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Amplified electrochemical DNA sensor using peroxidase-like DNAzyme

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ABSTRACT

A novel electrochemical DNA sensor was developed here by using peroxidase-like G-quadruplex-based DNAzyme as a biocatalytic label. A hairpin structure including the G-quadruplex-based DNAzyme in a caged configuration and the target DNA probe were immobilized on Au-electrode surface. Upon hybridization with the target, the hairpin structure was opened, and the G-quadruplex-based DNAzyme was generated on the electrode surface, triggering the electrochemical oxidization of hydroquinone by H_2O_2, which provide a quantitative measure for the detection of the target DNA. The DNA target was analyzed with a detection limit of 0.6 nM. This method is simple and easy to design without direct conjugation of redox-active element.

1. Introduction

A series of G-quadruplex-based DNAzymes which specifically bind to hemin and act with peroxidase-like activity have been selected via the systematic evolution of ligand by exponential enrichment (SELEX) process [1–4]. In the presence of potassium, the G-quadruplex-based DNAzyme could catalyze the oxidation of luminol or 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) by H_2O_2. These biocatalytic reactions were used to develop different DNA-detection assays [5,6]. With the high stability, easy synthesis and flexible modification, the DNAzymes have been widely used as novel catalytic beacons for the colorimetric [7] or chemiluminescent detection [8,9] of DNA and telomerase activity or the amplified analysis of small molecules [10] and proteins [11].

Electrochemical biosensors attract intensive attention for their simple, sensitive, selective and compatible with microfabrication technologies. Recently, labeling the termini of DNA with redox-active units [12,13], or intercalating the redox-active reagents to the double strands DNA (ds-DNA) [14,15], facilitated numerous electrochemical detection for various analytes. These electronic DNA-based sensors are particularly attractive since they are fast, accurate, compact, portable, and of high sensitivity and selectivity, simple instrumentation, and low production cost. However, the label of DNA probe needs complex procedures and high cost. Even intercalating redox active molecules to ds-DNA omit the conjugation steps, which usually leads to relatively higher background signal.

As such, G-quadruplex-based DNAzyme is an interesting DNAzyme with peroxidase-like activities which could catalyze the oxidation of some redox-active reagents. Herein, considering the advantages of DNA-based sensors and the catalytic effect of G-quadruplex-based DNAzyme, we developed a new electrochemical DNA sensor using peroxidase-like DNAzyme for DNA detection. A hairpin structure consisting of the DNAzyme and DNA probe was designed and immobilized on a gold electrode. Upon hybridization with the target DNA, the DNAzyme was released from the hairpin structure and triggered the electrochemical oxidization of hydroquinone by H_2O_2, providing a quantitative measure for the detection of the target DNA. In this strategy, the hairpin probe possesses dual functions, discriminating the target DNA and generating electrochemical signal by catalyzing. Furthermore, it saves the step of redox-active modification and allows the sensor to be not only signal amplification, but also lower cost.

2. Experimental

2.1. Reagents and materials

The oligonucleotides were commercially synthesized by TaKaRa Bio Inc. (Dalian, China). Sequences of the oligos are listed in Table 1. Hemin was purchased from Sigma–Aldrich, and used without further purification. A hemin stock solution was prepared in DMSO and stored in the dark at −20 °C. All H_2O used in the preparation of buffers and for rinse solution had a resistivity of 18.2 MΩ as produced by the Nanopure Infinity™ ultrapure water system

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Table 1
The hairpin probe and oligonucleotides.

Name	Sequence (5′ to 3′)
Hairpin probe	5′-(SH)-*CCTACCC*AGGATTGCATCAAAAAGATTAAGAG *TGGGTAGGG* CGGGTTGGG-3′
Target DNA	5′-CTCTTAATCTTTTTGATGCAATCC-3′
T_1	5′-CTCTTAATCTTTTT**C**ATGCAATCC-3′
T_2	5′-CTCTTAAT**G**TTTTT**C**ATGCAATCC-3′
T_3	5′-CTCTTAAT**G**TTTTT**C**AT**C**CAATCC-3′
T_r	5′-ATGGGCGCACCTCTCTTTACTGTG-3′

The italicized region of the hairpin probe identifies the stem sequence and the underlined region identifies the peroxidase-like G-quadruplex-based DNAzyme. The bold face identifies the mutant points.

(Barnstead Thermolyne Corp., Dubuque, IA, USA). Hydroquinone, H_2O_2 and other reagents were of analytical grade. The buffer used for the forming of hemin/G-quadruplex contains nonionic detergent Triton X-100 (0.05%, w/v) and DMSO (1%, v/v).

2.2. Instruments

Voltammograms were recorded at a CHI660 electrochemical work station (Shanghai Chenhua Instruments, Shanghai) with a three-electrode system consisting of a gold working electrode with a diameter of 2 mm, a saturated calomel reference electrode and a platinum wire auxiliary electrode in a 10 mL beaker.

2.3. Experimental details

2.3.1. Immobilization of the hairpin probe and hybridization with the target DNA

The gold electrode (2 mm diameter) was subsequently polished to a mirror face with 1.0, 0.3, and 0.05 μM alumina slurry. After washed by ultrasonicating in both ultrapure water and ethanol for 5 min, the electrode was next purified by immersing in piranha solution (3:1 H_2SO_4/H_2O_2) for 5 min and then thoroughly rinsing in deionized water. It was then scanned between −0.5 V and 1.5 V in 0.1 M sulfuric acid for more than 20 cycles until a steady current–voltage curve was obtained. The gold electrode was interacted with a solution containing 1.5 μM hairpin probes in Tris–HCl buffer (pH 8.1) in a humidified chamber for 2.5 h. The resulting electrode was washed with Tris–HCl buffer (pH 8.1). The electrode was then treated with 1 mM 1-mercaptohexanol in 0.1 M phosphate buffer (pH 7.4) for 20 min. Finally, the resulting monolayer functionalized electrode was treated with different concentration of target DNA in Tris–HCl buffer (pH 8.1), for 80 min, to yield the ds-DNA assembly on the electrode.

2.3.2. Formation of hemin/G-quadruplex complex

An identical volume of solution containing 12 μM hemin, 25 mM HEPES, 20 mM KCl, 200 mM NaCl, 0.1% Triton X-100, and 1% DMSO, pH 7.4, was added on the assembled surface for 12 h, to allow appropriate folding of the nucleic acid and form the super molecular hemin/G-quadruplex.

2.3.3. Amperometric measurements

A three-electrode system was employed in this measurement. In a 10 mL beaker, a modified gold electrode was used as working electrode. All electrochemical potentials are reported versus a saturated calomel reference electrode (SCE). And a platinum wire was used as the counter electrode. The electrolytes employed were 10 mL of 0.1 M phosphate buffer (pH 7.0) with 0.15 M NaCl and 1 mM hydroquinone. The solution was first bubbled thoroughly with pure nitrogen gas for 20 min, and then blown gently across the surface of the test buffer. Amperometric measurements were performed at a potential −0.15 V with unceasing stirring, until the transient current rose to a steady value. And then, 10 μL 30% H_2O_2 was added quickly to the buffer. The sharply increase of the oxidization currents indicates the formation of G-quadruplex-based DNAzyme. The cyclic measurements were conducted between −0.3 V and 0.1 V at a scan rate of 100 mV/s.

3. Results and discussions

3.1. Principle of the amplified DNA detection strategy

The principle of this electrochemical DNA sensor is shown in Scheme 1. A hairpin probe with 5′-end thioled is immobilized on Au-electrode surface. The hairpin probe consists of segments A, B, and C. Segment A is the peroxidase deoxyribozyme PS2.M evolved by Sen and coworkers [16]. In the presence of potassium ions, the PS2.M forms into a G-Quadruplex with hemin, with a high affinity (27 ± 2 nM), and could catalyze the oxidization of some redox reagents by H_2O_2 [16–18]. Segment B is complementary to target DNA, while segment C is complementary to the 5′-end of segment A with 8 nucleotides. In the absence of target, the segment A hybridized with segment C and was prevented from forming the G-quadruplex-based DNAzyme. Upon hybridization with the target, the hairpin configuration was opened and released the segment A, producing with hemin the G-quadruplex-based DNAzyme that oxidize hydroquinone by H_2O_2, and provided a quantitative measure for the detection of the target DNA.

3.2. Electrochemical characterization of the formation of peroxidase-like G-quadruplex-based DNAzyme

The activation of the DNA detection system is based on the formation of G-quadruplex-based DNAzyme in the presence of target DNA. To characterize the formation of G-quadruplex-based DNAzyme, cyclic voltammetry was employed. As shown in Fig. 1 (curve a), when applying a range of 0.6–0.1 V, a reduction peak was observed at −0.370 V, which was reported in a previous study [19]. The peak indicates the formation of G-quadruplex-based DNAzyme, while the peaks of hemin alone or hemoglobin are at −0.365 V, and −0.392 V, respectively. A control experiment was performed, which was treated with the same procedures except for adding hemin into the system. There was no peak observed at −0.370 V (curve b in Fig. 1). These results indicate

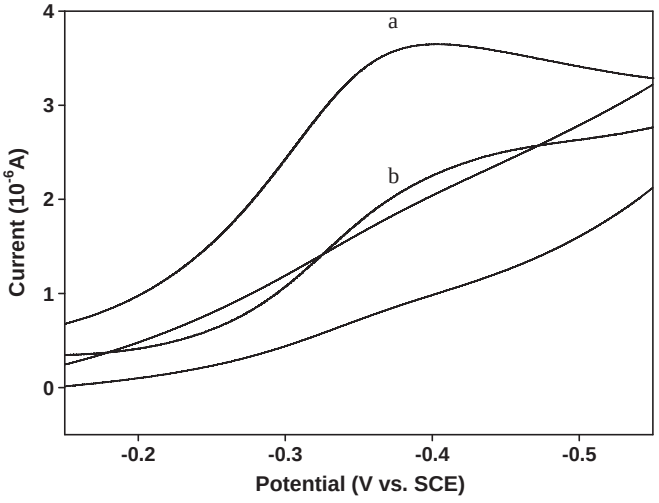
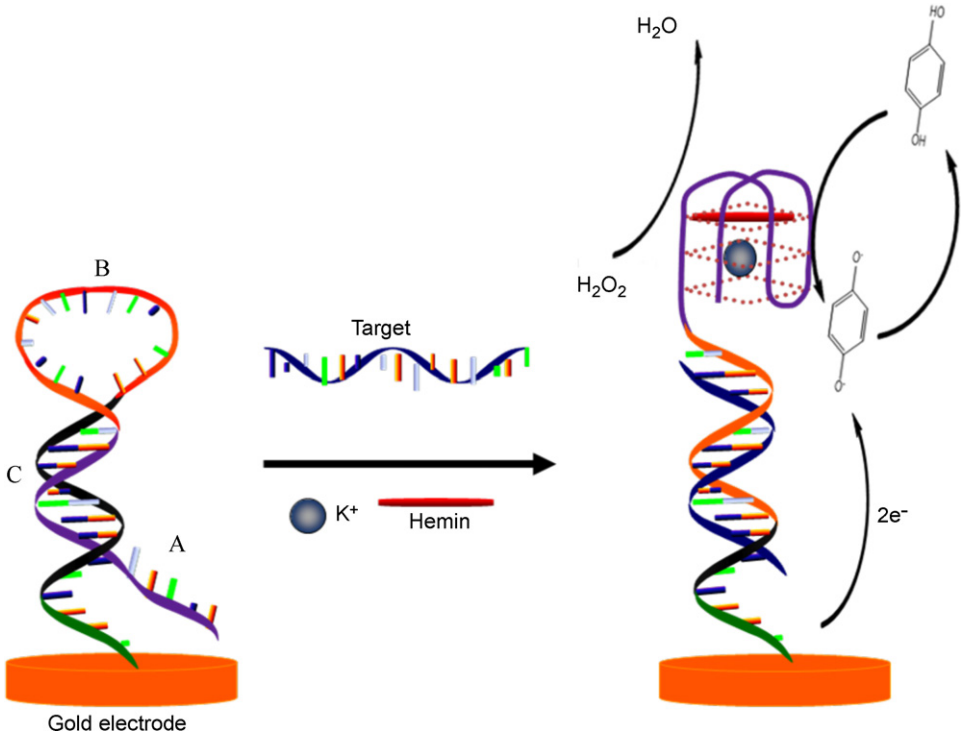


Fig. 1. Typically cyclic voltammograms in 0.1 M phosphate buffer (pH 7.0) containing 0.15 M NaCl. Hairpin probe was modified on electrode and incubated with target DNA (1 μM) with loading hemin (a) and without loading of hemin (b).



Scheme 1. Sensing target DNA by opening the hairpin probe immobilized on gold electrode and forming G-quadruplex-based DNAzyme that catalyze the oxidization of hydroquinone by H_2O_2.

that the peak characterized the formation of G-quadruplex-based DNAzyme.

3.3. Electrochemical behaviors of this DNA sensor

This method for DNA detection is based on the formation of hemin/G-quadruplex and its catalytic behavior. First, we investigated the feasibility of this DNA sensor. As shown in Fig. 2A, a reduction peak (curve a) is observed at −0.12 V in the presence of target DNA, indicating that the oxidization of hydroquinone was triggered by the G-quadruplex-based DNAzyme. While no reduction peak (curve b) is observed in the absence of target DNA, which indicates no oxidization of hydroquinone occurred. These results indicate that the hairpin probe recognized the target DNA and hybridized with it, leading to the formation of G-quadruplex-based DNAzyme and catalyzing the redox reaction in the presence of H_2O_2/hydroquinone.

The above results were further confirmed by amperometric measurements. As shown in Fig. 2B, we find the significant increase of the current upon adding H_2O_2 to the mixture in the presence of target DNA, which indicates that the hemin/G-quadruplex is formed and acts with peroxidase-like activity.

3.4. Optimization of the experimental conditions

Since the maximum loading amount and the spatial alignment of hairpin probe directly influence the detection sensitivity, the hairpin probe concentration for fabrication of the sensing interface has to be optimized. The gold electrode was interacted with a serial of solutions containing different concentrations of hairpin probe range from 0.1 nM to 4 μM, respectively. As shown in Fig. 3A, sensor gain is a complex function of probe concentration for modification. Maximum gain is observed when the concentration of hairpin probe is 1.5 μM and decreases at lower and higher concentrations. For example, at higher concentration of hairpin probe (4 μM), the gain is only one-third of the maximum signal (1.5 μM). Meanwhile, at lower concentration of hairpin probe (0.5 μM), the gain also decreases significantly. We presumed that this behavior occurred owing to the existence of two competing effects. One is that target accessibility might be restricted at high probe concentration, since the assembly of hairpin probe was too dense. Another effect is that the electrode might exchange electrons with the hydroquinone at low probe concentration, which increased the background current and thereby decreased signal intensity.

The efficiency of the hybridization between hairpin probe and target DNA on the electrode also influences the performance of this sensor. We expected that sufficient reaction time could guarantee the maximized amount of target DNA hybridizing to the hairpin probe on the electrode, which in turn improved the sensitivity in DNA detection. 1.0 μM target DNA was allowed to hybridize with the hairpin probe modified on the electrode with different time (40, 60, 80, 120, 160, 240 min) at room temperature, respectively. Fig. 3B shows the effect of the hybridization time between hairpin probe and target for DNA detection. The reduction current increases with the hybridization time, reaching a plateau at 80 min. Therefore, to ensure the hybridization completed, we set the hybridization time as 80 min throughout this study.

Since sufficient H_2O_2 could accelerate the oxidization of hydroquinone, we investigated the effect of the concentration of H_2O_2 for DNA detection. As shown in Fig. 3C, for 1 μM target DNA, the amperometric response increases with adding of the H_2O_2 (from 1 to 15 mM). At an H_2O_2 loading of 10 mM, the current increase reaches a steady level and then leveled off. Higher H_2O_2 loadings are not considered in order to not increase the biosensor cost.

3.5. Analytical performance of the DNA sensor

Fig. 4 shows the relative reduction current of this DNA sensor vs. the logarithm of concentration of target DNA range from 1 nM to 1000 nM. The inset figure shows that the reduction current increase is proportional to the logarithm of the concentration of target DNA in the range from 0.1 to 1000 nM (R^2 = 0.9889). A

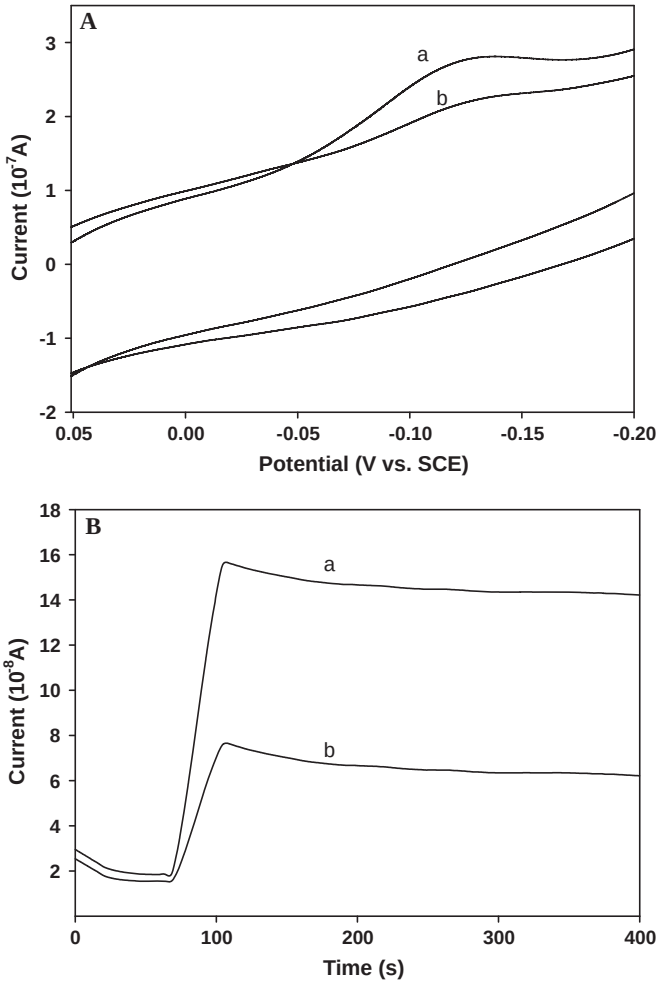


Fig. 2. (A) Cyclic voltammograms in 0.1 M phosphate buffer (pH 7.0) containing 0.15 M NaCl and 1 mM hydroquinone. The electrode modified with probe was treated with 5 μM target DNA (curve a) and no target DNA (curve b). (B) Chronoamperometric curves of electrode modified with hairpin probe, treated with 5 μM target DNA (curve a) and no target DNA (curve b) by adding of H_2O_2. Amperometric experiments were performed at a working potential of −0.15 V under unceasing stirring until the transient current decreased to a steady value.

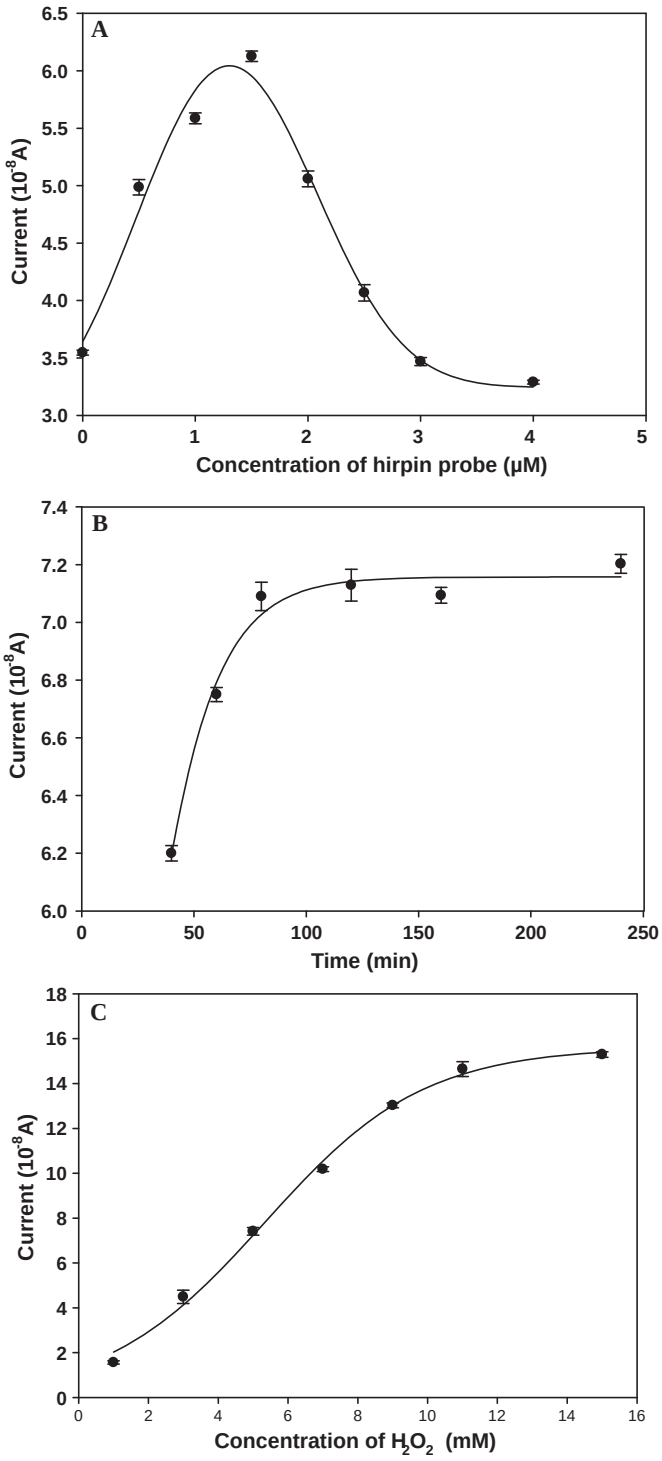


Fig. 3. Optimization of experimental conditions. (A) Concentration of hairpin probe modified on electrode. (B) Hybridization time with target DNA. (C) Concentration of H_2O_2.

detection limit of 0.6 nM was obtained (S/N > 3) by this sensor. Compared with the colorimetric assay which adopted the same hairpin probe and target DNA, and the absorbance was proportional to the concentration of target DNA, in the range from 0.2 μM to 4.3 μM [6], our electrochemical protocol based on peroxidase-like DNAzyme amplifying reaction improved the sensitivity in ~3 order and extended the range of detection by magnitudes. Moreover, in comparison with electrochemical method for DNA detection base on hybridization and with redox-active molecules tagged probe, which had a detection range from 2 nM to 100 nM [20], this sensor significantly enhances the sensitivity with the amplification effect of peroxidase-like DNAzyme. Nevertheless, this assay possesses a comparable sensitivity with the method employed with gold nanoparticles amplification and peroxidase-like DNAzyme amplification reaction for DNA detection, with detection limit 0.1 nM [5].

The RSD of this method across four electrodes is ~10% for the detection of 10 μM target DNA under the same experimental condition, indicating that the proposed sensing system could offer an acceptable reproducibility for the detection of target DNA. Moreover, random, and 1nt-, 2nt-, or 3nt-mismatched DNA sequences (T_r, T_1, T_2, and T_3) were synthesized to examine the selectivity of this DNA sensor. As seen in Fig. 5, when the signal for the perfect match DNA target is 100%, the same concentration of random DNA target yields a signal of only 2.3%, which is in the error for the perfect match DNA target (T). And the signals for 1nt-, 2nt-, and 3nt-mismatched DNA sequences for the same concentrations are 35.4%, 31.5%, 22.4% respectively, which indicate that this DNA sensor could selectively determine the target DNA from its analogous sequences.

Sensor regeneration is crucial to ensure that a signal is arising due to specific recognition of target instead of nonspecific modifi-

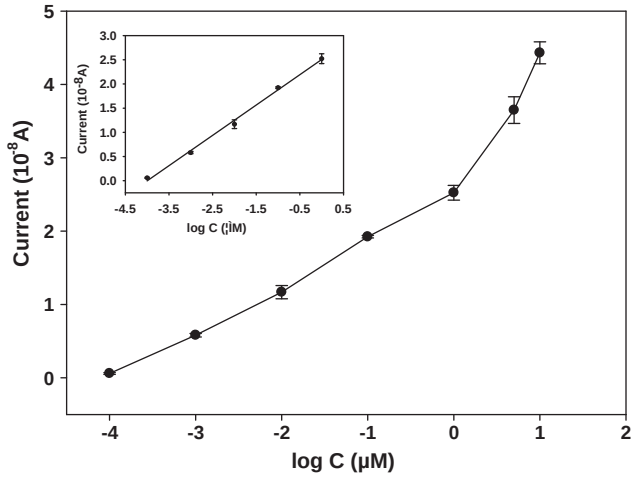


Fig. 4. Current responses of the electrode on concentration of target DNA.

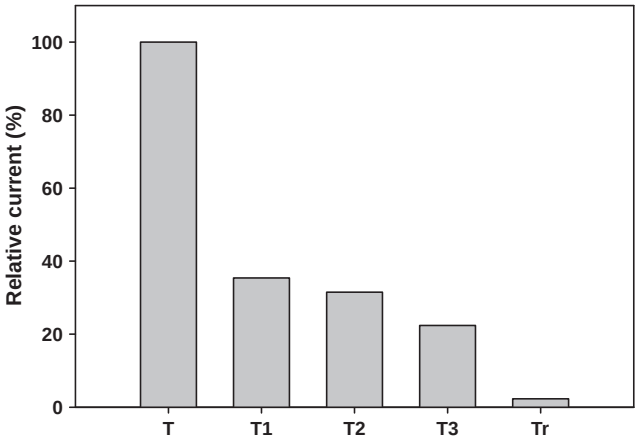


Fig. 5. Relative current responses to target DNA, T_1, T_2, T_3, and T_r (1.0 μM).

cation of the probe or sensor head. Fortunately, the sensor is stable enough to allow for ready regeneration, because the hairpin probe is a single-stranded nucleic acid sequence which is strongly chemoadsorbed to the gold electrode surface, To eliminate the effects of any secondary structure of the hairpin probe, the sensed electrode was immersed in the hybridization buffer at 90 °C with unceasing stirring, and the electrode was washed by hybridization buffer, followed to be gradually cool to room temperature. The resulted electrode was treated with the same concentration of target DNA. The regenerated sensor got 13% lower signal than that of previous one. But the background signal decreased in almost the same percentage. This result indicates that this DNA sensor could be easily and successfully regenerated. We stored one electrode at 4 °C for two weeks, and then the signal could still reach 91% of the original one.

4. Conclusions

An amplified electrochemical DNA sensor for sensitive detection of DNA was developed in this study. This DNA sensor emphasized the using of a region of DNA sequence in the probe design, which could act as an enzyme in the presence of hemin. It makes this sensor excellent for eliminating the procedures for enzymes or redox active molecules conjugation. Even though we could not make real comparison between this sensor and other reported ones [21–24] by using horseradish peroxidase as biocatalysts which were with the activity ~2 orders higher than the peroxidase-like G-quadruplex-based DNAzyme, we provided a simple and convenient strategy for DNA or other molecule detection. Furthermore, considering this modification-free feature and the valuable feature of electrochemical sensor, this sensor has the potential to be developed into a high sensitivity and selectivity, low cost, portable, and compact devices for nucleic acid detection.

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