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Short communication

Ultrasensitive signal-on DNA biosensor based on nicking endonuclease assisted electrochemistry signal amplification

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ABSTRACT

Combining the advantages of signal-on strategy and nicking endonuclease assisted electrochemistry signal amplification (NEAESA), a new sensitive and signal-on electrochemical DNA biosensor for the sequence specific DNA detection based on NEAESA has been developed for the first time. A Hairpin-shape probe (HP), containing the target DNA recognition sequence, is thiol-modified at 5′ end and immobilized on gold electrode via Au–S bonding. Subsequently, the HP modified electrode is hybridized with target DNA to form a duplex. Then the nicking endonuclease is added and nicks the HP strand in the duplex. After nicking, 3′-ferrocene (Fc)-labeled part complementary probe (Fc-PCP) is introduced on the electrode surface by hybridizing with the thiol-modified HP fragment, which results in the generation of electrochemical signal. Hence, the DNA biosensor is constructed successfully. The present DNA biosensor shows a wide linear range of 5.0×10^{-13}–5.0×10^{-8} M for detecting target DNA, with a low detection limit of 0.167 pM. The proposed strategy does not require any amplifying labels (enzymes, DNAzymes, nanoparticles, etc.) for biorecognition events, which avoids false-positive results to occur frequently. Moreover, the strategy has the benefits of simple preparation, convenient operation, good selectivity, and high sensitivity. With the advantages mentioned above, this simple and sensitive strategy has the potential to be integrated in portable, low cost and simplified devices for diagnostic applications.

1. Introduction

The detection of specific DNA sequences at ultralow concentration is extremely important in clinical diagnostics, mutation detection, environmental monitoring, and a variety of other biomedical researches (Chen et al., 2010; Cui et al., 2001; Hacia et al., 1996; Ricci et al., 2007). A lot of detection systems for DNA have thus been developed, such as surface plasmon resonance (SPR) (Milkani et al., 2010), quartz crystal microbalance (QCM) (Feng et al., 2007), fluorescence (Niu et al., 2010), colorimetry (Xia et al., 2010), and electrochemistry (Levine et al., 2009). Among them, electrochemical methods have been widely developed due to their advantages, such as simple and inexpensive instrumentation, fast analytical time, precise and sensitive measurements, and low production cost (Cash et al., 2009; Centi et al., 2009; Chen et al., 2008; Li et al., 2007; G. Liu et al., 2008; G.D. Liu et al., 2008; Z.Y. Liu et al., 2008; Lubin et al., 2009; Miao and Bard, 2004). In recent years, in order to improve sensitivity, various target and signal amplification methods have been developed, such as the polymerase chain reaction (PCR) (Mullis and Faloona, 1987), rolling-cycle amplification (RCA) (Lee et al., 2010; Lizardi et al., 1998), loop-mediated isothermal amplification (LAMP) (Notomi et al., 2000), and nucleic acid sequence based amplification (NASBA) (Compton, 1991). Nevertheless, these methods suffer from several disadvantages that include complex operation processes, easy contamination, and high cost (Chen et al., 2010). Therefore, alternative strategy is needed to overcome the above mentioned problems for ultrasensitive DNA detection.

In this study, we report a sensitive signal-on nicking endonuclease assisted electrochemistry signal amplification (NEAESA) strategy for DNA detection for the first time. A Hairpin-shape probe (HP) containing the target DNA recognition sequence is employed to fabricate the DNA biosensor, with a thiol at 5′ end functioning as the anchor on a gold electrode surface. The HP also carries the recognition sequence (5′-GAGTC-3′) and cleavage site (4 bases away from the 3′ end of its recognition site) for the nicking endonuclease N.BstNB I (New England Biolabs). Nicking endonucleases are a special species of restriction endonucleases. They recognize specific nucleotide sequence in double-stranded DNA and nick only one specific strand of the duplex (Bi et al., 2010; Chen et al., 2010; Cui et al., 2011; Higgins et al., 2001; Xu et al., 2009; Xue et al., 2010). Therefore, in the absence of target DNA, the nicking endonuclease cannot nick the HP. However, after a

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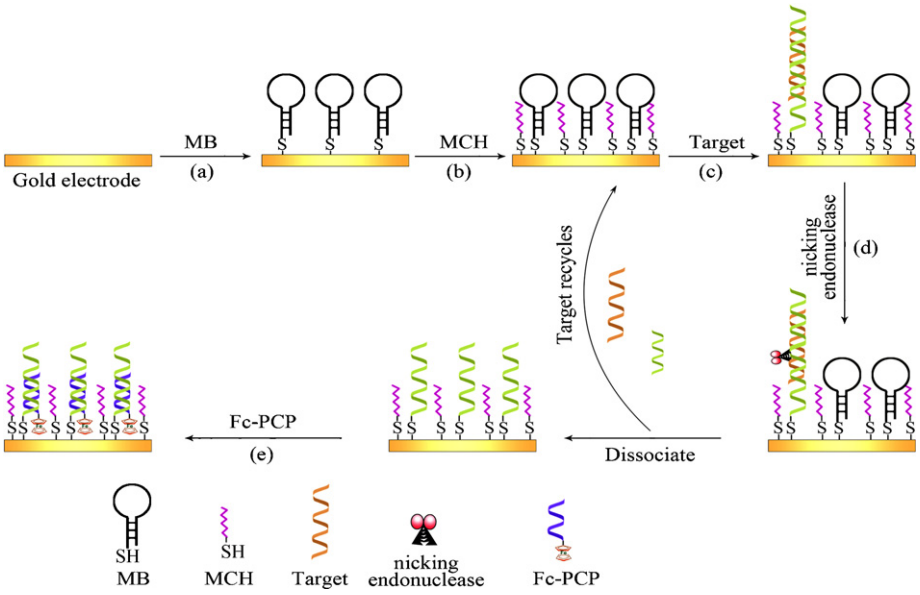

Scheme 1. Schematic for the preparation of the DNA biosensor. (a) Assembling the MB; (b) 6-mercaptohexanol (MCH) block to the electrode; (c) hybridizing with target DNA; (d) nicking reaction; (e) hybridizing with ferrocene-labeled part complementary probe (Fc-PCP).

fully matched target DNA hybridized to the HP, the stem of the HP is opened and a complete recognition site forms for the nicking endonuclease. Then the nicking endonuclease nicks the HP strand in the duplex. After nicking, the HP is cleaved into two fragments, and only the thiol-modified fragment remains on the electrode surface but the other one dissociates. Meanwhile, the target DNA dissociates from the HP. Subsequently, the released target DNA can be captured by another intact HP, which generates the cycle of nicking–dissociation–hybridization (Zou et al., 2011). Thus, each target strand can go through many cycles, resulting in the cleavage of many HPs. At the same time, many thiol-modified HP fragments remain on the electrode surface. Finally, 3′-ferrocene (Fc)-labeled part complementary probes (Fc-PCPs) are introduced onto the electrode surface by hybridizing with the thiol-modified HP fragments, resulting in the generation of electrochemical signals. The detailed concept of NEAESA is displayed in Scheme 1. Unlike most of other electrochemical amplification assays for DNA detection, the proposed strategy does not require any amplifying labels (enzymes, DNAzymes, nanoparticles, etc.) for biorecognition events. Moreover, in contrast to other template replication based amplification methods, the NEAESA based strategy amplifies the target-DNA-specific signals instead of target DNA itself, which decreases the risk of cross-contamination from amplicons to avoid false-positive results.

2. Experimental

2.1. Reagents and materials

All of the DNA oligonucleotides used in this study were obtained from Takara Biotechnology Co. Ltd. (Dalian, China) with the following sequences: (1) Hairpin-shape probe (HP): 5′-HS-$(CH_2)_6$-GCA CGC TAG ATG AGT CCG TCC TGC T GCG TGC-3′; (2) ferrocene (Fc) label part complementary probe (Fc-PCP): 5′-TAG CGT GC-$(CH_2)_6$-NHCOFc-3′; (3) target DNA (T1): 5′-AGC AGG ACG GAC TCA T-3′; (4) single-base mismatched probe (T2): 5′-AGC AGG AGG GAC TCA T-3′; (5) non-complementary probe (T3): 5′-GAT GAA GAA AGA GAG A-3′. Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) and 6-mercaptohexano (MCH) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). N.BstNB I was purchased from New England Biolabs (Beijing) Ltd (Beijing, China). All other chemicals and solvents were of analytical grade and used as received. Doubly distilled water was used throughout all experiments. Buffer for dissolution of HP was I-buffer (25 mM Tris–HCl, 100 mM NaCl, 100 mM $MgCl_2$, 10 mM TCEP, pH 8.23), buffer for dissolution of Fc-PCP and target DNA was H-buffer (25 mM Tris–HCl, 100 mM NaCl, pH 8.23). Phosphate buffer solutions (PBS, 10 mM, pH 7.4) containing 0.1 M $NaClO_4$ were used as the detection electrolyte in the experiment.

2.2. Apparatus

Cyclic voltammetric (CV) and differential pulse voltammogram (DPV) measurements were carried out in a conventional three-electrode cell with a CHI 840B electrochemistry work station (Shanghai CH Instruments Co., China), using an Ag/AgCl reference electrode (saturated KCl), a platinum wire counter electrode, and the modified gold electrode (Φ = 3 mm) as working electrode. The CV was taken from −0.2 to 0.6 V (vs. Ag/AgCl) at 50 mV/s in 10 mM PBS (pH 7.4) in the presence of a 5 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$. The DPV measurement was taken: the potential ranged from 0 to 0.6 V, modulation amplitude was 0.05 V, pulse width was 0.06 s, and sample width was 0.02 s. Electrochemical impedance spectroscopy (EIS) was done with a CHI 660C electrochemistry work station (Shanghai CH Instruments Co., China).

2.3. Fabrication of the sensing interface and detection principle of the DNA biosensor

Prior to surface modification, the gold electrode (Φ = 3 mm) was polished with 1.0, 0.3 and 0.05 μm alumina slurry repeatedly to obtain a mirror-like surface. After rinsing with doubly distilled water, the electrode was immersed into freshly prepared 2:1 mixture of H_2SO_4 and H_2O_2 for 30 s, and then cleaned by doubly distilled water and ethanol in an ultrasonic bath. Subsequently, the electrode was placed into an electrochemical cell with 0.1 M H_2SO_4 for electrochemical cleaning until background signal stabilized. Further, the electrode was rinsed thoroughly with doubly distilled water and dried in nitrogen stream.

To fabricate the biosensor, the pretreated gold electrode was immersed in 5 μM thiolated HP solution for 1 h at 37 °C to prepare HP-modified electrode through interaction of S–Au. In order to avoid non-specific adsorption, 10 μL of 10 mM 6-mercaptohexanol

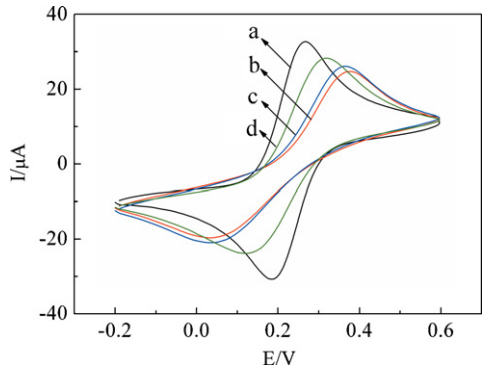

Fig. 1. CVs of the different electrodes in 10 mM PBS (pH 7.4) containing 5 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$: (a) a bare gold electrode; (b) MB modified gold electrode; (c) the electrode (b) after incubated in the target DNA and (d) the electrode (c) after nicked by N.BstNB I. Scan rate: 50 mV/s.

(MCH) was dropped on the modified electrode surface and incubated for 30 min at 37 °C to block the sensing interface. Subsequently, the modified electrode was incubated into target DNA solution for 1 h at 37 °C. Then the electrode was immersed in 0.5 U/μL N.BstNB I solution for 4 h at 55 °C for nicking reaction. After each step, the modified electrode was entirely cleaned with doubly distilled water and buffer to remove the non-chemically adsorbed species. At last, the electrode was incubated with 5 μM Fc-PCP for 1 h at 37 °C and then washed with distilled water. Accordingly, the DNA biosensor was constructed successfully.

In the last step, electrochemical signal was generated by the Fc-PCP hybridization with the thiol-modified HP fragments. The intensity of the electrochemical signal would increase with the increasing concentration of the target DNA, and thus it was able be used to quantify the target DNA content. By employing the signal-on NEAESA strategy, the proposed DNA biosensor is not only ultrasensitive but also highly specific since the nicking reaction requires complete complementarity between the MB and the target at the restriction site and sufficient complementarity to allow hybridization outside of the enzyme recognition site (Chen et al., 2010; Li et al., 2008).

3. Results and discussion

3.1. Electrochemical characterization of the modified electrode

CVs of the different modified procedures of gold electrode in 10 mM PBS (pH 7.4) in the presence of a 5 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ mixture as a redox probe are shown in Fig. 1. Compared with curve a (bare gold electrode), the peak current of curve b (MB modified electrode) decreases obviously and the peak-to-peak separation increases, which indicates that the MBs are immobilized on gold electrode surface successfully and reduce the electron-transfer efficiency (Liu et al., 2010). On hybridization with the target sequence, a slight increase in redox currents is observed (Fig. 1c). The reason of this phenomenon may be presumed as follows. In the absence of target DNA, MB forms a stem–loop structure on the electrode surface, which impedes the electron transfer seriously due to the large stereospecific blockade of the stem–loop structure. However, after hybridization with target DNA, the stem of the MB is opened and forms a duplex structure which may impede the electron transfer more slightly than the stem–loop structure of MB. After nicking reaction, the redox currents increase further because of the dissociation of target DNA and MB fragments (Fig. 1d). As shown in Fig. S1, electrochemical impedance spectroscopy (EIS) experiments also confirm the successful modification and show the effect of each modification step on electron-transfer kinetics.

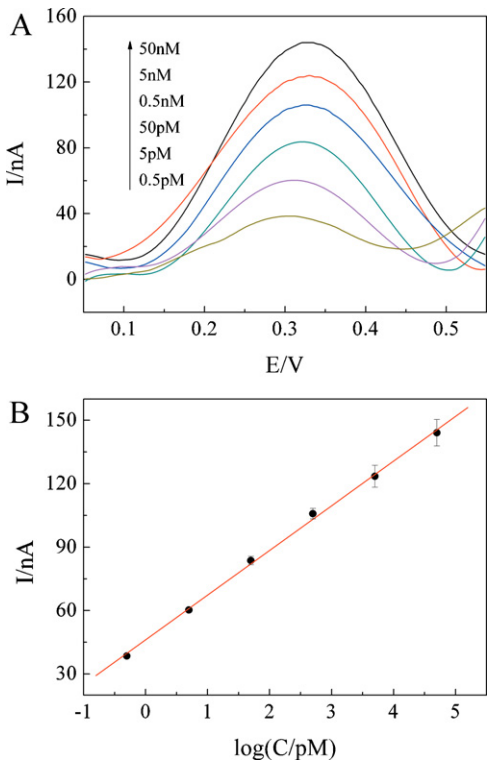

Fig. 2. (A) Background-subtracted DPVs for the electrochemical DNA biosensor at various target DNA concentrations, from 0.5 pM to 50 nM. (B) Linear relationship between the peak current and the logarithm of the target DNA concentration. The error bars represent the standard deviation of three measurements.

3.2. The quantificational detection of target DNA

In order to investigate the sensitivity of the prepared electrochemical DNA biosensor for the detection of the target DNA, the sensor was hybridized with different concentrations of the target and then the current response of Fc was measured with DPV in 10 mM PBS (pH 7.4) containing 0.1 M $NaClO_4$. As shown in Fig. 2b, the oxidation currents of Fc are proportional to the logarithm of the complementary target DNA concentration ranging from 5.0×10^{-13} to 5.0×10^{-8} M with a regression equation of the form $I = 21.134 \log C + 46.166$ and a linear correlation of $R^2 = 0.9982$. The detection limit (S/N = 3) is 0.167 pM, and the relative standard deviation (RSD) of the sensor for three replicate determinations of 5 pM is 1.72%. The result is comparable with those of the other reported methods (Table S1).

3.3. The selectivity, reproducibility, and stability of the DNA biosensor

The selectivity of the DNA biosensor was investigated, and the results are shown in Fig. S2. The prepared sensor was hybridized with various DNA sequences (complementary DNA T1, one base mismatch DNA T2 and non-complementary DNA T3) (Chen et al., 2010) and then the current response of Fc was measured respectively. After hybridization with T1 (5 nM), a visible current signal of Fc generates (Fig. S2a), while low signals generate after hybridization with T2 (5 μM) and T3 (5 μM) (Fig. S2b and c). The fact clearly demonstrates that the prepared DNA biosensor has not only excellent selectivity for the detection of target DNA sequence but also discrimination ability for a single-base mismatch.

The reproducibility of the DNA biosensor was also evaluated, and the relative standard deviation is 4.3% for the detection of 5 nM of target DNA at five DNA biosensors.

The stability of the DNA biosensor was evaluated on a 21-day period. When the sensor was stored in the refrigerator at 4 °C, the DPV peak current of the sensor retained 91.2% of its initial response after the first 7 days storage and 87.4% after 21 days storage. This indicates that the developed DNA biosensor has a good stability.

3.4. Detection of the noise signal

In this study, the current intensity of Fc-PCP/MCH/HP (Fig. S3a) and Fc-PCP/N.BstNB I/target DNA (5 nM)/MCH/HP (Fig. S3b) modified electrodes are recorded in 10 mM PBS (pH 7.4) containing 0.1 M $NaClO_4$. It can be found that low current signal response of the former electrode is obtained before hybridization with the target DNA, and the signal of the former electrode is much lower than the designed biosensor, suggesting the noise signal can be neglected.

3.5. Analytical application of the DNA biosensor

In order to evaluate the practical applicability of the proposed DNA biosensor, a series of samples were prepared by adding target DNA of different concentrations to diluted human blood plasmas (standard addition method, and the human blood plasmas were diluted with H-buffer 100 times prior to detection). Then, the proposed DNA biosensor was used to detect target DNA in these human blood plasma samples. The analytical results are described in Table S2 (see Supplementary material). These data show that the recoveries (between 91.6% and 92.2%) and relative standard deviation values (between 3.5% and 4.7%) are acceptable.

4. Conclusion

In summary, we have successfully developed a new sensitive and signal-on electrochemical DNA biosensor for the sequence specific DNA detection based on NEAESA. The proposed strategy does not require any amplifying labels (enzymes, DNAzymes, nanoparticles, etc.) for biorecognition events, and false-positive results will not frequently occur. Moreover, the strategy has the advantages of simple preparation, convenient operation, good selectivity, wide linear range and high sensitivity. With the advantages mentioned above, the simple and sensitive strategy has the potential to be integrated in portable, low-cost and simplified devices for diagnostic applications. Finally, the NEAESA based strategy can be used in immunosensors and aptamer-based sensors for sensitive detection of other targets with slight modification and improvement.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.07.076.

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