Signal-on electrochemiluminescence biosensor for thrombin based on target-induced conjunction of split aptamer fragments†

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Received 14th April 2010, Accepted 10th May 2010
First published as an Advance Article on the web 7th June 2010
DOI: 10.1039/c0cc00932f

A highly sensitive and selective electrochemiluminescence biosensor for detection of thrombin based on the strategy of target-induced conjunction of split aptamer fragments was developed.

Aptamers are specific DNA or RNA strands obtained from random–sequence nucleic acid libraries by an *in vitro* evolution process called SELEX (systematic evolution of ligands by exponential enrichment).[1,2] Aptamers could recognize a wide array of targets, including proteins, small molecules, peptides, and even cells with high affinity and specificity. In many aspects, the binding performance of aptamers could rival that of antibodies.[3,4] However, compared with antibodies, aptamers possess a number of advantages over antibodies such as simple synthesis, easy labeling and good stability. Hence, aptamers have attracted increasing interest as the biorecognition element in biosensors.

Hereinto, the thrombin-binding aptamer (15-mer,5′-GGTTGG-TGTG GTTGG-3′),[5] the first DNA aptamer isolated from vivo selection, has been extensively investigated and coupled to biosensors for thrombin detection.[6] Electrochemical,[7–12] fluorescence,[13–16] absorbance,[17] colorimetric[18,19] and electrochemiluminescence (ECL)[20–22] sensors have been reported, the majority of which rely on a conformational change of the aptamer induced by the specific target binding, resulting in the change of detection signals. Specially, the $Ru(bpy)_3^{2+}$-based ECL sensors,[20–22] which have many advantages such as simple equipment, low detection limits, have been paid more and more attention in recent years.[23]

Recently, conjunction of split aptamer fragments has been used to develop sensing, nanotechnology and logic gate applications.[24,25] Here, we designed a novel thrombin aptamer ECL sensor based on target-induced conjunction of split aptamer fragments. Ruthenium complexes, especially $Ru(bpy)_3^{2+}$ and its derivatives (such as *N*-hydroxysuccinimide (NHS) ester and phosphoramidite conjugates[26]) were usually used as the markers for aptasensors.[27–29] But the synthesis procedures for these reagents were difficult, which limited their application. Hence, a more simple and convenient complex needs to be developed. Because of their ease of synthesis and functionalization, and possessing evidently amplifying signal effect, $Ru(bpy)_3^{2+}$-doped silica nanoparticles (Ru-SNPs) have been paid much attention in recent years.[30–33]

In this work, Ru-SNPs were applied to develop an aptamer-based ECL sensor for thrombin detection. To fabricate the aptasensor, we chose the 15-base antithrombin aptamer that had been reported to bind with the Exosite I of thrombin with a high dissociation constant (Kd).[5,34,35] Here, we split the sequence of 15-base aptamer into two different fragments, capture probe and detection probe. The capture probe was modified with thiol and immobilized on the gold electrode by the interaction of S–Au; the detection probe functionalized with Ru-SNPs was conjuncted with the capture probe to form a G-quadruplex structure in the presence of thrombin. The principle of the ECL biosensor was shown in Fig. 1. The capture probe with an alkanethiol moiety at the 5′-terminus covalently immobilized on a gold electrode through Au–S bond, the detection probe was labeled with Ru-SNPs which was coupled with an amido at the 3′-terminus. In the absence of thrombin, the capture probe interacted weakly with the detection probe, the two DNAs exited mainly in the dissociated form and the detection probe with ECL reagent could be easily removed *via* washing, resulting in a weak ECL signal, so the background signals were low. In the presence of thrombin, the capture probe combined with the detection probe and bound with thrombin to form a G-quadruplex structure, allowing the ECL reagent to attach on the surface of the electrode, resulting in the enhancement of ECL signals on the electrode and which could be readily detected. Compared with previous methods,[20–22] this method could decrease the background signal since there is no ECL reagent in the system before the introduction of thrombin.

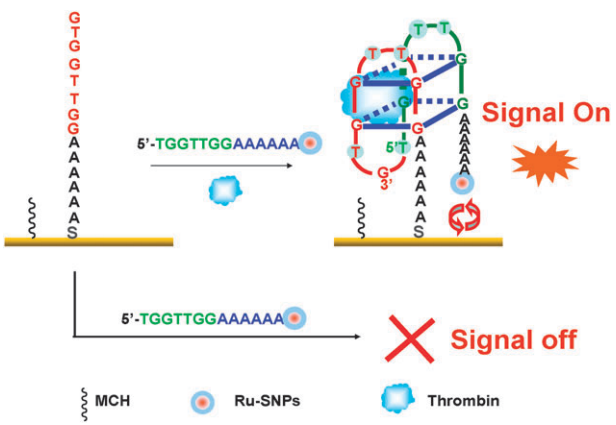

Fig. 1 Principle of the aptamer-based ECL sensor for thrombin detection.

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† Electronic supplementary information (ESI) available: Experimental details, sensor Preparation, ECL Measurements. See DOI: 10.1039/c0cc00932f

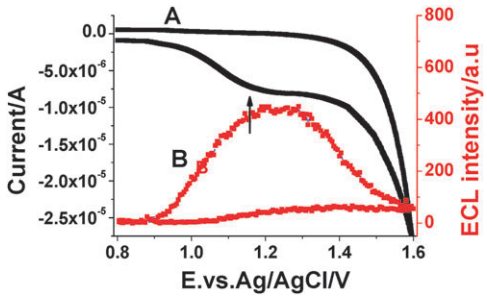

Fig. 2 CV (curve A) and the corresponding ECL-potential curve (curve B) of the Ru-SNPs on the modified electrode. Medium: Tris-HCl (pH 7.4), 5×10^{-3} mol L^{-1} DBAE. Scan rate: 50 mV s^{-1}.

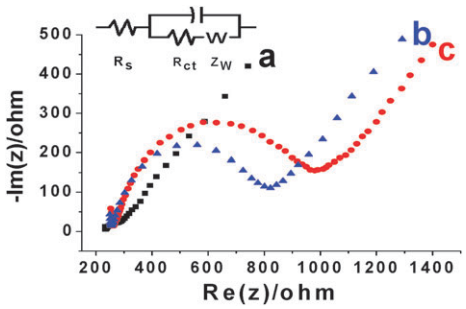

Fig. 3 Nyquist plots corresponding to the different Au electrodes (a) Bare gold electrode; (b) Capture probe modified gold electrode; (c) Capture probe/thrombin/detection probe modified gold electrode. The data was obtained in the presence of 5.0 mmol L^{-1} $[Fe(CN)_6]^{3-/4-}$ containing 0.5 mol L^{-1} KCL, and the biased potential was 0.218 V, applying amplitude 5 mV in the frequency range of 1–10kHz. Inset shows the equivalent circuit.

Cyclic voltammetry (CV) and ECL were used to show the characters of Ru-SNPs. As shown in Fig. 2, curves A and B were the CV curve and the corresponding ECL response of gold electrode modified with Ru-SNPs at the scan rate of 50 mV s^{-1} in Tris-HCl (pH 7.4) containing 5×10^{-3} mol L^{-1} 2-(dibutylamino) ethanol (DBAE, acts as a coreaction reagent[36]). No ECL response could be found by scanning the potential from 0.80 to 0.95 V, while the ECL intensity achieved its maximum when the potential reached 1.20 V, an obvious oxidation current could be seen (pointed by the arrow) at this potential because the $Ru(bpy)_3^{2+}$ was electrochemically oxidized. The above results implied that the electrochemical and ECL properties of Ru-SNPs were similar to that of $Ru(bpy)_3^{2+}$.

The difference stages of the electrode surface modification could be characterized by faradic electrochemical impedance spectroscopy (EIS). Fig. 3 showed the Nyquist plots of impedance spectra at different electrode surfaces in the 5.0 mmol L^{-1} $Fe(CN)_6^{3-/4-}$ containing 0.1 mol L^{-1} KCL. Curves a, b, and c are Nyquist diagrams of $Fe(CN)_6^{3-/4-}$ on the bare gold electrode, capture probe modified gold electrode, and the capture probe/detection probe modified gold electrode after reaction with 10^{-11} mol L^{-1} thrombin, respectively. Furthermore, the equivalent circuit, as the model of the work electrode (shown in the inset) was used to offer a direct way for us to get more information from EIS results. The equivalent circuit contained the electrolyte solution resistance R_s, the surface electron transfer resistance R_{ct} that reflected the surface property of the surface of the electrode, the Warburg impedance Z_w and the constant phase element related to double layer capacitance C_{dl}.[37] Curve a showed the Nyquist diagram of the bare gold electrode had almost no small semicircle domain at high frequencies and the value of R_{ct} was 51.4 Ω, indicating that the electrons on the surface of the electrode were going on very fast. When the capture probe through thiol–Au interaction immobilized on the electrode, the R_{ct} value was enhanced to a much larger value (curve b, 510.3 Ω), the reason lies in that the negative charged phosphate backbone of a single oligonucleotide assembled on the gold electrode could prevent the negative charged redox probe $Fe(CN)_6^{3-/4-}$ from reaching the electrode and inhibited interfacial charge transfer. After thrombin and the detection probe were captured on the surface of the Au electrode, there was an increase of the R_{ct} value (curve c, 753.5 Ω), originating from the attachment of thrombin and the detection probe caused more negative charges on the electrode surface and enhanced R_{ct}, leading to the response of Fe $(CN)_6^{3-/4-}$ anion decreasing, so the value of R_{ct} of curve c was larger than that of curve b.

The ECL intensity of the biosensor enhanced with the increase of thrombin concentration. Fig. 4(A) showed the ECL response of the sensor after reacting with the different concentrations of thrombin in the 2 mL Tris-HCl (pH 7.4) containing 5×10^{-3} mol L^{-1} DBAE under CV scanning. In the absence of thrombin, the ECL intensity was weak, showing that the capture probe and detection probe were in the dissociated form and few Ru-SNPs had been modified on the electrode surface. With increasing thrombin concentration, ECL intensity increased, which can be attributed to the G-quadruplex structure formation, resulting in Ru-SNPs immobilized on the electrode surface. The ECL intensity had a linear relationship with the logarithm of thrombin concentration in the range of 3.3×10^{-13}–3.3×10^{-11} mol L^{-1} (shown in Fig. 4(B)) and the regression equation was

$$I/a.u. = 185.54 \log C + 2387.97$$

Where I represented ECL intensity, and C was the thrombin concentration. The regression coefficient R of this regression equation was 0.9986 and detection limit was 2.0×10^{-13} mol L^{-1}, which was lower than the previous reported thrombin ECL aptsensor.[38]

The ECL responses (at 3.3×10^{-11} mol L^{-1} thrombin) of the modified electrode in Tris-HCl (pH 7.4) containing 5×10^{-3} mol L^{-1} DBAE under continuous CV scanning were examined (see Fig. S1 in supporting informations (SI) for detail†), the relative standard deviation (RSD) of the ECL response ($n = 6$) was found to be 5.2%. The reproducibility of 6 different modified electrodes was also examined, and the RSD was found to be 5.8%. Moreover, if the sensors were stored at 4 °C for several weeks, they still remained bioactive, and the ECL signal only reduced 5%, showing that this biosensor has good stability and high reproducibility.

In order to confirm that the ECL signal was caused just by the amount of thrombin and the aptasensor was just responsive to thrombin, bovine serum albumin (BSA), bovine hemoglobin (BHb) and immumnoglobulin G (IgG) were used to examine the selectivity of the sensor (see Fig. S2 in SI for detail†). The concentrations of the three interferents were

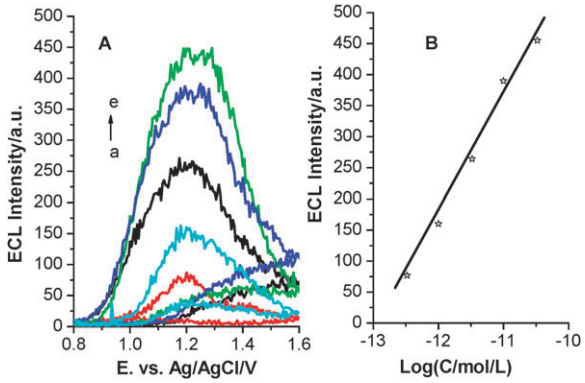

Fig. 4 (A) ECL–potential curves at different thrombin concentrations. (a)–(e): 3.3×10^{-13}; 1.0×10^{-12}; 3.3×10^{-12}; 1.0×10^{-11}; 3.3×10^{-11} mol L^{-1}. (B) the calibration curve between the ECL intensity and the thrombin concentration. The other conditions were the same as in Fig. 2.

5.0×10^{-8} mol L^{-1}, but the concentration of thrombin was only 3.3×10^{-11} mol L^{-1}, and we found that the ECL intensity in the presence of thrombin was much stronger than those of the others, which meant that BSA, BHb and IgG had no influence on the aptasensor for detection of thrombin, therefore this ECL biosensor has good selectivity for thrombin.

In summary, a novel highly sensitive and selective ECL biosensor was developed by splitting aptamer and target-induced conjunction of aptamer fragments which form a G-rich structure for the detection of thrombin. The thrombin aptamer was split into two suitable segments, the capture probe and the detection probe which was modified with an ECL reagent (Ru-SNPs). In the presence of thrombin, a guanine–quartet based "G-quartet structure" formed when both the DNA segments bound with thrombin and caused the attachment of Ru-SNPs onto the electrode surface, the ECL signal of the biosensor was increased. Compared to the target induced conformational change based aptasensors, it possesses distinct advantages, the sensing system is very easy to be constructed, the most important thing is that it has low background signal and low detection limit. It also expands the application of aptamer on biosensors and also explores a new way for other small molecules or protein based on the splitting of aptamer.

This project was financially supported by National Basic Research Program of China (No. 2010CB732403), NSFC (20735002, 40940026, 20905013), the Special Foundation for Young Scientists of Fujian Province, China (2008F3057).

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