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The potential application of a poly(3,4-ethylenedioxythiopene) modified platinum DNA biosensor in mutation analysis

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ABSTRACT

In this study, we have fabricated a label free DNA biosensor by modifying the platinum wire with electrochemically synthesized poly(3,4-ethylenedioxythiopene) and poly(*p*-aminobenzoic acid). A designed single-strand DNA oligo was immobilized with the carboxyl group of poly(*p*-aminobenzoic acid) and served as the probe, a target DNA was then hybridized with the probe under a proper condition. Differential pulse voltammetry was performed to characterize the hybridization efficiency in the presence of daunorubicin hydrochloride that was able to be intercalated into the hybridized double-strand DNA and possessed the redox activity. Our results revealed a satisfied linear correlation between the peak current of differential pulse voltammetry and the concentration of complementary target DNA. On the other hand, the mismatches between the target- and probe-DNA caused a significant reduction of electrochemical response, in which was correlated with the amount of mismatched base pairs, therefore the current DNA biosensor had potential applications not only in DNA quantification but also in mutation detection for clinical diagnostics and laboratory applications.

1. Introduction

The mutations of human genes have been implicated to be predisposed to various cancers (Cairns, 1975). Over the past decades, dramatic progresses have been achieved in cancer therapeutics (Tsuruo, 2003; Workman and de Bono, 2008), however, cancer is still one of leading causes of human deaths so far (Jemal et al., 2005), which may be greatly due to the belated diagnosis. With the accomplishment of human genome project, information on mutations of cancer related genes has lead us to develop novel methods for early diagnosis, in which have profound clinical impacts on cancer therapies and prevention, thereby probably reducing cancer mortality (Dang-Tan and Franco, 2007; Mao, 2002). Recently, DNA based biosensors have experienced notable development with good prospects by offering rapid, inexpensive and reliable analytic tools for DNA quantification and mutation detection (Kerman et al., 2004; Mao et al., 2009; Teles and Fonseca, 2008).

The mechanism of a DNA biosensor for DNA quantification and mutation analysis is based on the specific hybridization between the single stranded target DNA and the probe DNA that has been immobilized on the surface of an electrode. Techniques such as optical, piezoelectric, and electrochemical are available to assess the hybridization efficiency, which is greatly depended on the complementarities between target- and probe-DNA, and is also influenced by particular sequence, the length of DNA, and the hybridization conditions such as temperature, pH, and ionic strength (Cai et al., 2003; Li et al., 2001; Minunni et al., 2005; Vattanaviboon et al., 2008). Among them, the electrochemical approach is typically attractive because it is relatively sensitive, fast, and inexpensive (Brett et al., 2003; Chang et al., 2007; Mascini et al., 2001; Meric et al., 2002). An electroactive chemical that is able to be intercalated into the duplex of DNA is normally employed to enhance the electrochemical signal while monitoring the hybridization events. The detectable response in corresponding to the amount of intercalated indicator implies the hybridization efficiency.

In order to overcome the limitations of traditional DNA biosensors with gold or glass electrodes, such as low probe immobilization density, unavoidable noise, poor sensitivity and stability (Pang et al., 1996; Pividori et al., 2000), modifications of electrodes with conductive polymer films have become prospective fashions for DNA biosensors because the conductive polymers offer unique surface structures for probe immobilization and excellent electrochemical properties for detection. Poly(3,4-ethylenedioxythiopene), or PEDOT, is a fascinating conductive polymer exhibiting many superior properties to other intrinsic conducting polymers, including good thermal and environmental stabilities (Kim et al., 2005). As the result of doping, PEDOT may also achieve a higher conductivity that makes it suitable for a durable biosensor fabrication (Kim et al., 2005; Kros et al., 2005; Yamato

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et al., 1995). Meanwhile, PEDOT can be easily synthesized by either a chemical or an electrochemical polymerization approach, and its well-defined surface is adapted to immobilize biomolecules, such as proteins and oligonucleotides. The *p*-aminobenzoic acid (PABA) is a kind of carboxylated aniline derivatives and can be electrochemically synthesized to form a poly(PABA) film that provides carboxyl groups for probe DNA immobilization.

In this study, a platinum electrode was electrochemically modified by PEDOT and poly(PABA) in sequence. A DNA biosensor was constructed by crosslinking the single-stranded DNA probe with a 3′ amino terminal to the carboxyl group of poly(PABA) according to Sulfo-NHS (*N*-hydroxysulfosuccinimide) and EDC (ethyl(dimethylaminopropyl) carbodiimide) coupling protocol. The DNA biosensor was able to hybridize with a complementary single-stranded target DNA under an optimized condition and an electroactive chemical such as daunorubicin hydrochloride (DMN) was then intercalated into the duplex DNA. Differential pulse voltammetry (DPV) was performed to characterize the hybridization efficiency which was largely depended on the complementarities between the target- and probe-DNA. The linear correlation between the electrochemical response and the concentration of target DNA was finally established.

2. Material and methods

2.1. Chemicals

Carboxylate-terminated *p*-aminobenzoic acid (PABA), 3,4-ethylenedioxythiophene (EDOT), daunorubicin hydrochloride (DNM) were purchased from Sigma–Aldrich Corp. (St. Louis, USA). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and Sulfo *N*-hydroxysulfosuccinimide (NHS) were obtained from Pierce (Boston, MA, USA). All other reagents that used for buffers and solutions were purchased from various commercial sources and were of analytical grade.

2.2. Electrode modification and preparation

Before electrochemical modification, a bared Pt wire (0.6 mm in diameter) was polished in a sequential order by soaking in 3 M NaOH for 15 min, rinsing with distilled water, being transferred into 3 M HCl for 15 min, rinsing again with distilled water, followed by becoming dry under N_2 flow. The polished Pt wire was modified in a miniature electrochemical cell with a platinum wire as the counter electrode and an Ag/AgCl (3 M NaCl) electrode as the reference electrode (Ag/AgCl). The Pt wire was first immersed in 0.1 M $LiClO_4$ solution containing 0.01 M of EDOT with 0.5 cm in depth. EDOT was electrochemically polymerized on the submerged section of Pt wire by cyclic voltammogram technique under 25 °C, where the potential was swept between 0.0 and 1.0 V with a rate of 10 mV s^{-1} for five cycles. The PEDOT/Pt electrode with a total working area approximately 0.097 cm^2 was further modified with 3 mM of PABA in 0.1 M $LiClO_4$ solution by amperometric technique at 1.0 V for 4000 s under 25 °C. The surface morphologies of the electrodes were visualized by an ABT-150S SEM (TOPCON Corp., Tokyo, Japan). In order to evaluate the events of electrode preparation, we modified a thin Pt plate with the same procedure as that for the Pt wire, the surface morphologies of both Pt plate and Pt wire were confirmed to be similar by SEM, and the compositions and structures of modified Pt plates were subjected to PTIR analysis by a DA 8.3 FTIR spectrophotometer (Bomem Inc., Québec City, Canada). Meanwhile, its electrochemical property was evaluated by a PC-controlled CHI621B electrochemical analyzer (CH Instruments, Austin, USA).

Table 1
The single strand DNA sequences of probe and targets.

Name	Sequence (5′ → 3′)	T_m (°C)
Probe-1	CGCCGGCCACGAGAATAGC*aminoC6*	52.4
cTarget[a]	GCTATTCTCGTGGCCGGCG	52.4
mTarget-1[b]	GCTATTCTC**A**TGGCCGGCG	50.3
mTarget-3[b]	GCTATTCT**TAG**GGCCGGCG	50.3
mTarget-5[b]	GCTATTC**CTAGA**GCCGGCG	50.3

The mismatched bases in comparison with cTarget were bolded and underlined.
[a] Complementary.
[b] Mismatched.

In order to effectively attach the DNA probe (Probe-1) to the modified poly(PABA)/PEDOT/Pt electrode, the –COOH group of PABA on the electrode was further activated by immersing into 50 mM phosphate buffered saline solution (PBS, pH 7) containing 5 mM EDC and 8 mM NHS under 25 °C for 1 h. The activated electrode was then submerged into a solution containing 100 nM of DNA probe under 25 °C for 12 h. The constructed DNA biosensor was accomplished by rinsing with PBS solution completely.

2.3. Probe and target DNA

19-mer single-stranded DNA oligoes for probe or targets were synthesized by Mission Biotech (Taipei, Taiwan) and their sequences were listed in Table 1. The probe (named as Probe-1) was capped with an amino-modifier C6 at its 3′ terminal, which provided a free-NH_3 group for covalent binding with EDC/NHS activated –COOH group of PABA on the electrode. The targets included complementary DNA (named as cTarget) and those containing one, three, or five mismatches against the probe DNA sequence, in which were assigned to mTarget-1, mTarget-3, and mTarget-5, respectively). In addition, two oligoes, one was 56-mer THU-RT-P56: 3′aminoC6-ATCGTATGCCCCTAGGTACTGTGGCGAGCAGAGGG CGGACTCAGCAGACACCCGG T-5′ and the other was 64-mer THU-RT-P56: 5′-CGGGGATCCATGACACCGCTCGTCTCCGCCTGAGT-CGTCTGTGGGCCATCATGAGGAAGCCAC-3′, were used to evaluate the proposed protocol.

2.4. Electrochemical measurement

The target DNA was hybridized with constructed electrode in a 50 mM PBS solution (pH 7.4) under a proper temperature for 1 h, and free target DNA was washed away by PBS solution. The electrode was then immersed in a 0.5 mM PBS solution (pH 7.4) containing 1 μM daunorubicin hydrocholride (DNM) under 25 °C for 15 min with gentle agitation and washed with PBS solution thoroughly. Finally, the electrode was assembled in a miniature electrochemical cell to be served as the working electrode with a platinum wire as the counter electrode and an Ag/AgCl (3 M NaCl) electrode as the reference electrode (Ag/AgCl). Differential pulse voltammetry (DPV) was performed to characterize the hybridization efficiency in a 50 mM PBS solution (pH 7.4), where the potential was swept from 0.2 to 0.7 V with settled parameters (pulse potential: 0.05 V, pulse width: 0.06 s, and pulse period: 0.2 s).

3. Results and discussion

3.1. Characterizations of DNA biosensor

SEM was employed to reveal the surface morphology of Pt wire during the stepwise electrode fabrication as described in Section 2. Fig. 1A(a) displays the smooth surface of a polished Pt wire, whereas Fig. 1A(b) presents that the section of Pt wire was completely covered by the electrochemically synthesized PEDOT film with a typical compact and granular landscape. Fig. 1A(c) exhibits

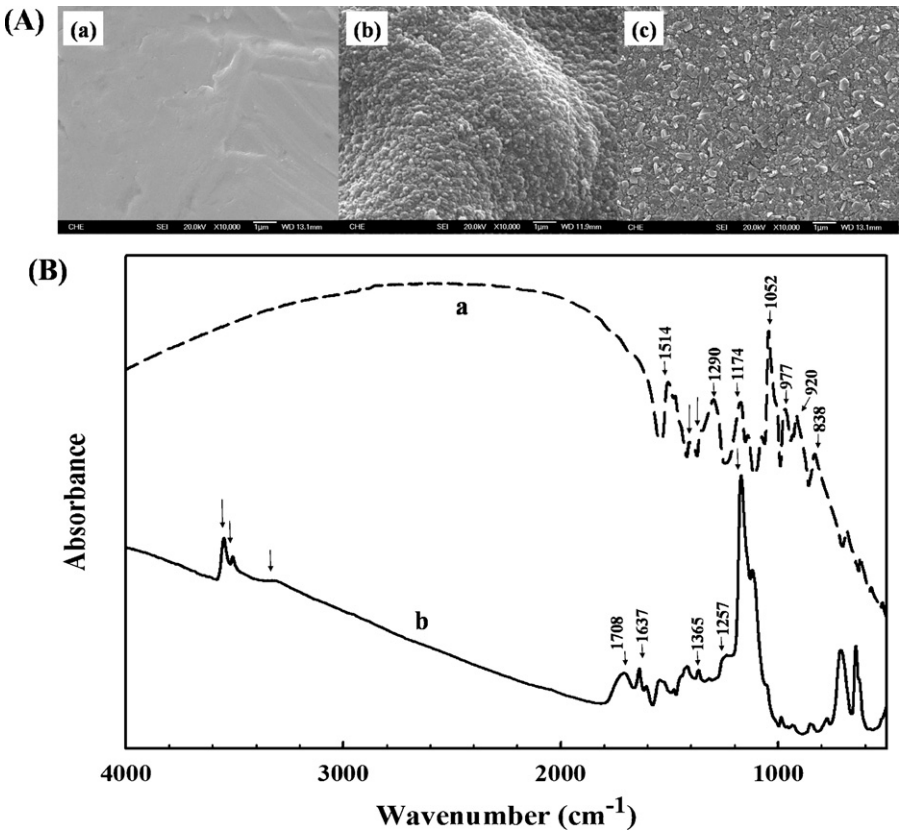

Fig. 1. (A) The SEM images for surfaces of (a) a polished Pt electrode; (b) a PEDOT/Pt electrode; and (c) a poly(PABA)/PEDOT/Pt electrode. (B) The FTIR spectra of (a) PEDOT film and (b) poly(PABA)/PEDOT film on respective electrodes.

the crystal nature of poly(PABA), while the background of PEDOT was still able to be visualized. The structure of poly(PABA) might implicate the formation of short oligoes of PABA on the PEDOT film due to the low reactivity of PABA (Thiemann and Brett, 2001). Accordingly, the Pt wire was successfully modified as the working electrode.

Fig. 1B demonstrates the FTIR spectra of PEDOT (line (a)) and poly(PABA)/PEDOT (line (b)), where both lines showed the characteristic spectra of PEDOT, which including bands at 1514, 1415, 1356 cm^{-1} (stretching of C=C and C–C in the thiophene ring), 1174 and 1052 cm^{-1} (stretching of ethylenedioxy group), 977 and 838 cm^{-1} (stretching of C–S bond in the thiophene ring), and 920 cm^{-1} that was probably due to the deformation of ethylenedioxy ring (Kvarnstrom et al., 1999; Li and Imae, 2004; Selvaganesh et al., 2007). The band at 890 cm^{-1} that was corresponding to the bending mode of C–H bond in EDOT monomer disappeared, indicating the formation of PEDOT molecular chains with α–α′ coupling (Selvaganesh et al., 2007; Zhan et al., 2008). On the other hand, line (b) in Fig. 1B exhibited extra pronounced bands that were assigned to the characteristic spectra of poly(PABA), which were situated between 3300 and 3500 cm^{-1} (stretching of N–H), 1708 cm^{-1} (stretching of C=O), 1637 cm^{-1} (stretching of C–N), and 1257 cm^{-1} (stretching of aromatic ester) (Alva et al., 1996; Thiemann and Brett, 2001). In comparison with line (a), the major spectra of PEDOT in line (b) became faint, however, the bands including 1174 cm^{-1} (stretching of C–H), 1365 cm^{-1} (deformation of aromatic ring) (Perry et al., 2009), were significantly enhanced after poly(PABA) modification.

In addition, the stepwise modifications were characterized by cyclic voltammetry in a 50 mM PBS solution (pH 7.4) containing 0.1 M potassium ferricyanide ($K_3Fe(CN)_6$). Fig. 2A exhibits the significant redox responses of PEDOT/Pt (line (a)) and poly(PABA)/PEDOT/Pt (line (b)) to $Fe(CN)_6^{3-/4-}$ ions in solution, which were probably attributed to the positively charged PEDOT film (Kowalewska et al., 2007). The presence of poly(PABA) did not significantly alternate the typical redox behavior of $Fe(CN)_6^{3-/4-}$ ions on the electrode, but slightly reducing the detectable current, suggesting poly(PABA) was less electroactive than PEDOT. Meanwhile, the free carboxyl groups of poly(PABA) is negatively charged under pH 7.4, which might repulse some $Fe(CN)_6^{3-/4-}$ ions from the positive charged PEDOT modified electrode.

Fig. 2B demonstrates the differential pulse voltammetry (DPV) profiles of poly(PABA)/PEDOT/Pt electrodes with or without DNA probe while measuring a complementary target (cTarget). There was no obvious peak for the absence of DNA probe (line (a)), indicating the target DNA was hardly captured by a bare poly(PABA)/PEDOT/Pt electrode and the PEDOT modification significantly eliminated the background interference. On the other hand, a major peak centered near 0.32 V was noticed for the electrode with the DNA probe, which was assigned to the redox response of daunorubicin hydrochloride (DNM) on the electrode. Therefore, the target DNA was successfully hybridized with the probe DNA to form a double strand structure where DNM was stably bounded. In addition, it has been suggested that the space between the terminal of the target DNA and the solid surface has an impact on the formation of perfect duplex (Watterson et al., 2002; Wong et al., 2005). In this study, the protrude crystals of poly(PABA) shown in Fig. 2C might be benefit for the hybridization between probe and target DNA by reducing the steric hindrance.

3.2. The sensitivity of DNA biosensor

The constructed DNA biosensor was further evaluated by quantifying the complementary target DNA in varied concentrations.

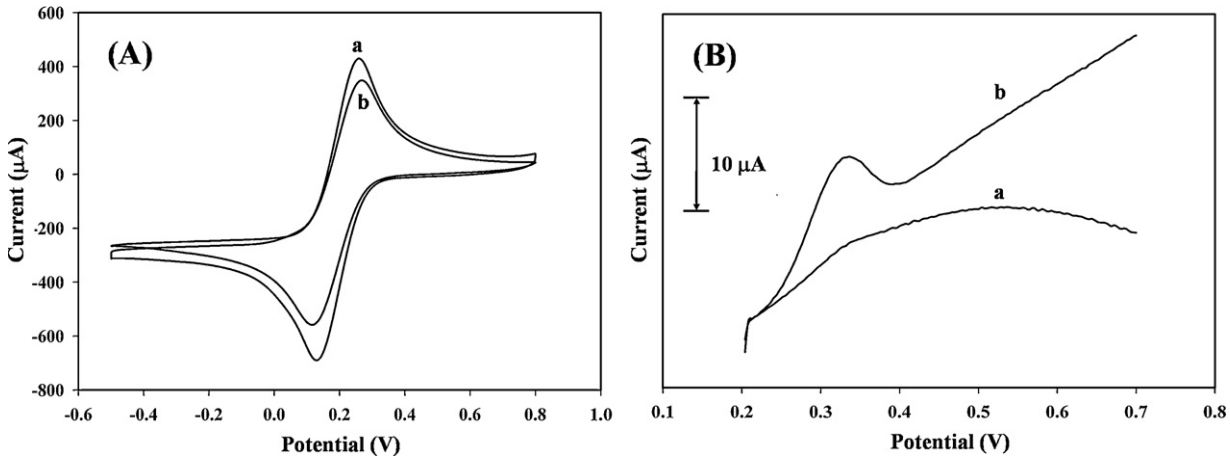

Fig. 2. (A) The cyclic voltammetry profiles of (a) PEDOT/Pt and (b) poly(PABA)/PEDOT/Pt electrodes in response to 0.1 M potassium ferricyanide ($K_3Fe(CN)_6$) in 50 mM PBS (pH 7.4). (B) The DPV profiles of poly(PABA)/PEDOT/Pt electrode (a) without or (b) with a DNA probe while measuring a complementary target (cTarget).

As shown in Fig. 3A, the peak current near 0.32 V increased with the target DNA concentration. Fig. 3B demonstrates the satisfied linear correlation between the DPV peak current and the concentration of a complementary target DNA (the regression coefficient was 0.9909). Accordingly, the sensitivity of our construct DNA electrode was approximately 1.39 $\mu A\,nM^{-1}\,cm^{-2}$.

3.3. The specificity of DNA biosensor

In order to evaluate the specificity of the constructed electrode, we performed experiments by comparing the signal response of matched and mismatched target DNA against probe DNA. As shown in Fig. 4A, the peak current of DPV profile for target DNA reduced successively when one, three, or five mismatched bases were introduced between probe and target DNA. The reduction of the peak current in the DPV profile indicated mismatched bases interfered with the DNA hybridization under a selected temperature. Although three or five continuous mismatched bases are not common in clinical sense, our results did suggest that the constructed DNA biosensor was able to distinguish the complementary target DNA from the target DNA with mismatches. Meanwhile, the discrepancy between mismatched target and complementary target was further emphasized by increasing the hybridization temperature (S1 in supplement material). In practice, a threshold of the peak current with a proper hybridization temperature will be established in order to determine whether the target DNA is fully complementary with the probe DNA or any mismatches exist.

In order to further investigate the specificity of the constructed DNA biosensor, we mixed 100 nM of complementary target DNA (cTarget) with one mismatch target DNA (mTarget-1) in varied concentrations. As shown in Fig. 4B, increasing the concentration of mTarget-1 caused the notable decrease of peak current. Therefore, the constructed DNA biosensor had the ability to identify mismatched DNA in a mixture with complementary DNA, and had the potential clinical application for mutation detection.

3.4. A proposed protocol for DNA analysis

Nevertheless, a practical DNA biosensor should be able to quantify a double strand DNA following a polymerase chain reaction (PCR). Therefore, we have proposed a procedure shown in Fig. 5A, whereas the double stranded PCR product was denatured and renatured by the heating and cooling procedures, respectively. During the renaturation, a designed oligo as the competitor was annealed to one separated strand and the other strand was later hybridized with the DNA probe on the electrode. To evaluate the protocol, we have constructed a DNA biosensor with a 56-mer probe DNA (THU-RT-P56) that was then used to monitor a 64-mer complementary target DNA (THU-RT-T64). In order to improve the target-probe DNA hybridization efficiency as discussed in Section 3.1, a seven-

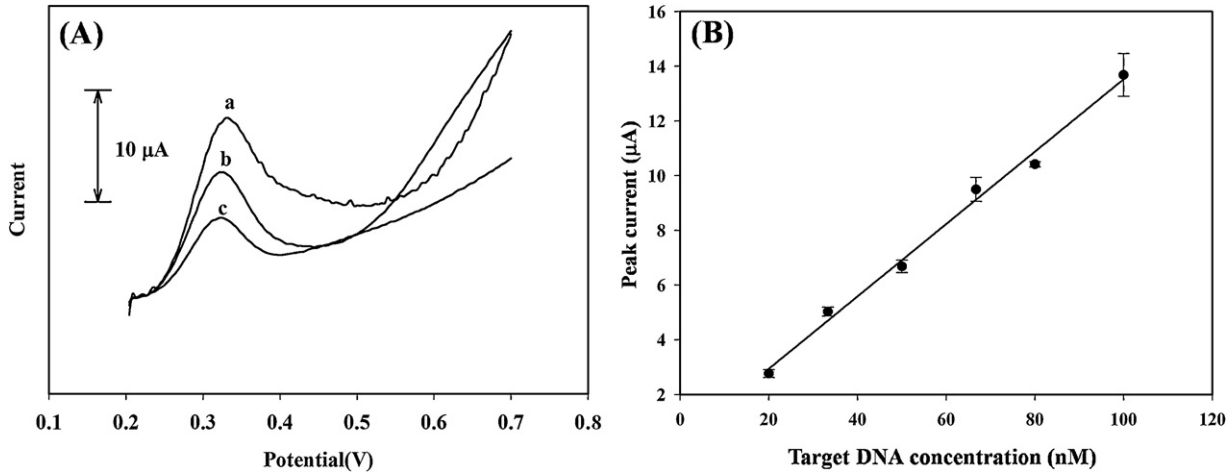

Fig. 3. (A) The effect of complementary target DNA (cTarget) concentration on the DPV currents, where (a) 100 nM; (b) 66.7 nM; and (c) 33.3 nM. (B) The linear correlation between the concentration of cTarget DNA and DPV current. The peak currents were obtained by subtracting the baseline value at the same potential.

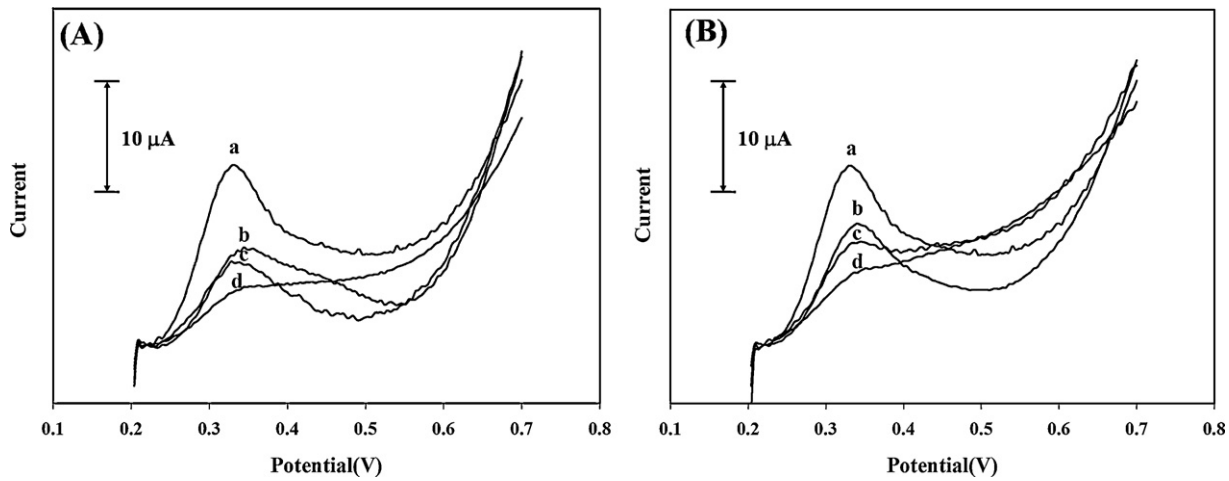

Fig. 4. (A) The influence of mismatches between Probe-1 and target-DNAs on DPV current, where the target DNA contained (b) one mismatch (mTarget-1); (c) three mismatches (mTarget-3); (d) five mismatches (mTarget-5) against the Probe-1 DNA. (a) represented no mismatch between probe and target DNA. The sequences of 19-mer oligoes were listed in Table 1 and their concentrations were all 100 nM. (B) The influence of mismatched target DNA (mTarget-1) on DPV current in the presence of 100 nM complementary target DNA (cTarget), where the concentrations of mTarget-1 were (a) 0; (b) 6.67 nM; (c) 20 nM; (d) 22.67 nM. The hybridization temperature was 55 °C.

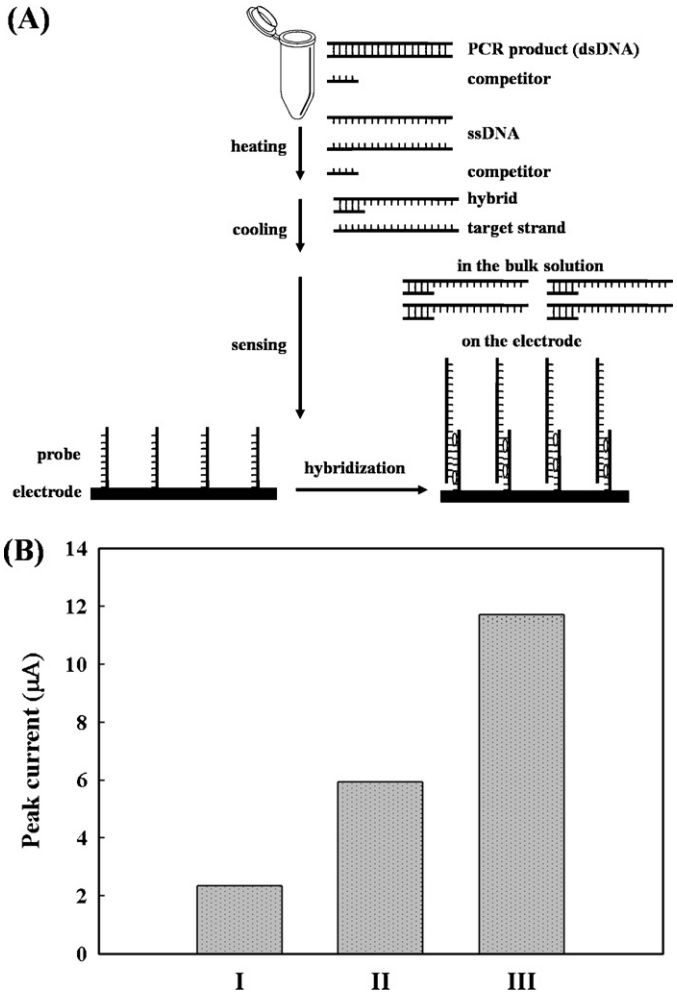

Fig. 5. (A) The scheme of the PCR coupled DNA biosensor for mutation analysis. (B) The influence of the heating–cooling procedure on a analysis of a double strand DNA (I) without the heating–cooling procedure; (II) after the heating–cooling procedure; and (III) after the heating and cooling procedure in the presence of the competitive oligo, PE1: 5′-CGGGGATCCATGACAC CG-3′.

nucleotide spacer was designed at one terminal of the probe closing to the electrode surface, which maintained single strand after hybridization. As shown in Fig. 5B, The DNA biosensor had a weak response to the double strand DNA (I in Fig. 5B), but the heating and cooling procedures brought about a notable response signal (II in Fig. 5B), and the response was further enhanced by the addition of the competitive oligo (III in Fig. 5B).

Meanwhile, we have amplified a 64 base-pair DNA product from a recombinant pET21 plasmid by PCR, the product was purified with QIGEN PCR purification kit and analyzed by gel electrophoresis as shown in Fig. S2A (supplement material). Following the procedure presented in Fig. 5A, we have obtained the significant response to the PCR product as shown in Fig. S2B (supplement material).

4. Conclusions

A label free DNA biosensor was constructed by modifying the platinum wire with electrochemically synthesized PEDOT and poly(PABA), and a designed DNA probe was then immobilized with the carboxyl group of poly(PABA). Differential pulse voltammetry was employed to characterize the hybridization efficiency between the target DNA and the probe in the presence of daunorubicin hydrochloride. Our results demonstrated a satisfied linear correlation between the peak current of DPV and the concentration of complementary target DNA with a sensitivity of 1.39 $\mu A\,nM^{-1}\,cm^{-2}$, approximately (Fig. 3). By comparing with other common used conductive polymers, such as polypyrrole (PPy) and polyaniline (PANI), PEDOT provides more homogeneous and smoother surface structure that may be benefit for further surface modification, probe immobilization, and probe-target hybridization. Fig. S3a and b in supplement material displays the SEM images of PPy and PANI modified Pt electrodes, where PPy/Pt displayed an uneven surface with larger nodules and PANI/Pt showed the typical sponge-like structure. Going through the similar fabrication protocol, we did not detect notable signal in responding to the applied target DNA (lines a and b in Fig. S4 of supplement material) for both poly(PABA)/PPy/Pt and poly(PABA)/PANI/Pt, suggesting the surface properties and structure of an electrode are critical for making the success of a DNA biosensor.

Meanwhile, we found that the mismatches between the target- and probe-DNA caused a significant reduction of electrochemical response, and the reduction was also corresponding to the

percentage of mismatched DNA (Fig. 4). In addition, we have compared the target DNA with different mismatches against the probe DNA and we scored the detected signal in the order of C/G > C/A ≧C/T ≧C/C (Fig. S5 in supplement material), which is similar to the ranking order of the base–base binding affinities (Naiser et al., 2008). Therefore the current DNA biosensor can be used not only for DNA quantification but also for mutation analysis. Nevertheless, a practical DNA biosensor should be able to quantify a double strand DNA following a polymerase chain reaction (PCR). Our results have suggested it can be accomplished by the procedure shown in Fig. 5A. During the heating and cooling procedure in the presence of a specific competitive oligo, one strand of PCR product forms a hybrid with the competitor and the other strand is later hybridized with the probe on the electrode. We are currently working on the further improvement of this PCR coupled biosensor for mutation analysis.

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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.2011.10.014.

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