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Cascade signal amplified assay of nucleic acids based on entropy-driven amplification strategy and Mg^{2+}-dependent DNAzyme cleavage

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ABSTRACT

Nucleic acids-based biosensors are extremely important in modern life sciences and have been widely used for the detection of many biomarkers of disease and extensively applied in many fields, such as medical analysis, gene therapy, and pathogen determination. Therefore, it is necessary to develop some sensitive and selective methods for rapid detection of nucleic acids. In this work, an ultrasensitive and non-enzyme electrochemical biosensor has been developed for nucleic acids detection based on entropy-driven amplification (EDA) strategy and Mg^{2+}-dependent DNAzyme cleavage method. In the presence of target DNA (T-DNA), the T-DNA could hybridize with the premade three-strand duplex (TD) through the toehold region to initiate the EDA process (Cycle I), leading to the generation of Mg^{2+}-dependent DNAzyme served for Cycle II. The newly formed Mg^{2+}-dependent DNAzyme could hybridize with the methylene blue (MB)-labeled hairpin DNA (MB-HP) on the gold electrode surface which induced the cleavage process of Mg^{2+}, resulting in the recycle of Mg^{2+}-dependent DNAzyme, accompanied by the release of MB-labeled DNA fragment from the gold electrode surface. Based on the proposed strategy, the developed electrochemical biosensor exhibited a wide linear relationship in the range from 5 fM to 1 nM with a limit of detection (LOD) of 2.7 fM (S/N = 3), which gave the developed electrochemical biosensor a great promising for the detection of nucleic acids in biomedical research and disease diagnosis.

1. Introduction

In the last few years, the research of nucleic acids-based biosensors has attracted substantial concerns for disease diagnosis, biomedical study and environmental monitoring [1–4]. To satisfy the requirements, a variety of detection technologies have been developed, such as photoelectrochemistry [5,6], electrochemiluminescence [7,8], colorimetry [9–11], fluorescence [12,13], and electrochemistry [14,15]. Among these biosensing technologies, electrochemical technique has drawn gradual attentions due to its rapid response, good sensitivity, low cost, and simplicity [16–18].

Recently, in order to obtain more excellent analytical performance, many sorts of signal amplification strategies have been introduced into the electrochemical biosensors. These signal amplification strategies can be divided into two main types: enzyme-regulated strategy and non-enzyme strategy. The enzyme-regulated strategy is usually related to the singular or combined use of multiple protein nucleases containing polymerase, exonuclease, and endonuclease to obtain the recycle of targets or the yield of copious target-assisted nucleic acid sequences for signal amplification [19–23]. Nevertheless, the enzyme-regulated signal amplification strategies may lead to nonspecific reaction and increase the risk of false-positive signal output, and in some cases utilizing multiple sorts of enzymes can increase the assay cost and reduce the portability of target determination. In addition, most enzymes are highly sequence-specific and impressionable to the test environment, and the enzymatic probe reactions require strict conditions (such as temperature, pH value, and salinity) to proceed, which may limit their extensive applications [24,25]. The non-enzyme signal amplification strategy is generally designed in light of the dynamic DNA assembly principle containing hybridization chain reaction (HCR), catalyzed hairpin assembly (CHA), and entropy-driven amplification (EDA) strategy. Although many sensitive biosensors have been developed for target detection based on HCR [26,27] or CHA strategy [28,29], these biosensors still have some drawbacks, such as background signal due to catalyst-independent by-reactions and environmental interference, which may result in relatively low signal-to-noise ratio and sensitivity. For example, the two hairpin nucleic acids in the CHA reaction can probably hybridize with each other nonspecifically even without target, and the nonspecific background

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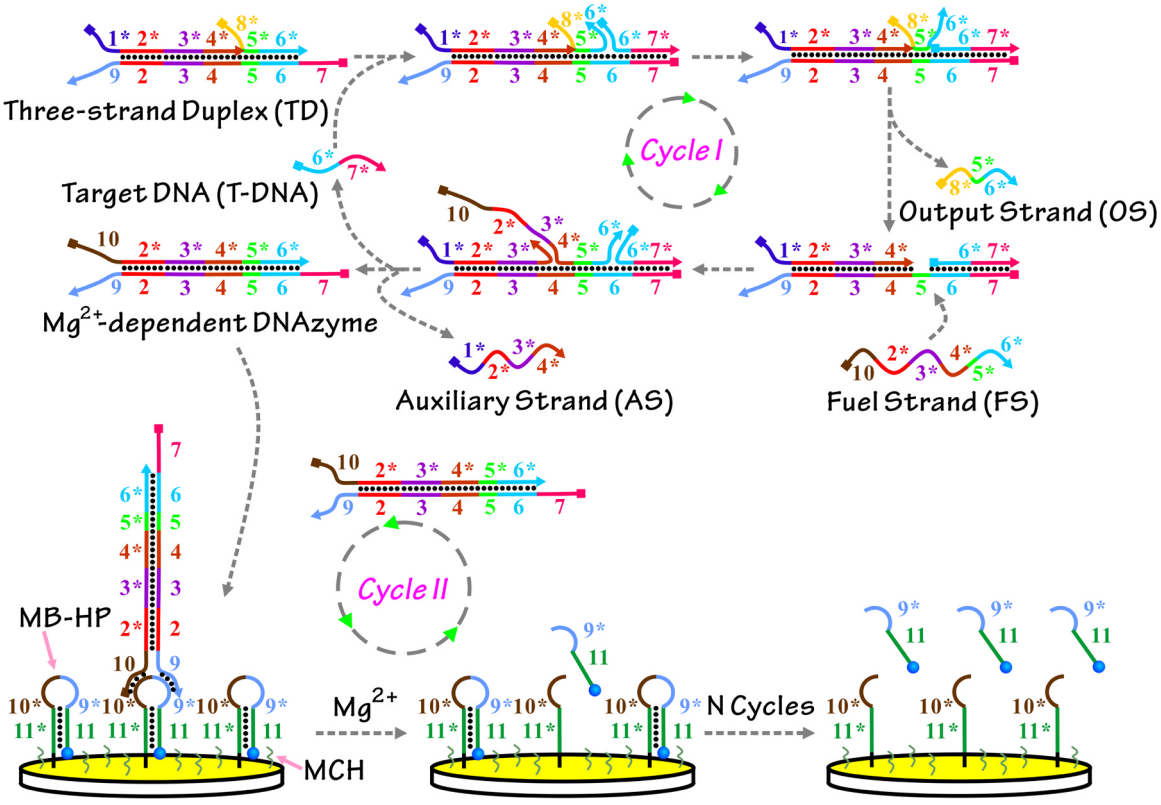
signal decreases the sensitivity of the sensing system [30]. As a landmark report in the field of DNA circuits, EDA strategy was constructed by Zhang *et al.*, which was driven forward by the increase of entropy in the reaction system and formed stable double-stranded complex, leading to an irreversible reaction [31]. Briefly, when two single-stranded DNA (ss-DNA) share the same domain, they will bind to the complementary-stranded domain competitively and the shorter ss-DNA is displaced by an external introduction of longer ss-DNA through its hybridization with a toehold domain to trigger the strand displacement process [32]. In addition, DNAzymes have been used as active elements for the signal amplification and information delivery to detect various metal ions, for instance, Zn^{2+}, Cu^{2+}, Mg^{2+}, Pb^{2+}, Hg^{2+}, Cd^{2+} and so on [33–38]. In order to obtain much more sensitive analytical performance, exploring new approaches based on multi-signal amplification strategies is still considered to be highly desirable. Inspired by the advantages of EDA strategy and metal ions-dependent DNAzyme cleavage method, it is very necessary to develop a biosensing platform for sensitive detection of nucleic acids based on these two strategies. This biosensing platform has three attractive features. First, the EDA strategy is an irreversible process, which helps the reaction to proceed in the forward direction. Second, compared with protein enzymes, most of DNAzymes can be denatured and renatured multiple times without the loss of their binding capability or activity toward substrates, which contributes to the recycle of DNAzymes and provide an effective route to achieve an excellent signal amplification. Third, the biosensing system is pretty easy to perform without any protein enzymes, or precise temperature cycling. To the best of our knowledge, it has rarely been reported an electrochemical biosensor coupled with the EDA strategy and metal ions-dependent DNAzyme cleavage method used for nucleic acids detection.

Herein, an ultrasensitive and non-enzyme electrochemical biosensor has been developed for the detection of nucleic acids based on EDA strategy and Mg^{2+}-dependent DNAzyme cleavage method (Scheme 1). In the presence of target DNA (T-DNA), the T-DNA could hybridize with the premade three-strand duplex (TD) through the toehold region to initiate the EDA process, leading to the release of output strand (OS) and expose a prelocked toehold region for the hybridization with a fuel strand (FS). The hybridization between FS and substrate strand (SS) resulted in the release of auxiliary strand (AS) and T-DNA used for Cycle I and the generation of Mg^{2+}-dependent DNAzyme served for Cycle II. The newly formed Mg^{2+}-dependent DNAzyme can hybridize with the methylene blue (MB)-labeled hairpin DNA (MB-HP) on the gold (Au) electrode surface which induced the cleavage process of Mg^{2+}, resulting in the recycle of Mg^{2+}-dependent DNAzyme, accompanied by the release of MB-labeled DNA fragment from the Au electrode surface. Thereby, the current response of MB decreased. Based on the proposed strategy, the developed electrochemical biosensor exhibited a wide linear relationship in the range from 5 fM to 1 nM with a limit of detection (LOD) of 2.7 fM (S/N = 3), which gave the proposed electrochemical biosensor a good promising for the detection of nucleic acids in molecular recognition and medical diagnosis.

2. Experimental section

2.1. Materials and reagents

All oligonucleotides were supplied by Sangon Biotech Co., Ltd (Shanghai, China) and listed in Table S1. Low molecular weight DNA ladder and gel loading dye, purple (6 ×) were supplied by New England Biolabs Inc. (Beijing, China). 40% acrylamide/bis solution, 19:1 was obtained from Bio-Rad Laboratories Inc. (Shanghai, China). SYBR Gold and 10 × Tris-borate-EDTA (TBE) buffer were supplied by Life Technologies (Guangzhou, China). Ammonium persulphate (APS), ethylenediaminetetraacetic acid (EDTA), 6-mercapto-hexanol (MCH), TEMED, human serum, tris base, urea, $MgCl_2$, KCl, and NaCl were obtained from Sigma-Aldrich Inc. (Shanghai, China). Deionized water was supplied by a Millipore water purification system (≥18 MΩ cm, Milli-Q, Millipore).



Scheme 1. Schematic diagram of the electrochemical biosensor.

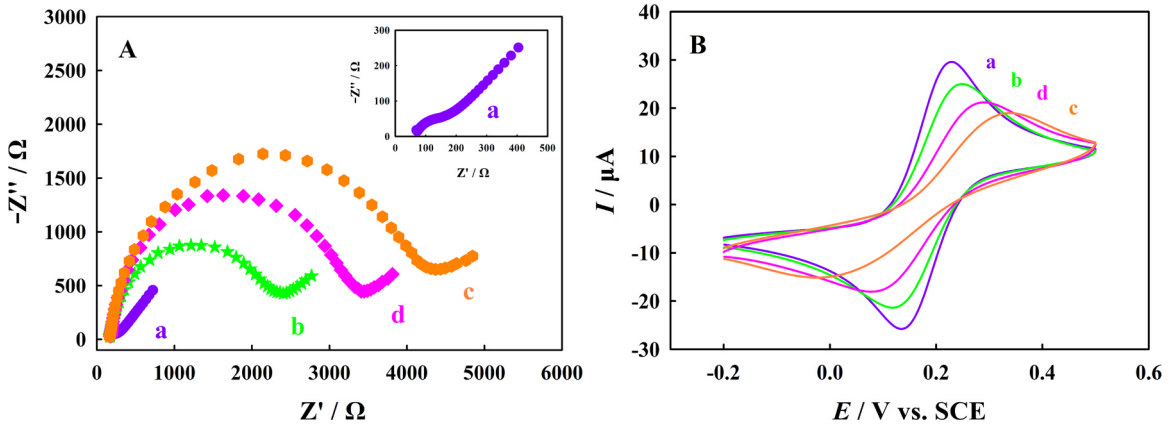


Fig. 1. (A) EIS and (B) CV responses of the different modified electrodes. Bare Au electrode (a), MB-HP/Au electrode (b), MCH/MB-HP/Au electrode (c), MCH/ MB-HP/Au electrode after Cycle I and Cycle II (d).

2.2. Apparatus

ChemiDoc MP Imaging System (Bio-Rad Laboratories Inc., Shanghai, China) and CHI660E Electrochemical Workstation (Shanghai, China) were used in this work. A traditional three-electrode system was employed with a planar Au electrode as the working electrode, a platinum wire as the auxiliary electrode, and a saturated calomel reference electrode (SCE).

2.3. Fabrication of the MCH/MB-HP/Au electrode

Au electrodes (2 mm in diameter) were polished carefully with 0.5 and 0.05 μm alumina oxide slurries separately for 5 min, and then successively sonicated in deionized water and ethanol for 5 min each. The above electrodes then were incubated in a fresh-made piranha solution (H_2SO_4:H_2O_2 = 3:1, *v/v*) for 10 min and completely washed by deionized water. After that, the resulted electrode was treated with 0.5 M H_2SO_4 solution and scanned at the potential from − 0.3 to 1.5 V till a steady cyclic voltammogram was gained. Eventually, the electrode was rinsed by deionized water and dried under a nitrogen atmosphere. Before the fabrication of MB-HP onto the Au electrode surface, it was treated with 10 mM TCEP in Tris-HCl buffer (pH 7.4) in the dark for 1 h. Afterwards, 10 μL of 1 μM MB-HP was added on the Au electrode surface and kept overnight to gain HP-MB/Au electrode. Finally, the resulting electrode was soaked in 2 mM MCH solution for 1 h to gain the MCH/MB-HP/Au electrode.

2.4. Electrochemical analysis of T-DNA

Prior to the amplification reaction, the TD probes were first prepared by mixing the three strands (SS, AS, and OS) with equal molar concentration (2 μM) in 1 × TAE/Mg^{2+} buffer (40 mM Tris-Acetate, 12.5 mM $MgCl_2$, 1 mM EDTA, pH 8.0) and then the mixture was heated to 90 °C for 5 min followed by slowly cooling to room temperature at a rate of 0.1 °C/s. The EDA process was performed in 20 μL of total reaction solution containing 1 μL of 2 μM TD, 1 μL of 3 μM FS, 1 μL of T-DNA with various concentrations, and 17 μL of 1 × TAE/Mg^{2+} buffer. The mixture was kept at 37 °C for 2 h, subsequently, the resulting solution was added on the MCH/MB-HP/Au electrode surface and kept at 37 °C for 2 h. After being rinsed, the final electrode was measured through differential pulse voltammetry (DPV) in 10 mM PBS buffer (50 mM NaCl, 5 mM $MgCl_2$, pH 7.4).

2.5. Native polyacrylamide gel electrophoresis analysis

Before performing the polyacrylamide gel electrophoresis (PAGE), 10% native PAGE was first prepared by using 40% acrylamide and bis-acrylamide solution (19:1) in 1 × TBE buffer containing 0.04% APS and 0.1% TEMED and then pre-run the gel at 250 V for 30 min. After the addition of samples in 1 × Gel Loading Dye, the gel was operated at 180 V for 2.5 h, followed by staining in SYBR Gold dye solution for 15 min, and then the gel was photographed by the ChemiDoc MP Imaging System.

3. Results and discussion

3.1. Characterization of the electrochemical biosensor

The measurements of electrochemical impedance spectroscopy (EIS) were performed to characterized the fabrication process by using the redox couple of 5 mM $[Fe(CN)_6]^{3-/4-}$ (1:1) in 0.1 M KCl solution. As shown in Fig. 1A, the bare Au electrode showed a very small semicircle diameter, indicating a very small value of interfacial electron-transfer resistance (R_{et}) (115 Ω, curve a). After fabrication of MB-HP, the MB-HP/Au electrode displayed a larger R_{et} value because of the strong repulsion effect between the negatively charged MB-HP and $[Fe(CN)_6]^{3-/4-}$ (R_{et} = 2452 Ω, curve b). When the above was further immersed with MCH, an obvious increase of R_{et} value was observed (R_{et} = 4478 Ω, curve c). After the cleavage process of Mg^{2+}-dependent DNAzyme, the R_{et} value decreased due to the release of MB-labeled DNA fragment from the MCH/MB-HP/Au electrode surface (R_{et} = 3406 Ω, curve d). Furthermore, to confirm the stepwise fabrication process, the cyclic voltammetry (CV) was also carried out at a scan rate of 0.1 V/s. As shown in Fig. 1B, the bare Au electrode gave a pair of well-defined peaks (curve a) due to its superior electric conduction. After the modification of MB-HP, a remarkable decrease in current responses and an augment of peak separation could be seen (curve b) because the negatively charged phosphate backbones of MB-HP prevented the access of $[Fe(CN)_6]^{3-/4-}$ on the MB-HP/Au electrode surface. Subsequent blocking with MCH, the current response of the MCH/MB-HP/Au electrode decreased dramatically (curve c). Furthermore, after the cleavage process of Mg^{2+}-dependent DNAzyme, the current response increased due to the release of MB-labeled DNA fragment from the MCH/MB-HP/Au electrode surface (curve d). These results obtained from Fig. 1B are in line with that showed in the EIS measurements (Fig. 1A), verifying the successful modification process of the developed electrochemical biosensor.

3.2. Feasibility of the electrochemical biosensor

To verify if the proposed biosensor was feasible, the DPV method was carried out. As displayed in Fig. 2A, compared with the current response of the MCH/MB-HP/Au electrode (curve a), there was only a negligible change of peak current (curve b) after the MCH/MB-HP/Au

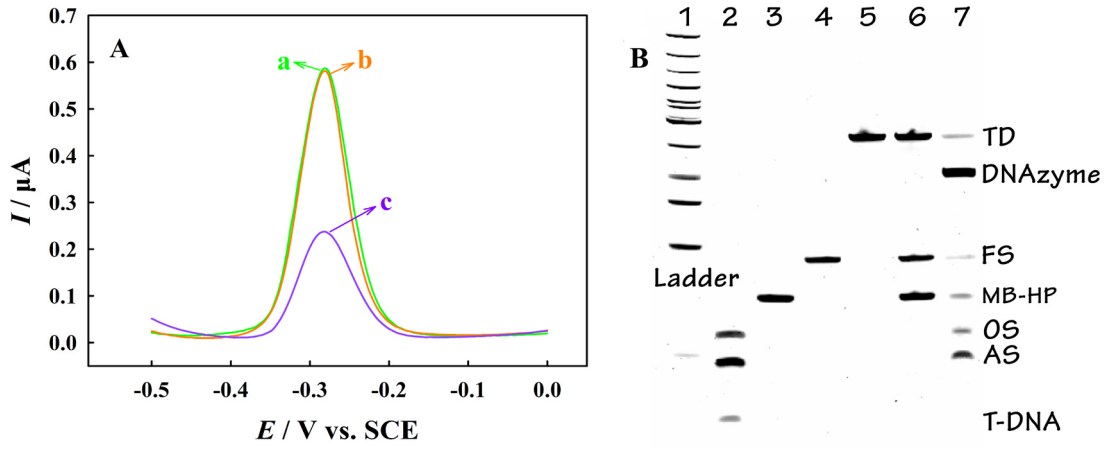


Fig. 2. (A) DPV responses in different experimental conditions. (a) the MCH/MB-HP/Au electrode, (b) the MCH/MB-HP/Au electrode immersed in the EDA process without T-DNA, (c) the MCH/MB-HP/Au electrode immersed in the EDA process with 10 pM T-DNA. (B) 10% native PAGE to analyze the biosensing system. Lane 1: 1 μM low molecular weight DNA ladder; lane 2: 2 μM OS + AS + T-DNA; lane 3: 2 μM MB-HP; lane 4: 2 μM FS; lane 5: 2 μM TD; lane 6: 2 μM TD, 3 μM FS, 2 μM MB-HP; lane 7: 10 pM T-DNA, 2 μM TD, 3 μM FS, 2 μM MB-HP.

electrode was immersed in the EDA process without T-DNA. In the presence of 10 pM T-DNA, a remarkable decrease of the peak current was observed (curve c), which should be contributed to the cleavage of the fabricated MB-HP because of the formation of the Mg^{2+}-dependent DNAzyme through the EDA process. The EDA process was also confirmed by 10% native PAGE image. As shown in Fig. 2B, lane 1 was the band of low molecular weight DNA ladder and lane 2 was the band of OS, AS and T-DNA. Land 5 was the band of TD with lower migration rate than the strands of MB-HP (lane 3) and FS (lane 4). In the absence of T-DNA, no new band could be seen (lane 6). In the presence of T-DNA, it could be seen that an intense, new band appeared and the bands of FS and MB-HP almost disappeared (lane 7), which should be contributed to the formation of Mg^{2+}-dependent DNAzyme and the consumption of FS and MB-HP. These results indicated the potential of the signal amplification approach for amplified and selective determination of T-DNA.

3.3. Optimization of experimental conditions

The assay capability of the sensing system is greatly affected by experimental conditions, so two important experimental parameters containing the reaction temperature and incubation time were optimized. As shown in Fig. 3A, the effect of reaction temperature was evaluated firstly, the ΔI value ($\Delta I = I_0 - I$, I_0 represented the current response in the absence of T-DNA; I represented the current response of 1 pM T-DNA) increased with the increase of temperature from 25 °C to 37 °C. However, when the reaction temperature rose to 45 °C, the ΔI value decreased because the high temperature was not beneficial to the binding of toehold. Thus, 37 °C was selected as the optimum reaction temperature.

Furthermore, the incubation time was also optimized due to its effect on the amounts of both formed Mg^{2+}-dependent DNAzyme and cleaved MB-HP. As shown in Fig. 3B, the ΔI value enhanced with the augment of incubation time and reached a plateau at 2 h. Therefore, 2 h of incubation time was adopted in the whole experiments.

3.4. Detection sensitivity of the electrochemical biosensor

Under optimal experimental conditions, the detection sensitivity of the developed electrochemical biosensor was evaluated with various T-DNA concentrations in the range from 0 to 10 nM. As displayed in Fig. 4A, the peak currents decreased with the augment of T-DNA concentrations. Fig. 4B displayed a good linearity between the currents and the logarithmic concentrations of T-DNA from 5 fM to 1 nM. The regression equation was $I = -0.1012 \lg C_{T\text{-}DNA} + 0.3537$ ($R^2 = 0.9961$) with a LOD of 2.7 fM (S/N = 3), which meant that the detection sensitivity of the proposed biosensor was superior to some existing T-DNA analytical methods (Table S2).

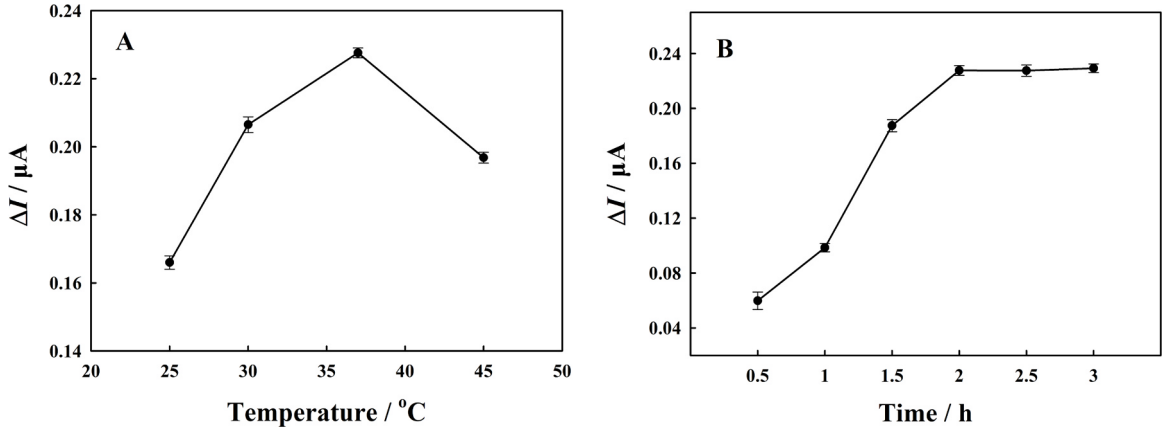


Fig. 3. Effects of (A) reaction temperature and (B) incubation time. The error bars were derived from three separate assays.

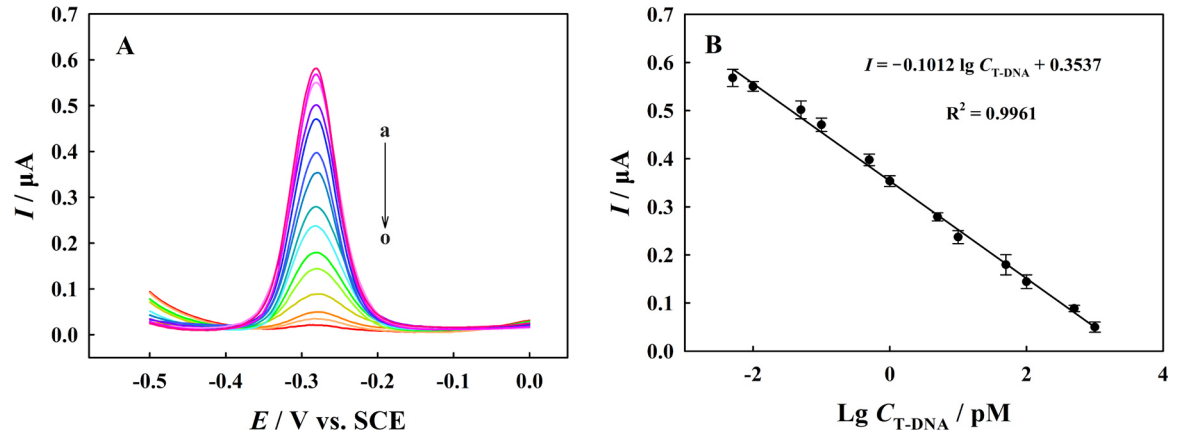


Fig. 4. (A) DPV responses for the assay of various T-DNA concentrations. These concentrations are 0, 5 fM, 10 fM, 50 fM, 100 fM, 500 fM, 1 pM, 5 pM, 10 pM, 50 pM, 100 pM, 500 pM, 1 nM, 5 nM, 10 nM (from a to o). (B) Linear relationship between the currents and the logarithmic concentrations of T-DNA. The error bars were derived from three separate assays.

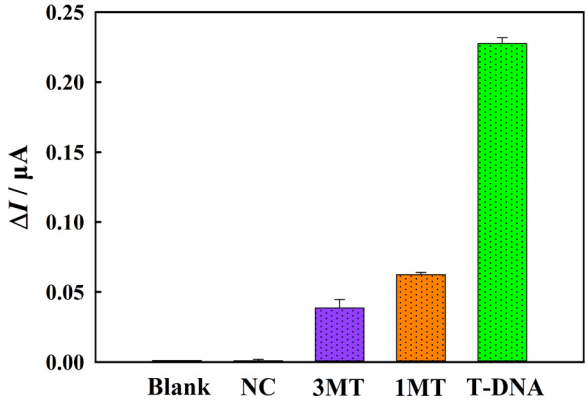


Fig. 5. Specificity of the developed electrochemical biosensor in the blank solution, or in the presence of NC, 3MT, 1MT, and T-DNA. The error bars were derived from three separate assays.

Table 1
Recovery analysis of T-DNA in 10% diluted human serum samples.

Sample (Nos.)	Added	Found	Recovery (%)
1	5 fM	4.87 ± 0.34 fM	97.4
2	50 fM	49.73 ± 1.87 fM	99.46
3	100 fM	104.51 ± 3.28 fM	104.51
4	1 pM	1.08 ± 0.13 pM	108.0
5	5 pM	4.89 ± 0.21 pM	97.8
6	10 pM	10.34 ± 0.56 pM	103.4
7	50 pM	51.37 ± 2.02 pM	102.74

3.5. Stability, reproducibility, and specificity of the proposed electrochemical biosensor

The stability of the electrochemical biosensor was studied by measured five modified electrodes with 1 pM T-DNA and the relative standard deviation was only 4.1%. Moreover, the reproducibility of the biosensor was also evaluated, three parallel measurements indicated that the modified electrodes could keep about 96.8% of their original currents for the determination of T-DNA after the storage of two weeks at 4 °C. All the results showed that the proposed electrochemical biosensor had acceptable reproducibility and stability.

Specificity is a critical factor to study the success of the developed biosensor. Thus, the specificity was evaluated by measuring the DPV responses to four different sorts of DNA sequences, containing T-DNA (1 pM), single-base mismatched DNA (1MT, 10 pM), three-base mismatched DNA (3MT, 10 pM), and non-complementary DNA (NC, 10 pM). As shown in Fig. 5, the NC displayed the similar ΔI value with the blank solution. For 3MT and 1MT, the ΔI values were only about 16.9% and 27.4% of that toward T-DNA, respectively, verifying a good performance to distinguish the perfect complementary target and the base-mismatched targets.

3.6. Real samples analysis

To evaluate the applicability of the developed method for real samples, T-DNA with various concentrations (5 fM, 50 fM, 100 fM, 1 pM, 5 pM, 10 pM, and 50 pM) were added in 10% diluted serum samples. It could be seen from Table 1, the recoveries for T-DNA were 97.4%, 99.46%, 104.51%, 108.0%, 97.8%, 103.4%, and 102.74%, respectively, which was satisfactory for sensitive assays performed in relatively complex biological samples.

4. Conclusions

In summary, this work developed a cascade signal amplified electrochemical biosensor for nucleic acids determination based on EDA strategy and Mg^{2+}-dependent DNAzyme cleavage method. The developed biosensing system demonstrated considerable advantages over the most of conventional nucleic acids-based biosensors, such as enzyme-free, good stability and specificity, as well as a lower LOD of 2.7 fM (S/N = 3). Importantly, it would be easily extended for the determination of various nucleic acids targets through changing the corresponding sequences. Hence, the developed biosensor has a good promising for the determination of nucleic acids in molecular recognition and medical diagnosis.

Acknowledgements

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.talanta.2019.02.008.

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