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A signal-on electrogenerated chemiluminescent biosensor for lead ion based on DNAzyme

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ABSTRACT

A highly reproducible and sensitive signal-on electrogenerated chemiluminescence (ECL) biosensor based on the DNAzyme for the determination of lead ion was developed. The ECL biosensor was fabricated by covalently coupling 5′-amino-DNAzyme-tagged with ruthenium bis (2,2′-bipyridine) (2,2′-bipyridine-4,4′-dicarboxylic acid)-ethylenediamine (Ru1-17E′) onto the surface of graphite electrode modified with 4-aminobenzoic acid, and then a DNA substrate with a ribonucleotide adenosine hybridized with Ru1-17E′ on the electrode. Upon binding of Pb^{2+} to the Ru1-17E′ to form a complex which catalyzed the cleavage of the DNA substrate, the double-stranded DNA was dissociated and thus led to a high ECL signal. The signal linearly increases with the concentration of Pb^{2+} in the range from 5.0 to 80 pM with a detection limit of 1.4 pM and a relative standard derivation of 2.3%. This work demonstrates that using DNAzyme tagged with ruthenium complex as an ECL probe and covalently coupling method for the fabrication of the ECL biosensor with high sensitivity, good stability and significant regeneration ability is promising approach.

1. Introduction

Lead ion is a toxic metal ion due to its causing a number of adverse health effects [1]. Once introduced into the body, lead ion is a potential neurotoxin that can cause chronic inflammation of the kidney and heart, inhibit brain development, and decrease nerve conduction velocity [2,3]. It is highly desirable to develop sensitive methods for the detection of Pb^{2+}. The methods currently available for the detection of Pb^{2+} are atomic absorption spectrometry [4], inductively coupled plasma (ICP)-atomic emission spectrometry [5], ICP-mass spectrometry [5], atomic fluorescence spectrometry [6], and electrochemical stripping analysis [7]. Although these methods have low detection limits, they are sophisticated, time-consuming, and inappropriate for on-site field analysis and rather costly for instruments used. Recent years, Pb^{2+} biosensors based on DNAzyme which is catalytically active DNA molecule and can catalyze a number of reactions in the manner seen for protein- or RNA-based biocatalysts [8], have been received much attention due to their simple, inexpensive and suitable for on-site field analysis.

Some efforts have been made to develop biosensors based on the DNAzyme for the determination of Pb^{2+}, including optical, electrochemical and electrogenerated chemiluminescence (ECL) biosensors. Lu et al first developed a series of fluorescent and colorimetric methods for the determination of Pb^{2+}, based on Pb^{2+}-dependent DNAzyme [8–11]. The homogeneous fluorescent methods were based on the DNAzyme as a molecular recognition element and a fluorescent quench reaction. When the DNAzyme strand (17E) tagged with a fluorescence quencher at the 3′-end of 17E hybridized a DNA substrate with a ribonucleotide adenosine (17DS) tagged with the fluorophore at the 5′-end of 17DS, the fluorescence was effectively quenched and little signal was observed. Upon addition of Pb^{2+}, the 17DS at the site of ribonucleotide adenosine was cleaved, releasing the fluorescent portion for detection. Recently, Lu, et al reported a new fluorescent biosensor designed by using the A C mismatch or G T wobble pair in the 8–17 DNAzyme and dual quenchers. This biosensor achieved a much low detection limit and its performance in lower temperature [12]. These optical methods have high sensitivity. However, they suffer from possible drawbacks including the potential for false signals arising from contaminating colorants, fluorophores, quenchers and expensive instruments. Plaxco et al. [13] developed an electrochemical DNAzyme-based Pb^{2+} biosensor fabricated by thiol-assembling a methylene blue-tagged thiol containing DNAzyme with a detection limit of 0.3 μM. Shao et al. [14] also developed a novel free-label electrochemical DNAzyme-based biosensor using thiol-assembly technique and DNA-Au bio-bar code amplification with the detection limit of 1.0 nM. Yang et al. [15] recently reported an electrochemical sensor using an amplification strategy based on DNAzyme functionalized gold nanoparticles for the detection of Pb^{2+} with a detection limit

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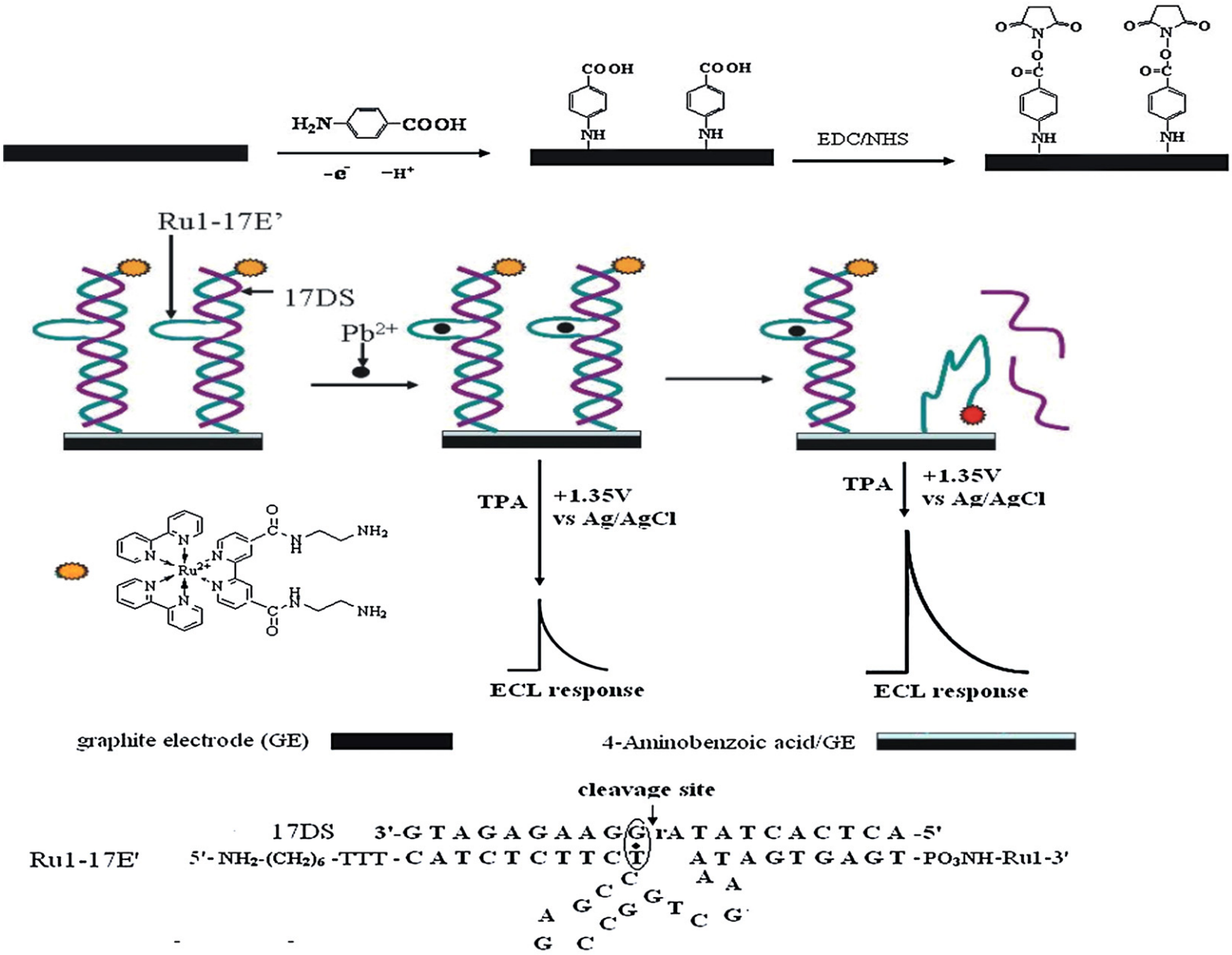

Fig. 1. Schematic diagram of the fabrication of DNAzyme-based ECL biosensor for the detection of lead ion.

of 0.028 nM. Chen et al. [16] first reported an ECL DNAzyme-based Pb^{2+} biosensor with a detection limit of 11 pM Pb^{2+}. This biosensor was fabricated by self-assembling a 5′-thiol-DNAzyme on the surface of a gold electrode, and then a DNA substrate, which was tagged with a $Ru(bpy)_3{}^{2+}$-N-hydroxylsuccinimide ester, hybridized with the DNAzyme on the electrode. When the biosensor was immersed into a solution containing Pb^{2+}, the 17DS was cleaved at the site of ribonucleotide adenosine, resulting that the portion of the ECL tag of $Ru(bpy)_3{}^{2+}$-N-hydroxylsuccinimide ester left from the surface of the electrode, led to decrease the ECL intensity. The ECL intensity of the biosensor decreased with the increase of Pb^{2+} concentration, which is known as a "signal off" mode. However, this ECL biosensor suffers from some drawbacks such as high background and limited linear range due to using a "signal off" mode [17] and limited reproducibility due to a breaking of the S–Au bond at working potentials (+0.90 V to +1.2 V) [18]. The "signal-on" mode of the sensor in which the signal increases with the increase of analyte concentration can avoid the pitfalls of the "signal off" mode [17].

The aim of the present work is to develop a highly reproducible and sensitive signal-on ECL DNAzyme-based Pb^{2+} biosensor. The principle scheme of the designed ECL biosensor for the determination of Pb^{2+} is demonstrated in Fig. 1. This signal-on ECL DNAzyme-based Pb^{2+} biosensor was designed by using a 5′-amino-DNAzyme (17E′)-tagged with ruthenium bis (2,2′-bipyridine) (2,2′-bipyridine-4,4′-dicarboxylic acid)-ethylenediamine (Ru1) at terminal 3′ (Ru1-17E′). Ru1 was used as the ECL tag due to its high ECL efficacy and regeneration on the electrode surface. The biosensor was fabricated by covalently coupling Ru1-17E′ on a graphite electrode, and then a DNA substrate (17DS) with a ribonucleotide adenosine (rA) hybridized with Ru1-17E′ on the electrode to form a double-stranded DNA (Ru1-17E′ 17DS). In the absence of Pb^{2+}, the double-stranded DNA on the electrode stayed a fixed rigid conformation, allowing Ru1 to be far away from the surface of the electrode, and thus led to a low ECL signal. It is well-known that the ECL emission was electrochemically generated from the excited state, $Ru1^{2+*}$. $Ru1^{2+*}$ was generated from the electron transfer reaction between $Ru1^{3+}$ generated electrochemically upon the oxidation of $Ru1^{2+}$ and a strongly reducing species $TPA^{\bullet}$ generated upon the oxidation of TPA [19]. Upon binding of Pb^{2+} to the Ru1-17E′ 17DS to form a complex (Ru1-17E′ 17DS Pb^{2+}), the complex catalyzed the hydrolytic cleavage of the 17DS at the site of ribonucleotide adenosine and the ds-DNA was presumably dissociated to form a flexible single stranded DNA, allowing Ru1 to be close to the surface of the electrode, and thus led to a high ECL signal. In this paper, the fabrication and characterization of the ECL biosensor for the determination of Pb^{2+} were presented.

2. Experimental

2.1. Reagents and apparatus

4-Aminobenzoic acid (4-ABA) was purchased from Bio. Basic Inc. (Canada). Lead(II) acetate trihydrate ($(CH_3COO)_2Pb{\cdot}3H_2O$), imidazole, N-(3-dimethylaminopropyl)-N-ethyl carbodiimide hydrochloride (EDC), and N-hydrosuccinimide (NHS) were obtained from Sigma–Aldrich (USA). Ethanolamine and tri-*n*-propylamine (TPA) was obtained from First Reagent Corporation of Shanghai (China). Ruthenium bis (2,2′-bipyridine) (2,2′-bipyridine-4,4′-dicarboxylic acid)-ethylenediamine (Ru1) was synthesized by our lab [20]. All reagents were of analytical grade. Millipore Milli-Q water (>18 MΩ cm) was used throughout.

Lead-dependent DNAzyme, 17E′, was designed to be 5′-amino-$(CH_2)_6$-TTT-17E-phosphate group-3′, 5′-NH_2-$(CH_2)_6$-TTT CAT CTC TTC TCC GAG CCG GTC GAA ATA GTG AGT-$HPO_4{}^{-}$-3′ according to the reference [13], and synthesized by Sangon Inc. (China). The substrate 17DS, 5′-ACT CAC TAT rAGG AAG AGA TG-3′, was synthesized by Integrated DNA Technologies Inc. (USA). The store solutions of DNAzyme and the substrate were prepared by the DNA buffer, which was composed of 500 mM NaCl and 25 mM tris-acetate (pH 7.5).

The ECL experimental set-up consisted of a CHI 600B workstation (Shanghai Chenhua Instruments, China) and an ultra-weak chemiluminescence analyzer controlled by a personal computer with the BPCL program (Institute of Biophysics, Chinese Academy of Sciences, China). A commercial cylindroid glass cell was used as an ECL cell. A three-electrode system composed of a biosensor or a graphite electrode (GE, $\Phi \approx 5$ mm) as working electrode, a platinum plate as counter electrode, and a Ag/AgCl (saturated KCl) as reference electrode, was used. All potentials in this work were referred to the reference electrode. For detecting ECL, the cell was placed directly in front of a photomultiplier (PMT, operated at −850 V) and the PMT window was only opened toward the working electrode to eliminate the blank chemiluminescence and the ECL from the counter electrode. The UV–Vis spectrum was recorded using a UV–Vis spectrophotometer (TU-1900, Beijing Puxi, China).

2.2. Synthesis of ruthenium bis (2,2′-bipyridine)(2,2′-bipyridine-4,4′-dicarboxylic acid)-ethylenediamine-17E′ (Ru1-17E′).

2 OD of 17E′ was dissolved in 400 μL of DNA buffer. 500 μL of 0.01 M EDC was added to above 17E′ solution. 500 μL of 0.1 M imidazole and 500 μL of 0.93 mM Ru1 were immediately added to the above mixture solution, allowed to shaking at low speed overnight at room temperature [21–23]. And then, 200 μL of 3 M sodium acetate and 4 mL of ethanol were sequentially added to the mixed solution. The precipitate reaction was carried out in refrigerator over 12 h. The resulting solution was chilled over 12 h at −16 °C and then centrifuged for 30 min at 12,000 rpm. The precipitate was rinsed with 70% cold ethanol at several times and then was dissolved in 200 μL of DNA buffer and stored under −16 °C prior to use. The concentration of Ru1-17E′ solution was estimated to be 26.7 μM according to the value of UV absorption of Ru1 at 457 nm [24]. The mole ratio of the 17E′ to the Ru1 tag within the complex was calculated to be approximately 1:1 on the basis of the UV–visible absorption spectra with the absorbance value ($A = 0.3783$) at $\lambda_{max} = 260$ nm for 17E′ and the absorbance value ($A = 0.035$) at $\lambda_{max} = 457$ nm for Ru1 tag as well as their respective ε values ($\varepsilon^{260nm} = 1.4 \times 10^5\ M^{-1}\ cm^{-1}$, $\varepsilon^{454nm} = 1.4 \times 10^4\ M^{-1}\ cm^{-1}$).

2.3. Fabrication of the ECL biosensor

Fabrication of the Pb^{2+}-DNAzyme-based ECL biosensor includes three steps. Firstly, the electrochemical modification of 4-ABA on the surface of a clean GE was carried out in ethanol solution containing 3 mM 4-ABA and 0.1 M $LiClO_4$ by cyclic scanning between 0 and +0.9 V for 4 cycles with a scan rate of 10 mV s^{-1} [25]. The activation of the 4-ABA modified GE (4-ABA/GE) was performed by immersing 4-ABA/GE in 5 mM tris-acetate buffer (pH 7.5) containing 5 mM EDC and 8 mM NHS for 0.5 h and then rinsed with 5 mM tris-acetate buffer. In second step, the ECL probe Ru1-17E′ was immobilized on the surface of the activated 4-ABA/GE. The activated electrode was immersed in 400 μL of 2.0 μM Ru1-17E′, allowing to stay for 1 h in a water-saturated atmosphere. The resulting electrode was thoroughly rinsed with DNA buffer. 30 μL of 5 mM tris-acetate buffer containing 1 mM ethanolamine was dropped onto the surface of Ru1-17E′/4-ABA/GE, allowing to stay for 15 min to deactivate the remaining succinimide groups and to block unreacted active sites on the surface of the electrode [26]. In third step, the substrate 17DS was hybridized on the electrode. 30 μL of 2.5 μM 17DS was dropped on the surface of Ru1-17E′/4-ABA/GE, allowing to stay for 10 min

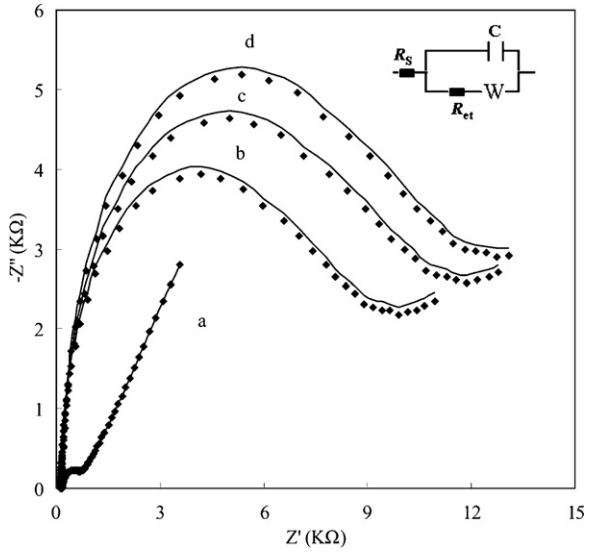

Fig. 2. Electrochemical impedance spectra of the electrodes in each step of the fabrication of the ECL biosensor. (a) Bare GE; (b) 4-ABA/GE; (c) Ru1-17E′/4-ABA/GE; (d) Ru1-17E′/17DS/4-ABA/GE. All measurements were performed in 5 mM $K_3Fe(CN)_6$–5 mM $K_4Fe(CN)_6$–0.1 M PBS–0.1 M KCl (pH 7.2). Bias potential, 0.22 V; frequency, 100 kHz to 1.0 Hz; amplitude, 5.0 mV.

in a 65 °C water bath and then to cool slowly to room temperature over 1 h and to keep for 16 h at room temperature. The obtained electrode was rinsed thoroughly with DNA buffer and then used as the ECL sensor and stored in air at room temperature in the dark.

2.4. ECL measurement

The ECL biosensor fabricated was immersed in a solution containing 5 mM tris-acetate buffer (pH 7.5) and a fixed concentration of Pb^{2+} for 1 h in a 37 °C water bath and then washed with DNA buffer. The ECL measurement was performed using the ECL biosensor which had interacted with Pb^{2+} as working electrode at a constant potential of +1.35 V in 3.0 mL of 5 mM tris-acetate buffer containing 0.10 M TPA (pH 7.5). The concentration of Pb^{2+} was quantified by the increased ECL peak intensity, $\triangle I$ ($\triangle I = I - I_0$), where I_0 and I were the ECL peak intensity before and after the ECL biosensor was interacted with Pb^{2+}, respectively.

3. Results and discussion

3.1. Characteristics of the ECL biosensor

Fig. 2 shows the electrochemical impedance spectra of the electrodes in each step of the fabrication of the ECL biosensor. From Fig. 2, it can be seen that the experimental impedance data (dot) are consistent with a fitted data (line) with commercial software (CHI 660). The electrochemical impedance spectra of the bare GE (line a) exhibits a very small semicircle domain, suggesting a very low electron-transfer resistance (R_{et}, 0.76 KΩ). When the 4-ABA was electrochemically modified on the GE (line b), the R_{et} increased from 0.76 KΩ to 9.16 KΩ, attributed to the fact that 4-ABA modified on the GE surface blocks the electrode surface and the full deprotonation of carboxyl group of the 4-ABA makes an electrostatic repulsive force to $[Fe(CN)_6]^{3-/4-}$ [25]. As the Ru1-17E′ designed was covalently coupled to carboxyl group of the 4-ABA (line c), the R_{et} increased 14.3% from 9.16 KΩ to 10.68 KΩ. This is attributed to the fact that the Ru1-17E′ having a single strand nucleic acid with negative charges on its phosphate backbone makes an electrostatic repulsive force and a space hindrance to $[Fe(CN)_6]^{3-/4-}$. This indicates that the Ru1-17E′ probe has been covalently coupled onto the surface of the 4-ABA. After 17DS was hybridized to the Ru1-17E′ on the surface of the electrode (line d), the R_{et} increased 10.6% from 10.68 KΩ to 11.81 KΩ. This indicates that the Ru1-17E′ 17DS double strands complex is formed. It was found that the increased ratio of R_{et} in this system is smaller than that (20–60%) obtained in the hybridization of a single strand DNA self-assembled on the surface of gold electrode with a complementary single strand DNA [27,28]. A possible explanation for this small increased ratio of R_{et} is that the density of Ru1-17E′ on the surface of the electrode maybe reduced, attributed to the fact that the Ru1-17E′·17DS double strands on the surface of the electrode stay a fixed rigid conformation with a big bump in 17E′ strand after a long time hybridization. However, it is difficult to obtain the density of Ru1-17E′ on the surface of the electrode using electrochemical method on the basis of the passed charge [29] since the amount of Ru1-17E′ on the surface of the electrode is too small and electrochemical active component Ru1 in Ru1-17E′ is far away from the surface of the electrode. A more reasonable explanation for this small increased ratio should be further studied.

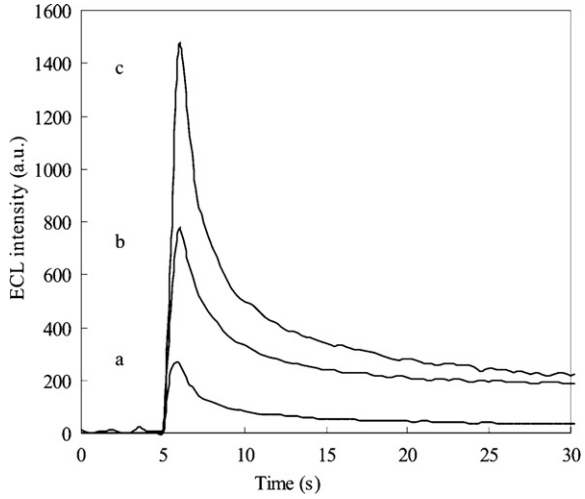

Fig. 3. ECL responses of ECL biosensors at +1.35 V in 5 mM tris-acetate buffer (pH 7.5) containing 0.10 M TPA. (a) without interaction of Pb^{2+}; (b) with interaction of 70 pM Pb^{2+}; (c): with interaction of 0.3 nM Pb^{2+}.

Fig. 3 shows ECL kinetic profiles of the ECL biosensor fabricated before (line a) and after interaction with 70 pM Pb^{2+} (line b) and 0.3 nM Pb^{2+} (line c). Compared line a with line b, the ECL biosensor with interaction of 70 pM Pb^{2+} displayed a higher signal. This

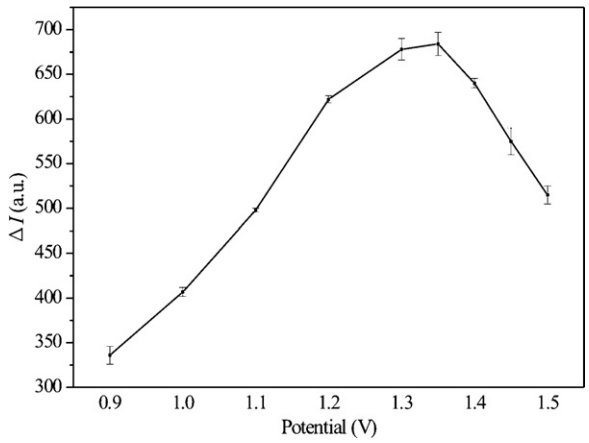

Fig. 4. Dependence of the increased ECL peak intensity ($\triangle I$) of the ECL biosensor on applied potential after the interaction with 0.2 nM Pb^{2+}, obtained in 5 mM tris-acetate buffer (pH 7.5) containing 0.10 M TPA.

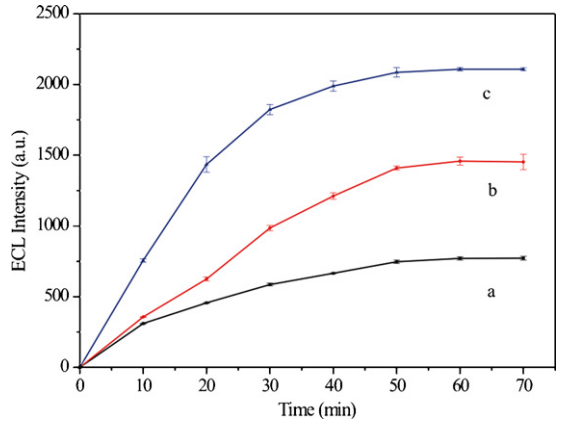

Fig. 5. Dependence of the ECL intensity of ECL biosensors on incubation time. (a) interaction with 70 pM Pb^{2+}; (b) interaction with 0.3 nM Pb^{2+}; (c) interaction with 2 nM Pb^{2+}. The ECL measurement condition is same as Fig. 3.

indicates that the ECL biosensor has a response to analyte Pb^{2+}. Compared line b with line c, it can be clearly observed that the ECL intensity increases from 771 to 1459 as the Pb^{2+} concentration is elevated. This indicates that the increased ECL intensity is consistent with the increasing the concentration of Pb^{2+}. Therefore, the ECL sensor fabricated possibly can be used to detect Pb^{2+}.

3.2. Optimization of test conditions

Important experimental parameters including applied potential and incubation time for the ECL biosensor were optimized. The dependence of $\triangle I$ of the ECL biosensors on applied potential without and with interaction of 0.2 nM Pb^{2+} from +0.9 V to +1.5 V was examined. The result is showed in Fig. 4. It can be seen that $\triangle I$ increases from 336 to 684 with the applied potential increasing from +0.9 to +1.35 V and reaches the maximum at about +1.35 V, attributed to increasing number of excited-state molecules produced during an electrochemically initiated reaction. The potential must be positive enough to oxidize both $Ru(bpy)_3^{2+}$ ($E^\circ \sim +1.1$ V vs. Ag/AgCl) and TPA ($E_p \sim +0.9$ V vs. Ag/AgCl) at a GE without damaging the 4-ABA monolayer and producing any undesired species from the electrolyte solution. A positive potential shift of 0.25 V from the reversible redox potential of $Ru1^{2+}$ in solution could result from the effect of the electrode material as well as the minor electronic resistance formed between the GE and Ru1 redox center due to the 4-ABA monolayer and the Ru1-17E′ linkage that contains 6 $-CH_2-$ groups. With a further increase of the applied potential, however, $\triangle I$ decreased. The decrease in ECL intensity beyond + 1.35 V vs. Ag/AgCl remains unclear but could be related to the oxidation of water that produces extra amounts of oxygen, resulting in the consumption of $TPA^{\bullet}$ radicals needed for the generation of the $Ru1^{2+*}$ species [30]. Therefore, a constant potential of +1.35 V was chosen in the following experiments to obtain a high sensitivity and good reproducibility.

The incubation time of the ECL biosensor with different concentration of Pb^{2+} was also optimized. Fig. 5 shows the dependence of the ECL intensity on incubation time in three concentrations of Pb^{2+}. From Fig. 5, it can be seen that the ECL intensity increases with an increase of the incubation time and then reaches a plateau. The slope of the ECL intensity with the incubation time and the time of reaching the maximum ECL intensity are different in the different concentration of Pb^{2+}. It was found that the lower concentration of Pb^{2+}, the lower slope and the longer time required for reaching the maximum ECL intensity. The ECL intensity can reach the maximum at 50–60 min in 70 pM Pb^{2+} testing solution, indicting that the time of reaching the maximum ECL intensity is

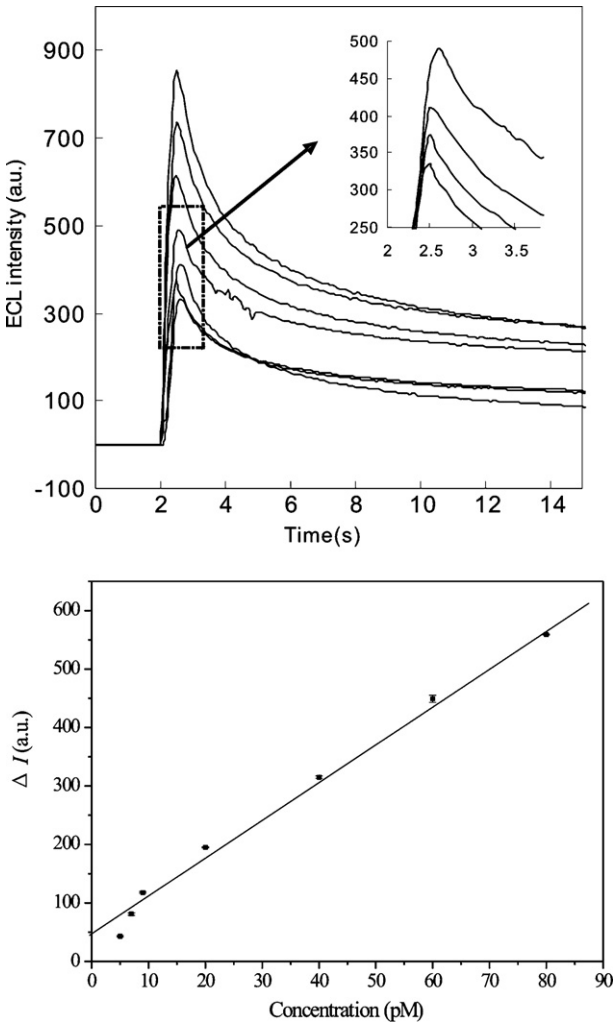

Fig. 6. ECL responses of the ECL biosensors with interaction of Pb^{2+} in different concentrations. (a) 5.0 pM; (b) 7.0 pM; (c) 9.0 pM; (d) 20 pM; (e) 40 pM; (f) 60 pM; (g) 80 pM. The ECL measurement condition is same as Fig. 3.

60 min. This time is the same as the used time reported in the "signal off" ECL [16], the fluorescent [31], the electrochemical [13,14] biosensors based on DNAzyme for Pb^{2+} detection. This incubation time is much longer than that required in homogenous fluorescent and colorimetric methods (only a few minutes) [11], indicting that the reaction of the surface-confined DNAzyme with Pb^{2+} is much

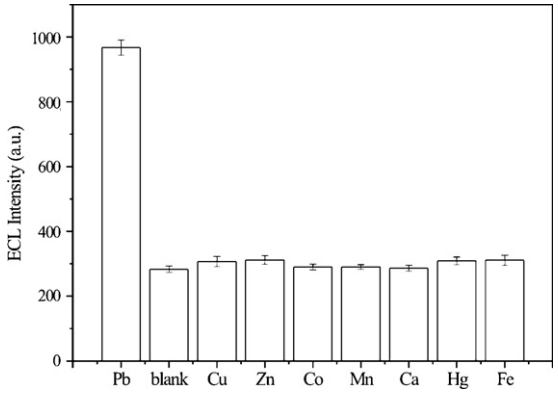

Fig. 7. ECL intensity of the ECL biosensors after individual interaction with 0.2 nM Pb^{2+} or 20 nM metal ion (including Cu^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Ca^{2+}, Hg^{2+}, Fe^{3+}). The ECL measurement condition is same as Fig. 3.

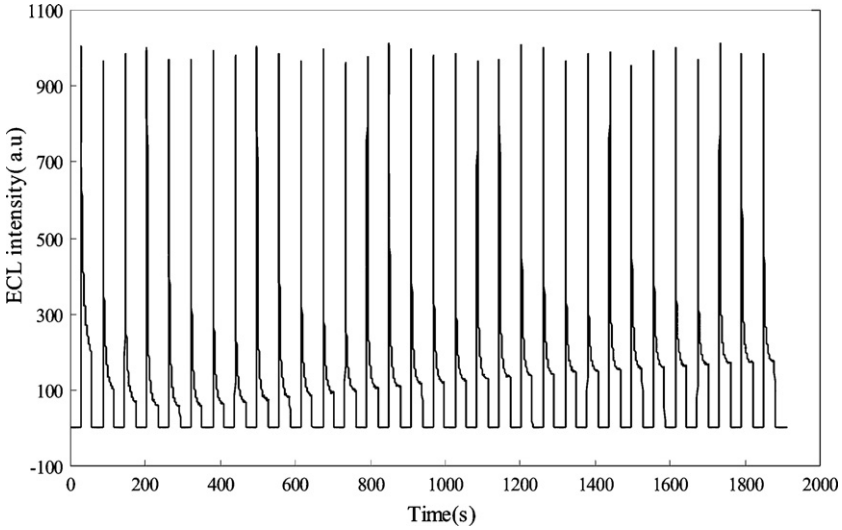

Fig. 8. ECL continual response of the ECL biosensor obtained at +1.35 V in 0.10 M TPA-5 mM tris-acetate buffer (pH 7.50) after the sensor was interacted with 0.2 nM Pb^{2+}.

slower than that of the DNAzyme with Pb^{2+} in solution. Therefore, an incubation time of 60 min was chosen in the following experiments to obtain a high sensitivity. It should be noted that 60 min of the incubation time was too long to be practical on-site detection. The incubation time of this ECL biosensor should be reduced.

3.3. Performance of ECL biosensors

Fig. 6 shows the ECL profiles of the ECL biosensors after interaction with different concentrations of Pb^{2+} under the optimized conditions. From inset of Fig. 6, it can be seen that $\triangle I$ increases with an increase of the concentration of Pb^{2+} in the range from 5.0 pM to 80 pM. The linear regression equation was $S = 6.65C + 41.4$ (unit of C is pM) with a regression coefficient (R) of 0.9954. The detection limit obtained is 1.4 pM (3σ) [32], which is much lower than the lowest one reported using "signal off" ECL biosensor so far [16]. The lowest detection limit obtained in this work is attributed to the fact that the "signal on" mode and the C–N covalently coupling method are used for the design of the ECL biosensor. This greatly benefits super-trace analysis of Pb^{2+}.

The selectivity of the biosensor was evaluated by challenging it with several metal ions. The ECL intensity of the biosensor fabricated was measured after it was interacted with each cation individually. The result is showed in Fig. 7. From Fig. 7, it can be seen that the ECL intensity of the biosensor with interaction of Cu^{2+}, or Zn^{2+}, Co^{2+}, Mn^{2+}, Ca^{2+}, Hg^{2+}, Fe^{3+} in the concentration of 20 nM is nearly same as that in the absence of Pb^{2+} (blank) and is much lower than that with interaction of 0.2 nM Pb^{2+}. This indicates that the ECL biosensor has a good selectivity for discriminating Pb^{2+} from other metal ions tested.

Fig. 8 shows the ECL response of the ECL biosensor fabricated after interaction with 0.2 nM Pb^{2+} at a constant potential of + 1.35 V for 32 cycles (time remaining 32 min). The relative standard deviation of the ECL intensity of the ECL biosensor for 32 cycles was 6.0%. Compared with the "signal off" ECL biosensor reported [16] which kept a stable ECL at 1.15 V only for 3 min, the ECL biosensor fabricated using C–N covalently coupling method in this work showed a good stability and fine reproducibility.

The regeneration ability of the ECL biosensor was tested using the reference method [31]. The used biosensors (after interaction with Pb^{2+}) were subsequently soaked in water for 18 h to remove the substrate strand (both cleaved and uncleaved), and rinsed with water for 5 min, and then hybridized with substrate strand 17DS and measured as described in experiment 2.4. The result showed that the average ECL intensity of a regenerated biosensor for three times was 304 (RSD = 2.8%), which was very close to the ECL intensity (281) of an initial ECL biosensor, without interacting with Pb^{2+}. And the average ECL intensity of a regenerated biosensor for three times was 920 (RSD = 1.0%), which was very close to the ECL intensity (955) of an initial ECL biosensor, after interacting with 0.2 nM Pb^{2+}. The signal response recovered up to 91% of the original signal response. This recovery is higher than that reported (81%) using a Pb^{2+}-specific DNAzyme fluorescent biosensor [31]. It is attributed to the fact that the ECL probe Ru1-17E′ is covalently coupled onto the surface of the 4-ABA/GE. Therefore, a good regeneration ability of the ECL biosensor fabricated was obtained.

The storage stability of the ECL biosensors fabricated was also checked. After being stored in air at room temperature for 3 days, the biosensor showed that the average ECL intensity of stored biosensor was 95.7% of the ECL intensity of the initial ECL biosensor after interacting with 0.06 nM Pb^{2+}. The storage stability of the present biosensor is improved compared with that (1 day) reported in a preliminary result of a Pb^{2+}-specific DNAzyme fluorescent biosensor fabricated by assembling thiolated DNAzyme and mercaptohexanol on Au surface [31]. This indicates that the ECL biosenor fabricated has good storage stability.

3.4. Dissociation constant of immobilized complex with Pb^{2+}

In order to understand the fundamental of metal-binding sites in nucleic acids and the performance of designed biosensors, the dissociation constant of the immobilized complex consisting of Ru1-17E′ and 17DS with Pb^{2+} was determined by the ECL method proposed in this work. It is hoped to know whether the binding strength of the complex with Pb^{2+} is affected by tagging 17E′ with Ru1. To end this aim, we supposed that the cleave reaction consists two steps. The first step is represented by binding of the cofactor Pb^{2+} to the complex of Ru1-17E′ 17DS immobilized on the surface of the 4-ABA, to form a Ru1-17E′ 17DS Pb^{2+} complex. In second step, the substrate (17DS) is cleaved by the complex, following releasing two products (17DS1′, 17DS2′) in solution and regenerating the free-binding Ru1-17E′ on the electrode. The ECL intensity (I) is directly proportional to unbinding Ru1-17E′ on the electrode. Supposed that the interaction of the binding Ru1-17E′ 17DS with Pb^{2+} meets the requirements of the following Eq. (1), the dissociation constant K_d can be derived from the reaction equation based on the rate equation and steady state approximation. k represents the rate constant for cleavage of the substrate within the Ru1-17E′

17DS Pb^{2+} complex. k_1 and k_{-1} express the association rate constant and the dissociation rate constant between Ru1-17E′ 17DS and the Pb^{2+}, respectively.

$$\mathrm{Ru1-17E'\cdot 17DS + Pb^{2+} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} Ru1-17E', \cdot 17DS\cdot Pb^{2+} \xrightarrow{k} 17DS'_1}$$
$$+17DS'_2 + \mathrm{Ru1-17E' + Pb^{2+}} \quad (1)$$

According to Eq. (1), following rate equation is obtained.

$$k_1[\mathrm{Ru1-17E'\cdot 17DS}][\mathrm{Pb^{2+}}] = k_{-1}[\mathrm{Ru1-17E'\cdot 17DS\cdot Pb^{2+}}] + k[\mathrm{Ru1-17E'\cdot 17DS\cdot Pb^{2+}}] \quad (2)$$

When the rate of cleavage is the limiting factor, the ECL intensity should be a direct measurement of the cleavage rate. Thus, the rate of cleavage reaction (v) can be expressed by the ECL complex as:

$$v = \frac{\Delta[\mathrm{Ru1-17E'}]}{\Delta t} = \frac{\Delta I}{\Delta t} = k[\mathrm{Ru1-17E'\cdot 17DS\cdot Pb^{2+}}] \quad (3)$$

Based on the Eq. (1), the K_d can be expressed as:

$$K_d = \frac{k_{-1}+k}{k_1} = \frac{[\mathrm{Ru1-17E'\cdot 17DS}][\mathrm{Pb^{2+}}]}{[\mathrm{Ru1-17E'\cdot 17DS\cdot Pb^{2+}}]} \quad (4)$$

The second key assumption is that the total concentration of Ru1-17E′ 17DS, $[\mathrm{Ru1\text{-}17E'\ 17DS}]_0$, on the electrode does not change over time. Thus, we can write $[\mathrm{Ru1\text{-}17E'\ 17DS}]_0$ as the sum of the free [Ru1-17E′ 17DS] and the bound [Ru1-17E′ 17DS·Pb^{2+}] on the electrode:

$$[\mathrm{Ru1-17E'\cdot 17DS}]_0 = [\mathrm{Ru1-17E'\cdot 17DS}] + [\mathrm{Ru1-17E'\cdot 17DS\cdot Pb^{2+}}]$$
$$[\mathrm{Ru1-17E'\cdot 17DS}] = [\mathrm{Ru1-17E'\cdot 17DS}]_0 - [\mathrm{Ru1-17E'\cdot 17DS\cdot Pb^{2+}}] \quad (5)$$

Substituting Eq. (5) into Eq. (4), we obtain an expression for K_d:

$$K_d = \frac{\left([\mathrm{Ru1-17E'\cdot 17DS}]_0 - [\mathrm{Ru1-17E'\cdot 17DS\cdot Pb^{2+}}]\right)[\mathrm{Pb^{2+}}]}{[\mathrm{Ru1-17E'\cdot 17DS\cdot Pb^{2+}}]} \quad (6)$$

$$[\mathrm{Ru1-17E'\cdot 17DS\cdot Pb^{2+}}] = \frac{[\mathrm{Ru1-17E'\cdot 17DS}]_0[\mathrm{Pb^{2+}}]}{(K_d + [\mathrm{Pb^{2+}}])} \quad (7)$$

Substituting Eq. (7) into Eq. (3), we obtain an expression for v:

$$v = \frac{k\cdot[\mathrm{Ru1-17E'\cdot 17DS}]_0[\mathrm{Pb^{2+}}]}{(K_d + [\mathrm{Pb^{2+}}])} \quad (8)$$

If the concentration of Pb^{2+} is much high, the v asymptotically approaches the maximum rate v_{max} and expresses as:

$$v_{max} = \frac{\Delta[\mathrm{Ru1-17E'}]_{max}}{\Delta t'} = k[\mathrm{Ru1-17E'\cdot 17DS}]_0 \quad (9)$$

Substituting Eq. (9) into Eq. (8), the v can be described as follows:

$$v = \frac{\Delta I}{\Delta t} = \frac{(I_{max}/\Delta t')[\mathrm{Pb^{2+}}]}{(K_d + [\mathrm{Pb^{2+}}])} \quad (10)$$

This makes it easier to determine the K_d from measured data.

$$\frac{1}{I/\Delta t} = \frac{K_d}{I_{max}/\Delta t'\cdot[\mathrm{Pb^{2+}}]} + \frac{1}{I_{max}/\Delta t'} \quad (11)$$

Therefore, the plot of $(1/(I/\Delta t))$ vs. $1/[\mathrm{Pb^{2+}}]$ should give a straight line when the concentration of Pb^{2+} is low. And the K_d can be gained from the values of the intercept and the slope.

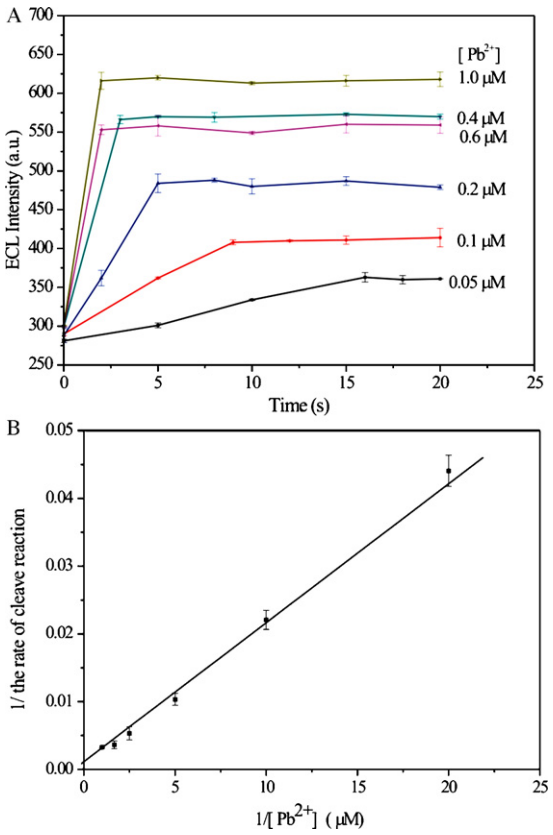

Fig. 9. (A) Dependence of the ECL intensity on incubation time after the sensor was interacted with Pb^{2+} in different concentrations. (B) Dependence of reciprocal of rate of cleavage reaction on reciprocal of concentration of Pb^{2+}. The ECL measurement condition is same as Fig. 3 except the PMT is biased at −800 V.

Fig. 9A displayed the dependence of the ECL intensity on the time in different Pb^{2+} concentrations. Note that a lower concentration of substrate strand, 0.5 nM 17DS was used in order to meet the single-turnover condition for this kinetics study [32]. From each line in Fig. 9A, we can get the different slops $\triangle I/\triangle t$ in different Pb^{2+} concentrations. Fig. 9B shows the plot of $(1/(I/\Delta t))$ vs. $1/[\mathrm{Pb^{2+}}]$. This curve was fit to Eq. (11) with an intercept of 0.0001 and a slope of 0.0022 (R = 0.9993). An apparent K_d of 2.22×10^{-5} M was calculated by the obtained intercept and slope. This K_d is slightly higher than that (1.35×10^{-5} M) measured using gel-based assay at pH 6.0 [33]. This was contributed to the fact that a spacer of six $-CH_2-$ was added into 17E′ in our design and Ru1 made a steric hindrance and the electrostatic repulsion for Ru1-17E′ 17DS binding with Pb^{2+}. This K_d value suggests that under present experimental condition the binding strength of the complex Ru1-17E′ 17DS with Pb^{2+} is only slightly affected by tagging Ru1. The biosensors employing a DNAzyme tagged with $Ru(bpy)_3^{2+}$ derivatives as an ECL probe can be designed for more applications

4. Conclusion

A signal-on ECL biosensor for the determination of Pb^{2+} has been developed on the basis of DNAzyme. The ECL biosensor was designed by employing a DNAzyme tagged with ruthenium complex as an ECL probe and a covalently coupling method for immobilizing the ECL probe Ru1-17E′ onto the surface of 4-ABA/GE. The ECL biosensor fabricated has the lowest detection limit of Pb^{2+} (1.4 pM) so far, good stability and selectivity as well as regeneration ability. The apparent dissociation constant of Ru1-17E′ 17DS with Pb^{2+} was measured by the proposed ECL method, indicting that the binding strength of the complex Ru1-17E′ 17DS with Pb^{2+} is only

slightly affected by tagging 17E′ with Ru1. This work demonstrates that employing a DNAzyme tagged with $Ru(bpy)_3^{2+}$ derivatives as an ECL probe and a covalently coupling ECL probes onto the surface of GE/4-ABA is a promising approach in constructing ECL biosensors with high sensitivity, good stability, and significant regeneration ability. The strategy could be easily extended to various analytical platforms including ECL and electrochemical biosensors as well as lab on chips for the detection of other metal ions.

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