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Immobilizing DNA on Cetyltrimethyl Ammonium Bromide Cationic Membrane for the Detection of Specific Gene Related to NPT II

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Carbon paste electrode (CPE) was fabricated using solid paraffin as binder. Cetyltrimethyl ammonium bromide (CTAB) was self-assembled on the surface of the CPE to form compact CTAB monolayer with high density of positive charges oriented outside. In weak alkaline medium, both ssDNA and dsDNA could be immobilized on the CTAB/CPE *via* electrostatic adsorption. The immobilized ssDNA could selectively hybridize with its complementary DNA sequence. The optimization of the factors affecting the property of CPE, stability of self-assembled CTAB, immobilization of ssDNA and electrochemical detection of the hybridization were monitored by cyclic voltammetry and differential pulse voltammetry using methylene blue or $K_3Fe(CN)_6$ as indicator. This kind of DNA biosensor was successfully used to detect the gene sequence related to the exogenous NPT II gene from the transgenic cole with a detection limit of 1.0×10^{-8} M. Two base-mismatch gene sequence could also be recognized by this method.

Key Words: Cetyltrimethyl ammonium bromide, Carbon paste electrode, DNA Electrochemical biosensor, NPT II gene.

INTRODUCTION

The electrochemical DNA biosensors with the probe directly immobilized on an electrode surface for hybridization have been widely used for identification of pathogens, monitoring of gene expression, diagnosis of a variety of infectious diseases and analysis of genetically modified organisms owing to their advantages such as low cost, simple design, small dimensions, rapid and direct detection[1-4]. It is very important to select an appropriate basal electrode for the preparation of electrochemical DNA biosensor. Carbon paste electrode (CPE), composed of a matrix of graphite powder and a pasting binder, has been widely and successfully

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employed in DNA electrochemical sensors for its many advantages, such as wide potential window, very low background current, high sensitivity, low cost, easily prepared, renewed and modified surface[5-8]. It's a typical example to immobilize DNA based on the electrostatic adsorption of negatively charged DNA molecules on the positively charged CPE surface pretreated by anodic polarization at +1.7 V for several minutes[9-11]. Using paraffin oil as binder, Hu and his coworker[12] developed a CTAB cationic membrane modified CPE and pointed out that the high density of positive charges on CTAB/CPE surface could be used to immobilize dsDNA. Other cationic membranes, such as chitosan[13,14] and polylysine[15] were also used to immobilize DNA on glassy carbon electrode or Au electrode. This simple method of DNA immobilization by electrostatic adsorption does not require special reagents or nucleic acid modifications and thus it is easy and economic for the application.

Ordinarily, CPE is made up of liquid binder such as mineral oil. The carbon grain on the electrode surface was prone to fall off due to the poor binding property of the liquid binder, which limited the repetition and the further application of the CPE. On the contrary, the CPE made up of solid paraffin possesses the virtues of the solid membrane electrode such as stable glossy surface, finer repetition, convenient application and preservation[16,17]. In this work, solid paraffin was used as binder to prepare CPE with high mechanical stability and perfect repetition. ssDNA was immobilized on the CTAB modified CPE *via* electrostatic adsorption. Using $K_3Fe(CN)_6$ or methylene blue (MB) as an indicator, factors affecting the immobilization of DNA were optimized and the hybridization property of the immobilized ssDNA was studied by the techniques of cyclic voltammetry (CV) and differential pulse voltammetry (DPV). The results showed that both ssDNA and dsDNA could be immobilized on the CTAB/CPE and the immobilized ssDNA could selectively hybridize with its complementary sequence DNA. This kind of DNA sensor was successfully used to detect the specific sequence related to the exogenous NPT II gene (neomycin-3'-phosphotransferase gene) from the transgenic code.

EXPERIMENTAL

The cyclic voltammetry (CV) and the differential pulse voltammetry (DPV) were performed with a CHI832 Electrochemical Analyzer (CH Instruments, Shanghai, China). A three-electrode cell, consisting of a modified carbon paste electrode as working electrode, a saturated calomel electrode (SCE) as reference electrode and a platinum wire auxiliary electrode, was used in electrochemical experiments. A model pHS-25 digital pH meter (Shanghai Leici Instrument Factory, China) was used for pH measurement.

Three 20-base synthetic oligonucleotides were obtained from SBS Genetech Co., Ltd. (Beijing, China) and they had the following sequences: target A, namely NPT II (NPT II is an exogenous gene in transgenic code) gene sequence: 5'- AGT ACG TGC TCG CTC GAT GC -3', immobilized probe B (complementary with target A): 5'- GCA TCG AGC GAG CAC GTA CT -3' and two-base mismatched target A': 5'- AGT ATG TGC TCG CTC GTT GC -3', the two mismatched bases of which were indicated by the underline. 15 μM probe B solution of pH 8.1 was prepared using TE solution (10 mM *tris*-HCl, 1 mM EDTA). Target A and target A' stock solutions (15 μM) were prepared using 2 × SSC buffer (0.3 M NaCl + 0.03 M sodium citrate, pH 7). All the solutions were kept in 4ºC oven, more diluted solutions were obtained *via* diluting the stock solution with TE or 2 × SSC buffer prior to use.

Double-strand herring fish sperm DNA (dsDNA) from Sigma was used as received. The stock solution of 0.1 g/L DNA (pH 8.1) was prepared in TE solution and stored at 4ºC. Denatured single-strand DNA (ssDNA) was obtained by heating dsDNA in a water bath at 100ºC for 10 min, followed by rapidly cooling in an ice bath. Cetyltrimethyl ammonium bromide (CTAB) and *tris*(hydroxymethyl) aminomethane were obtained from Sigma. Methylene blue, $K_3Fe(CN)_6$ were all purchased from Shanghai Chemical Reagent Company (Shanghai, China). Other chemicals were of analytical grade. All solutions were made up in double distilled water.

Preparation of CTAB modified carbon paste electrode: 3 g Graphite powder and 0.75 g solid paraffin were heated at 80ºC for 2 h and ground intensively to obtain uniform carbon paste. The hot carbon paste was tightly packed into the cave of a glass tube (Φ 4 mm) and a copper wire was introduced for electrical contact. The carbon paste cool down to the ambient temperature under certain pressure. The obtained electrode (CPE) was smoothed on a weighing paper, subsequently 20 μL 0.01 M CTAB solution was dropped onto the surface of the CPE. After 1 h, the electrode was completely rinsed with double distilled water to remove unadsorbed CTAB and air-dried to obtain CTAB modified CPE (CTAB/CPE).

Preparation of DNA modified carbon paste electrode: 20 μL DNA solution was dropped on CTAB/CPE at ambient temperature for 0.5 h and then the electrode was immersed in 0.2 % SDS solution for 5 min and then rinsed with water to remove the unimmobilized DNA. Thus, the DNA modified electrode (probe B/CTAB/CPE, ssDNA/CTAB/CPE or dsDNA/CTAB/CPE) was ready for use. The contrast electrodes were prepared by directly immobilizing ssDNA or dsDNA on the surface of the bare CPE and denoted as ssDNA/CPE or dsDNA/CPE. Piping 20 μL 2 × SSC buffer solution containing the complementary DNA on the ssDNA/CTAB/CPE or probe B/CTAB/CPE for 1 h performed the hybridization.

Then the electrode was washed with 2 × SSC buffer solution containing 0.2 % SDS for 5 min. The obtained electrode was denoted as hybrid/CTAB/CPE.

Electrochemical detection: The scan rate in CV was 100 mV/s and the pulse amplitude, the pulse width and the pulse period in DPV were 50 mV, 50 ms and 0.2 s, respectively. The electrode was immersed in 1.5 mM $K_3Fe(CN)_6$ containing 0.1 M NaCl solution for 2 min and then the CV curve was recorded.

Methylene blue sensing procedure A: The modified CPE was soaked into 15 μM MB in pH 6 B-R buffer solution containing 20 mM NaCl for 5 min and then the voltammetric measurement was performed in the same solution.

Methylene blue sensing procedure B: The modified CPE was soaked into the MB solution above for 5 min and then washed with deionized water for 5 s with vortexing, then the electrode was subjected to electrochemical study in the same B-R buffer solution without addition of MB.

RESULTS AND DISCUSSION

Cyclic voltammetry of methylene blue at DNA/CTAB/CPE

The CV peaks of 15 μM MB at DNA/CTAB/CPE increased with increasing the accumulation time until 5 min. They leveled off when the accumulation time exceeded 5 min. 5 min of accumulation time was chosen in all experiments.

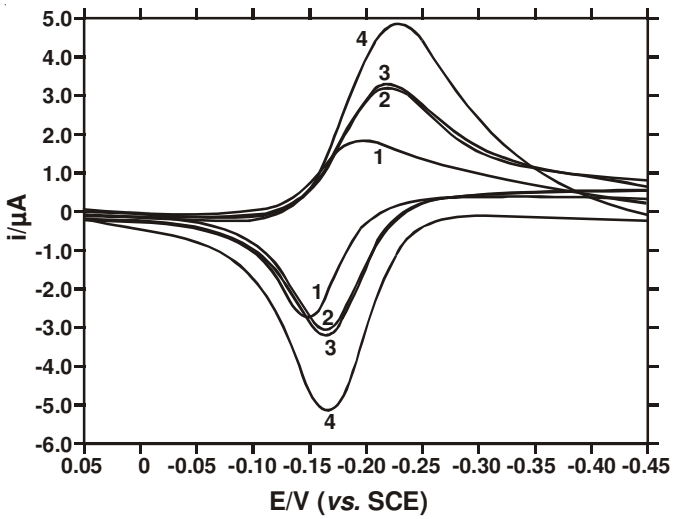

Fig. 1. Cyclic voltammograms of methylene blue at: (1) CTAB/CPE; (2) dsDNA/CTAB/CPE; (3) hybrid/CTAB/CPE; (4) ssDNA/CTAB/CPE

Fig. 1 shows the cyclic voltammograms of MB at different modified CPE by sensing procedure A. A couple of well-defined redox peaks of MB were obtained at the CTAB/CPE. CTAB was self-assembled on the surface of the CPE by the hydrophobic adsorption to form compact CTAB mono-layer with high density of positive charges oriented outside, which had an electrostatic repulsion toward MB^+ and made MB^+ difficult reach to the electrode surface. Simultaneously, it was difficult for the hydrophobic MB molecule to adsorb onto the hydrophilic electrode surface with high density of positive charges. Thus the peak currents of MB were the smallest at CTAB/CPE. The electrostatic repulsion of the positive charges on the electrode surface towards MB^+ was much bigger than that towards MB, therefore the anodic peak current of MB (i_{pa}, 3.158 μA) was bigger than the cathodic peak (i_{pc}, 1.917 μA) and $i_{pa}/i_{pc} = 1.65$.

DNA could be immobilized on the positively charged surface of CTAB/CPE through electrostatic interaction[12,18]. Besides, MB has a strong affinity to the free guanine bases exposed to the exterior of ssDNA. Thus much more MB molecules were accumulated on the surface of the ssDNA/CTAB/CPE[19], which made the peak currents, especially i_{pc} of MB rise markedly at the ssDNA/CTAB/CPE. The biggest peak currents of MB (i_{pa}, 5.045 μA; i_{pc}, 5.109 μA) were obtained. The smaller affinity of MB to the guanine bases packed between the bulky double helix of dsDNA resulted in the smaller CV peak currents of MB at the dsDNA/CTAB/GCE (i_{pa}, 3.502 μA; i_{pc}, 3.385 μA). But the negatively charged sugar-phosphate backbone of dsDNA could accumulate enough MB^+ onto the electrode surface leading to the bigger peak currents of MB at the dsDNA/CTAB/CPE than those at the CTAB/CPE. In addition, the formal potential [$E_f = (E_{pa} + E_{pc})/2$] of MB at the DNA/CTAB/CPE exhibits *ca.* 20 mV negative shift compared with that at the CTAB/CPE, which indicated that MB was associated with DNA through electrostatic binding in nature.

The cathodic peak current was examined as a function of the scan rate (ν) from 50 to 400 mV/s with the technique of cyclic voltammetry at both the ssDNA/CTAB/CPE and dsDNA/CTAB/GCE. Excellent linear relationships of i_{pc} with ν were obtained with the regression equations $i_{pc} = 24.440\ \nu + 2.314$ ($R^2 = 0.9971$) and $i_{pc} = 15.336\ \nu + 1.680$ ($R^2 = 0.9984$), respectively, which indicated that the redox reactions of MB at the electrodes are controlled by adsorption. Furthermore, i_{pa}/i_{pc} at ssDNA/CTAB/CPE and dsDNA/CTAB/CPE are 0.99 and 1.03, respectively (both are close to 1.0), indicating that the redox reactions of MB at both electrodes are controlled by adsorption. However, the i_{pc} of MB at the CTAB/CPE is proportional to the square root of ν (*i.e.* $\nu^{1/2}$) with the regression equation $i_{pc} = 5.163\ \nu^{1/2} + 0.184$ ($R^2 = 0.9924$), which indicates that the redox reaction of MB is controlled by diffusion. The change of the characteristic of the electrode

reaction after immobilizing DNA on the surface of the CTAB/CPE proves that DNA have been immobilized on the surface of the CTAB/CPE. The contrast experiment showed that the peak currents and peak potentials of MB at ssDNA/CPE and dsDNA/CPE were almost the same as those at the bare CPE, which demonstrated that DNA could not be immobilized on the bare CPE. The surface of the bare CPE without CTAB is highly hydrophobic, which is unfavourable for the immobilization of the hydrophilic DNA molecule.

Optimization in the preparation of DNA/CTAB/CPE

Preparation of the carbon paste electrode: The electrochemical properties of the CPE were characterized with the technique of CV after immersing the electrodes into 15 μM MB solution for 5 min. With the decreasing of the content of solid paraffin, the binding properties of the carbon paste and the mechanical stability of the electrode decreased and the carbon grain on the electrode surface was prone to fall off. At the same time, MB was easy to penetrate into the carbon paste and the remainder MB signal at the test electrode was difficult to eliminate. On the other hand, the peak current of MB decreased and the background current and the peak-to-peak separation (ΔE_p) increased frequently upon the rising of the percentage of solid paraffin. In order to meet both the requirements for the mechanical stability and electrochemical properties, the mass ratio of carbon powder to solid paraffin employed in all the subsequent work was 4:1. The uniform carbon paste must be packed into the glassy tube while it was hot and then cooled down to the ambient temperature under certain pressure. Otherwise the shrink of the carbon paste would induce small gaps between the carbon paste and the wall of the glassy tube. For the same reason the CPE should be used at relatively steady temperature, avoiding exquisite changes of the temperature. The electrode could be regenerated after being polished on the weighing paper. The background current would become higher after the electrode being used 3-4 times. This electrode should be heated up in 50ºC oven for 10 min to soften the carbon paste and then the contaminative carbon paste was extruded out of the tube. The CPE could be used again after cooling down to the ambient temperature under certain pressure. Thus one piece of CPE could be repetitively used for 15-20 times in all.

Effect of the assembling time of CTAB: The electrochemical signal of 1.5 mM $K_3Fe(CN)_6$ in 0.1 M NaCl solution at the bare CPE was too small to obtain measurable redox peaks (Fig. 2, curve 3). However, a couple of well-defined redox peaks of $K_3Fe(CN)_6$ were obtained at the CTAB/CPE. For the positive charges on the CTAB/CPE surface could accumulate the negatively charged $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ (Fig. 2, curve 1).Thus $K_3Fe(CN)_6$ could be applied to detect the assembling effect of CTAB.

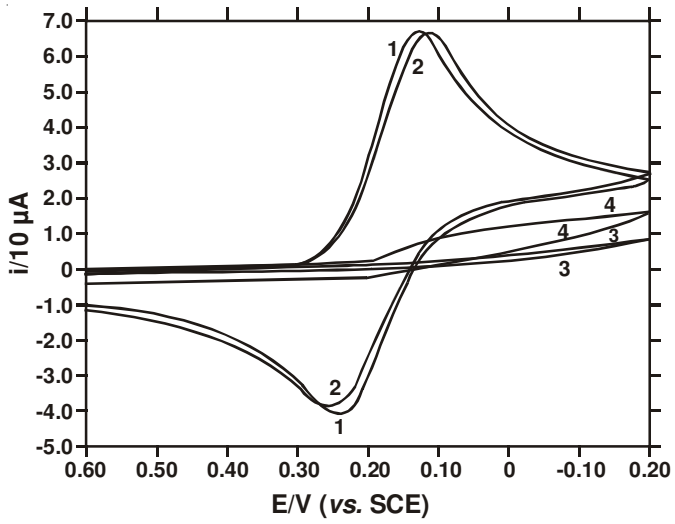

Fig. 2. Cyclic voltammograms of 1.5 mM $K_3Fe(CN)_6$ in 0.1 M NaCl at: (1) CTAB/CPE; (2) CTAB/CPE after being soaked in 0.01 M TE buffer solution of pH 8.1 for 1 h; (3) CPE; (4) ssDNA/CTAB/CPE

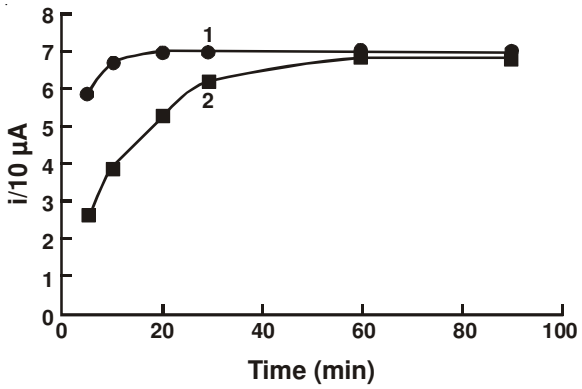

Fig. 3. Effect of self-assembling time on the i_{pc} of $K_3Fe(CN)_6$ at: (1) CTAB/CPE; (2) CTAB/CPE after being treaded with 0.01 M TE buffer solution of pH 8.1 for 1 h

20 µL of 0.01 M CTAB was dipped onto the surface of the bare electrode at ambient temperature for different periods of time. Then the electrodes were completely rinsed with double distilled water to obtain a set of CTAB/CPEs. The other set of CTAB/CPEs obtained as above were soaked in 0.01 M TE buffer solution of pH 8.1 (the blank solution of the DNA immobilizing solution) for 1 h and then rinsed with double distilled water. The CV curves of 1.5 mM $K_3Fe(CN)_6$ at the two sets of electrodes

were recorded, respectively. As shown in Fig. 3, when the assembling time of CTAB exceeded 10 min, the peak currents of $K_3Fe(CN)_6$ at the CTAB/CPE leveled off. However, after being treated with TE buffer solution for 1 h, the peak currents of $K_3Fe(CN)_6$ at the CTAB/CPE increased with the assembling time of CTAB up to 1 h and then tended to remain constant. This indicated that within certain period, the longer the assembling time of CTAB is, the firmer the binding between the hydrophobic cetyl and hydrophobic surface of the CPE. In other words, when the assembling time exceeded 1 h, the immobilized CTAB could not be washed off after being treated with TE buffer solution for 1 h. Thus 1 h was selected as the assembling time of CTAB.

Effect of the pH of DNA immobilizing solution: CTAB is a strong electrolyte and thus the pH of the solution can not affect its positively charged property. However the pH of the solution affects the negatively charged property of DNA by changing the existent states of the bases and the 5'-terminal phosphate group of the DNA molecule, and thus affects seriously the immobilization of DNA. The results are shown in Table-1. When the pH of the immobilizing solution was 3.2, H^+ could bind to the exposed bases of ssDNA and thus weaken the negatively charged property of the whole ssDNA molecule. The weakened electrostatic interaction between DNA and the positively charged CTAB resulted in the small quantity of the immobilized ssDNA. The shape of the CV curve of MB at the ssDNA/CTAB/CPE looked like that at the CTAB/CPE and the peak current only increased slightly. However, pH has little effect on dsDNA/CTAB/CPE because the bases of dsDNA are packed between the bulky double helix of dsDNA, so dsDNA could be immobilized effectively even when the pH of immobilizing solution was 3.2. As a result, the peak current of MB at the dsDNA/CTAB/CPE was bigger than that at the ssDNA/CTAB/CPE and the ratio of i_{pa}/i_{pc} was more closed to 1.0 at the former electrode. When the pH of the DNA solution increased to 6.5, the capability of the bases of ssDNA binding to H^+ decreased, and thus the quantity of the immobilized ssDNA increased. The peak currents of MB at ssDNA/CTAB/CPE were closed to that at dsDNA/CTAB/CPE. When the pH of the DNA solution exceeded 7.5, the quantity of immobilized ssDNA further increased and the peak currents of MB at the ssDNA/CTAB/CPE were remarkably bigger than those at the dsDNA/CTAB/CPE. pH 8.1 was selected as the pH of DNA immobilizing solution.

The influence of the immobilizing time of DNA on the property of the DNA/CTAB/CPE was also tested. The peak currents of MB at the DNA/CTAB/CPE increased with increasing the immobilizing time of DNA up to 0.5 h and then tended to remain constant. 0.5 h of the immobilizing time of DNA was selected in all experiments.

TABLE-1
PROPERTIES OF DNA/CTAB/CPE PREPARED WITH THE IMMOBILIZING SOLUTION OF DIFFERENT pHs

pH of DNA immobilizing solution	i_p of MB	3.2	6.5	7.5	8.1	9.0
ssDNA/CTAB/CPE	i_{pc} (μA)	2.184	3.229	4.192	5.109	5.068
	i_{pa} (μA)	3.377	3.767	4.212	5.045	4.987
dsDNA/CTAB/CPE	i_{pc} (μA)	3.514	3.467	3.395	3.385	3.384
	i_{pa} (μA)	3.864	3.667	3.424	3.502	3.519

Two CTAB/CPEs fabricated under optimal conditions were chosen for the contrast experiment. One CTAB/CPE was treated with 20 μL of 0.01 M TE buffer solution (pH 8.1) for 1 h and the other CTAB/CPE was treated with the same buffer solution containing 0.1 g/L ssDNA to prepare ssDNA/CTAB/CPE. As shown in Fig. 2, i_{pc} and i_{pa} of $K_3Fe(CN)_6$ at the former CTAB/CPE were 6.523 and 4.727 μA, respectively, almost the same as those at the CTAB/CPE before the treatment (curve 1: i_{pc} = 6.577 μA; i_{pa} = 4.869 μA). However, no measurable redox peaks of $K_3Fe(CN)_6$ were obtained at the ssDNA/CTAB/CPE, because the negatively charged sugar-phosphate backbone of ssDNA had an electrostatic repulsion toward the similarly negatively charged $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ and thus made $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ difficult reach to the surface of the electrode. These results also showed that ssDNA could be effectively immobilized on the CTAB/CPE.

Comparison of different sensing methods

CV and DPV were carried out using DNA/CTAB/CPE as working electrode by sensing procedure A. The ratio of $i_{pc(ssDNA/CTAB/CPE)}/i_{pc(dsDNA/CTAB/CPE)}$ of MB was 1.51 in CV and 1.57 in DPV. No measurable signal of MB was obtained in CV by sensing procedure B. This may be due to the fact that the interaction between DNA and MB was too weak to bind enough MB on the surface of the DNA/CTAB/CPE. However, DPV technique could effectively increase the detection sensitivity. Well-defined DPV curves of MB at the MB-DNA/CTAB/CPE were obtained when the electrode was in the MB-free B-R buffer solution. i_{ps} of MB bound to the ssDNA/CTAB/CPE and dsDNA/CTAB/CPE were 4.08 and 1.75 μA, respectively. The ratio (2.33) of $i_{p(ssDNA/CTAB/CPE)}/i_{p(dsDNA/CTAB/CPE)}$ was much higher than that obtained by sensing procedure A. In sensing procedure A, the prevailing redox reaction by MB presented in large excess in the bulk solution but not bound to the electrode surface partly concealed the different interaction between MB-dsDNA and MB-ssDNA, which leaded to the lower resolution than sensing procedure B.

Detection of the specific sequence related to the exogenous NPT II gene from the genetically modified code

ssDNA/CTAB/CPE was hybridized with 0.1 g/L homologous pyrolytic ssDNA at ambient temperature for 1 h to obtain hybrid/CTAB/CPE, the CV signal of MB at which was almost the same as that at the directly fabricated dsDNA/CTAB/CPE (Fig. 1, curve 3 and 2). This demonstrated that the ssDNA immobilized on the electrode could effectively hybridize with the complementary ssDNA, in other words, the ssDNA/CTAB/CPE prepared by this method could be used to detect specific sequence gene by hybridization.

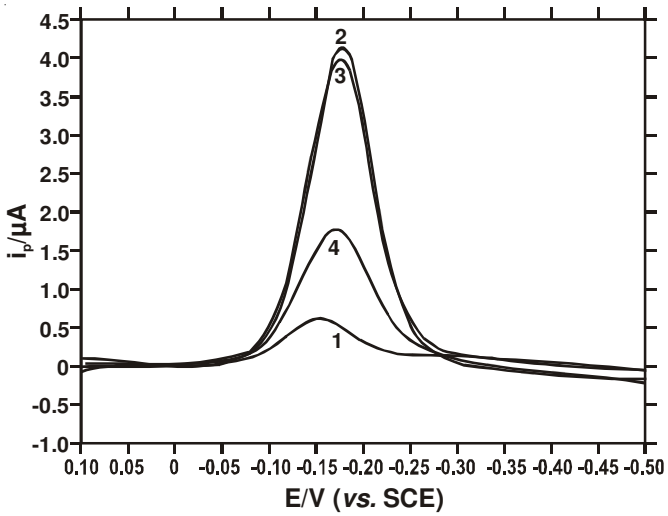

Fig. 4. Differential Pulse voltammograms of MB at: (1) CTAB/CPE; (2) ssDNA/CTAB/CPE; (3) MM-hybrid/CTAB/DNA; (4) hybrid/CTAB/DNA

Fig. 4 shows the results of the detection of specific sequence related to NPT II gene from the transgenic cole using DPV technique by sensing procedure B. A small quantity of MB could be bound to the surface of the CTAB/CPE due to the binding of MB to the hydrophobic area of the CTAB mono-layer. A cathodic current of 0.53 μA at -0.152 V was observed at CTAB/CPE. After immobilizing probe B on the CTAB/CPE, the peak potential of MB shifted to -0.176 V. Furthermore, the peak current of MB at the probe B/CTAB/CPE increased to 4.169 μA for much more MB molecules accumulating on the electrode surface. Probe B immobilized on the

CTAB/CPE could selectively hybridize with target A, the gene sequence related to the NPT II gene, to obtain hybrid/CTAB/CPE. The peak current of MB at the hybrid/CTAB/CPE decreased to 1.785 μA and thus the specific sequence related to the NPT II gene could be detected. Target A' was instead of target A for hybridizing with the immobilized probe B to obtain MM-hybrid/CTAB/CPE. The peak current of MB at the MM-hybrid/CTAB/CPE only slightly decreased compared with that at the probe B/CTAB/CPE. This indicated that two-base mismatched target DNA virtually could not hybridize with the immobilized probe B, namely, the immobilized probe B still preserved high hybridization selectivity.

Target A also could be quantitatively detected by this method. MB peak currents for the different concentration of target A were obtained according to sensing procedure B. The decrease of the peak current of MB was directly proportional to the concentration of target A. Regression equation ranging from 2.0×10^{-8} to 5.0×10^{-7} M was $\Delta i_p = 0.4125\ c_{\text{target A}} + 0.2275$ ($R^2 = 0.9974$), where $c_{\text{target A}}$ was the concentration of target A in 10^{-7} M and Δi_p the decrease of the DPV peak current ($i_{\text{pssDNA/CTAB/CPE}} - i_{\text{p hybrid/CTAB/CPE}}$) in μA. A detection limit of 1.0×10^{-8} M of target A was estimated using 3σ (where σ is the standard deviation of a blank solution, n = 11).

Conclusion

The objective in DNA genosensor research is to make them cheaper and easier to use. In this report, the carbon paste electrode (CPE) was prepared using solid paraffin as binder with stable glossy surface and finer repetition. 0.01 M CTAB was self-assembled on the surface of the CPE at 25ºC for 1 h to form compact CTAB cationic membrane with high density and stability. DNA in 0.01 M TE buffer solution (pH 8.1) was immobilized through electrostatic adsorption to obtain DNA/CTAB/CPE. ssDNA immobilized on the electrode could effectively hybridize with its complementary ssDNA. The developed method was successfully used to detect the specific sequence related to the exogenous NPT II gene from the transgenic cole with a detection limit of 1.0×10^{-8} M. The main advantage of this DNA genosensor is its cost-effectiveness because it is cheap, handy, disposable and needless of expensive or noxious reagents and further modification of nucleic acid. The developed method also has a sufficient detection limit for real-world analysis.

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