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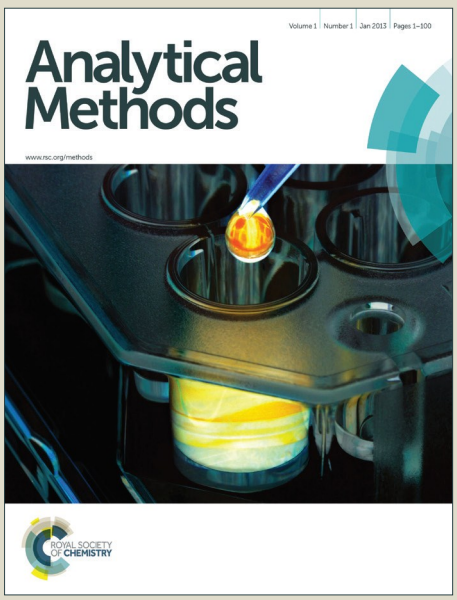

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Application of titanium dioxide nanowires and electroreduced graphene oxide modified electrode for the electrochemical detection of specific *tlh* gene sequence from vibrio parahemolyticus

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In this paper an electrochemical DNA biosensor was prepared by using electroreduced graphene oxide, titanium dioxide nanowires and chitosan modified carbon ionic liquid electrode as the substrate electrode, which was further used for the electrochemical detection of the specific *tlh* gene sequence related to vibrio parahemolyticus. The presence of nanocomposite on the electrode surface can increase the surface area and fix the ssDNA probe sequence by electrostatic attraction. After the hybridization with the target ssDNA sequence under the selected conditions, methylene blue (MB) was used as the electrochemical indicator for monitoring the hybridization reaction. Under the optimal conditions the reduction peak current of MB was proportional to the concentration of *tlh* gene sequence in the range from 1.0×10^{-12} to 1.0×10^{-6} mol L^{-1} with a detection limit as 3.17×10^{-13} mol L^{-1} (3σ). This electrochemical DNA sensor exhibited good discrimination ability to one-base and three-base mismatched ssDNA sequences, and the polymerase chain reaction amplification product of the *tlh* gene from oyster samples were further detected with satisfactory results.

Introduction

Electrochemical DNA sensors have been widely reported due to their higher sensitivity, wider dynamic range, fast responses and low cost devices, which have the potential applications in molecular diagnostics and drug screening.[1] In general electrochemical DNA sensors are composed of three procedures, including the selection of basal electrode, the immobilization of probe ssDNA sequence and the electrochemical indication of hybridization reaction. And the sensitivity and lifetime of an electrochemical DNA biosensor are greatly influenced by the immobilization of probe ssDNA sequence on the surface of basal electrode.[2] Therefore various kinds of modified electrodes have been devised for the ssDNA immobilization. In recent years nanomaterials modified electrodes have been fabricated and used for the DNA sensors due to the specific properties such as large surface area, excellent conductivity and good biocompatibility. Xu et al. reviewed the applications of different nanomaterials for the electrochemical DNA sensors.[3] Li et al. applied gold nanoparticles as the immobilization matrix and labels for the electrochemical DNA sensor.[4] Zhang et al. presented a label-free electrochemical detection of DNA hybridization based on gold nanoparticles/ poly(neutral red) modified electrode.[5] Weber et al. developed a chemically modified single-walled carbon nanotube electrode for the electrochemical impedance-based DNA sensor.[6] As a new carbon nanomaterial, graphene (GR) has attracted considerable attentions due to its unique electrical properties, large surface area, tunable surface functionality and certain catalytic activity.[7] Different methods have been proposed for the synthesis of GR, including micromechanical cleavage of graphite,[8] chemical vapor deposition,[9] chemical reduction of exfoliated graphite oxide (GO) [10] and so on. Among them electrochemical reduction of GO has been proven to be a green and facile way to synthesis GR film directly on the electrode surface. By controlling the reduction potential, the oxygen-containing groups can be thoroughly removed from GO plane via electrochemical reduction, and the reaction exhibits the properties such as green, fast without the contamination of the reduced materials.[11] Therefore

electrochemical reduced GO (ERGO) modified electrode has been used in the fields of electrochemical sensor and electroanalysis. Zhou et al. reported the controlled synthesis of large-area and patterned ERGO modified electrode.[12] Chen et al. applied an ERGO modified GCE for the simultaneously detection of hydroquinone and catechol.[13] Gao et al. used ERGO modified electrode for the sensitive voltammetric detection of rutin.[14] Recently our group also applied an ERGO modified electrode for the preparation of electrochemical DNA sensor and used for the detection of transgenic maize MON810 sequence.[15] GR and its related nanomaterials have also been used in the field of electrochemical DNA sensor. Hu et al. applied a GO modified electrode for the electrochemical detection the conserved sequence of the human immunodeficiency virus 1 pol gene fragments.[16] They also proposed gold nanoparticles (AuNPs) decorated GR sheets for label-free electrochemical impedance DNA hybridization biosensing.[17] Han et al. applied a gold nanorods decorated GO sheets for the electrochemical DNA detection.[18] Sun et al. prepared a AuNPs and GR modified electrode for the electrochemical detection of Listeria monocytogenes.[19] Wang et al. used a reduced GO-modified glassy carbon electrode to detect the methicillinresistant Staphylococcus aureus DNA by using electrochemical impedance spectroscopy.[20] Zhang et al. developed a hybrid biosensor composed of reduced GO sheets decorated with gold nanoparticles for sequence-specific DNA detection.[21]

TiO_2 nanomaterials have been widely reported in the preparation of chemically modified electrodes due to the excellent properties such as low toxicity, thermal stability, large surface area and good biocompatibility.[22] Nanostructured TiO_2 with various morphography had been used for electrode modification. Lunsford et al. used a nanostructured TiO_2 modified sonogel carbon electrode for the determination of catechol.[23] Bao et al. fabricated uniformly porous nanostructured TiO_2 for direct electrochemistry of glucose oxidase and glucose sensing.[24] Song et al. investigated the direct electrochemistry of hemoglobin (Hb) with TiO_2 whisker.[25] Therefore TiO_2 nanomaterials have many potential applications in the field of electroanalysis. Lu et al. devised a photoelectrochemical strategy for hairpin DNA hybridization with TiO_2 electrode as the anchor and signal transducer.[26] Lu et al. applied a AuNPs labeled DNA as probe onto TiO_2 substrate electrode for the photoelectrochemical detection of DNA hybridization.[27] Zhang et al. prepared a AuNP/TiO_2 hollow microsphere membranes modified carbon paste electrode for the enhancing the sensitivity of electrochemical DNA hybridization detection.[28] Recently Bai et al. reviewed the application of TiO_2 nanomaterials for the various sensors.[29]

In this paper a new electrochemical DNA sensing platform was constructed by using ERGO, TiO_2 nanowires and chitosan (CS) modified electrode. By using carbon ionic liquid electrode (CILE) as the substrate electrode, the nanomaterials modified electrode was fabricated and used for the probe ssDNA sequence immobilization. CILE has been proven to exhibit the advantages such as large electrochemical windows, high electron conductivity and good anti-fouling ability,[30] which has been used in the field of electrochemical sensors.[31] The presence of nanomaterials on the electrode surface can enhance the electron transfer rate, increase the electrode surface and provide a suitable microenvironment for the immobilization of ssDNA probe. The electrochemical DNA sensor was further applied to the *tlh* gene sequence that related to vibrio parahemolyticus detection. Vibrio parahemolyticus is a kind of gram-negative bacterium that often found in the marine environment, which can result in the disease of human.[32] The *tlh* gene sequence is present in all of the vibrio parahemolyticus strains and can be regarded as the target for vibrio parahemolyticus.[33] The proposed electrochemical DNA sensor was successful used to detect the *tlh* gene sequence of vibrio parahemolyticus and its PCR products from oyster samples.

Experimental

Apparatus and reagents

A CHI 750B electrochemical workstation (Shanghai Chenhua Instrument, China) was used to carry out all the electrochemical experiments including cyclic voltammetry, differential pulse voltammetry (DPV) and electrochemical impedance spectroscopy (EIS). A conventional three-electrode system was used in the experiments, which was composed of a saturated calomel electrode (SCE) as reference electrode, a platinum wire as auxiliary electrode and a modified CILE as working electrode. Scanning electron microscopy (SEM) was obtained by a JSM-7100F scanning electron microscope (Japan Electron Company, Japan). X-ray diffracton (XRD) was performed on a Rigaku Ultima IV X-ray diffractometer with Cu Kα radiation (Japan Rigaku Company, Japan).

1-Hexylpyridinium hexafluorophosphate ($HPPF_6$, >99%, Lanzhou Greenchem. ILS. LICP. CAS., China), chitosan (CS, Dalian Xindie Ltd. Co., China), graphite powder (Shanghai Colloid Chemical Plant, particle size of 30 μm), graphene oxide (GO, Taiyuan Tanmei Co., China), and methylene blue (MB, Shanghai Chemical Plant, China) were used as received. TiO_2 nanowires were synthesized according the reported method.[34] The DNA extraction kit was purchased from Beijing Tiangen Biotech. Ltd. Co. (China). Different kinds of buffers such as 0.2 mol L^{-1} PBS (pH 8.0), 0.05 mol L^{-1} PBS (pH 7.0), 50.0 mmol L^{-1} Tris-HCl buffer solution (pH 7.0) and 1×TAE buffer (40.0 mmol L^{-1} Tris+1.0 mmol L^{-1} EDTA+40.0 mmol L^{-1} acetate, pH 8.0) were used in the experiment. All the solutions were prepared with doubly distilled water and other chemicals used were of analytical reagents grade.

Various 23-base oligonucleotides sequences, which were selected from *tlh* gene sequence of vibrio parahemolyticus,[35] were purchased from Shanghai Sangon Biological Engineering Tech. Ltd. Co. (China) and their sequences were listed as follows.

Probe ssDNA sequence: 5'-GATGACACTGCCAGATGCGACG A-3';

Target ssDNA sequence: 5'-TCGTCGCATCTGGCAGTGTCAT C-3';

One-base mismatched ssDNA sequence: 5'-TCGTCGCATCTAGC AGTGTCATC-3';

Three-base mismatched ssDNA sequence: 5'-TAGTCGCATCTAG CAGTGTCAGC-3';

Non-complementary ssDNA sequence: 5'-ATCCTTTGCAATTG CCCAGTCGG-3'.
The DNA sample for polymerase chain reaction (PCR) amplification was extracted from oyster. The PCR reaction was performed on an Eppendorf Mastercycler Gradient PCR system (Eppendorf, Germany) using oligonucleotide primers for *tlh* gene with the following sequences:
Primer F: 5'-AAAGCGGATTATGCAGAAGCACTG-3';
Primer R: 5'-CGATCTCTTCTTGTGTTGAGTACTTAAACTG-3'.

Fabrication of the modified electrode

Based on the reported procedure CILE was fabricated with $HPPF_6$ as the binder and the modifier.[36] In brief 3.2 g of graphite powder and 1.6 g of $HPPF_6$ was mixed thoroughly in a mortar to get the IL modified carbon paste, which was inserted into a glass tube (Φ=4 mm) tightly with a copper wire inserted to one end to establish an electrical contact. The surface of CILE was smoothed on a piece of weighing paper just before use. Afterwards the newly prepared CILE was put into a 1.0 mg ml^{-1} GO solution and electrochemical reduction was performed at the potential of -1.3 V for 600 s to get the ERGO modified CILE. Then 6.0 μL of 1.0 mg ml^{-1} TiO_2 nanowires suspension solution were further casted onto the surface of ERGO/CILE and dried in the air, which was denoted as TiO_2/ERGO/CILE. Finally, 6.0 μL of 1.0 mg ml^{-1} CS solution (in 1.0% HAc) was spread evenly onto the surface of TiO_2/ERGO/CILE and dried to get a uniform film modified electrode, which was denoted as CS/TiO_2/ERGO/CILE.

Immobilization of the probe ssDNA sequence

By using CS/TiO_2/ERGO/CILE as the basal electrode, probe ssDNA sequence was immobilized with the following procedure. 10.0 μL of 1.0×10^{-6} mol L^{-1} probe ssDNA solution (in 50.0 mmol L^{-1} pH 7.0 PBS) was directly pipetted onto the surface of CS/TiO_2/ERGO/CILE. Based on the electrostatic adsorption of negatively charged probe ssDNA sequence with the positively charged CS film, the probe oligonucleotide can be immobilized on the surface of CS/TiO_2/ERGO/CILE. Then the modified electrode was washed with 0.5% sodium dodecyl sulfate (SDS) solution and doubly distilled water for 3 times successively to remove unadsorbed probe ssDNA sequence on the electrode surface. This probe ssDNA captured electrode was denoted as ssDNA/CS/TiO_2/ERGO/CILE.

Hybridization reaction

The hybridization experiments were further carried out by dropping 8.0 μL of target ssDNA sequence directly on the surface of the probe ssDNA sequence modified electrode. The hybridization between the target ssDNA sequence in the solution and the probe ssDNA sequence on the electrode was allowed to proceed for 30 min at room temperature. Then the electrode was washed with 0.5% SDS solution and doubly distilled water for 3 times to remove the unhybridized target ssDNA sequence. This hybridized electrode was further named as dsDNA/CS/TiO_2/ERGO/CILE.

Electrochemical detection

The hybridized dsDNA modified electrode was immersed into a 2.0×10^{-5} mol L^{-1} MB solution for 10 min to accumulate MB molecules on the electrode surface. Then the electrode was taken out and washed by 50.0 mmol L^{-1} PBS for 3 times. Electrochemical measurement was performed in a 50.0 mmol L^{-1} Tris-HCl buffer solution (pH 7.0) and electrochemical responses of MB were recorded by DPV method with the experimental parameters set as: the initial potential 0.2 V, the finial potential -0.70 V, the pulse amplitude 0.008 V, the pulse width 0.05 s and the pulse period 0.2 s.

PCR amplification

The DNA template for PCR amplification was extracted from the oyster samples, which were minced into tiny pieces, added into 10 mL concentrated digestion solution and incubated for 48 h in water bath at 25 °C. Then the solution was centrifuged 4 min at 12,000 rpm to obtain the precipitate, which was dispersed in 100 μL sterilized distilled water immediately. Then DNA sequence was extracted according to the procedure of DNA extraction kit. The amplification of *tlh* gene was performed in a final volume of 25 μL containing 200.0 nmol L^{-1} each primer of *tlh* gene sequence (primer F and primer R), 10×reaction buffer B (Promega, USA), 2.0 mmol L^{-1} $MgCl_2$, 200.0 nmol L^{-1} each of dATP, dCTP, dGTP and dTTP, 1.5 units of Taq DNA polymerase (Promega, USA), and 1.0 μL DNA template sequence extracted from samples (oysters). During the PCR procedure, DNA was initially denatured at 94 °C for 30 s, and PCR experiment was performed for 35 cycles of amplification (94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s) and final extension at 72 °C for 5 min. Then 6.0 μL of the PCR products were analyzed by electrophoresis separation at 5 V cm^{-1} for 40 min on a 2% agarose gel, which contained 0.5 μg mL^{-1} of ethidium bromide in 1×TAE buffer (pH 8.0). Finally the PCR products of *tlh* gene were kept at 4 °C before use.
Before electrochemical detection the products were diluted with 20.0 mmol L^{-1} PBS (pH 7.0) and heated in a boiling water bath for 10 min with the further cooling in an ice water bath for 2 min. By this procedure the dsDNA formed in PCR products was denatured to ssDNA, which could be further used for the electrochemical measurement by the proposed procedure.

Results and discussion

Characteristics of TiO_2 nanowires

SEM image of the synthesized TiO_2 nanowires were shown in Fig. 1A, which indicated that the product was composed of a large quantity of thin wires with a length in the range of several micrometers to several tens of micrometers. The XRD pattern of as-prepared TiO_2 nanowires was given in Fig. 1B. All the peaks could be indexed as TiO_2-B phase (JCPDS 46-1237) with some of the diffraction peaks being broadened. [37-39] Therefore the as-synthesized sample is high purity TiO_2-B, as no additional diffraction peaks are observed.

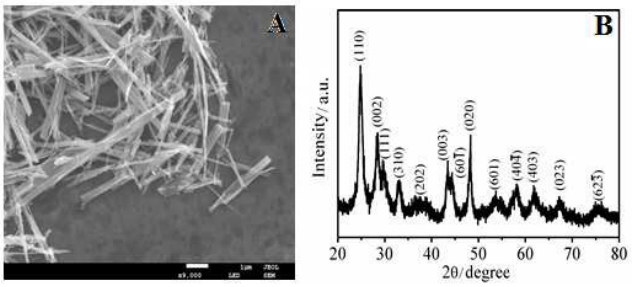

Fig. 1 SEM image (A) and XRD pattern (B) from as-prepared TiO_2 nanowire.

SEM images of the modified electrodes

Fig. 2 showed the SEM images of different modified electrodes. On CILE (Fig.2A) a flat surface could be observed, which was attributed to the high viscosity of IL in the carbon paste that bind the carbon powder together. After the electroreduction of GO on the surface of CILE, a rough surface with many folds and pores could be observed (Fig. 2B). Electrochemical reduction of GO to the formation of GR has been elucidated to be a facial and controllable method to synthesize GR nanosheets, which exhibits green nature with controllable procedure.[40] While on TiO_2/ERGO/CILE (Fig. 2C), TiO_2 nanowires could be clearly observed on the surface of electrode, which distributed randomly and the interface roughness was further increased. On CS/TiO_2/ERGO/CILE (Fig. 2D) a stable film was present on the electrode surface with the increase of the size of TiO_2 nanowires, indicating that CS had been present on the TiO_2 nanowire. CS is a commonly used biocompatible natural biopolymer for the electrode modification, which can form a stable membrane with good stability. Then the nanomaterials on the electrode surface can be tightly fixed on the electrode surface with the increase of the surface area.

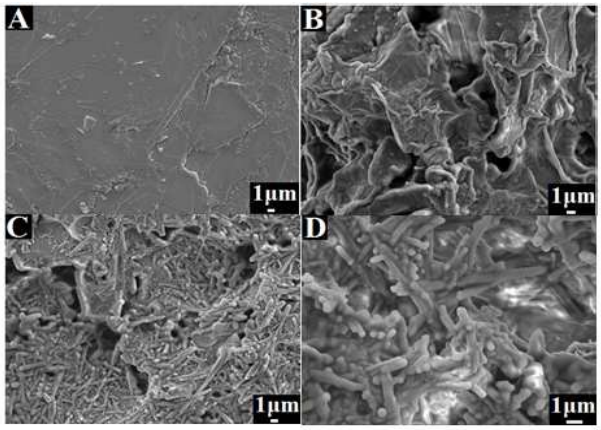

Fig. 2. SEM images of CILE (A), ERGO/CILE (B), TiO_2/ERGO/CILE (C) and CS/TiO_2/ERGO/CILE(D).

Electrochemical characterization of the modified electrodes

Cyclic voltammetry is an effective method to monitor the preparation procedure of the modified electrodes. As shown in Fig. 3A, cyclic voltammograms of different modified electrodes in a 1.0 mmol L^{-1} $[Fe(CN)_6]^{3-/4-}$ and 0.5 mol L^{-1} KCl solution were recorded. On CS/CILE a pair of redox peak currents appeared with the smallest value (curve a) and the addition of TiO_2 nanowires resulted in the increase of the redox peaks (curve b), which may be due to the increase of the interface area with the presence of TiO_2 nanowires. On CS/ERGO/CILE the redox peak currents also increased (curve c), which can be attributed to the presence of high conductive ERGO on the electrode surface. Electrochemical reduction is often used for the synthesis of GR nanosheet and ERGO also exhibits large specific surface area, excellent electrical conductivity and high electron transfer capacity. So the electrochemical responses were increased on CS/ERGO/CILE. On CS/TiO_2/ERGO/CILE, the biggest redox peak currents appeared (curve d), which could be ascribed to the presence of nanocomposite that combined the properties of TiO_2 nanowires and ERGO simultaneously. The changes of the redox peak currents in cyclic voltammograms proved the modified electrodes were successful prepared.

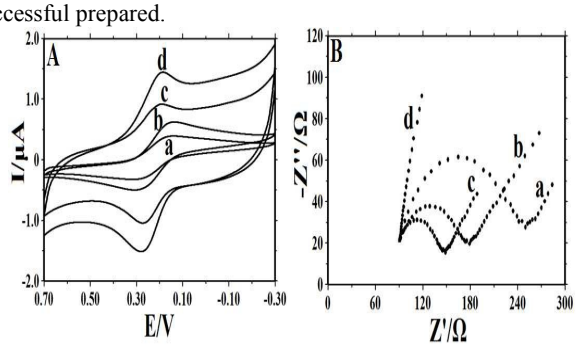

Fig. 3. (A) Cyclic voltammograms of CS/CILE (a), CS/TiO_2/CILE (b), CS/ERGO/CILE (c) and CS/TiO_2/ERGO/CILE (d) in a 1.0 mmol L^{-1} $[Fe(CN)_6]^{3-/4-}$ and 0.5 mol L^{-1} KCl solution with the scan rate of 100 mV s^{-1}. (B) Electrochemical impedance spectra of CS/CILE (a), CS/TiO_2/CILE (b), CS/ERGO/CILE (c) and CS/TiO_2/ERGO/CILE (d) in 10.0 mmol L^{-1} $[Fe(CN)_6]^{3-/4-}$ and 0.1 mol L^{-1} KCl solution with the frequency sweep from 10^4 to 1.0 Hz.

EIS can provide interfacial information of the modified electrode during the modification process, which is commonly used for the evaluation of the electrochemical information such as electrolyte resistance, electron transfer resistance and faradaic capacitance of a give system. The diameter of the semicircle usually equals to the electron transfer resistance (Ret), which controls the electron transfer kinetics of the redox probe at the electrode interface. The EIS experiments were perform in a 10.0 mmol L^{-1} $[Fe(CN)_6]^{3-/4-}$ and 0.1 mol L^{-1} KCl solution with the frequency swept from 10^4 to 1.0 Hz, and the results were shown in Fig. 3B. On CS/CILE the Ret value was got as 150.3 Ω (curve a), and after TiO_2 nanowires were modified on CILE surface, the Ret value was got as 90.1 Ω (curve b), which may be due to the presence of TiO_2 nanowires on the electrode surface. TiO_2 exhibited the specific characteristics such as large surface area and certain conductivity, which resulted in more $[Fe(CN)_6]^{3-/4-}$ participating in the reaction. And that of ERGO/CILE was further decreased to 58.2 Ω (curve c), which indicated the formation of high conductive ERGO on the electrode surface could increase the interfacial conductivity and decrease the

resistance. While on CS/TiO_2/ERGO/CILE a straight line appeared with the Ret value close to 0 Ω (curve d), which could be attributed to the synergistic effects of TiO_2 nanowires and ERGO on the electrode surface that could further decrease the interfacial resistances. The EIS results were in good accordance with CV data, indicating the successful preparation of the nanocomposite modified electrode.

Electrochemical behaviours of MB on different DNA modified electrodes

MB is a commonly used electrochemical indicator in DNA biosensor, which belongs to the phenothiazine family that exhibits excellent electrochemical performance. MB can interact with ssDNA and dsDNA by different interaction model, which is influenced by the experimental conditions. The negatively charged phosphate framework of ssDNA can be electrostaic bound with the positively charged MB molecules. MB can intercalate into the major or minor helix grooves of dsDNA structure. Also MB can bind specifically to the guanine bases on the ssDNA sequence.[41-44] After the hybridization reaction with the formation of dsDNA structure on the electrode surface, the electrochemical responses of MB is decreased due to the inaccessibility of MB to the guanine bases of dsDNA. Electrochemical behaviors of MB on different DNA modified electrodes were investigated with the results shown in Fig. 4, which was performed by using probe ssDNA/CS/TiO_2/ERGO/CILE to hybridize with 1.0×10^{-6} mol L^{-1} different ssDNA sequences. The biggest reduction peak of MB appeared on ssDNA/CS/TiO_2/ERGO/CILE (curve a), which was attributed to the electrochemical reaction of MB that interacted with the guanine bases of ssDNA on the electrode surface. The presence of ssDNA on the electrode surface can expose more guanine bases for the interaction and accumulation of MB molecules, so the biggest electrochemical response appeared. After hybridized with complementary ssDNA sequence, the electrochemical response decreased greatly with the smallest reduction peak current appeared (curve e). The result indicated the successfully hybridization of probe ssDNA sequence with target ssDNA sequence and the dsDNA structure was formed on the electrode surface. Then guanine bases were wrapped up in the duplex structure of dsDNA and the specific interaction of MB with guanine bases was prevented after the hybridization reaction finished on the electrode surface. So the smallest electrochemical response appeared. After incubated with the noncomplementary ssDNA sequence (curve b), the electrochemical response of MB was a little smaller than that of the target ssDNA sequence. The probe ssDNA sequence cannot hybridize with noncomplementary ssDNA sequence, so the ssDNA sequence still remained on the electrode surface with the ability to interact with MB molecules by the guanine bases in its structure. The little change may be due to the adsorption of some noncomplementary ssDNA sequence on the electrode surface that blocked the guanine bases of the probe ssDNA sequence. After hybridized with one-base mismatched ssDNA sequence (curve c) and three-base mismatched sequence (curve d), the reduction peak currents of MB decrease gradually, which indicated the partially formation of dsDNA duplex structure with the mismatched sequence on the electrode surface. So the amount of guanine bases that could bind to MB decreased gradually, and the electrochemical responses decreased. Also the reduction peak current of MB after hybridization with three-base mismatched ssDNA sequence was obviously higher than that of one-base mismatched ssDNA sequence, which demonstrated that this electrochemical DNA biosensor exhibited excellent selectivity and good distinguish ability for the hybridization detection of different ssDNA sequence.

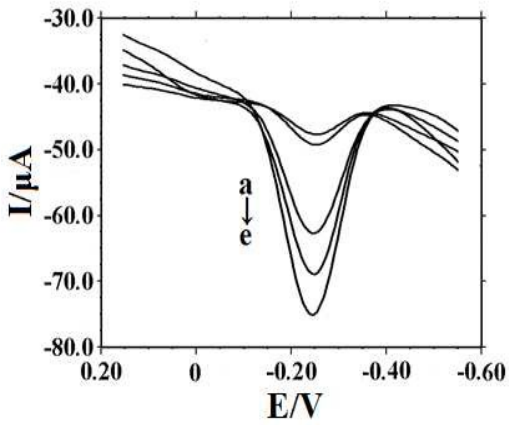

Fig. 4. Differential pulse voltammograms of 2.0×10^{-5} mol L^{-1} MB after hybridization with 1.0×10^{-6} mol L^{-1} the target ssDNA sequence (a), noncomplementary ssDNA sequence (b), one-base mismatched ssDNA sequence (c), three-base mismatched ssDNA sequence (d) and at the probe ssDNA modified electrode (e).

Optimization of the conditions of electrochemical detection

In order to maximize the efficiency and sensitivity of the DNA biosensor, the optimization of the experimental conditions was investigated. The concentration of MB used in the experiment can affect the amount of MB interacted on the probe ssDNA and then influence the sensitivity of biosensor. The current response increased with the MB concentration increasing from 2.0×10^{-6} to 2.0×10^{-5} mol L^{-1} and intended to be a stable value at the concentration of 2.0×10^{-5} mol L^{-1} or above. Therefore, 2.0×10^{-5} mol L^{-1} of MB was employed as the optimal concentration for this DNA biosensor. The accumulation time of MB was further investigated. The highest current signal of MB was sampled after 10 min accumulation and a signal plateau appeared when the accumulation time was more than 10 min. Therefore 10 min was chosen as the optimum accumulation time of MB in this paper.

Analytical performance of the electrochemical DNA biosensor

The electrochemical DNA sensor was applied to detect different concentrations of the complementary target ssDNA sequence with the results shown in Fig. 5. After hybridization the reduction current of MB decreased with the concentration of complementary target ssDNA sequence in the range from 1.0×10^{-12} to 1.0×10^{-6} mol L^{-1} and then tended to a stable value, indicating that all the probe ssDNA sequence on the electrode surface had been involved in the hybridization reaction. The formation of dsDNA molecules on the electrode resulted in the inaccessibility of guanine bases to MB, so

less MB molecules were present on the electrode and the electrochemical responses of MB were decreased correspondingly. The changes of the reduction peak currents of MB before and after hybridization exhibited good linear relationship with the logarithmic value of the complementary target ssDNA sequence with the linear regression equation as $\Delta I(\mu A)=1.79\log[C/(\text{mol L}^{-1})]+23.2$ (n=8, γ=0.996), where C is the concentration of target ssDNA sequence, ΔI is the difference of the reduction peak current of MB before and after hybridization. The detection limit was calculated as 3.17×10^{-13} mol L^{-1} (3σ), which was lower than the previous reported values such as Fe_3O_4 microsphere-GR composite modified CILE (3.59×10^{-13} mol L^{-1}),[45] multi-walled carbon nanotubes/ZrO_2/CS modified GCE (7.5×10^{-11} mol L^{-1}),[46] multi-walled carbon nanotubes and gold nanoparticles modified gold electrode (7.5×10^{-12} mol L^{-1}),[47] chitosan/nano-V_2O_5/ multi-walled carbon nanotubes composite modified CILE (1.76×10^{-12} mol L^{-1}) [48] and the Cu@Au alloy nanoparticle modified glassy carbon electrode (5.0×10^{-12} mol L^{-1}).[49] The relative standard deviation (RSD) of the reduction peak current for the six repeated detections of 1.0×10^{-6} mol L^{-1} target ssDNA sequence was calculated as 3.7%, indicating the good reproducibility of this method. The stability of ssDNA/CS/TiO_2/ERGO/CILE was investigated after 10 days storage at 4 °C and further used to hybridize with the target ssDNA sequence, 96.3% of the initial sensitivity remained, indicating this modified electrode was a stable platform as electrochemical DNA biosensor.

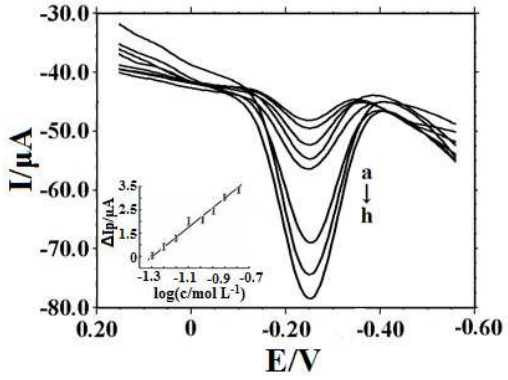

Fig. 5. Differential pulse voltammograms of MB on ssDNA/CS/TiO_2/ERGO/CILE after hybridization with different concentrations of target ssDNA sequence (from a to h were 1.0×10^{-6} mol L^{-1}, 1.0×10^{-7} mol L^{-1}, 1.0×10^{-8} mol L^{-1}, 1.0×10^{-9} mol L^{-1}, 1.0×10^{-10} mol L^{-1}, 1.0×10^{-11} mol L^{-1}, 1.0×10^{-12} mol L^{-1} and 0 respectively). Inset: plots of Ip versus logarithm of target ssDNA sequences concentration.

Detection of PCR products of vibrio parahemolyticus gene sequence

The PCR products of *tlh* gene sequence from oyster were treated to obtain the sample solution that containing target ssDNA sequence. Then 6.0 μL of sample solution was applied on the surface of ssDNA/CS/TiO_2/ERGO/CILE with the hybridization reaction and electrochemical detection performed under the experimental procedures. As shown in Fig. 6, the biggest electrochemical response of MB appeared on ssDNA/CS/TiO_2/ERGO/CILE (curve c), which was due to the binding of MB with guanine base of ssDNA sequence on the electrode. After hybridization with the sample solution the electrochemical signal of MB decreased greatly (curve b), which was due to the formation of dsDNA structure on the electrode. The guanine bases were wrapped inside the duplex structure of dsDNA and could not interact with MB, so the electrochemical response of MB was decreased. The significant difference of the MB signals between ssDNA/TiO_2/GR/CILE (curve a) and the hybridized dsDNA/CTS/TiO_2/GR/CILE (curve b) proved that this electrochemical DNA biosensor could effectively detect the PCR product of *tlh* gene sequence from vibrio parahemolyticus. While the smallest responses of MB appeared at CS/TiO_2/ERGO/CILE (curve c), indicating that the electrode reaction of MB was slow on the electrode and the presence of ssDNA sequence on the electrode surface could accumulate more MB on the electrode with the responses increased.

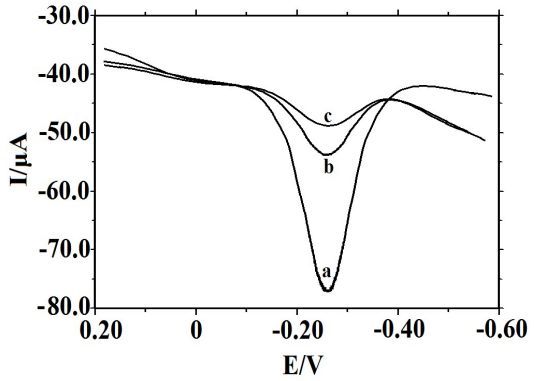

Fig. 6. Differential pulse voltammograms of MB at ssDNA/CS/TiO_2/ERGO/CILE (a), hybridized with PCR product of *tlh* gene sequence (b) and CS/TiO_2/ERGO/CILE (c).

Conclusion

In this paper electrochemical reduced GO nanosheets, TiO_2 nanowires and CS were modified on the surface of CILE step-by-step to get a modified electrode (CS/TiO_2/ERGO/CILE), which was further used as the basal electrode for the immobilization of probe ssDNA sequence. The presence of ERGO and TiO_2 nanowires on the electrode surface resulted in the increase of effective surface area and enhanced electron conductivity. After hybridization with the target *tlh* gene sequence from vibrio parahemolyticus, the decrease of the reduction peak current of MB was proportional to the concentration of target ssDNA sequence in the range from 1.0×10^{-12} to 1.0×10^{-6} mol L^{-1} with a detection limit of 3.17×10^{-13} mol L^{-1} (3σ). The electrochemical DNA sensor showed excellent discrimination ability to the detection of different ssDNA sequence and was further applied to the PCR product of *tlh* gene with satisfactory results.

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