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A label-free electrochemical biosensor for highly sensitive and selective detection of DNA via a dual-amplified strategy

Rong-Mei Kong [a,b], Zhi-Ling Song [b], Hong-Min Meng [b], Xiao-Bing Zhang [b,*], Guo-Li Shen [b], Ru-Qin Yu [b]

[a] The Key Laboratory of Life-Organic Analysis, College of Chemistry and Chemical Engineering, Qufu Normal University, Qufu Shandong 273165, PR China
[b] State Key Laboratory for Chemo/Biosensing and Chemometrics, College of Chemistry and Chemical Engineering, Hunan University, Changsha 410082, PR China

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ABSTRACT

In this work, by combining the enzymatic recycling reaction with the DNA functionalized gold nanoparticles (AuNPs)-based signal amplification, we have developed an electrochemical biosensor for label-free detection of DNA with high sensitivity and selectivity. In the new designed biosensor, a hairpin-structured probe HP was designed to hybridize with target DNA first, and an exonuclease ExoIII was chosen for the homogeneous enzymatic cleaving amplification. The hybridization of target DNA with the probe HP induced the partial cleavage of the probe HP by ExoIII to release the enzymatic products. The enzymatic products could then hybridize with the hairpin-structured capture probe CP modified on the electrode surface. Finally, DNA functionalized AuNPs was further employed to amplify the detection signal. Due to the capture of abundant methylene blue (MB) molecules by both the multiple DNAs modified on AuNPs surface and the hybridization product of capture DNA and enzymatic products, the designed biosensor achieved a high sensitivity for target DNA, and a detection limit of 0.6 pM was obtained. Due to the employment of two hairpin-structured probes, HP and CP, the proposed biosensor also exhibited high selectivity to target DNA. Moreover, since ExoIII does not require specific recognition sequences, the proposed biosensor might provide a universal design strategy to construct DNA biosensor which can be applied in various biological and medical samples.

1. Introduction

Developing biosensors for highly sensitive detection of sequence-specific oligonucleotides with an equally strong capability to identify single nucleotide polymorphisms (SNPs) has attracted considerable interests (Lubin and Plaxco, 2010). The research upsurge proved the increasing importance of DNA detection in modern life sciences such as gene identification, molecular diagnostics, pathogen detection, forensic investigation, and environmental monitoring (Mo et al., 2013; Weile and Knabbe, 2009; Takamiya et al., 2013; Rodriguez-Mozaz et al., 2006). Hence, development of highly sensitive analytical methods coupling with the specificity to detect unique target DNA sequences has been a major goal of biosensor technology. The polymerase chain reaction (PCR) amplification is the most widely used method for detection of specific DNA sequences due to its excellent performance in amplifying oligonucleotide targets and making even single-molecule detection possible (Ye et al., 2003; Ju et al., 2003). However, the PCR-based thermal cycling amplification methods are limited by its own complexity, as well as contamination and false-positive signals (Lie and Petropoulos, 1998; Dirks and Pierce, 2004; Mason et al., 2006).

In recent years, an isothermal cyclic signal amplification strategy based on the reaction of a single target DNA molecule with multiple signal probes has attracted increasingly interests due to its efficiently improvement of the detection sensitivity without sacrificing the specificity (Zhu et al., 2012; Gao et al., 2013a, 2013b). In this research strategy, the hybridization between target DNA and signal probe can trigger the selective enzymatic cleavage or digestion of signal probe, resulting in the release of the full target DNA to hybridize with another signal probe and trigger the next round of cleavage or digestion. Therefore, it is possible to produce multiple signals to achieve the signal amplification detection purposes. The using of nicking endonucleases, exonuclease, polymerase and the DNAzyme for amplified detection of DNA have been reported in the literatures (Kong et al., 2011; Gao et al., 2013c; Zuo et al., 2010; Freeman et al., 2011; Guo et al., 2009; Lu et al. 2011; Zhang et al., 2013). These reported biosensors based on various signal transduction mechanisms, including fluorescence (Peng et al., 2012; Liu et al., 2013),

* Corresponding author. Tel.: +867 318 882 2577; fax: +867 318 882 2782.
E-mail addresses: xiaobingzhang89@hotmail.com, xbzhang@hnu.cn (X.-B. Zhang).

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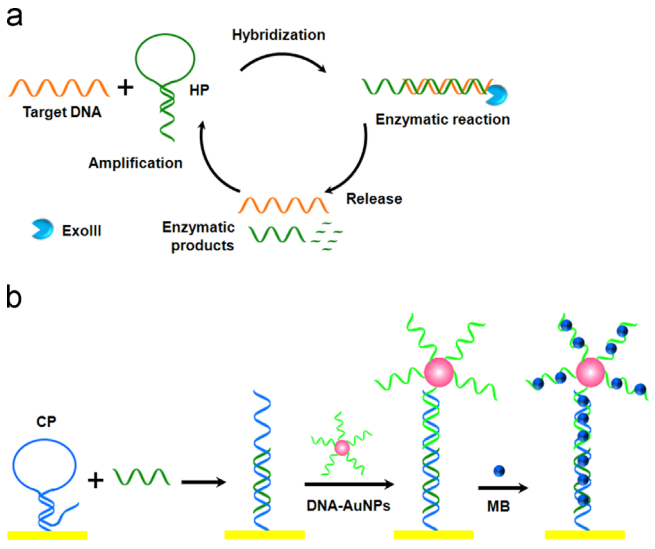
colorimetry (Xu et al., 2009), and electrochemistry. Among them, electrochemical methods have received increasing attention due to their remarkable features, such as high sensitivity, simple instrumentation and low production cost. However, the enzymatic reactions were performed on the electrode surfaces for most of the reported electrochemical biosensors which suffer from the low cleavage or digestion efficiency (Hsieh et al., 2010; Fan et al., 2012). In contrast to linear probes, hairpin structure probes show improved ability to distinguish mismatches, and they have been widely used in DNA/RNA detections (Bonnet et al., 1999). Exonuclease III (ExoIII) is a double-strand-specific 3′ to 5′ exodeoxyribonuclease which catalyzes the stepwise removal of mononucleotides, while it is less active on single-stranded DNA or 3′-overhang termini of double-stranded DNA (Richardson et al., 1964; Wang et al., 2005). In contrast to the sequence-specific nicking endonuclease, ExoIII does not require the specific recognition sequence. Therefore, ExoIII can directly digest any double-stranded DNA at the blunt or recessed 3′ end, which possesses more beneficial of developing universal signal amplification strategies for DNA detection (Wu et al., 2011; Ju et al., 2012).

Nanomaterials offer excellent prospects for biosensing because of their unique properties Wang and Lu (2009). Owing to the large surface to volume ratio and excellent biocompatibility, gold nanoparticles (AuNPs) can be modified with a large number of DNA strands onto their surfaces. Therefore, DNA functionalized AuNPs have been employed for designing biosensors to improve the detection sensitivity due to their efficient signal amplifying properties (Kong et al., 2009; Li et al., 2009). Herein, we reported the development of a novel label-free electrochemical assay for sensitive and selective detection of DNA via ExoIII and nanoparticle dual signal amplification technology (Scheme 1). The assay is first carried out in a homogeneous solution to realize the hybridization of hairpin probe with target DNA and the ExoIII enzymatic amplification reaction, which can both avoid the drawback of low enzymatic efficiency of interfacial reaction and provide a better ability to distinguish mismatches by using the hairpin probe. Then, the released enzymatic product hybridize with the capture probe on electrode surface, the hairpin structure of capture probe can further improve the base–mismatch recognition ability. Finally, DNA functionalized AuNPs was employed for further electrochemical signal amplification. Since methylene blue (MB) molecules can specifically bind with guanine bases to form MB–guanine complexes and result in a significant increase in the electrochemical signal, MB was used as the electrochemical indicator (Tuite and Norden, 1994; Rohs et al., 2000). Due to the capture of abundant MB molecules by both the multiple DNAs modified on AuNPs surface and the hybridization product of capture DNA and enzymatic products, the proposed biosensor shows an improved sensitivity and specificity for target DNA.



Scheme 1. Schematics of the nanoparticle and enzymatic dual signal amplification technology based DNA electrochemical sensing platform: (a) ExoIII digestion amplification; (b) electrochemical detection of target DNA.

2. Experimental section

2.1. Materials and Reagents

Exonuclease III (ExoIII) and NEB buffer 1 were purchased from New England Biolabs Inc. $HAuCl_4 \cdot 4H_2O$ and 6-mercaptohexanol (MCH, 98%) were purchased from Sigma-Aldrich (Sigma-Aldrich Co. LLC., China), Methylene blue (MB) was purchased from Shanghai Reagents (Shanghai, China), and all other chemicals were of analytical reagent grade and used without further purification or treatment. Buffers involved in this work were as followed: 1 × NEB buffer 1 (10 mM Bis Tris Propane-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.0 @ 25 °C) was used for enzymatic experiments; 10 mM PBS (10 mM Na_2HPO_4–NaH_2PO_4, 300 mM NaCl, pH 7.4) was used for DNA hybridization experiments; 100 mM PB (100 mM Na_2HPO_4–NaH_2PO_4, pH8.0) was used for the preparation of AuNPs functionalized reporter DNA; 10 mM PB (10 mM Na_2HPO_4–NaH_2PO_4, pH 7.4) was used for the preparation of MB solution. All solutions were prepared with Milli-Q water (resistance > 18 MΩ cm) from a Millipore system.

All electrochemical measurements were carried out at room temperature in a laboratory-made measuring cell using a CHI 760B electrochemical workstation (Shanghai Chenhua Equipments, China). The three electrode system used is consisted of a gold working electrode (2 mm diameter), a saturated calomel reference electrode, and a platinum foil auxiliary electrode.

DNA oligonucleotides used in this work were synthesized and purified by Takara Biotechnology Co., Ltd. (Dalian, China), and their sequences are shown in Table S1.

2.2. Synthesis of AuNPs

AuNPs of ~13 nm in diameter were prepared according to the literature method (Grabar et al., 1995). Briefly, 100 mL of 1.0 mM $HAuCl_4$ solution was heated to reflux under vigorous stirring; the rapidly addition of 10 mL of 38.8 mM sodium citrate solution into the boiling solution accompanied with a color change from pale yellow to wine-red. After boiling for 10 min under stirring, the solution was continuously stirred for an additional 15 min to cool down, and stored in a refrigerator at 4 °C before being used. The concentration of AuNPs was estimated by UV/vis spectroscopy based on an extinction coefficient of 2.7×10^8 M/cm at $\lambda = 520$ nm for 13-nm particles (Demers et al., 2000).

2.3. Preparation of DNA functionalized AuNPs

The DNA functionalized AuNPs were freshly prepared by addition of thiolated DNA (3 μM SH-DNA, 100 μL) in sterilized water into 1 mL AuNPs aqueous solution. After incubation in the dark at 4 °C for 16 h, the DNA-AuNPs conjugates were “aged” by gradually addition of 2 M NaCl and 100 mM PB buffer every 8 h until the solution contained 0.3 M NaCl and 10 mM PB. Then the solution was centrifuged for 25 min at 4 °C (14,000 rpm) with the supernatant being removed. The red oily precipitate was washed with 10 mM PBS and centrifuged. This centrifugation process was repeated for 2 times to remove most of the free DNA in solution. The final solution was re-dispersed in 10 mM PBS and stored at 4 °C.

2.4. Sensing interface fabrication

The gold electrode was polished sequentially with 0.3 and 0.05 μm alumina powder and soaked in an ultrasonic bath successively with ultrapure water, absolute alcohol, and ultrapure water for 5 min each. Then, the gold electrode was immersed in piranha solution (H_2SO_4/H_2O_2, 7:3 by volume) for 20 min. Finally, the electrode was rinsed with ultrapure water and dried under nitrogen stream. To construct the electrochemical sensing interface, the cleaned electrode was interacted with thiolated capture probe (CP, 1 μM) for 12 h at 4 °C. Afterward, the modified electrode was dipped in MCH (1 mM) for 20 min at 4 °C to block the nonspecific sites and obtain an optimal oligonucleotide orientation. Then, the electrode surface was rinsed with 10 mM PBS for 5 min to remove the nonspecific adsorbed DNA and MCH and obtain the sensing interface.

2.5. Target DNA detection

The enzymatic reaction was conducted in 100 μL 1 × NEB buffer 1 (10 mM Bis Tris Propane-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.0). For the sensing of target DNA, the hairpin probe HP was annealed by warming the solution to 95 °C for 5 min and slowly cooling to room temperature for 1 h before use. The HP with a final concentration of 200 nM was mixed with target DNA at different concentrations first, followed by adding ExoIII with a final concentration of 0.4 U/μL, and then incubated for 60 min at 37 °C. After the enzymatic reaction, the solution was heated to 70 °C for 20 min to inactivate the ExoIII, and then the solution was cooled to room temperature for the electrochemical measurements.

The electrochemical measurements were performed as follows: first, 20 μL enzymatic reaction solution was dropped onto the prepared sensing interface and incubated at 37 °C for 1 h. After incubation, the electrode was rinsed with 10 mM PBS buffer to reduce the nonspecific DNA adsorption. Then, 20 μL DNA-AuNPs was dropped onto the electrode interface and further incubated at 37 °C for 1 h. After rinshing of the electrode interface with 10 mM PBS buffer, 30 μL MB solution (60 μM, prepared in 10 mM PB buffer, pH 7.4) was dropped onto the electrode interface and further incubated at room temperature for 30 min at room. Finally, the electrode was washed with 10 mM buffer under stirring for 5 min. In order to detect the electrochemical responses, the electrode was immersed in 15 mL 10 mM PBS buffer and differential pulse voltammetry (DPV) was performed from 0 to −0.5 V with a 50 mV pulse amplitude and 5 ms pulse width to measure the reduction peak current of MB. Electrochemical impedance measurements were performed in 5 mM $K_3Fe(CN)_6/K_2Fe(CN)_6$ solution containing 0.1 M KCl at a scan rate of 100 mV/s.

3. Results and discussion

3.1. Mechanism for the proposed biosensor

Scheme 1 illustrates the general principle of ExoIII and nanoparticle dual signal amplification technology based electrochemical biosensor for the detection of target DNA. ExoIII can catalyze the stepwise removal of mononucleotides of double-stranded DNA from 3′ to 5′ with no requirement of specific recognition sequence, and it is less active on single-stranded DNA or 3′ protruding termini of double-stranded DNA. Therefore, ExoIII was chosen as the enzymatic amplification unit for the proposed biosensor. Since the hairpin structure probes show improved ability to distinguish mismatches, the target recognition probe and the capture probe immobilized on the electrode interface were all designed as hairpin structure. In the absence of target DNA, ExoIII cannot catalyze the hairpin probe HP digestion due to the 3′ protruding end of its stem sequence. As a consequence, the HP cannot hybridize with the hairpin-structured capture probe CP, and the DNA-AuNPs cannot hybridize with CP due to the steric hindrance. Accordingly, few MB molecules can specifically bind with guanine bases of CP to form MB-guanine complexes, resulting in a weak electrochemical signal. However, the introduction of target DNA can hybridize with the hairpin probe HP and open its stem-loop structure to form double-stranded DNA, as well as form the 3′ recessed end recognition sequence for ExoIII. Then, ExoIII can catalyze the stepwise removal of mononucleotides from the 3′ to 5′ of the hybridized double-stranded DNA, resulting in the releases of target DNA and the enzymatic products of HP. The released target DNA can then hybridize with another HP and trigger the second cycle of digestion. Eventually, each target DNA can undergo many cycles to trigger the digestion of many HPs, providing many enzymatic products of HP. The enzymatic sequence products of HP will hybridize with CP and open its stem-loop structure, the released 3′ sequence of CP can hybridize with the DNA-AuNPs. Therefore, much more MB molecules are bound to the capture probe CP and DNA-AuNPs, resulting in a great amplified increase of electrochemical signal.

3.2. Estimation of signal amplification function

To estimate the signal amplification function of the proposed biosensor, we have investigated and compared the performances of the nanoparticle and enzymatic dual signal amplification technology and only one signal amplification technology. The amplification strategy based on the nanoparticle and ExoIII led to a dramatic increase in the final DPV intensity upon the addition of the target DNA. As shown in Fig. 1, in the presence of 1 nM target DNA, a 453 ± 27% signal increase was observed by introducing nanoparticle and enzymatic dual signal amplification technology (curve *a*). In contrast, taking nanoparticle amplification technology and enzymatic signal amplification technology, only a 117 ± 12% (curve *b*) and a 116 ± 14% (curve *c*) signal increase was observed, respectively. Therefore, the proposed nanoparticle and enzymatic dual signal amplification technology could be applied in the amplifying detection of target DNA.

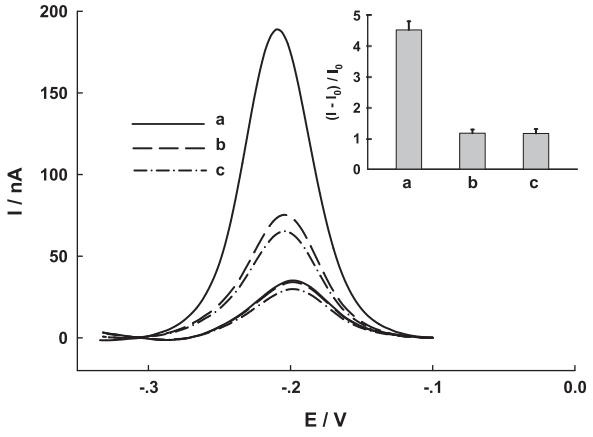


Fig. 1. DPV responses of the biosensor under different conditions: (a) the DPV responses by introducing nanoparticle and enzymatic dual signal amplification technology, (b) the DPV responses by introducing nanoparticle signal amplification technology, (c) the DPV responses by introducing enzymatic dual signal amplification technology. Inset shows the enhancement times of DPV intensity under different conditions, I_0 and I are the peak current of the sensor in the absence and presence of 1 nM target DNA. Error bars were estimated from three replicate measurements.

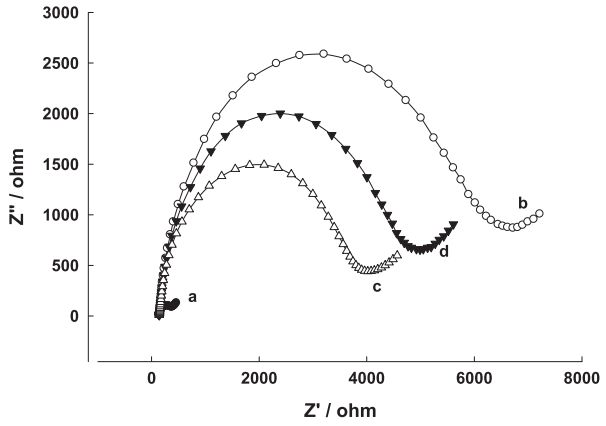


Fig. 2. Nyquist plots of the gold electrode corresponding to different conditions. (a) The bare electrode, (b) after immobilization of capture probe, (c) after hybridization of capture probe with the enzymatic products of HP probe in the presence of 1 nM target DNA, (d) after further hybridize with DNA-AuNPs. The impedance detections were performed in 10 mM PB buffer (pH7.4) containing 5 mM $[Fe(CN)_6]^{3-}$/$[Fe(CN)_6]^{4-}$ and 0.1 M KCl.

3.3. Impedimetric characterization of the sensor

Impedance spectroscopy is a common and effective method for probing the features of surface-modified electrodes. Thus, faradic impedance spectroscopy was carried out to study the gold electrodes modified by different procedures. Fig. 2 depicts a Nyquist plot of impedance for the stepwise modification process with gold electrode. One can observed that the impedance intensity for bare gold electrode (Fig. 2, curve *a*) is very small, as characteristics of an electrochemical diffusion limiting process. Faraday impedance increased significantly because of the thiolated DNA capture probe modification (Fig. 2, curve *b*). Electron transfer became more difficult because of the immobilization DNA resulting in a negatively charged interface which electrostatically repels the negatively charged redox probe $[Fe(CN)_6]^{3-}$/$[Fe(CN)_6]^{4-}$ and inhibits interfacial electron transfer. However, the capture probe hybridization with the enzymatic products of HP probe induced decreased electrochemical impedance (Fig. 2, curve *c*) in the presence of 1 nM target DNA. The results may be due to the hairpin structure of capture probe can more effectively inhibits electron transfer. However, when the hairpin structure is open and hybridizes with the enzymatic products of HP probe to form double-stranded DNA which is relatively ordered arrangement and equivalent to the formation of electronic channels, resulting in the decrease of electron transfer resistance and the decline of impedance. After the further hybridization with DNA-AuNPs, the impedance increased again (Fig. 2, curve *d*). Here the AuNPs are no longer good conductors for $[Fe(CN)_6]^{3-}$/$[Fe(CN)_6]^{4-}$ due to that the surface of AuNPs has been covered with a large amount of DNA and interfered electron transfer on its surface. The above results confirmed that proposed biosensor can be feasibly applied for the detection of target DNA.

3.4. Optimization of detection conditions

To achieve the best performance of the designed biosensor, the concentration of the hairpin probe HP was optimized. As shown in Fig. S1, an increasing concentration of HP resulted in the increase of the signal-to-background ratio (SBR) of the DPV signal until it reached 200 nM. However, when the concentration of HP exceeded 200 nM, the SBR of the DPV signal decreased due to the nonspecific hybridization between HP and CP resulted in the increasing background signal. Therefore, 200 nM of HP was chosen for the biosensor for further experiments.

The effects of the concentration of and ExoIII, and the enzymatic time on the performance of the biosensor were also investigated. As shown in Fig. S2a, the SBR of the DPV signal of the biosensor increases significantly upon introduction of increased concentration of ExoIII. When the concentration of ExoIII reached 0.4 U/μL, the performance of the biosensor reached the maximum SBR of the DPV signal. However, the continued increasing of the HP concentration resulted in the decreasing of the SBR of the DPV signal. This can be explained that the ExoIII maybe can act on the double-stranded stem portion of hairpin probe HP to produce single chain shearing gap and open the HP stem, causing the non-target DNA induced hybridization between HP and CP, thereby resulting in increased DPV background signal. Therefore, 0.4 U/μL of ExoIII was selected as the optimized enzyme concentration for the biosensor. The effect of enzymatic time on the performance of the biosensor was further investigated and the results were shown in Fig. S2b. One can observe that the SBR of the DPV signal increased rapidly with the increase of enzymatic time and tended to stabilize after more than 50 min. To ensure the enzymatic reaction be performed thoroughly, 60 min was selected for the following studies.

3.5. Analytical performance of the biosensor for target DNA

To evaluate the sensitivity of the proposed biosensor, the DPV signal enhancements in the presence of different concentrations of target DNA were recorded under optimized conditions. As shown in Fig. 3a, the DPV signal was increased with the increase of target DNA concentration. Fig. 3b depicts the relationship between the DPV intensity and the different concentrations of target DNA. A dynamic range from 1 pM to 10 nM for target DNA was achieved with a response plateau at 10 nM. The inset figure shows the linear relationship between the DPV intensity and the LogC of target DNA at low concentration from 1 pM to 0.5 nM, and a detection limit of 0.6 pM (based on 3σ/slope) was estimated. The detection limit is much lower than that of the previous ExoIII-based and gold nanoparticle-assisted electrochemical DNA sensor (Fan et al., 2012). The high sensitivity indicates the success of the proposed nanoparticle and enzymatic dual signal amplification technology for electrochemical detection of target DNA.

A high specificity for target analyte is a necessity for a new designed biosensor with potential applications in complicated samples. The specificity test of the biosensor was then carried out by investigating the ability to distinguish mismatches. In order to investigate its capability of identifying SNPs, the DPV responses induced by different DNA strands, including single-base mismatch DNAs and four-base mismatch DNA (the mismatch sequences are shown in Table S1) were compared with that of target DNA at same concentration. As shown in Fig. 4, in contrast to the significant enhancement of DPV intensity induced by target DNA, the addition of base-mismatches strands trigger much less DPV enhancements of the biosensor. These results indicated that the employment of two hairpin probes, HP and CP, for the proposed amplified biosensor can significantly improve the ability of SNPs identification.

The precision and reproducibility is very important for an electrochemical biosensor and has also been investigated by comparing the same batch and batch-to-batch DNA detection DPV results at different concentrations. The experimental results demonstrated that the DPV responses of the biosensor with the same batch were 5.2%, 6.7%, and 7.9% at 5 pM, 50 pM, and 500 pM target DNA ($n=3$), respectively. The batch-to-batch DPV results were 7.3%, 9.1% and 8.8% at the above DNA concentrations, respectively. Therefore, the results indicated that the precision and reproducibility of the proposed biosensor was acceptable and

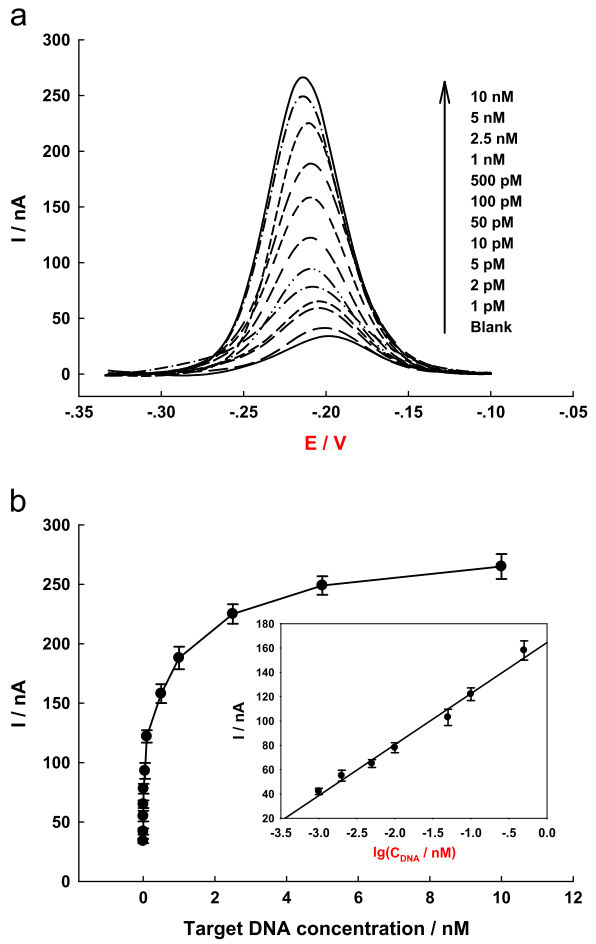


Fig. 3. The sensitivity of the proposed biosensor for target DNA detection. (a) The DPV responses in the presence of different concentrations of target DNA. (b) The relationship of the DPV responses with the target DNA concentration. Inset shows the relationship of the DPV intensity with the LogC of target DNA at low concentration. Error bars were estimated from three replicate measurements.

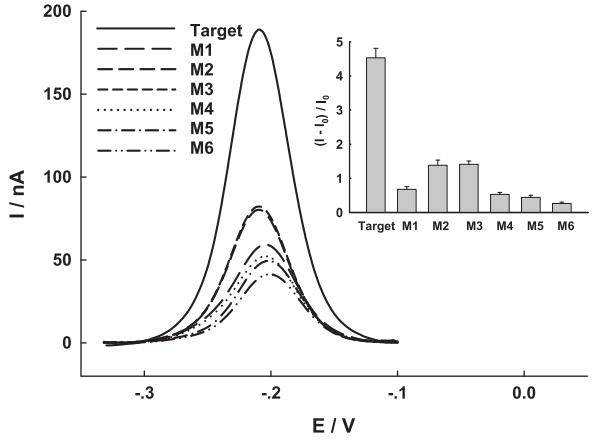


Fig. 4. DPV responses of the biosensor toward perfectly matched and mismatched DNA targets. Inset shows the enhancement times of DPV intensity toward different DNA sequences, the descriptors I_0 and I are the DPV peak currents in the absence and presence of 1 nM of the DNA. Error bars were estimated from three replicate measurements.

comparable to the reported electrochemical DNA biosensor (Fu et al., 2013; Zhuang et al., 2013).

Reusability is also an important issue for sensors. In the present study, the sensing interface could be easily regenerated by immersing the electrode into 1 M NaOH at 50 °C for 15 min, which attributed to great affects of the high pH of alkaline solution on the stability of hybridized DNA. It was found that the sensing interface could retain about 90.6% of its initial response toward target DNA after three times regeneration. Therefore, the results indicate that the developed sensing interface would attain a sufficient stability.

4. Conclusions

In summary, we have developed label-free electrochemical biosensor for sensitive and selective detection of DNA based on ExoIII and nanoparticle dual signal amplification technology. The hairpin probe HP based enzymatic amplification reaction was performed in homogeneous solution to detect of target DNA, which can effectively avoid the drawback of low enzymatic efficiency of interfacial reaction. The enzymatic products could then hybridize with the hairpin capture probe CP on the electrode surface. DNA functionalized AuNPs was further employed to amplify the electrochemical detection signal. These designs together allow a high sensitivity for target DNA, and a detection limit of 0.6 pM was achieved. The proposed biosensor also exhibited high specificity for target DNA which attributes to the employment of two hairpin probes, HP and CP. Moreover, since ExoIII does not require specific recognition sequences, the proposed biosensor is expected to hold wide applications such as SNPs detection and clinical diagnostics in various biological and medical samples in the future.

Acknowledgments

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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.2013.11.041.

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