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Homogeneous Electrochemiluminescence Biosensor for the Detection of RNase A Activity and Its Inhibitor

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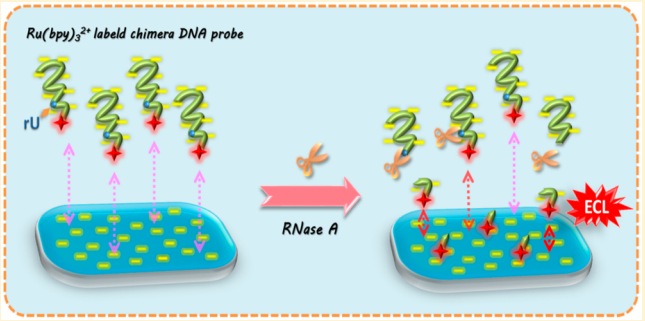
ABSTRACT: Ribonuclease A (RNase A) is increasingly considered as a biomarker for tumor diagnosis, and it is of great significance to develop an ultrasensitive, cost-effective assay for RNase A detection. Electrochemiluminescence (ECL) technology has distinctive advantages in the development of biosensors for diverse targets. However, most of the ECL biosensors require the complex process of electrode modification, which is laborious and time consuming. In this work, an immobilization-free homogeneous ECL assay was developed for the highly sensitive detection of RNase A activity for the first time. On the basis of the fact that RNase A can specifically hydrolyze RNA at the site of ribonucleotide uracil (rU), a rU-containing chimeric DNA probe is designed and labeled with $Ru(bpy)_3^{2+}$ (act as ECL indicator). The chimeric DNA probe hardly diffuses to the surface of negatively charged indium tin oxide (ITO) electrode due to the strong electrostatic repulsion between the negatively charged DNA and ITO electrode, resulting in a weak ECL signal detected. When the RNase A is present, the chimeric DNA probe is hydrolyzed into small fragments, which contains little negative charge and can diffuse easily to the ITO electrode surface due to the decreased electrostatic repulsion. In this case, an enhanced ECL signal can be detected. Under the optimal conditions, there is a linear relationship between the ECL signal and the concentration of RNase A in the range of 0.001−0.10 ng/mL, and the detection limit is 0.2 pg/mL. In addition, the proposed ECL sensing system is also applied to detect the RNase A inhibitor, taking As^{3+} as an example. The proposed homogeneous ECL sensing system provides a new approach for the highly sensitive and convenient detection of RNase A as well as other ribonucleases only by redesigning a responding chimeric DNA probe.



Ribonuclease A (RNase A), a key endonuclease (13686 Da; 124 amino acids), is involved in gene duplication, cellular metabolism, and cancer therapy.[1−4] The overexpression of RNase A is closely related to many kinds of cancers.[5,6] RNase A is increasingly considered as a new biomarker for malignant tumors diagnosis and employed as a specific target for new drug discovery. Traditional methods for the RNase A detection include radiolabeling assay,[7] gel electrophoresis,[8] and zymography,[9] which often suffer from time consuming and complicated operations. It is of great significance to develop new approaches for cost-effective detection of RNase A. Ribonucleotide cytosine and uracil (rC and rU) are the enzyme cutting sites for RNase A,[10−12] so a chimeric DNA probe that contains rC or rU base can be specifically hydrolyzed into small fragments by RNase A too. According to such a biochemical mechanism, some biosensors based on electrochemical and fluorescent technology were developed.[13−16] For example, Takenaka et al.[14] designed a rC containing chimeric DNA probe which was immoblized on the gold electrode; the presence of RNase A would hydrolyze the probe and release ferrocenyl-labeled fragment, leading to a decreasing current response for quantification.

Electrochemiluminescence (ECL) technology, which combines the sensitivity of luminescence and the flexibility of electrochemistry,[17,18] has been widely used in the construction of biosensors.[19−23] However, most of the DNA-based ECL biosensors are heterogeneous assays which require the probes to be immobilized on the surface of the electrode by a laborious procedure. More importantly, the efficiency of the probe−target conjunction is thus influenced due to the steric hindrance of immobilized probes. To overcome these disadvantages, homogeneous electrochemical biosensors were

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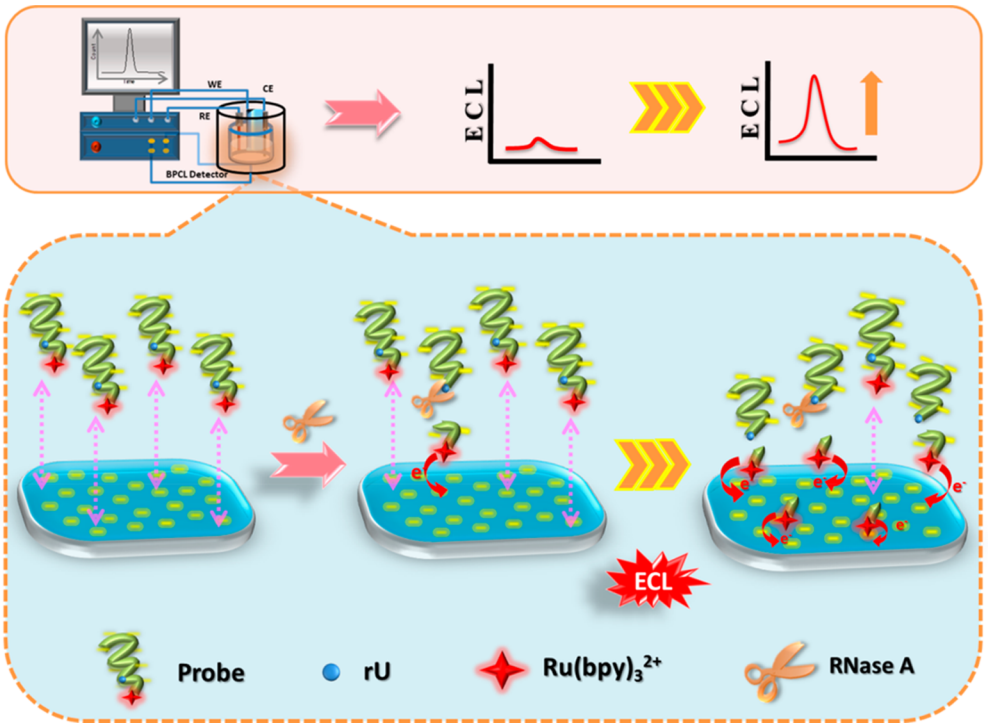
Scheme 1. Schematic Illustration for the Homogeneous ECL Assay in Response to RNase A



developed on the basis of the fact that there are different electrostatic repulsions of the negatively charged indium tin oxide (ITO) electrode toward the short chain and long chain of DNA. This characteristic has been applied to develop many DNA-based homogeneous electrochemical biosensors.[24−26] Our group first employed this principle to develop homogeneous ECL biosensors in which the ECL indicators of $Ru(phen)_3^{2+}$ were embedded into the groove of dsDNA.[27] However, a relatively high background signal had been detected since many $Ru(phen)_3^{2+}$ are free in the solution. To overcome this issue, $Ru(bpy)_3^{2+}$ can be labeled on the DNA similar to electrochemical biosensors labeling of the electroactive compounds (e.g., ferrocene[28] and methylene blue[29]) on the DNA.

In this study, a homogeneous ECL assay was developed to detect RNase A activity for the first time. A chimeric DNA probe containing rU base was designed; $Ru(bpy)_3^{2+}$ was labeled on the chimeric DNA and acted as ECL indicator. The probe was immoblization free and directly used as the substrate of RNase A. By the specific hydrolysis of RNase A, the negative long probes were cut into small fragments, which easily diffused to the surface of negatively charged ITO electrode due to the decreased electrostatic repulsion. In this case, an increasing amount of $Ru(bpy)_3^{2+}$-labeled fragments reached the electrode surface and enhanced ECL signal can be detected, which was proportional to the concentration of RNase A. The proposed ECL assay has been applied for the detection of toxic metal ion inhibitor on RNase A.

EXPERIMENTAL SECTION

Reagents and Materials. All synthetic DNA used and RNase A in the project were provided by Takara Biotechnology Co., Ltd. (Dalian, China). Their sequences are shown as follows. Probe 1 (P1): 5′-NH_2-$(CH_2)_6$-AGAG-rU-AGAGAGAGAGAGAGAGAGAG-3′. Probe 2 (P2): 5′-NH_2-$(CH_2)_6$-AGAG-rC-AGAGAGAGAGAGAGAGAGAG-3′.

Ribonuclease H (RNase H), deoxyribonuclease I (DNase I), trypsin (Try), human serum albumin (HSA), bovine serum albumin (BSA), *cis*-dichlorobis (2,2′-bipyridine)ruthenium(II) ($Ru(bpy)_2Cl_2$), 2,2′-bipyridine-4,4′-dicarboxylic acid (dcbpy), sodium hexafluorophosphate ($NaPF_6$), NHS, and *N*,*N*′-dicyclohexylcarbodiimide (DCC) in (*N*,*N*′-dimethylformamide (DMF)) and other chemicals were purchased from Sigma-Aldrich. All reagents were of analytical reagent grade and used without further purification except where noted. Ultrapure water (18.2 MΩ·cm) was prepared with a Milli-Q system (Millipore, Bedford, MA, USA) and used throughout the study.

Apparatus and Electrode Pretreatment. The ECL detection system consists of a CHI660E electrochemical workstation (Chenhua Instruments, Shanghai, China) and a BPCL ultraweak luminescence analyzer (Institute of Biophysics, Chinese Academy of Science, Beijing, China). The photomultiplier tube (PMT) of the BPCL analyzer was operated at a voltage of −900 V. The three-electrode system contains an ITO working electrode, a platinum counter electrode, and an Ag/AgCl reference electrode. The surface area of the ITO working electrode was 5 mm × 3 mm (Huanan Xiangcheng, Shenzhen, China, resistance ≤ 6 Ω). The bare ITO electrode can be negatively charged after a proper pretreatment (through sequentially sonicating the ITO electrode in an alconox solution (10 g/L alconox of ultrapure water) for 15 min, in propan-2-ol for 15 min, and twice in ultrapure water for 15 min).[30,31]

Polyacrylamide Gel Electrophoresis (PAGE) Assay. PAGE was carried out to verify the hydrolysis of RNase A on the chimeric DNA probe. The concentrations of all DNA sequences were 5.0 μM, and the concentration of RNase A was settled as 1.0 ng/mL. All reaction samples were incubated at 37

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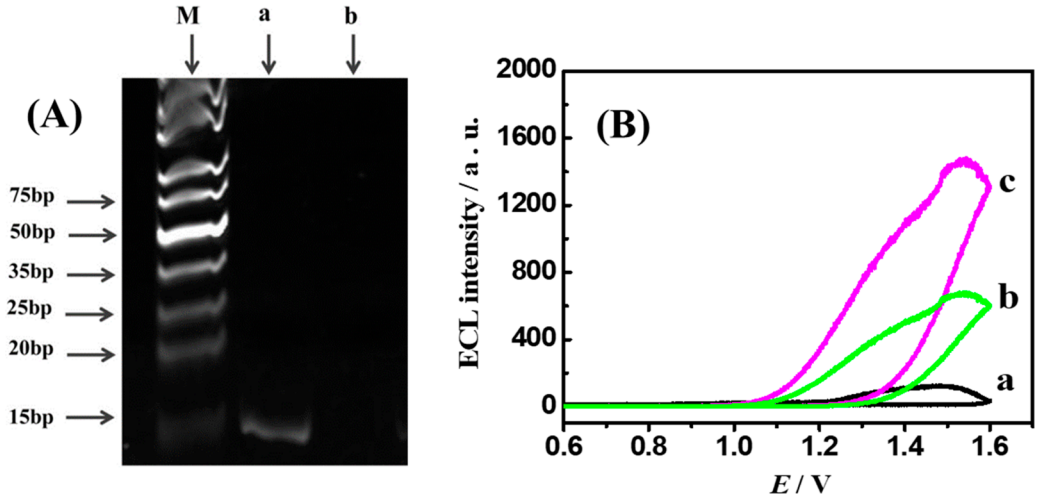

Figure 1. (A) Hydrolysis of RNase A on the chimeric DNA probe by PAGE (Lane M, marker; Lane a, P1; Lane b, P1+ RNase A). RNase A was 1.0 ng/mL, and P1 was 5.0 μM. (B) ECL responses to (a) P1 only, (b) P1 hydrolyzed by RNase A, and (c) P2 hydrolyzed by RNase A. P1 and P2 were 1.0 μM, and RNase A was 0.05 ng/mL.

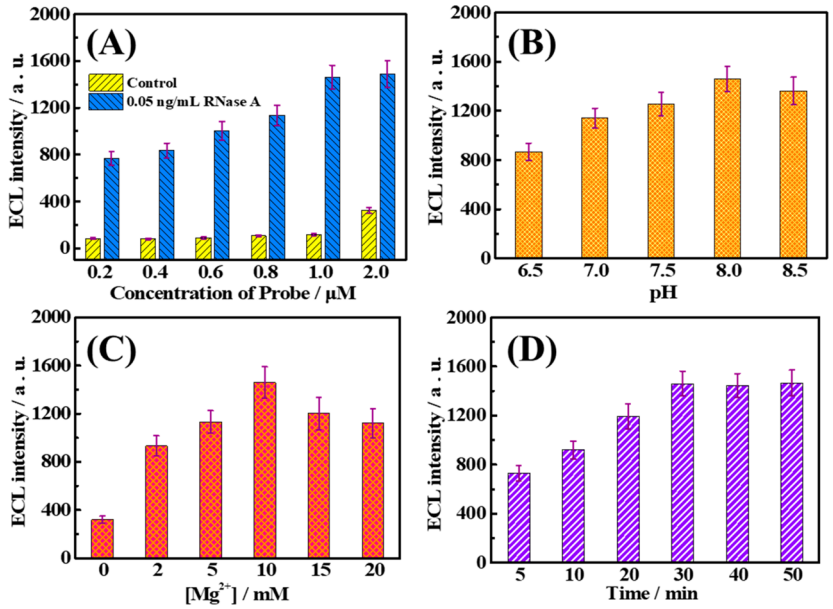

Figure 2. Optimizations of the homogeneous ECL sensing system for RNase A detection. (A) Effect of the chimeric DNA probe concentration. (B) Effect of pH on the RNase A activity. (C) Effect of the Mg^{2+} concentration on the RNase A activity. (D) Effect of the hydrolysis time of RNase A. [RNase A] = 0.05 ng/mL. [P1] = 1.0 μM. All ECL signals were measured in buffer solution containing TPA (0.02 M) by scanning the potential from 0.6 to 1.6 V at a scanning rate of 100 mV/s.

°C for 1 h. Then the products of the reaction were mixed with SYBR Green II (100×) and incubated at room temperature for 10 min. The project was performed in Trisborate-EDTA (TBE, 100×) buffer at a constant voltage of 130 V for 1.5 h at room temperature. The gels were photographed by a gel image system (Amersham imager 600, GE, USA).

Synthesis of $Ru(bpy)_3^{2+}$−NHS and Process of Probe Labeling. $Ru(bpy)_3^{2+}$−NHS was synthesized and labeled on the probes according to the previous literature with little modification.[32] In brief, $Ru(bpy)_2(dcbpy)(PF_6)_2$ was obtained by refluxing $Ru(bpy)_2Cl_2$ with excess dcbpy, and $NaPF_6$ solution was added in the filtrate to isolate the ruthenium complexes as PF_6 salts. Then the $Ru(bpy)_2(dcbpy)(PF_6)_2$ was activated by reacting with NHS and DCC in DMF. The final product of $Ru(bpy)_3^{2+}$−NHS ester was added to the amino-modified DNA (P1, P2) (sodium borate buffer, pH = 8.5) at a molecular ratio of 30:1. Also, the mixture was incubated to accomplish sufficient ligation. Purification, cooling, and centrifugal sedimentation of the ligation products were performed four times using ethyl alcohol to ensure the purity. Then $Ru(bpy)_3^{2+}$-labeled probes (P1−Ru, P2−Ru) were obtained and stored at 4 °C for further use.

Analytical Procedures for RNase A Detection. For the RNase A detection, 1.0 μM P1−Ru with various levels of RNase A were incubated at 37 °C for 30 min in working buffer (50 mM Tris-HCl (pH = 8.0), 10 mM $MgCl_2$, 100 mM NaCl, and 1 mM EDTA). Afterward, ECL measurements were carried out in the resulting solution containing TPA (0.02 M), the potential range was 0.6−1.6 V, and the scan rate was 100 mV/s. Each sample was detected five times, and the averaged value was adopted as the result. The error bars showed the standard deviation of five replicate determinations. RNase H

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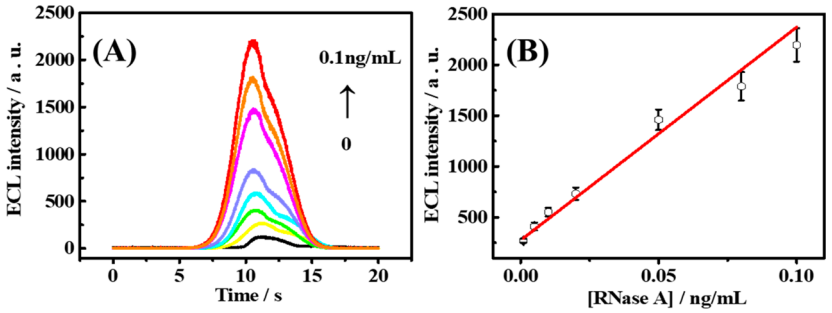

Figure 3. (A) ECL signals in response to different concentrations of RNase A (0, 0.001, 0.005, 0.01, 0.02, 0.05, 0.08, and 0.1 ng/mL). (B) Calibration curve between the ECL intensity and the RNase A concentration. Error bars represent the standard deviation of five repetitive measurements. [P1] = 1.0 μM. Experiments were carried out at 37 °C of the buffer solution (pH = 8.0), and ECL signals were measured in the solution containing TPA (0.02 M) by scanning the potential from 0.6 to 1.6 V at a scanning rate of 100 mV/s.

(0.5 ng/mL), DNase I (0.5 ng/mL), Try (0.5 ng/mL), HSA (0.5 ng/mL), and BSA (0.5 ng/mL) were employed as the interference for the specificity test.

Inhibitor Detection. For the inhibition assay, 1.0 μM P1−Ru and various amounts of inhibitor (Arsenic(III) ion) were incubated at 37 °C for 10 min in the buffer (50 mM Tris-HCl (pH = 8.0), 10 mM $MgCl_2$, 100 mM NaCl, and 1 mM EDTA). Then 0.05 ng/mL RNase A was added, and the sample was continued to incubate for 30 min. The ECL intensities of samples were measured as described above.

RESULTS AND DISCUSSION

Principle of the Proposed ECL Sensing System. First, the rU base was synthetically embedded into a ssDNA 5 bases away from the 5′ terminus to obtain the chimeric DNA probe, which was then labeled with $Ru(bpy)_3^{2+}$ at the 5′ terminus (for details see the Experimental Section).[32] Also, the labeled probe was employed for the following ECL detection of RNase A. As shown in Scheme 1, DNA contains negatively charged phosphate,[33] so the long chain of the probe is far away from the negatively charged ITO electrode due to the strong electrostatic repulsion (step a). Therefore, diffusion of the probe toward the ITO electrode not only depends the concentration gradient but also is controlled by the particular electrostatic repulsion. In this case, the ECL indicator of $Ru(bpy)_3^{2+}$ labeled on the intact probe cannot diffuse easily to the surface of ITO electrode, and the ECL response is weak. When the RNase A is present, RNase A can specifically hydrolyze the chimeric DNA probe at the site of rU (blue point) to produce numbers of short DNA fragments including the $Ru(bpy)_3^{2+}$-labeled one (step b). Since the released DNA fragments are smaller and contain little negative charge, the $Ru(bpy)_3^{2+}$-labeled fragments can diffuse easily toward the ITO electrode surface due to the decreasing electrostatic repulsion. Thus, an enhanced ECL signal can be detected. Meanwhile, owning to the inherent catalytic activity, one RNase A can efficiently hydrolyze numerous probes and induce plenty of released $Ru(bpy)_3^{2+}$-labeled fragments, resulting in an improved sensitivity of the ECL detection (step c). Because the enhanced ECL signal depends on the enzymolysis of RNase A on the chimeric probe, the activity of RNase A can be accordingly detected. Compared with the traditional DNA-based ECL biosensors, the proposed biosensor does not need the complex process of electrode modification.

Table 1. Comparison of Different Methods for RNase A Detection

method	LOD	linear range	ref
electrochemistry	0.20 pg/mL	0.20 pg/mL to 10 ng/mL	35
electrochemistry	4.0 pg/mL	37 pg/mL to 0.37 μg/mL	13
electrochemistry	10 pg/mL	10 pg/mL to 0.10 μg/mL	14
fluorescence	3.8 μg/mL	3.8 μg/mL to 38 μg/mL	36
fluorescence	0.13 μg/mL	0.13 μg/mL to 20 μg/mL	15
fluorescence	5.5 pg/mL	5.5 pg/mL to 0.88 ng/mL	16
fluorescence	50 pg/mL	50 pg/mL to 1.0 μg/mL	34
chemiluminescence	0.32 pg/mL	1.0 pg/mL to 0.10 ng/mL	1
electrochemiluminescence	0.20 pg/mL	1.0 pg/mL to 0.10 ng/mL	this work

Feasibility Test. PAGE was used to prove the hydrolysis of RNase A on the chimeric DNA probe. As shown in Figure 1A, a band was observed for the long chain of P1 without hydrolysis of RNase A (lane a). Upon introduction of RNase A, no bands were observed (lane b), indicating the long chain of P1 had been hydrolyzed into the short segments by RNase A. These results suggest the highly efficient hydrolysis of RNase A on the fabricated probe.

ECL measurement was further performed to verify the feasibility of the homogeneous ECL biosensor for RNase A detection, and the results are shown in Figure 1B. In the presence of probe P1−Ru only, a weak ECL response was detected (curve a), suggesting the $Ru(bpy)_3^{2+}$ -labeled probe hardly reaches the surface of the ITO electrode due to the strong electrostatic repulsion. In the presence of RNase A, the ECL response was clearly enhanced (curve b), which might arise from the fact that the long chain P1−Ru had been hydrolyzed and the $Ru(bpy)_3^{2+}$-labeled short fragment easily reaches the ITO electrode surface. The chimeric probe (P2) that contains rC base was also investigated to optimize the specificity of the biosensor. It was found that the ECL response of P2−Ru was weaker than that of P1−Ru, indicating the rU-containing probe was superior to the rC-containing one for RNase A detection. To testify the particular electrostatic repulsion of the ITO electrode toward DNA, other bare electrodes (e.g., bare glassy carbon electrode and gold electrode) instead of the ITO electrode had also been

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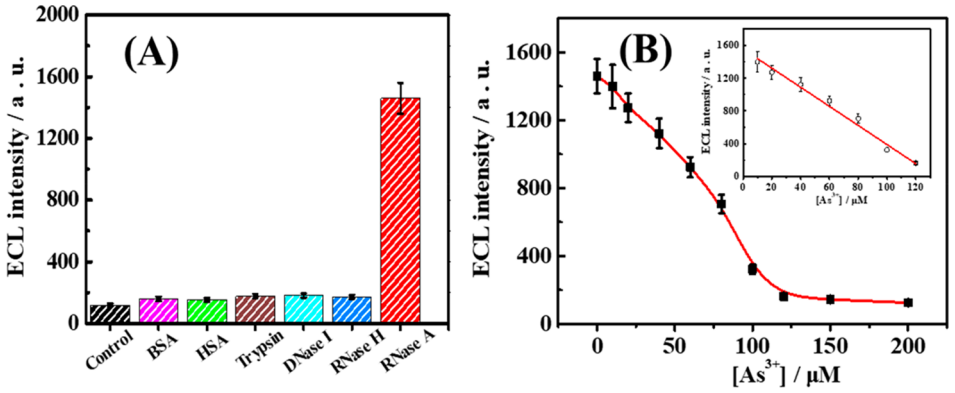

Figure 4. (A) Selectivity of the homogeneous ECL sensing system for RNase A assay. Concentrations of RNase A and interferences were 0.05 ng/mL and 0.5 ng/mL, respectively. (B) Inhibitory effect of As^{3+} (10, 20, 40, 60, 80, 100, and 120 μM) on the activity of RNase A. [P1] = 1.0 μM, [RNase A] = 0.05 ng/mL. Experiments were carried out at 37 °C of the buffer solution (pH = 8.0), and ECL signals were measured in the solution containing TPA (0.02 M) by scanning the potential from 0.6 to 1.6 V at a scanning rate of 100 mV/s.

employed to perform detection under the same operations, but no obvious enhanced signal was observed.

Optimization of the Sensing System toward RNase A. To establish the homogeneous ECL assay responding toward RNase A, relevant conditions were investigated. As a limited concentration of P1−Ru might result in a low ECL response, excessive amounts of P1−Ru might lead to a high background signal; the concentration of P1−Ru was optimized first. As shown in Figure 2A, the ECL signal increased with the increase of P1−Ru concentration and then reached a plateau at 1.0 μM. However, when the concentration of P1−Ru exceeded 1.0 μM, the background signal was also enhanced. Therefore, 1.0 μM P1−Ru was chosen as the optimized condition in this work.

It was reported that the composition of the reaction buffer can influence the activity of RNase A.[34] To obtain the optimized reaction buffer, the pH and metal ions were investigated. As Figure 2B shows, the ECL response increased as the value of the pH rose up and reached a plateau after 8.0. Mg^{2+} in the buffer has an influence on the sensing system (Figure 2C), while Na^{+} showed no marked effects to RNase A activity. Thus, the optimal buffer for the ECL assay for RNase A in this experiment was 50 mM Tris-HCl (pH = 8.0) with 10 mM Mg^{2+}, 100 mM NaCl.

It is well known that the catalytic ability of enzyme is closely related to the reaction time and temperature, which were optimized. As presented in Figure 2D, the ECL signal increased with extension of the digestion time and reached a plateau after 30 min. Therefore, 30 min was selected as the optimal digestion time for the following study. Furthermore, the ECL response increased as the incubation temperature rose up to 37 °C and then decreased after that (data not shown); the possible reason could be attributed to the fact that the activity of RNase A decreased at higher temperature. Thus, 37 °C was selected as the reaction temperature in the following study.

Performance of the Proposed ECL Sensing System. Various levels of RNase A were added to the proposed ECL sensing system, and the responding ECL signals were measured. As shown in Figure 3A, the ECL intensities were enhanced with increasing levels of RNase A (0−0.1 ng/mL). The ECL intensities were linearly related to the RNase A concentrations at the range of 0.001−0.10 ng/mL (Figure 3B). The regression equation can be described as follows

$$\text{ECL/counts} = 274.9 + 20\,957[\text{RNase A}]\text{ng/mL} \quad (1)$$

Its corresponding regression coefficient (R^2) was 0.9714, and the limit of detection (LOD) was determined to be as low as 0.2 pg/mL (S/N = 3). These results demonstrate that this proposed ECL sensing system is sensitive to RNase A activity. Compared with other methods for RNase A detection (Table 1), the novel ECL sensing system not only shows sufficient sensitivity toward RNase A but also avoids tedious operations such as the complex electrode modification in the electrochemical methods and additional substances in the fluorescent methods.

Selectivity and Reproducibility of the Proposed ECL Sensing System. To test the selectivity of this ECL sensing system, some potential interferences including RNase H, DNase I, Trypin, HSA, and BSA were investigated under the same conditions. As shown in Figure 4A, only RNase A induced a significant ECL enhancement, whereas addition of other species led to almost no ECL change. These results confirm that the ECL sensing system has an outstanding selectivity toward RNase A and a prominent capacity of resisting disturbance. The reproducibility of the ECL sensing system was also tested, and the relative standard deviation (RSD) was 6.9% (n = 5), indicating the ECL assay for RNase A had acceptable accuracy and reproducibility.

Investigation of Inhibitor on the Activity of RNase A. The proposed ECL sensing system can be used to detect trace amounts of inhibitors and evaluate the inhibition efficiency based on the half-maximal inhibitory concentration (IC_{50}). Arsenic(III) (As^{3+}) was selected as an example to evaluate the practicality of this ECL sensing system. As shown in Figure 4B, the ECL intensities were gradually decreased with the increasing As^{3+} concentration and showed a good linear relationship in the range of 10−120 μM. The regression equation can be described as

$$\text{ECL/counts} = 1428 - 10.64[\text{As}^{3+}]\mu\text{M} \quad (2)$$

Its corresponding regression coefficient (R^2) was 0.9786, and the LOD was determined to be as low as 2.5 μM (S/N = 3). Using the double reciprocal plot method we further investigated the IC_{50} of As^{3+} toward RNase A, and the value was calculated to be 73.25 ± 0.57 μM. These results indicate inhibitor detection of RNase A based on the proposed ECL sensing system is feasible.

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CONCLUSION

In summary, a homogeneous ECL biosensor was developed for detecting RNase A activity in the combination of the high enzymolysis of RNase A on the chimeric DNA probe and the high sensitivity of ECL method. On the basis of the different electrostatic repulsion between long and short chain of probes toward the negatively charged ITO electrode, the homogeneous ECL strategy avoided the tedious and time consuming procedures. This is the first time using ECL technology for RNase A detection, and the detection limit was as low as 0.2 pg/mL. The proposed ECL sensing system was also applied for inhibitor detection, and the corresponding IC_{50} for As^{3+} was determined to be 73.25 ± 0.57 μM. Compared with previous DNA-based ECL biosensors, the innovative biosensor not only avoids the complex process of electrode modification and immobilization but also has the merits of simplicity, high sensitivity, and specificity. Given all of these advantages, we are optimistic that the developed ECL sensing system can be further adopted to test diverse ribonucleases.

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Notes

The authors declare no competing financial interest.

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