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A nano-porous CeO_2/Chitosan composite film as the immobilization matrix for colorectal cancer DNA sequence-selective electrochemical biosensor

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Abstract

CeO_2/Chitosan (CHIT) composite matrix was firstly developed for the single-stranded DNA (ssDNA) probe immobilization and the fabrication of DNA biosensor related to the colorectal cancer gene. Such matrix combined the advantages of CeO_2 and chitosan, with good biocompatibility, nontoxicity and excellent electronic conductivity, showing the enhanced loading of ssDNA probe on the surface of electrode. The preparation method is quite simple and inexpensive. The hybridization detection was accomplished by using methylene blue (MB), an electroactive lable, as the indicator. The differential pulse voltammetry (DPV) was employed to record the signal response of MB and determine the amount of colorectal cancer target DNA sequence. The experimental conditions were optimized. The established biosensor has high detection sensitivity, a relatively wide linear range from 1.59×10^{-11} to 1.16×10^{-7} mol L^{-1} and the ability to discriminate completely complementary target sequence and four-base-mismatched sequence.

Keywords: DNA; Immobilization; Methylene blue; Composite film; CeO_2; Chitosan

1. Introduction

With the development of the biomedical studies of genetic diseases, the detection of DNA sequence has attracted increasing attention. Various techniques including enzymatic [1], fluorescent [2], electrochemical [3], surface plasmon resonance spectroscopy [4] and quartz crystal microbalance [5] have been proposed for DNA hybridization detection. Electrochemical methods possess the advantage of simplicity, low cost and high sensitivity [6–8]. The surface-immobilization of single-stranded DNA (ssDNA) probe on electrodes is a key step for fabricating the electrochemical DNA biosensor. Many methods including adsorption [9], covalent-binding [10,11], self-assembly [12,13], entrapment in a polymer matrix [14] and biotin–avidin interaction [15] have been used, though some of these methods are relatively complex and expensive, without the ability of fully retaining the activity ssDNA probe. The detection limit of reported methods rarely reached p mol L^{-1} level.

Recently, ceramic oxide materials have attracted considerable interest and have been successfully employed in DNA immobilization. Liu et al. reported the DNA immobilization based on ZrO_2 gel [16]. Despite of some advantages of sol–gel immobilization matrix, the brittleness of such matrix often limited its practical application. Zhu et al. applied electro-deposited ZrO_2 thin films on gold electrodes for fabricating DNA hybridization biosensor [17], though the linear range was relatively narrow. Inspired by the success of using oxides of tetravalent metals such as ZrO_2, one would expect that oxides of some other tetravalent metals such as CeO_2 could also be explored as candidate matrix. Lanthanide oxides possess affinity for oxygen and nitrogen atoms and cerium dioxide has been employed in various applications including fast ion conductors, oxygen storage capacitors and catalysts [18,19]. Recent years have seen efforts of combining the advantage of ceramic materials and organic polymers for the fabrication of biosensors. The using of organic polymer could overcome some limitation of ceramic materials in practical application. At the same time, the use of ceramic

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materials could also improve the chemical and thermal stability of organic membranes [20]. The potential of combined use of ceramic material and organic polymers as composite matrix for ssDNA probe immobilization deserves investigation.

Chitosan (CHIT), a natural cationic polymer, with excellent characteristic properties such as film-forming ability, non-toxicity and biocompatibility has been applied to immobilize biomolecules [21–23]. With the aim to explore the excellent film-forming ability of CHIT and the affinity of nano-CeO_2 toward the oxygen of DNA, the present authors investigated the use of CeO_2/CHIT composite film as immobilization matrix to fabricate an electrochemical DNA biosensor.

In this study, ssDNA probe sequences associated with colorectal cancer gene were immobilized on the surface of GC electrode modified with CeO_2/CHIT composite film. After hybridization reaction, the target DNA sequences were detected using an electroactive lable, methylene blue (MB) which possesses different affinity for dsDNA and ssDNA. The signal response of MB was measured by differential pulse voltammetry (DPV). This paper reports the experimental results of the preparation of the designed sensor and its analytical performance for the colorectal cancer DNA sequence detection.

2. Experimental

2.1. Reagents and materials

Table 1 shows the oligonucleotide sequences used in this study synthesized by Dalian Biotechnology Co., Ltd. (Liaoning, China). The target DNA sequence is the gene sequence associated with colorectal cancer. The probe DNA is the sequence completely complementary with the target DNA. The mismatch DNA is shown with four-mismatched bases underlined. The hybridization buffer solution was prepared with 0.1 mol L^{-1} sodium chloride, 1.0×10^{-2} mol L^{-1} Tris–HCl, 1.0×10^{-3} mol L^{-1} EDTA (STE, pH 8.8) and kept refrigerated. Phosphate buffer (pH 7.0) was used. Chitosan (CHIT, MW $\sim 1 \times 10^6$, 85% deacetylation) was supplied by Sigma (St. Louis, Mo, USA). Cerium oxide was produced by Nano Material Research Center (Zhejiang, China). Methylene blue and other chemicals were of analytical reagent grade. All solutions were prepared with doubly distilled water.

2.2. Apparatus

Cyclic voltammetry (CV) and differential pulse voltammetry experiments were carried out using a CHI 760b Electrochemical Analyzer (Chen Hua Instrument Inc., Shanghai, China). Scanning electron microscope (SEM) image of CeO_2/CHIT matrix was taken with JSM-5600LV (JEOL, Japan), using an accelerating voltage of 20 kV. The three-electrode system consisted of glassy carbon (GC) electrode (3 mm diameter) as the working electrode, a SCE as the reference electrode and a Pt foil as the counter electrode.

Table 1
Oligonucleotide sequences used in this study

Target DNA	5′-AAAACTTGTGGTAGTTGGAGCT GATGGCGTAGGCAAGAGTGCCC-3′
Probe DNA	5′-GGGCACTCTTGCCTACGCCATCAGCT-CCAACTACCACAAGTTTT-3′
Mismatch DNA	5′-AAA<u>T</u>CTTGTGGTAGTTG<u>T</u>AGCT GATGGCG<u>C</u>AGGCAAGAGTGC<u>G</u>C-3′

2.3. Preparation of CeO_2/CHIT solution

Appropriate amount of nano-sized CeO_2 was dispersed in 0.1% CHIT solution (1.0% acetic acid), and the mass ratio of CeO_2:CHIT was 1:100. The mixture was sonicated for 15 min with a high-dispersed colloidal solution obtained.

2.4. Electrode modification

Before each experiment, the GC electrode was polished with 0.05 μm α-alumina powder and rinsed thoroughly with absolute alcohol and water in ultrasonic bath and dried in air. CeO_2/CHIT composite solution (10 μL) was pipetted onto the surface of inverted GC electrode and air-dried at room temperature overnight. The electrode was rinsed with phosphate buffer (pH 7.0).

2.5. Immobilization of ssDNA probe on modified GC electrode

The CeO_2/CHIT-modified GC electrode was inverted and 10 μL of ssDNA probe (232.5 nmol L^{-1}) was pipetted onto the electrode surface. The probe droplet was air-dried at room temperature for 1 h to obtain a probe-modified GC electrode. After that, the electrode was washed with water and rinsed with phosphate buffer (pH 7.0) before hybridization.

2.6. Hybridization on the electrode surface

Hybridization reaction was conducted by pipetting 10 μL of hybridization buffer solution (0.1 mol L^{-1} NaCl, 1.0×10^{-2} mol L^{-1} Tris–HCl, 1.0×10^{-3} mol L^{-1} EDTA) containing the target DNA of different concentration onto the surface of GC electrode. This droplet was kept at 45 °C for 1 h. The electrode was washed with phosphate buffer (pH 7.0) to remove the unhybridized DNA. Similar procedures were also repeated by using the four-base-mismatched DNA sequence.

2.7. Reaction with MB

MB was accumulated onto the surface of dsDNA-modified electrode by immersing the electrode into the stirred phosphate buffer (pH 7.0) containing 2.0×10^{-5} mol L^{-1} MB with 20 mmol L^{-1} NaCl for 25 min without applying potential. After accumulation of MB, the electrode was rinsed in phosphate buffer (pH 7.0) for 10 s.

2.8. Electrochemical detection

The response signal of MB was measured by using differential pulse voltammetry in phosphate buffer (pH 7.0) with the

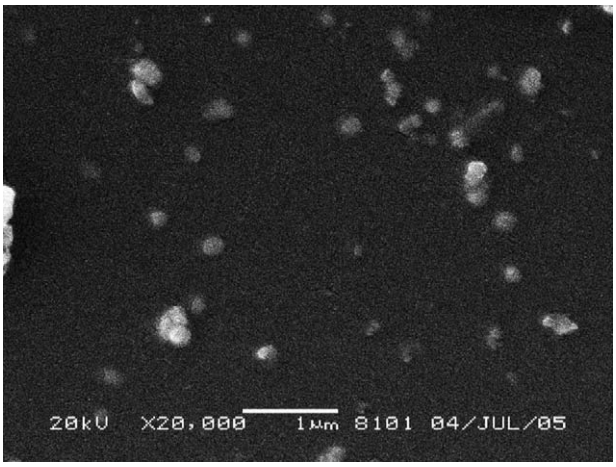

Fig. 1. SEM image of CeO_2 composite film.

hybridized working electrode with accumulated MB, the SCE reference electrode, and the platinum wire counter electrode. The scan rate was 50 mV s^{-1}.

3. Results and discussion

3.1. Morphology of CeO_2/chitosan composite film

CeO_2/CHIT composite matrix can form a film on the surface of GC electrode for the ssDNA probe immobilization. Its morphology was observed by scanning electron microscopy (SEM) as shown in Fig. 1. The CeO_2 particles with a few hundred nanometers more or less uniformly dispersed in CHIT film with a porous structure, which provided an enhanced loading interface for the ssDNA probe immobilization.

3.2. Electrochemical characteristics of modified GC electrodes

Electrochemical characteristics of modified GC electrodes are shown in Fig. 2. The cyclic voltammograms were obtained in 5 mmol L^{-1} ferricyanide/ferrocyanide solution containing 10 mmol L^{-1} KCl with bare GC electrode (GC), CeO_2/CHIT-modified GC electrode (CeO_2/CHIT/GC) and CeO_2/CHIT-modified GC electrode treated with ssDNA probe (ssDNA/CeO_2/CHIT/GC). One notices that the peak current increased and the peak-to-peak separation decreased in the order of GC, ssDNA/CeO_2/CHIT/GC and CeO_2/CHIT/GC electrodes. The CeO_2/CHIT-modified electrode shows the excellent electrochemical performance. This indicated that the CeO_2/CHIT film could enhance the electrochemical response of the ferricyanide/ferrocyanide, which could be attributed to the excellent electronic conductivity of cerium oxide and the accumulation ability of chitosan with amino groups for the negatively charged ferricyanide ions [24]. After the ssDNA probe immobilized on the CeO_2/CHIT-modified electrode, the peak current decreased. The reason seems to be related to the electrostatic repulsion between the polyanionic DNA

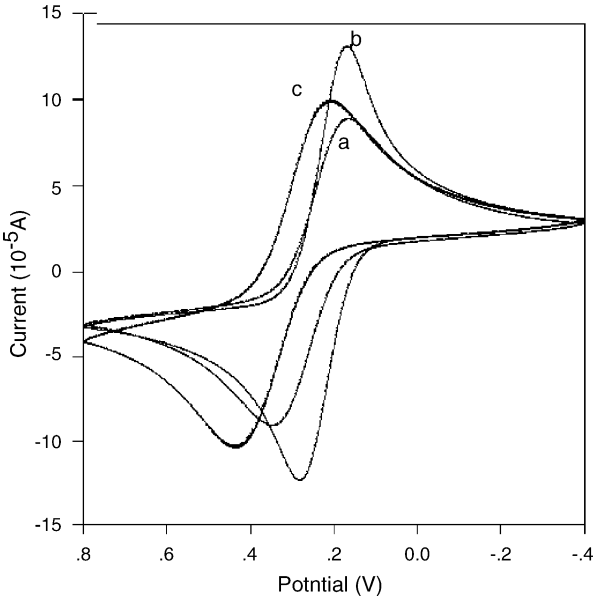

Fig. 2. Cyclic voltammograms of 5 mmol L^{-1} ferricyanide/ferrocyanide in 10 mmol L^{-1} KCl at 50 mV s^{-1} using (a) the bare GC electrode; (b) the CeO_2/CHIT-modified GC electrode (CeO_2/CHIT/GC) and (c) the CeO_2/CHIT-modified GC electrode treated with ssDNA probe (ssDNA/CeO_2/CHIT/GC), scan rate was 50 mV/s from −0.4 to 0.8.

immobilized on the CeO_2/CHIT/GC electrode and the anionic redox couple ions [25].

3.3. The loading of ssDNA on the electrode

DNA biosensor with MB as hybridization indicator was ever reported in previous literatures [26,27], which is based on the affinity of MB for the guanine bases of DNA. Thus, the increased loading of ssDNA probe can enhance the signal response of MB. Fig. 3 displays the DPV response of MB in phosphate buffer (pH 7.0) obtained with two different electrodes immobilized with ssDNA probe. The two electrodes were that the electrode modified only with 0.1% CHIT (CHIT/GC) (a) and the electrode modified with CeO_2/CHIT composite film (CeO_2/CHIT/GC) as described above (b). The preparation of CHIT/GC electrode used the same procedure as that of the CeO_2/CHIT/GC electrode, except that CeO_2/CHIT composite solution was substituted with 0.1% CHIT solution. As can be seen, the electrode modified only with CHIT film could also provide the enhanced MB signal, which was owing to the ability of CHIT, a polycationic polymer to bind with DNA and other polyanions [28]. Compared with the signal obtained with the ssDNA/CHIT/GC electrode, the ssDNA/CeO_2/CHIT/GC electrode offered an enhanced signal. This indicated that the CeO_2/CHIT composite film effectively increased the loading of the ssDNA probe, which was ascribed to the affinity of the oxygen atoms of CeO_2 to DNA and the high surface area of nano-CeO_2 dispersed in CHIT film. The value of the surface area of the constructed film was estimated to be 0.45 cm^2 according to the Randles–Sevcik equation [29]. The experimental results showed that the CeO_2/CHIT composite film is more propitious to increase the loading of ssDNA probe and improve the sensitivity of the analysis.

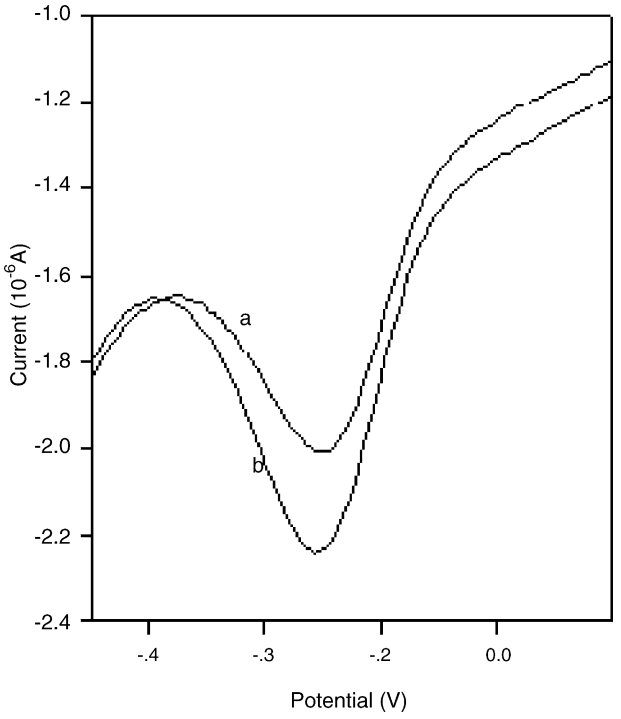

Fig. 3. The DPV response of MB as indicator in phosphate buffer (pH 7.0) recorded for the CHIT film modified GC electrode (a) and the CeO_2/CHIT composite film modified GC electrode (b) after immobilization with the same concentration (232.5 nmol L^{-1}) ssDNA probe. Stirred in phosphate buffer (pH 7.0) containing 2.0×10^{-5} mol L^{-1} MB with 20 mmol^{-1} NaCl for 5 min. Scan rate was 50 mV/s.

3.4. Optimization of experimental condition

Experimental condition can influence the biosensor characteristics. In order to maximize the sensitivity of the biosensor prepared, some parameters including the concentration of MB, the accumulation time with MB and the hybridization temperature were optimized.

The concentration of MB employed in the experiments directly affected the amount of MB accumulated with ssDNA probe and had a pronounced effect on the sensitivity of biosensor. The signal of MB increased with its concentration increased from 4.0×10^{-6} to 1.6×10^{-5} mol L^{-1}. A signal plateau appeared at 1.6×10^{-5} mol L^{-1} or above. Therefore, 2.0×10^{-5} mol L^{-1} MB was used as the optimum concentration.

The accumulation time of MB was also investigated. The hybridized-electrode was incubated in phosphate buffer (pH 7.0) containing 2.0×10^{-5} mol L^{-1} MB and 20 mmol L^{-1} NaCl, and the peak current of MB increased with the increasing accumulation time. The response signal tended to stabilize when the accumulation time reached 25 min or above. Therefore, the optimum accumulation time was 25 min.

The effect of the hybridization temperature on the response signal was investigated from 25 to 65 °C. The peak current reached the minimum value at the hybridization temperature of 45 °C, which showed that the highest hybridization efficiency was obtained at 45 °C. As a result, 45 °C was selected in the subsequent work.

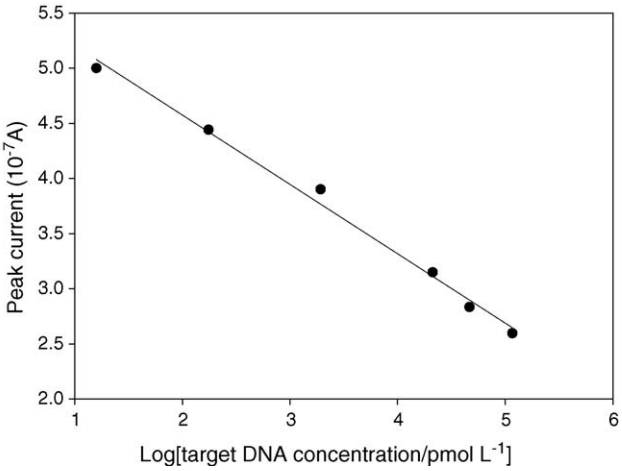

Fig. 4. Calibration curve obtained with different amounts of target DNA sequences for hybridization. The experiments were carried out under the optimal conditions.

3.5. Calibration curve

To obtain the calibration curve, the peak current values of MB were measured by DPV under the optimum conditions after ssDNA probe hybridized with the target DNA sequences of different concentrations according to the procedure described. As can be seen from Fig. 4, the reduction peak current values were linear with the logarithmic value of the complementary target DNA sequence concentration ranging from 1.59×10^{-11} to 1.16×10^{-7} mol L^{-1}, with a detection limit of 1.0×10^{-11} mol L^{-1}. The regression equation was $y = -0.6288 \log x + 5.833$, where y ($\times 10^{-7}$ A) was the reduction peak current value of MB by DPV, x (pmol L^{-1}) was the concentration value of target DNA. The correlation coefficient was 0.9929.

3.6. Selectivity of the DNA biosensor

The selectivity of the DNA biosensor was investigated by using the ssDNA/CeO_2/CHIT/GC electrode to hybridize with different kinds of DNA sequences. Fig. 5 shows the DPV curves of MB recorded for the ssDNA/CeO_2/CHIT/GC electrode without hybridization (a) and after hybridized with 1.93 nmol L^{-1} complementary sequences (b) or four-base-mismatched DNA sequences (c). All the peaks obtained were well defined. One can see that ssDNA/CeO_2/CHIT/GC electrode without hybridization presented the highest signal due to the strong affinity of MB for the free guanine bases. After hybridization with complementary target, the signal decreased obviously because of the inaccessibility of MB to the guanine bases. While incubation with four-base-mismatched sequence, the reduction signal of MB changed very small. The results show that the biosensor has good selectivity for hybridization detection.

3.7. Reproducibility of the DNA biosensor

The reproducibility of the proposed method was investigated by using 1.93 nmol L^{-1} target DNA sequence. The response

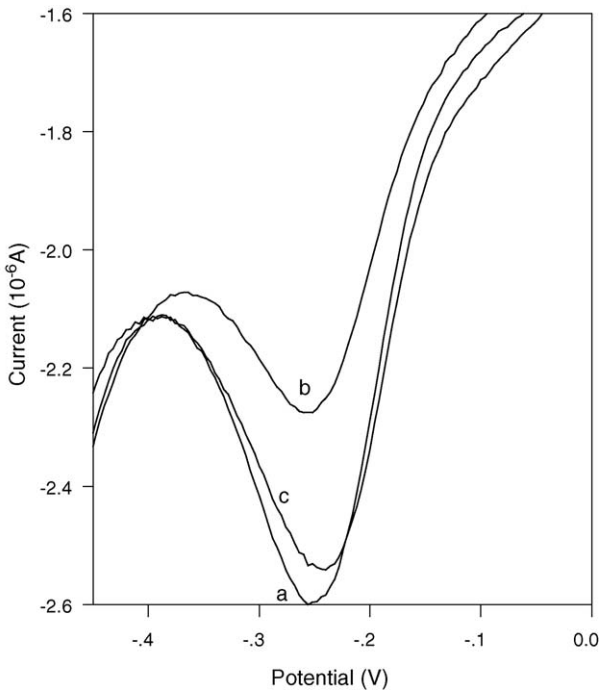

Fig. 5. Differential pulse voltammograms of MB as indicator in phosphate buffer (pH 7.0) using ssDNA probe-modified CeO_2/CHIT/GC electrode (a), the same after hybridization with the complementary target DNA sequence (1.93 nmol L^{-1}) (b) and after exposure to 1.93 nmol L^{-1} four-base-mismatched DNA sequence (c).

currents by using five electrodes prepared independently under the same conditions were 4.17, 4.03, 3.90, 3.88 and 3.75 ($\times 10^{-7}$ A). The relative standard deviation (R.S.D.) was 4.04% ($n = 5$). It indicated that a satisfactory reproducibility could be obtained by this system.

4. Conclusion

In this article, an efficient DNA immobilization matrix based on a nano-porous CeO_2/chitosan composite film was firstly developed for the fabrication of colorectal cancer DNA biosensor. The composite matrix incorporated the advantages of nano-CeO_2 and chitosan, which could form a nano-porous thin film on the surface of the GC electrode for the immobilization of ssDNA probe. The proposed method was very simple and practical. The experimental results showed that the electrode modified with chitosan doped with nano-CeO_2 could offer an enhanced signal than the electrode only modified with chitosan. The composite film could effectively increase the loading of ssDNA probe and enhance the biosensor response performance. The established DNA biosensor can discriminate completely complementary target sequence and four-base-mismatched sequence. The sensor for the detection of the target sequence associated with colorectal cancer gene has relatively wide linear range and low detection limit, high sensitivity and satisfactory reproducibility.

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