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Label-Free Electrochemical Detection of Short Sequences Related to the Hepatitis B Virus Using 4,4′-Diaminoazobenzene Based on Multiwalled Carbon Nanotube-Modified GCE

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A novel label-free electrochemical DNA biosensor based on 4,4′-diaminoazobenzene (4,4′-DAAB) and multiwalled carbon nanotube (MWNT)-modified glassy carbon electrode (GCE) for short DNA sequences related to the hepatitis B virus (HBV) hybridization detection was presented. Differential pulse voltammetry (DPV) was used to investigate hybridization event. The decrease in the peak current of 4,4′-DAAB was observed on hybridization of probe with the target. This electrochemical approach was sequence specific as indicated by the control experiments, in which no peak current change was observed when a noncomplementary DNA sequence was used. Numerous factors affecting the target hybridization were optimized to maximize the sensitivity. Under optimal conditions, this sensor showed a good calibration range between 7.94×10^{-8} M and 1.58×10^{-6} M, with HBV DNA sequence detection limit of 1.1×10^{-8} M.

Introduction

In the recent years, electrochemical DNA biosensors based on nucleic acid hybridization have been rapidly developed due to their increasing importance in the diagnosis of disease. DNA hybridization biosensor holds an enormous potential for pharmaceutical, clinical and forensic application (Wang, 2000; Lin et al., 2007; Odenthal and Gooding, 2007). DNA biosensors for recognition of DNA hybridization offer a new approach for rapid, sensitive, simple and low-cost detection of specific nucleic acid sequences (Palecek and Fojta, 2001; Zhang et al., 2004; Lazerges et al., 2006; Kim et al., 2007; Sassolas et al., 2008). Many protocols have been proposed for electrochemical monitoring of DNA hybridization. Electrochemical DNA biosensors are generally based on one or more hybridization and washing steps, with the capture probe immobilized on the electrode. Amplification of the signal arising from the hybridization event between capture and target probes is desirable for achieving low detection limits (Castaneda et al., 2007; Hanaee et al., 2007). These sensors can be prepared by immobilizing single-stranded DNA (ssDNA) probes on different electrodes and using electroactive indicators to measure the hybridization events between the DNA probes and their complementary DNA fragments.

The immobilization of DNA onto the transducer surface plays an important role in the overall performance of the DNA biosensors. However, traditional methods in immobilization of DNA onto electrode surfaces have poor hybridization efficiency (Levicky et al., 1998; Pividori et al., 2000). A useful method to retain the biological activities of DNA, including its function of hybridization and its bio-affinity property is covalent coupling, whereby DNA strands are anchored onto the transducer surfaces (Zhou et al., 1999). Various procedures have been developed for the immobilization of DNA on conducting polymers (Thompson et al., 2003; Riccardi et al., 2006; Peng et al., 2007). However, this approach has some disadvantages, with the localization of the ODN probes in the bulk copolymer leading to steric and kinetic barriers to DNA recognition events. Also, the ODN probes may be degraded during electropolymerization.

4,4′-Diaminoazobenzene (4,4′-DAAB) is useful as a model compound for studies of the chemistry of colored diamines, specifically for the preparation of colored diisocyanates. The electrochemical reduction of a series of aromatic azo compounds in dimethylformamide solutions is generally similar to that of the aromatic hydrocarbons in aprotic media. The reduction occurs in two one-electron steps. The product of

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the first electron transfer is a stable anion radical. The second electron transfer to the dianion is followed by a chemical reaction producing a protonated species which is oxidizable to the parent azo compound (Sadler and Bard, 1968).

Since the discoveries of multiwalled carbon nanotubes (MWNTs) and single-walled carbon nanotubes (SWNTs) in 1991 (Iijima, 1991) and 1993 (Iijima and Ichihashi, 1993), respectively, much attention has been attracted because of their unusual structures and properties (Daniel et al., 2007; Zhao et al., 2008). Carbon nanotubes have aroused increasing interests of many researchers due to their remarkable tensile strength, high resilence, flexibility, and other unique structural, mechanical, brilliant electrical, and physicochemical properties (Hu et al., 2005; Wang and Ozkan, 2008). Various applications of carbon nanotubes have been investigated, such as batteries (Che et al., 1998), electrochemistry of protein (Wang et al., 2002; Zhang et al., 2007), chemical sensors (Kong et al., 2000; Ma et al., 2006), etc.

Electrochemical characteristics of the immobilization of calf thymus DNA molecules on the surfaces of MWNTs have been investigated by cyclic voltammetry and electrochemical impedance analysis. Most of calf thymus DNA are covalently immobilized on MWNTs via diimide-activated amidation between the carboxylic acid groups on the carbon nanotubes and the amino groups on DNA bases, though the direct adsorption of the DNA molecules on MWNTs can be observed (Guo et al., 2004). An MWNT-enhanced electrochemical DNA biosensor has been fabricated by Fang and used in DNA hybridization detection (Cai et al., 2003).

In our previous reports, we have focused on the development of new electrochemical indicators for monitoring of DNA hybridization events (Li et al., 2007; Li et al., 2008). In the present paper, a novel label-free electrochemical DNA biosensor for short DNA sequences related to the hepatitis B virus (HBV) hybridization detection was presented. 4,4′-DAAB was used as electrochemical active indicator as well as substrates for probe DNA immobilization to obtain information about the preparation of a new MWNT-enhanced DNA biosensor. It was found that the resultant DNA functionalized SWNT multilayer films exhibit excellent specificity and chemical stability under the conditions of DNA hybridization. Changes in the voltammetric signals resulting from the interaction of 4,4′-DAAB with double-stranded DNA (dsDNA) and ssDNA were used to sequence-specific detection of short sequences related to the HBV. The use of 4,4′-DAAB in our work as a new indicator will extend the range of DNA indicator.

Materials and Methods

Apparatus and reagents

All electrochemical measurements were carried out at room temperature, using a conventional three-electrode cell. Modified glassy carbon electrodes (GCEs) were used as working electrode and Pt wire served as auxiliary electrode. All potentials are reported against an Ag/AgCl/KCl (sat) reference electrode. Cyclic voltammetric (CV) and Differential pulse voltammetric (DPV) studies were carried out with a Potentiostat/Galvanostat Model 263A Electrochemical Workstation (Princeton Applied Research, USA).

4,4′-DAAB (0.1 mol/L) was purchased from Alfa Aesar and solved in anhydrous methanol and used without further purification. Carboxyfunctionalized MWNT (MWNT-COOH) with an outside diameter of 10–20 nm, a COOH content of 2%, a special surface area of >200 m^2/g and a length of ~50 μm were received from Chengdu Organic Chemicals Co. Ltd. (Chengdu, P.R. China). A solution of the MWNT-COOH with a concentration of 1 g/L was prepared by solving 1 mg MWNT-COOH in 10 mL dimethylformamide (DMF). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide solution (EDC) and *N*-hydroxysuccinimide solution (NHS) from Sigma were used without further purification. Britton-Robinson buffer (BR), trishydroxymethylaminomethane hydrochloride (Tris–HCl), phosphate-buffered solution (PBS) and acetate buffer (HAc-NaAc) were used in this study.

The synthetic oligonucleotides were purchased from Shanghai Huashun Biological Engineering Company for the HBV derived from HBV-DNA subtype adr nucleotide position 1734–1754 (Erdem et al., 2000). The base sequences for HBV were as below: immobilized probe(21-mer sequence S_1): 5′- GAG-GAG-TTG-GGG-GAG-CAC-ATT-3′;

target (21-mer sequence S_2): 5′-AAT-GTG-CTC-CCC-CAA-CTC-CTC-3′;

noncomplementary sequence (sequence S_3): 5′-ATG-TGG-AAA-ATC-TCT-AGC-AGT-3′.

All oligonucleotides were dissolved in 10 mM Tris–HCl, 1 mM EDTA, pH 8.00 (TE buffer) and kept frozen. Other chemicals employed were all of analytical grade and double-distilled water (DDW) was used throughout.

The preparation of surface of biosensor and its modification with DNA GCE pretreatment

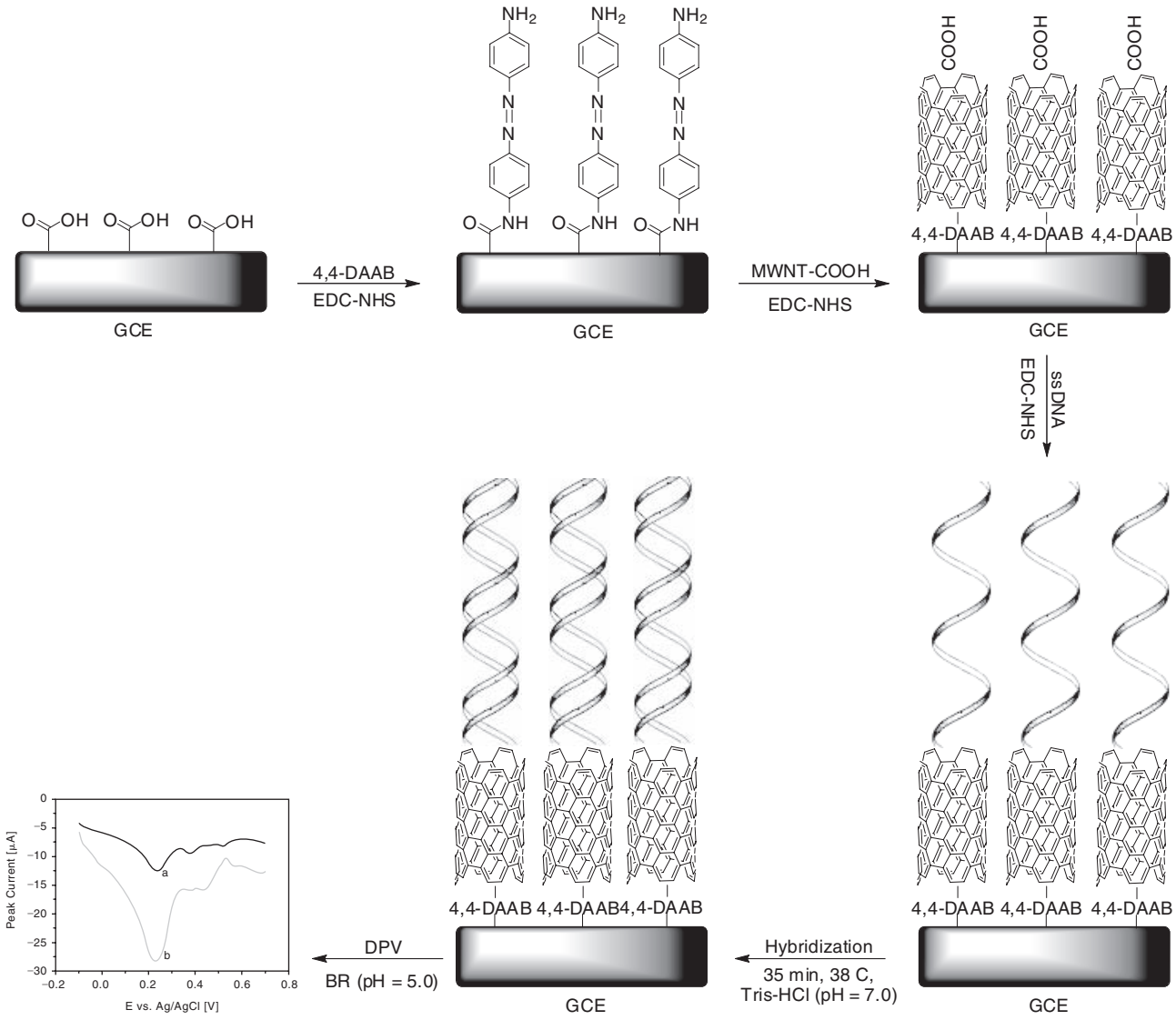
The GCE was polished with 1.0, 0.3, and 0.05 μm alumina power, sonicated in DDW for 2 minutes. The pretreated GCE was first oxidized at +0.50 V for 1 minute in 50 mM PBS (pH 7.40). After the oxidation step, the electrode was rinsed with DDW.

Covalent immobilization of 4,4′-DAAB on GCE

The DNA sensor fabrication method, such as the immobilization of MWNTs, the hybridization method and the conditions control, was described (Scheme 1). Direct covalent modification of GCEs with 4,4′-DAAB was conducted according to the following procedure (Pang et al., 1996). Modification of the electrode was achieved by dropping 20 μL PBS containing 5 mM EDC and 8 mM NHS on the electrode surface. After air-drying of this solution, the electrode was rinsed with DDW. 4,4′-DAAB (10 μL) was pipetted onto the GCE surface and air-dried to dryness.

Immobilization of MWNT-COOH and ssDNA on the modified GCEs

MWNT-COOH (10 μL) was added to the surface of GCE and air-dried to dryness. A 20 μL PBS (pH 7.40) containing 5 mM EDC and 8 mM NHS was dropped on the MWNTs-modified electrode surface and was evaporated to dryness for the activation of the carboxylic acid groups on the carbon



SCHEME 1. Schematic diagram illustrating the target DNA detection based on 4,4′-DAAB-MWNT layer.

nanotubes. Finally, ssDNA solution was dropped on the modified GCE surface. Then the electrode was rinsed with DDW to eliminate the DNA adsorbed.

Hybridization on oligonucleotide probe-modified electrode

The ssDNA-modified electrode was immersed in 20 mM Tris–HCl buffer (pH 7.00) containing ssDNA for 35 minutes at 38°C with shaking to form dsDNA at the electrode surface. After hybridization, the dsDNA electrodes were washed with Tris–HCl buffer and then with water to remove oligonucleotides that were bound nonspecifically.

Voltammetric transduction

The dsDNA GCE was immersed in 0.2 M BR buffer (pH 5.00) and differential pulse voltammograms were collected from −0.1 to 0.6 V, scan rate was 20 mV/s, pulse height was 50 mV for 60 ms, step height was 2 mV, and step time was 0.1 s.

Results and Discussion

Electrochemical study of 4,4′-DAAB

Figure 1 showed the cyclic voltammograms of bare GCE in 0.2 M BR (pH 5.00) containing 5.0×10^{-4} mol/L 4,4′-DAAB at 50 mV/s. It can be seen that, 4,4′-DAAB has well defined voltammetric peaks at 0.203 V (peak a) and 0.453 V(peak b), and ΔE_p is 43 mV and 33 mV, respectively. It indicated that this process might be a reversible, two-proton, two-electron redox reaction. The reaction scheme was given in Scheme 2 (Sadler and Bard, 1968; Yu et al., 1996). Because the peak a (0.203 V) has a better peak shape in the DPVs, so peak a was chosen as the research object.

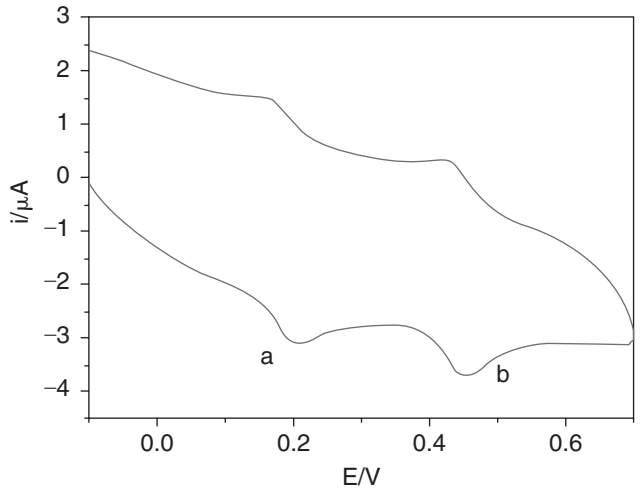


FIG. 1. The cyclic voltammograms of 5.0×10^{-4} mol/L[1] 4,4′-DAAB in a BR solution (pH 5.00) at 50 mV/s.

Buffer solution selection

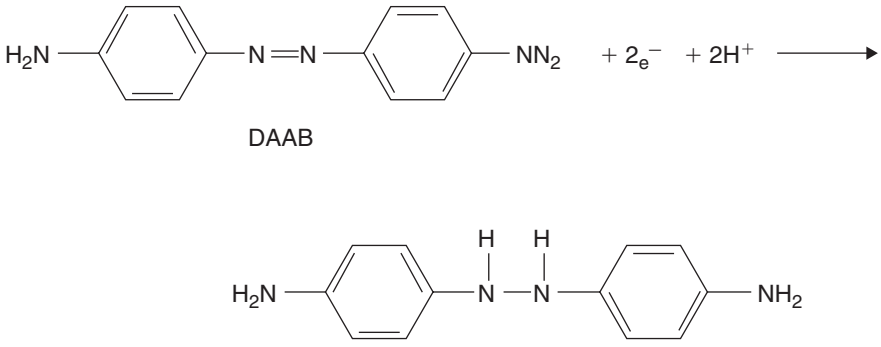
To search for the best conditions for the measurements, four different buffer solutions were tested. The tested buffer solutions were PBS, BR, Tris–HCl and Ac-NaAc. To 5 mL of every buffer solution 4,4′-DAAB (10 μL) was added, before DPVs were recorded. By comparison the DPVs of the different buffer solutions, the BR buffer showed the best results in the form of the peak current as well as the peak shape (spectra not shown) and was chosen for further measurements. Figure 2 showed the development of the peak current with the pH value. Thus, pH 5.00 was chosen as the most optimal pH value.

Interaction of ssDNA/GCE and dsDNA/GCE with 4,4′-DAAB

Figure 3 showed the DPV curves of 4,4′-DAAB at ssDNA-modified GCE (ssDNA/GCE) and after hybridization with the increasing amount of complementary DNA sequence (dsDNA-modified GCE, dsDNA/GCE). The ssDNA/GCE displayed significantly larger current than that of dsDNA/GCE. The voltammetric reduction signals of 4,4′-DAAB at ssDNA-modified electrode decreased after hybridizing with complementary target DNA. The decrease in the peak current of dsDNA/GCE may be related to a change of electron transfer after the immobilization of dsDNA on the carbon nanotubes. The DNA helices may block the electron transfer between the redox compounds and the electrode surface due to the low conductivity of DNA molecules. A relatively smaller peak current was observed when compared with the one obtained with the ssDNA/GCE. Thus 4,4′-DAAB can be used as an electroactive indicator for recognition of the surface hybridization process.

Hybridization detection

Because it was observed that there is a significant difference between the voltammetric signals of 4,4′-DAAB obtained with ssDNA and dsDNA on the probe electrode, subsequently the capture probe was investigated for the response of noncomplementary oligonucleotide. Figure 4 displayed the DPV for the signal of 4,4′-DAAB at synthetic single 21-mer sequence of the HBV probe–immobilized electrode (Fig. 4A), after hybridization with the complementary DNA sequence (Fig. 4C), and the noncomplementary sequence (Fig. 4B). The highest 4,4′-DAAB reduction signal was observed with the probe-modified GCE, indicating that the ssDNA/GCE have a faster rates of electron transfer than that on the dsDNA/GCE surface. Significant decreases in the voltammetric signals were observed after hybridization with the complementary ssDNA. This may be attributed to a continuous DNA double helix formed on the electrode surface, which hides the electron transfer between the electroactive species and the electrode surface (Moulton et al., 2003; Wong and Gooding, 2006). The peak current almost did not decrease when the capture probe was exposed to the noncomplemtentary sequence in the control experiment. This result indicated that only a complementary sequence could form a dsDNA on the probe-modified GCE and cause a significant decrease of the signal. No significant change in signal was observed for noncomplementary sequence, showing the high selectivity of the hybridization detection.



SCHEME 2. Redox reaction of 4,4′-DAAB.

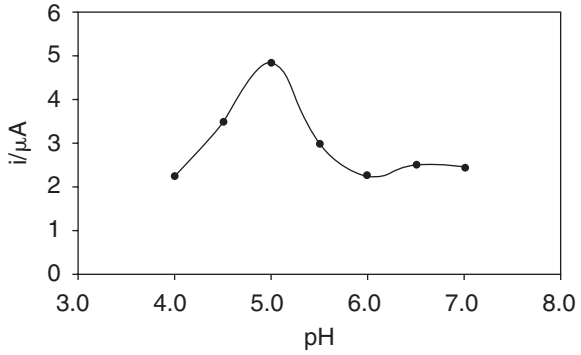


FIG. 2. Influence of pH value to the peak currents of 4,4′-DAAB in 0.2 M BR buffer, $c_{DAAB} = 5.0 \times 10^{-4}$ mol/L.

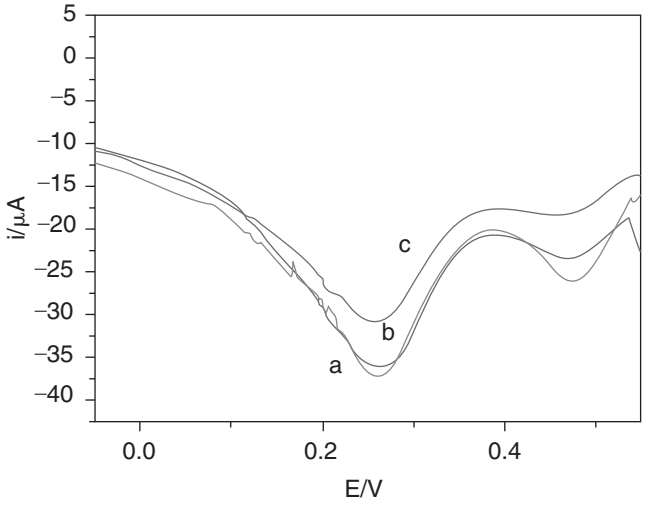


FIG. 4. Differential pulse voltammograms of 4,4′-DAAB on (a) S_1/GCE, $C_{S1} = 5.02 \times 10^{-5}$ mol/L; (b) $S_1 - S_3$/GCE, $C_{S3} = 5.28 \times 10^{-7}$ mol/L; (c) $S_1 - S_2$/GCE, $C_{S2} = 5.28 \times 10^{-7}$ mol/L. The scan rate, pulse amplitude, and pulse period were 20 mV/s, 50 mV, and 60 ms, respectively.

Electrochemical response of the complementary target with different concentration

Under constant 4,4′-DAAB, the response in the presence of hybridization between probe and increasing concentration levels of target was presented in Figure 5. The DPV peak currents of the proposed DNA sensor decreased with increasing target concentration in the hybridization solution after the hybridization reaction. The decrease of DPV peak current was proportional to target concentration in the range of 7.94×10^{-8} M to 1.58×10^{-6} M and the linear regression equation is $i = 0.7165c + 0.3202$ (c is the concentration of target DNA, 10^{-7} M; i is the cathodic current of 4,4′-DAAB, 10^{-6} A) and the regression coefficient $r = 0.9905$. In this concentration, all of the available probes on the electrode surface were involved in hybridization. The relative standard deviation over three independently modified capture probe electrodes measured at 5.4×10^{-7} M of target HBV-DNA was 4.78%, implying a remarkable reproducibility of the detection method, and the detection limit was 1.1×10^{-8} M.

Conclusion

A novel and sensitive electrochemical DNA biosensor based on MWNTs-COOH for covalent DNA immobilization and hybridization detection is described. The MWNTs-COOH-modified GCE was fabricated and oligonucleotides were covalently bonded to the carboxyl group of carbon

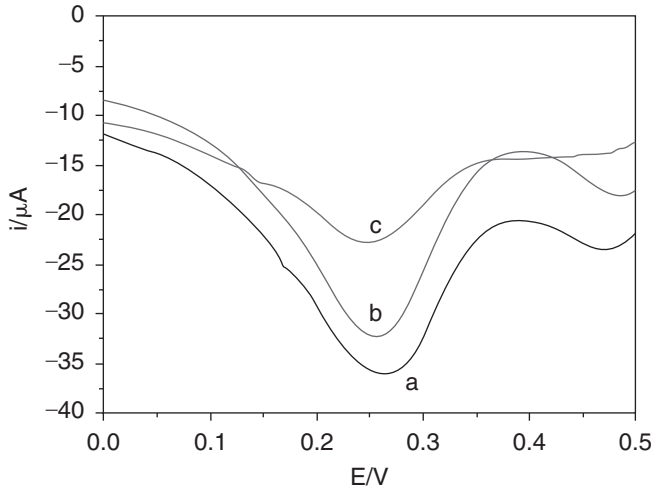


FIG. 3. Differential pulse voltammograms of 4,4′-DAAB on (a) S_1/GCE, $C_{S1} = 5.02 \times 10^{-5}$ mol/L; (b) $S_1 - S_2$/GCE, $C_{s2} = 2.64 \times 10^{-7}$ mol/L; (c) $S_1 - S_2$/GCE, $C_{s2} = 1.01 \times 10^{-6}$ mol/L.

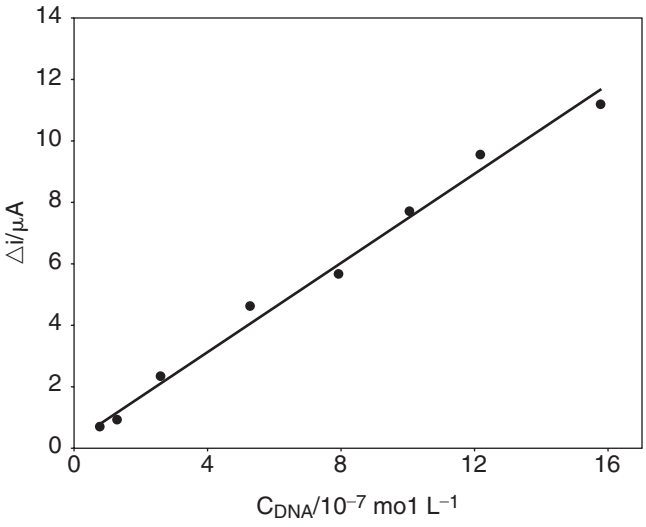


FIG. 5. Plot of peak current vs. the concentration of complementary target from 7.94×10^{-8} M to 1.58×10^{-6} M in BR buffer (pH 5.00).

nanotubes. The hybridization reaction on the electrode was monitored by DPV analysis using an electroactive compound 4,4′-DAAB. With this protocol, both the probe and target DNA were label-free, avoiding the site-specific labeling of both termini with thiol group and ferrocene which was difficult to obtain and still relatively expensive.

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