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A novel ECL biosensor for β-lactamase detection: Using RU(II) linked-ampicillin complex as the recognition element

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ABSTRACT

In this work, $Ru(phen)_2(cpaphen)^{2+}$ linked-ampicillin (Ru-Amp), as the novel specific recognition element, was proposed to construct a simple and sensitive electrogenerated chemiluminescence (ECL) biosensor for the determination of β-lactamase. Here, Ru-Amp complex acted not only as a novel specific recognition element for β-lactamase but also as the ECL Luminescent reagent. Through electrostatic adsorption and the intermolecular π–π interactions, a large amount of Ru-Amp was immobilized to gold nanoparticles (TA@AuNPs) prepared by thiophenemalonic acid (TA) to obtain Ru-Amp/TA@AuNPs nanocomposites. The nanocomposites, which can produce very stable films exhibiting excellent ECL behaviors, were self-assembled on the CNTs-Nf modified glassy carbon electrode surface. The presence of the target β-lactamase resulted in autonomous hydrolysis reaction of Amp, achievement of the efficient ECL emission and highly sensitive detection of β-lactamase. The biosensor for β-lactamase detection was developed with excellent sensitivity of a concentration variation from 50 pg mL^{-1} to 100 ng mL^{-1} with a low detection limit of 37 pg mL^{-1}. An ECL assay offers the proposed method opportunities for designing new Ru-based ECL luminophores for biosensing applications.

1. Introduction

β-Lactamases, found in nosocomial isolates of *Escherichia coli, Klebsiella pneumoniae* and the family *Enterobacteriaceae*, plays pivotal roles in bacterial resistance of many β-lactam drugs (Pérez-Pérez and Hanson, 2002; Rydzik et al., 2014). The increased levels of β-lactamases in bacterial chromosome or plasmid of patients could generate "super bugs", which threaten almost all β-lactam antibiotics (Girlich et al., 2000; Kumarasamy et al., 2010). However, the level of β-lactamases in human bodies may increase because of the ill-use of β-lactam antibiotics in clinical treatment and the deliberate addition of β-lactamases in dairy industry. The traditional analytical methods of β-lactamases is PCR analyses (Pérez-Pérez and Hanson, 2002; Senda et al., 1996) and chromogenic method (Huang et al., 2010; Livermore et al., 2007), which indeed give reliable and satisfactory results. But the sensitivity of these methods was limited. Therefore, it is desirable to develop a rapid, sensitive and inexpensive detection method for β-lactamases detection in dairy industry, clinical treatment and food safety.

Electrogenerated chemiluminescence (ECL), owing to its advantages of sensitivity, rapidity, control ability and low background, has been extensively used as a powerful tool in many analytical chemistry related areas such as environmental monitor (Xu and Yu, 2010; Yang et al., 2013; Li et al., 2015), food safety (Long et al., 2011; Zhou et al., 2014), pharmaceutical analysis (Huang et al., 2015; Ye et al., 2013) and bioassays (Hou et al., 2014; Jin et al., 2015). Among them, ECL based biosensors have attracted extremely interesting for detection of protein, which are usually developed based on biorecognition element such as antibody or aptamer (Ding et al., 2012; Feng et al., 2014). For example, Xiao et al. (2014) have used α-fetoprotein antibody for sensitive detection of α-fetoprotein (AFP) with a detection limit of 0.2 pg mL^{-1}. In our previous work, aptamer has been used as biorecognition element for ultrasensitive detection of thrombin with a detection limit of 0.3 fM (Gui et al., 2014). Although those immunosensors and aptasensors can successfully detect proteins, they have suffered from some disadvantages of the need of complicated biological systems for the production of antibody and the difficulty of selecting the proper aptamer to efficiently fit the target (Tombelli et al., 2005; Guo et al., 2010). Thus, it is necessary to explore effective recognition element for the detection of protein in bioassays.

Ampicillin (Amp), one of β-lactam antibiotic contained the structure of β-lactam ring, could be quickly hydrolyzed to generate

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a new secondary amine with the highly specific catalysis of β-lactamase. Interestingly, Martin group (Liang et al., 1996) has reported that β-lactam antibiotic could act as the amine component in the ECL reaction. More importantly, in the presence of β-lactamase, the hydrolyzed β-lactam antibiotic can enhance the ECL signal of ruthenium complex as coreactant. In our previous work, a ruthenium complex (Ru-Amp), including two regions of $[Ru(phen)_2(cpaphen)]^{2+}$ and ampicillin (Amp), was present as a self-enhanced luminophore. Based on the novel signal amplification of the hydrolyzed enzymatic reaction by β-lactamase, an ECL aptasensor was constructed for the detection of thrombin (Gui et al., 2014).

Inspire by above research achievements, Ru-Amp was prepared and used as a novel recognition element of β-lactamases to design an alternative charming method for β-lactamases assays with low cost and high sensitivity. For sensing interface construction, the carbon nanotubes (CNTs) were doped into the Nafion (Nf) film for active surface area increasing (Harmer et al., 1996). Then the CNTs-Nf composite was coated on the electrode to obtain the CNTs-Nf modified electrode (CNTs-Nf/GCE). Gold nanoparticles (TA@AuNPs), reduced by 3-thiophenemalonic acid (TA), have not only protonated carboxylic acid groups but also π-type bonds around nano-Au (Sun et al., 2006), which were employed as carriers for immobilization of Ru-Amp to form Ru-Amp/TA@AuNPs composites through the electrostatic interactions and the strong intermolecular π–π interactions. As expected, the as-prepared Ru-Amp/TA@AuNPs can produce very stable films on the surface of modified electrode (Ru-Amp/TA@AuNPs/CNTs-Nf/GCE). After incubated with the target of β-lactamases, the ECL signal of modified electrode was extremely amplified because the hydrolyzed enzymatic reaction was occurred on the surface of modified electrode.

2. Experimental sections

2.1. Reagents and material

Hydrogen tetrachloroaurate ($HAuCl_4 \cdot 4H_2O$), β-lactamase, ampicillin (Amp), 3-thiophenemalonic acid (TA), nafion (Nf) and thrombin (TB, enzymatic activity of 40–300 NIH units/mg, lyophilized power) were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). Carbon nanotube (CNTs, 95% purity) was purchased from Nanjing Xianfeng nano Co. (Nanjing, China). Sodium citrate (CA) was purchased from Chengdu Chemical Reagent Company (Chengdu, China). $Ru(phen)_2(cpaphen)(PF6)_2$ was prepared according our previous work (Gui et al., 2014). Phosphate-buffered saline (PBS, pH 7.4) was prepared by using 0.1 M Na_2HPO_4, 0.1 M KH_2PO_4, 0.1 M KCl. Double distilled water was used throughout every experiment.

The ECL emissions were monitored by a MPI-A electrochemiluminescence analyzer (Xi'an Remax Electronic Science & Technology Co. Ltd., Xi'an, China) in PBS (0.1 M, pH 7.4) with the voltage of the photomultiplier tube (PMT) at 800 V. Cyclic voltammetry (CV) was carried out with a CHI Instruments Model 660D electrochemical workstation (Shanghai Chenhua Instrument Co., Ltd., China). Experiments were performed by a conventional three-electrode system with a modified glassy carbon electrode (GCE, ϕ=4 mm) as the working electrode, a platinum wire as the counter electrode and Ag/AgCl (saturated KCl) as the reference electrode. UV–vis spectra of various nanoparticles were monitored by UV 2450 spectrophotometer (Shimadzu, Japan) spectrometer. The morphologies of various nanoparticles were analyzed by a scanning electron microscopy (SEM) system (SEM, S-4800, Hitachi) with an acceleration voltage of 10 kV.

2.2. Preparation of RU-amp/TA@AuNPs complex

Firstly, TA@AuNPs was prepared according to the literature (Sun et al., 2006) with a little modification. Briefly, 25 mL of the mixture solution, containing 500 μL 1% $HAuCl_4$ and 250 μL 0.3 M TA, was heated at 100 °C for 20 min, giving a dark red solution to obtain TA@AuNPs. Secondly, $[Ru(phen)_2(cpaphen)]^{2+}$ linked-ampicillin (Ru-Amp) complex was synthesized by the linkage reagents of EDC and NHS. Lastly, 200 μL 0.05 M Ru-Amp was added droplet into 20 mL as-prepared TA@AuNPs solution with stirring. After several minutes, a large quantity of black precipitate was obtained. Then the black precipitate was collected by centrifugation and washed several times by double distilled water. The resulting Ru-Amp/TA@AuNPs was dispersed in double distilled water and used in subsequent experiments.

2.3. Fabrication of the modified electrode

First of all, the bare electrodes were pretreated according to the previous literature (Gui et al., 2013). Then, 3 μL of CNTs-Nf was dropped onto the pretreated electrode and dried in the air to obtain CNTs-Nf modified electrode. Subsequently, 3 μL of as-prepared Ru-Amp/TA@AuNPs was casted onto the modified electrode surface and dried in the air to obtain Ru-Amp/TA@AuNPs/CNTs-Nf modified electrode. The modified electrode could be used as an ECL biosensor for the detection of β-lactamases. Schematic presentation of the fabrication of the ECL biosensor and the proposed mechanism of β-lactamases detection is shown in Scheme 1. When the target of β-lactamases was existed, the ECL signal of Ru-Amp was extremely amplified because the hydrolyzed enzymatic reaction caused a structure transformation of an amide bond into a secondary amine.

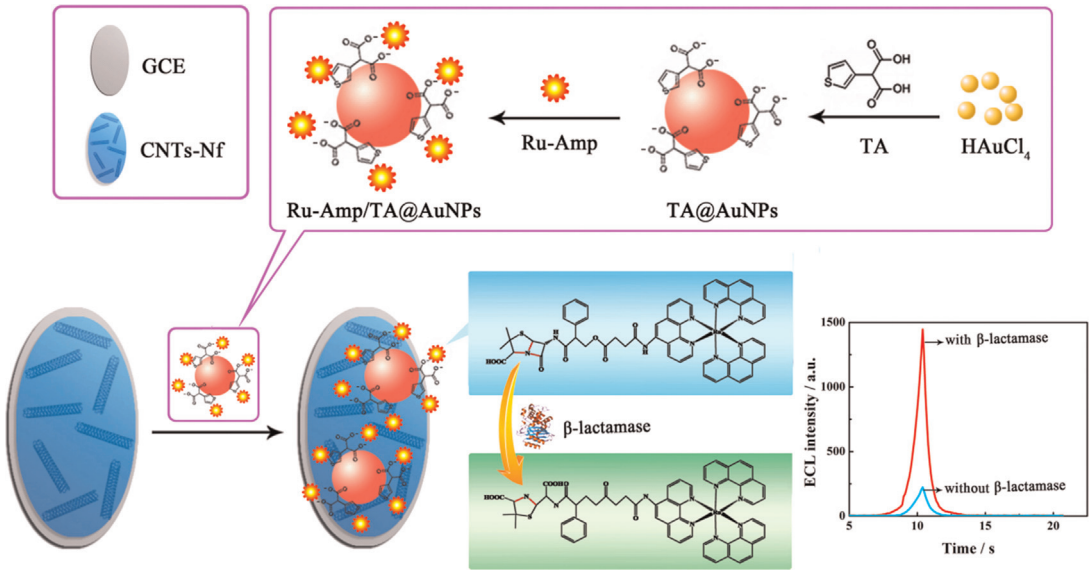
3. Results and discussion

3.1. SEM characterization of different nanomaterials

The morphologies of the as-prepared composites were investigated by SEM. As we can see from Fig. 1A, the SEM image of TA@AuNPs showed small bright points with a diameter of 22 ± 4 nm, and the size of which was bigger than that of CA@AuNPs (SEM image in supporting information, Fig. S4A). For comparison, the SEM image of Ru-Amp/TA@AuNPs (Fig. 1B) showed that many small bright points were accumulated together. The reason may be that Ru-Amp was attached with TA@AuNPs through electrostatic interactions and π–π interactions. In order to further confirm the successful synthesis of Ru-Amp/TA@AuNPs nanocomposites, the morphological changes in the synthetic process were also monitored using transmission electron microscopy (TEM). The TEM image of TA@AuNPs (Fig. S2A) showed small globular features. And the TEM images of Ru-Amp/TA@AuNPs (Fig. S2B) showed as clusters of small bolus. Significantly, the results of TEM were very similar with that of SEM.

3.2. UV–vis characterization of different composite

The UV–vis absorption spectrum was employed to further characterize the as-formed composite of Ru-Amp/TA@AuNPs. As shown in Fig. 2A, there was an obvious absorption peaks for TA@AuNPs at 524 nm (Fig. 2A, curve a). And three characteristic absorption peaks of pure Ru-Amp (Fig. 2A, curve b) were appeared since the complex of Ru-Amp has several benzenes and heterocyclic. The absorption spectrum of Ru-Amp/TA@AuNPs (Fig. 2A, curve c) at 528 nm slightly red-shifted compared with TA@AuNPs at 524 nm, which could prove the electrostatic interactions and π–



Scheme 1. The schematic diagrams of the biosensor and the mechanism of target detection.

π interactions between Ru-Amp and TA@AuNPs. The result indicated that the Ru-Amp/TA@AuNPs composite was successfully synthesized.

3.3. CV behavior of the biosensor

The cyclic voltammogram (CV) was employed to characterize the electrochemical behaviors of the modified electrode in each step in ferricyanide ($[Fe(CN)_6]^{3-/4-}$) solution. As we can see from Fig. 2B, a couple of quasi-reversible redox peak of $[Fe(CN)_6]^{3-/4-}$ was observed on the bare electrode (Fig. 2B, curve a). When the CNTs-Nf was immobilized on the electrode, there was no obvious redox peak current (Fig. 2B, curve b) observed due to the bulky Nf perturbed the interfacial electrons transfer. After the Ru-Amp/TA@AuNPs modified onto the electrode, the redox peak current (Fig. 2B, curve c) increased due to good conductivity of TA@AuNPs.

3.4. Effect of different gold nanoparticles on the response of the biosensor

We investigated the effect of gold nanoparticles prepared by different reducing agent. The proposed gold nanoparticles were reduced by TA, named TA@AuNPs. Another conventional gold nanoparticle prepared by using citric acid (CA) as reducer (Carpay and Cense, 1973), named CA@AuNPs. The two kinds of nanoparticles were used as carriers for immobilization of Ru-Amp as signal probe to construct biosensors. The different biosensors were incubated with 1 ng mL^{-1} β-lactamase under the same experimental conditions. As we can see from Fig. 3A, the proposed biosensor exhibited a higher ECL signal of 1935 a.u. compared with 1152 a.u. of Ru-Amp/CA@AuNPs/CNTs-Nf modified GCE. Compared with CA@AuNPs, TA@AuNPs have π-type bonds expect for protonated carboxylic acid groups around nano-Au. The intermolecular π–π interactions between TA and Ru-Amp also contribute to the formation of the aggregates via self-assembly (Sun et al., 2005). From the SEM images of TA@AuNPs and CA@AuNPs, we also found that the size of TA@AuNPs obviously larger than that of CA@AuNPs. It is reported that the ECL intensity increases with the increasing diameter of Au nanoparticle (Devadoss et al., 2011). Considering all of above, TA@AuNPs was chosen as carrier for immobilization of Ru-Amp.

3.5. The activity analysis of β-lactamase

The activity of β-lactamase plays an important role in ECL behavior of this biosensor. It is reported that the product generated from hydrolysis of β-lactam antibiotics by β-lactamases can readily react with molecular iodine (Tamilselvi and Mugesh, 2010), which can prevent the occurrence of chromogenic reaction between molecular iodine and starch. In this work, iodine–starch system

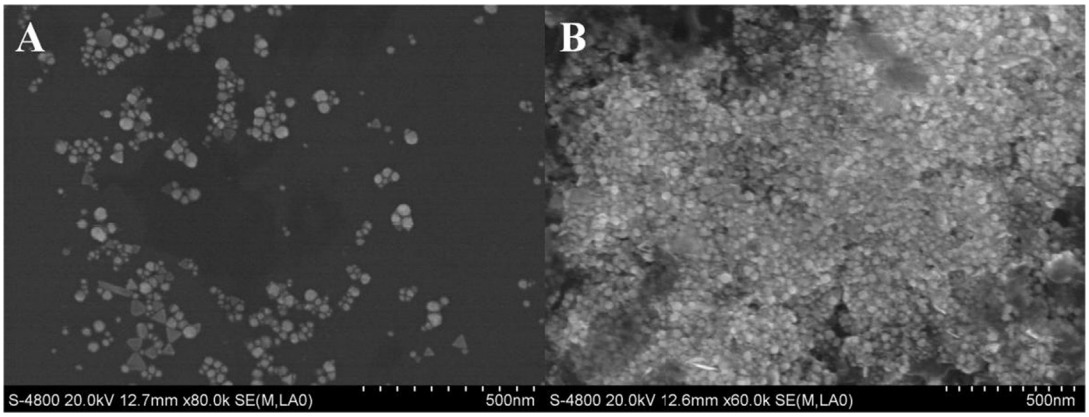


Fig. 1. SEM of TA@AuNPs (A) and Ru-Amp/TA@AuNPs (B).

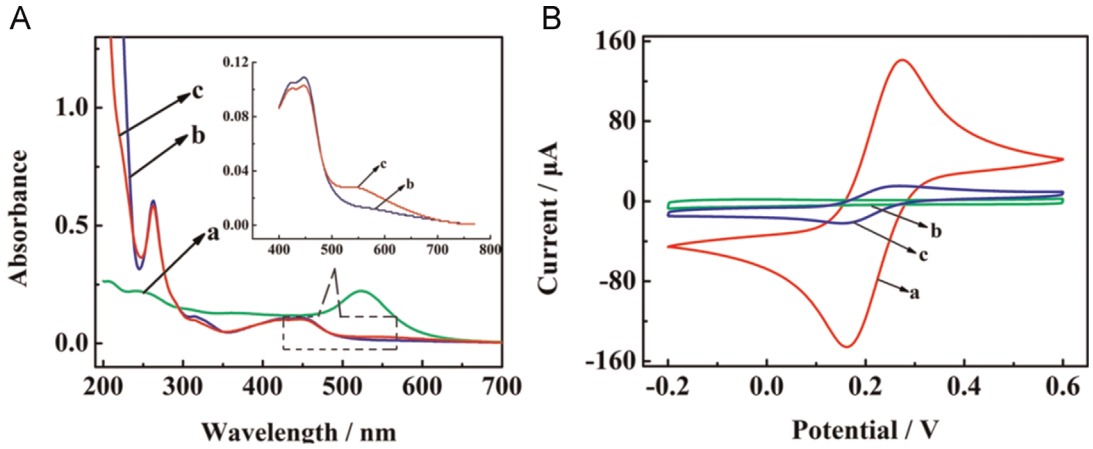


Fig. 2. (A) UV–vis absorption spectra of (a) TA@AuNPs; (b) Ru-Amp and (c) Ru-Amp/TA@AuNPs composite in double distilled water. (B) Cyclic voltammograms of the electrode at different stages in 2.5 mM $[Fe(CN)_6]^{3-/4-}$ solution for (a) bare electrode, (b) CNTs-Nf/GCE, (c) Ru-Amp/TA@AuNPs/CNTs-Nf /GCE.

was used to investigate the influence of TA@AuNPs and Ru complex for the activity of β-lactamase (the details expressed in supporting information). The results of the experiment are shown in Figs. S6 and S7. As we can see, the color of solution (No. 1-1 and 2-1) changed from colorless to blue in the absence of β-lactamase as expected. The colors of solution (No. 1-2 and 2-2) are kept colorless because of the reaction of hydrolysis between Amp and β-lactamase, which were used as reference solution. The colors of solution containing TA@AuNPs or Ru complex (No. 1-3 to 1-7 and 2-3 to 2-7) were shown no significant differences before and after adding iodine and starch. The results indicated that β-lactamase treated with TA@AuNPs or Ru complex for 24 h also had the excellent activity for the hydrolysis reaction.

3.6. Detection of β-lactamase with biosensor

The main aim of the present investigation was to determine β-lactamase, so the performance of the prepared biosensor was assessed by detecting standard β-lactamase solutions with ECL technique. The ECL signals relevant to the different β-lactamase concentrations are shown in Fig. 3B. As expected, the ECL intensity increased with the increasing concentration of β-lactamase. (Fig. 3B, curves a–h). β-Lactamase could be quantitatively measured over a concentration range from 50 pg mL^{-1} to 100 ng mL^{-1} with a detection limit of 37 pg mL^{-1} and the signal-to-noise ratio was 3. The regression equation was I (a.u.)=682.8lg c+125.0 with a correlation coefficient of 0.9911. The result indicated that this strategy is feasible and has great potential for detection of β-lactamase.

3.7. Stability and selectivity of the biosensor

We also evaluated the stability of the proposed biosensor by employing one prepared biosensor incubated with 10 ng mL^{-1} β-lactamase for consecutive cyclic potential scans. As we can see from Fig. 4A, under continuous cyclic potential scans for 14 cycles at a scan rate of 100 mV s^{-1}, the relative standard deviation (RSD) of ECL was 0.89%. The result suggested that the proposed biosensor had acceptable stability. The repeatability of the biosensor was tested by using five proposed biosensors incubated with 10 ng mL^{-1} β-lactamase. The prepared electrodes exhibited closely ECL responses and the RSD of 1.75%, which was acceptable for detection of β-lactamase. The storage stability of the prepared biosensor was also tested by monitoring of its ECL response. It was found that the ECL response of the biosensor decreased 1.4% one week later. Thus, the proposed biosensor was sensitive and stable for the detection of β-lactamase.

To investigate the selectivity and specificity of the present biosensor, it was incubated with different interfering substances such as ALP and TB. As shown in Fig. 4B, the control experiments were performed by using ALP (100 ng mL^{-1}) and TB (100 nM) to replace β-lactamase (1 ng mL^{-1}), respectively. We can see that the ECL signal of ALP and TB exhibited a small decrease compared with the blank. The biosensor was also incubated with 1 ng mL^{-1} β-lactamase containing ALP (100 ng mL^{-1}) and TB (100 nM), compared with the ECL response obtained from the 1 ng mL^{-1} β-lactamase only, no remarkable difference was found. The result suggested that the ECL intensity response to β-lactamase was much higher than those of the others, which meant that ALP and TB had no obvious influence on the ECL response to β-lactamase.

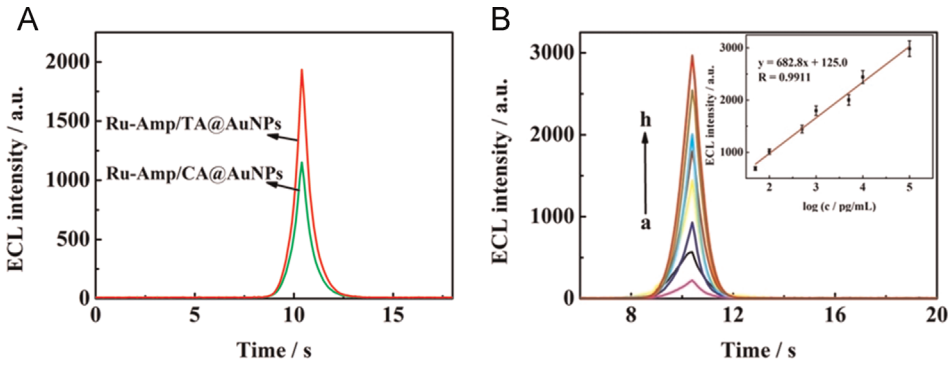


Fig. 3. (A) ECL-time curves of the biosensor incubated with 1 ng mL^{-1} β-lactamase with different signal probe. (B) ECL-time curves of the biosensor with different concentrations of β-lactamase. β-Lactamase concentration: (a) 0 pg mL^{-1}, (b) 50 pg mL^{-1}, (c) 100 pg mL^{-1}, (d) 500 pg mL^{-1}, (e) 1 ng mL^{-1} (f) 5 ng mL^{-1}, (g) 10 ng mL^{-1}, (h) 100 ng mL^{-1}. The inset is calibration curve for β-lactamase determination. Test solution: 2 mL 0.1 M PBS (pH 7.4).

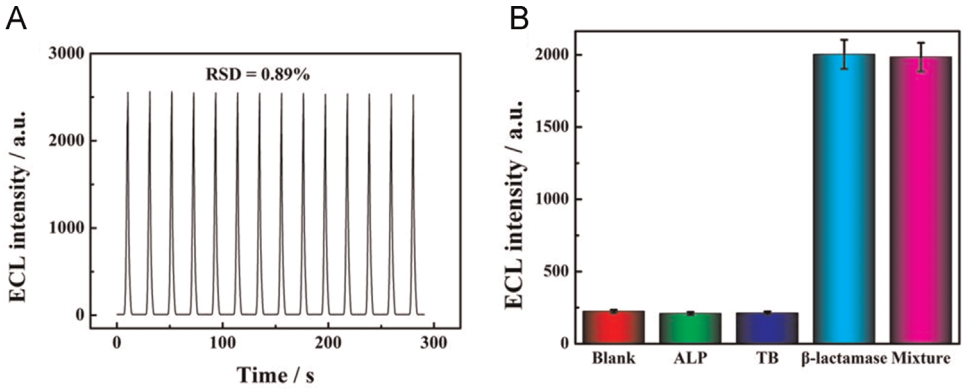


Fig. 4. (A) The stability of the proposed ECL biosensor incubated with 10 ng mL^{-1} β-lactamase under consecutive cyclic potential scans for 14 cycles. (B) Selectivity investigation of the proposed ECL biosensor for the detection of β-lactamase against the different interference sample: blank, ALP (100 ng mL^{-1}), TB (100 nM) and β-lactamase (1 ng mL^{-1}), a mixture (1 ng mL^{-1} β-lactamase + 100 ng mL^{-1} ALP + 100 nM TB).

Thus, the biosensor displayed good selectivity for the determination of β-lactamase.

4. Conclusions

A straightforward and sensitive ECL biosensor was developed for the detection of β-lactamase based on the novel recognition element of Ru-Amp, which was one of hydrolyzed substrates of the β-lactamase. A novel Ru-Amp complex here acted not only as ECL reagent for the ECL emitters but also as specific recognition element of β-lactamase. And Ru-Amp/TA@AuNPs nanomaterials generated a very stable film, which exhibited excellent ECL behaviors. On the basis of their excellent performance, this biosensor for the detection of β-lactamase achieved a detection limit of 37 pg mL^{-1} and exhibited excellent selectivity. The simple ECL tactics would be promising in applications of biotechnology and clinical diagnosis.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2015.03.023.

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