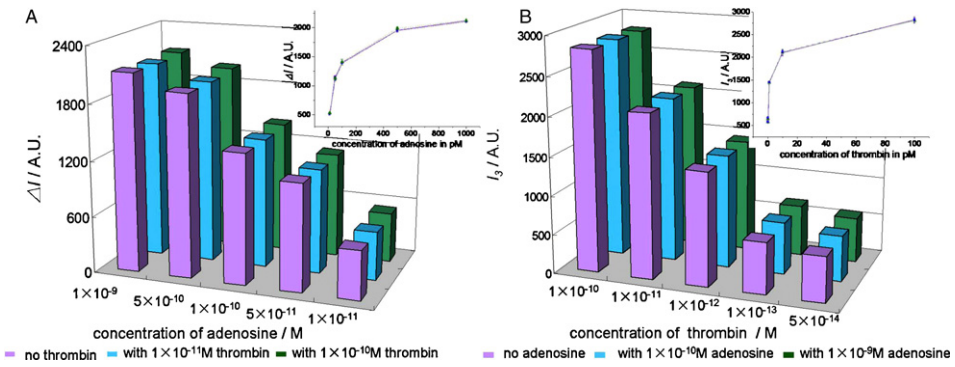

Fig. 3. (A) Effect of thrombin with different concentration on ΔI intensity. (B) Effect of adenosine with different concentration on I_3 intensity. Initial potential, 0 V; pulse period, 30 s; pulse time, 0.1 s; pulse potential, 0.8 V. All ECL signals were measured in 0.02 M CBS (pH 10.2) solution containing 1.75 mM H_2O_2.

in pM) with a correlation coefficient of $R = 0.998$. The detection limit for adenosine (3σ, $n = 5$) was estimated to be 2.2×10^{-12} M, which was more sensitive than most available adenosine aptasensors [24,32]. And the quantitative behavior of the ECL biosensor for thrombin was assessed by measuring the dependence of I_3 upon the concentration of thrombin under the optimized experimental conditions (Fig. 4B). The I_3 was linear with the logarithm of concentration of thrombin over the range of 5.0×10^{-14}–5.0×10^{-10} M. The linear regression equation was $I_3 = 1374 + 696 \times \log C$ (where C was thrombin concentration in pM) with a correlation coefficient of $R = 0.999$. The detection limit for thrombin (3σ, $n = 5$) was estimated to be 1.2×10^{-14} M, which was 1000-fold lower than literature value achieved by ECL-quenching mechanism based aptasensor using $[Ru(bpy)_3]^{2+}$ as a label [33]. It was notable that the high sensitivity of the ECL biosensor was owing to that the enriched ABEI on ABEI-AuNPs amplified the ECL signal and the AuNPs greatly enhanced the ECL of ABEI [34]. A series of five repetitive measurements of the 1.0×10^{-10} M adenosine and 1.0×10^{-12} M thrombin solution yielded reproducible ECL responses with coefficients of deviation of 2.1% and 4.5%, respectively, reflecting the good reproducibility of the biosensor.

3.5. Selectivity of the ECL biosensor

The ECL biosensor was assumed to be highly selective towards adenosine and thrombin as the sensing was relied on target–aptamer recognition. If other small molecules or proteins instead of adenosine or thrombin were employed on the modified electrode, ATA would not form a tertiary complex for the target sensing. Thus, the selectivity of the ECL biosensor was investigated as shown in Fig. 5. Guanine, thymine, uridine belong to the nucleoside family and were employed to assess the selectivity of the ECL biosensor for the detection of adenosine. As shown in Fig. 5A, for the same concentration (0.1 nM), only adenosine exhibited strong ΔI, while ΔI of only 27.2% for guanine, −5.1% for thymine, −20.7% for uridine, respectively, was obtained compared with ΔI from adenosine. The ECL signal shown in Fig. 5A curve f was ECL response I_1. As we can see, there was no distinct difference between the signals of blank measurement and I_1, which indicated that no non-target-induced displacement of ABEI-AuNPs labeled hybridization probe happened in our ECL biosensor for the detection of adenosine. On the other hand, Hb, PDGF-BB, GOx, hIgG were used as control proteins for the detection of thrombin. As can be seen in Fig. 5B, a strong ECL signal I_3 was observed for thrombin, whereas I_3 of only 18.8% for Hb, 18.3% for PDGF-BB, 16.5% for GOx, 10.1% for hIgG was observed on the modified electrode incubated with Hb, PDGF-BB, GOx, hIgG (all in 1.0 pM), respectively. These results demonstrate that the developed biosensor is selective for the detection of adenosine and thrombin.

3.6. Application of the ECL biosensor for the detection of adenosine and thrombin in human plasma sample

To test the validation of the ECL biosensor for the real sample matrix, analysis of adenosine and thrombin in human plasma samples were implemented. It has been reported that adenosine

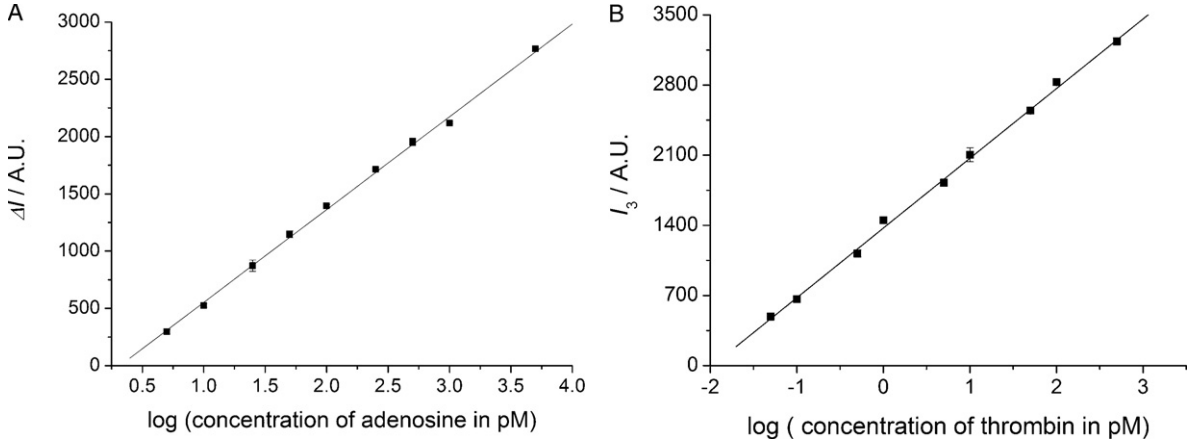

Fig. 4. (A) The linear relationship between the ECL response and logarithm of the adenosine concentration in pM. (B) The linear relationship between the ECL response and logarithm of the thrombin concentration in pM. Initial potential, 0 V; pulse period, 30 s; pulse time, 0.1 s; pulse potential, 0.8 V. All ECL signals were measured in 0.02 M CBS (pH 10.2) solution containing 1.75 mM H_2O_2.

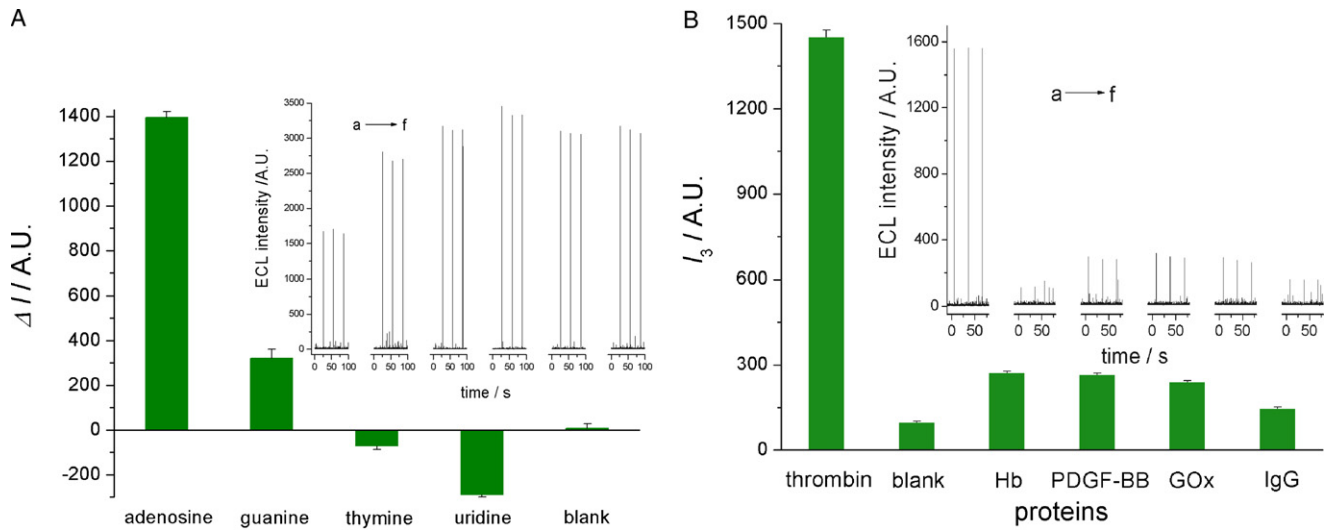

Fig. 5. Selectivity of the proposed ECL biosensor. (A) A comparison of ΔI intensity for adenosine, guanine, thymine, uridine, blank. Insert: ECL response of the modified electrode incubated with (a) adenosine, (b) guanine, (c) thymine, (d) uridine, (e) blank, and (f) the ECL signals of I_1. The concentrations of adenosine, guanine, thymine, uridine were all 0.1 nM. (B) A comparison of I_3 intensity for thrombin, blank, Hb, PDGF-BB, GOx, hIgG. Insert: ECL response of the modified electrode incubated with (a) thrombin, (b) blank, (c) Hb, (d) PDGF-BB, (e) GOx, and (f) hIgG. The concentrations of thrombin, Hb, PDGF-BB, GOx, hIgG were all 1 pM. Initial potential, 0 V; pulse period, 30 s; pulse time, 0.1 s; pulse potential, 0.8 V. All ECL signals were measured in 0.02 M CBS (pH 10.2) solution containing 1.75 mM H_2O_2.

concentration in normal human blood ranges between 0.05 and 0.1 μM [35], while the physiological concentrations of thrombin in resting and activated blood range from low nonomolar to low micromolar [24,36]. Considering the high sensitivity of the method, the pretreated human blood plasma sample was diluted 1000 times before analysis. 10 human plasma samples collected from 10 people at the Hospital of University of Science and Technology of China (pretreated, see in Supplementary information) were measured using the proposed ECL biosensor. For the five samples shown in Table 1, adenosine was observed in the range of 57.9–97.7 nM and thrombin was detected in the range of 3.3–5.2 nM. The results for the determination of adenosine by the present ECL sensor were compared with HPLC analysis (see the Supplementary information for details) [37,38] as shown in Table 1, the relative deviations of the results between these two methods were less than 5.5%, indicating the two methods were comparable. On the other hands, the thrombin content in the same five samples was also measured by human thrombin ELISA Kit as the reference method. As shown in Table 1, the thrombin concentration obtained by the ECL sensor was also in good agreement with those determined by the ELISA method and the relative deviations were not more than 9.1%. These results indicated that the proposed ECL biosensor is feasible to detect adenosine and thrombin in real human plasma samples. The recovery of the method in plasma samples was also investigated in another five human plasma samples (data shown in Table 2). The five samples in Table 2 were diluted and the standard solutions of adenosine and thrombin with appropriate concentration were added. Adenosine was observed in the range of 49.6–99.1 nM and thrombin was detected in the range of 3.4–4.9 nM in these samples. The recoveries were from 98% to 105% for adenosine detection and from 98% to 103% for thrombin detection, respectively. The results demonstrate that this ECL biosensor is applicable for the

Table 1
A comparison of the proposed ECL biosensor for the detection of adenosine with HPLC method and for the detection of thrombin with ELISA method in human plasma samples.

Sample[a]	Adenosine concentration (nM)		Relative deviation (%)	Thrombin concentration (nM)		Relative deviation (%)
	ECL sensor[b]	HPLC		ECL sensor[b]	ELISA	
1	71.7 ± 2.0	75.8	5.5	4.8 ± 0.4	4.4	9.1
2	65.3 ± 3.7	65.9	0.9	3.3 ± 0.2	3.4	2.9
3	57.9 ± 5.4	55.3	4.7	5.2 ± 0.8	5.0	4.0
4	60.9 ± 2.1	61.2	0.5	3.7 ± 0.6	4.3	5.7
5	97.7 ± 7.2	94.0	3.9	4.3 ± 0.2	3.5	0

[a] The five samples were from patients at the Hospital of University of Science and Technology of China.
[b] The data were given as average value ± SD obtained from five independent experiments, $n = 5$.

Table 2
The recovery assay of adenosine and thrombin using the ECL sensor in plasma samples.

Plasma sample[a]	Adenosine found (pM)[b]	Adenosine added (pM)	Total adenosine detected (pM)[a]	Recovery (%)	Thrombin found (pM)[b]	Thrombin added (pM)	Total thrombin detected (pM)[a]	Recovery (%)
6	53.7 ± 2.4	500.0	559.7 ± 19.5	101	3.4 ± 0.5	20.0	23.7 ± 1.6	102
7	49.6 ± 2.7	500.0	575.4 ± 6.7	105	3.5 ± 0.5	20.0	23.1 ± 1.6	98
8	83.2 ± 2.8	500.0	581.0 ± 23.6	99	3.7 ± 0.6	20.0	23.6 ± 1.1	100
9	99.1 ± 2.5	500.0	588.8 ± 23.7	98	3.7 ± 0.5	20.0	24.3 ± 2.6	103
10	63.1 ± 2.9	500.0	572.8 ± 23.2	102	4.9 ± 0.5	20.0	25.1 ± 3.9	101

[a] The five samples were from patients at the Hospital of University of Science and Technology of China.
[b] The data given are the detection results from 1000-fold diluted human plasma samples.

simultaneous determination of adenosine and thrombin in real sample matrix.

4. Conclusions

In conclusion, a bifunctional aptamer and ABEI-AuNPs based ECL biosensor for simultaneous detection of adenosine and thrombin in one sample has been developed. For adenosine detection, the linear range was 5.0×10^{-12}–5.0×10^{-9} M and the detection limit was 2.2×10^{-12} M. For thrombin detection, the linear range was 5.0×10^{-14}–5.0×10^{-10} M and the detection limit was 1.2×10^{-14} M. The proposed ECL biosensor has several advantages. (1) The bifuntional aptamer probe was not only linked on electrode as capture probe, but also used as recognition element for both adenosine and thrombin sensing, which was for the first time applied to ECL biosensor. (2) ABEI-AuNPs labeled on signal probes acted as signal reporters for the detection of both adenosine and thrombin. ABEI-AuNPs exhibited excellent ECL signal amplification as number of ABEI molecules were enriched on the surface of AuNPs. Compared with other labeling method in multi-analytes assay, such as enzyme labels and "bio-bar code" method, ABEI-AuNPs labeling was highly sensitive, simple, stable, low-cost and time-saving. (3) The sensing of two targets has no cross talk and the ECL biosensor can be used for the detection of both adenosine and thrombin coexisted in one sample. Due to the wide target recognition range of aptamer, this strategy provides a new approach for ECL aptasensor design and brings a new model for simultaneous detection of small molecules and proteins coexisted in one sample. Having been challenged in real plasma samples, the ECL biosensor also showed applicability in real biological samples. Therefore, it is believable that the present ECL biosensor could make a significant contribution to the multi-analytes assay of biological small molecules and proteins in clinical and research fields.

Acknowledgements

The support of this research by the National Natural Science Foundation of PR China (Grant Nos. 20625517, 20573101 and 21075115), the Fundamental Research Funds for the Central Universities (Grant No. WK2060190007) and the Overseas Outstanding Young Scientist Program of Chinese Academy of Sciences are gratefully acknowledged.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aca.2011.12.006.

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