Electrochemical DNA Sensor for adh 1 Gene Sequence from Corn Endogenous with Carbon Microsphere Modified Electrode

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In this paper carbon sphere (CS) and chitosan (CTS) were used to modify carbon ionic liquid electrode (CILE). Then CTS-CS/CILE was further used to immobilize probe single-stranded DNA (ssDNA) sequence to get an electrochemical DNA sensor. The presence of CS provides a big surface area and CTS exhibits strong electrostatic affinity to the negatively charged ssDNA, therefore oligonucleotides probe sequence could be fixed on CTS-CS/CILE tightly. Target adh 1 ssDNA sequence from corn endogenous in the solution was measured by this electrochemical sensor with methylene blue (MB) as the hybridization indicator, which could distinguish ssDNA and dsDNA on the electrode surface. At the optimal conditions the reduction current of MB had a linear relationship with adh 1 gene sequence concentration from 1×10^{-13} to 1.0×10^{-6} mol L^{-1} with the detection limit as 6.42×10^{-14} mol L^{-1} (3σ). This electrochemical DNA sensor could discriminate different mismatched ssDNA sequences, and further applied to the detection of polymerase chain reaction amplification product of adh 1 gene sequences of corn endogenous from edible oil.

Keywords: Carbon sphere; chitosan; electrochemical DNA biosenor; carbon ionic liquid electrode; adh 1 gene.

1. INTRODUCTION

Electrochemical DNA sensor is based on the usage of probe single-stranded DNA (ssDNA) as the recognition device, which has exhibited widely applications in the field of genetic measurement,
bacterial analysis and food monitoring [1]. Compared with other methods for DNA detection, electroanalysis has the properties including high sensitivity, cheap equipment, fast response with good selectivity [2]. In general the detection procedure is composed of the immobilization of probe ssDNA on working surface, and the examination of electrochemical signals before and after the hybridization reaction. Various procedures have used for the ssDNA sequence immobilization and nanomaterials modified electrodes can enlarge the loading concentration of probe ssDNA sequence on the sensing interface [3, 4].

As a new kind of carbon nanomaterials, carbon sphere (CS) has been reported to be synthesized by hydrothermal method or template method with various carbon sources [5, 6]. CS has many excellent characteristics including good thermal stability, big surface area and fast electron conductivity, which has been widely studied in different fields including lithium ion batteries, supercapacitors, adsorbent and electrochemical immunosensors [7]. Chen et al. developed a colloidal CS array for the fabrication of disposable electrochemical immunosensor [8]. Sheng et al. prepared a carbon nanodot and chitosan (CTS) composite as the immobilization matrix to entrap hemoglobin with direct electrochemistry and bioelectrocatalysis realized [9]. Xu et al. designed a CS/gold nanoparticle composite as label for protein marker by a disposable electrochemical immunosensor [10]. Chen et al. synthesized monodispersed CS with glucose solution and applied to fabricate supercapacitor [11].

As organic compounds that are entirely composed of ions with wide electrochemical windows and high ionic conductivity. Ionic liquids (ILs) show excellent electrochemical performances, therefore the applications of ILs in electrochemistry and electroanalysis had been reviewed [12, 13]. As a new kind of binder and modifier, IL based carbon paste electrode (CPE) had been reported, which is often denoted as carbon ionic liquid electrode (CILE) [14]. CILE exhibits some specific advantages including fast electron transfer rates, inherent catalytic activity, and good resistivity to electrode fouling. Safavi et al. fabricated 1-octylpyridinium hexafluorophosphate modified CPE and investigated the electrochemical applications [15]. Our groups applied various CILEs to detect guanosine-5'-monophosphate and guanosine-5'-triphosphate [16, 17]. Different kinds of modifiers or biomolecules have been modified on CILE to get various electrochemical sensors [18-20].

In this paper CS was used to modify CILE with the help of CTS film. As a biopolymer CTS processes primary amine group at the C-2 position of the glucosanamine residues. It owns abundant reactive amino and hydroxyl groups in its molecular structure, and can establish a suitable biocompatible microenvironment for enzymes. CTS can also disperse nanomaterials homogeneously, and further apply to the electrode modification, which shows the properties including stable film forming ability, good biocompatibility, mechanical stability and water permeability [21-23]. The mixture of CTS and CS can result in a stable composite, which is casted on CILE and used for ssDNA sequence immobilization in this paper. The positive charged CTS can adsorb negative charged ssDNA sequence on its surface. The presence of CS can improve the roughness and the effective interface area. Also the conductivity of interface is enhanced with high conductive CS. The adh 1 gene of corn endogenous is used as the analytical target in the experiment and more specific probe ssDNA sequence can be fixed on the surface of CTS-CS/CILE. The hybridization process is monitored by the voltammetric response of methylene blue (MB), a commonly used electrochemical hybridization indicator. Under the selected conditions, this electrochemical DNA sensor could be used to the
detection of a 27-base oligonucleotides sequence related to corn endogenous with high sensitivity and selectivity.

2. EXPERIMENTAL PART

2.1. Apparatus and reagents

All the electrochemical responses were recorded on CHI 750B electrochemical analyzer (Shanghai CH Instrument, China). The three-electrode system used was consisted of a reference electrode (saturated calomel electrode, SCE), an auxiliary electrode (platinum wire electrode) and a working electrode (modified CILE). Scanning electron microscopy (SEM, JSM-7100F, Japan Electron Company, Japan) was used to obtain the morphologies of different materials. DNA extraction kit was purchased from Beijing Tiangen Biotechnology Ltd. Co. (China). Eppendorf mastercycler gradient polymerase chain reaction (PCR) system (Eppendorf, Germany) was used for PCR amplification.

1-Hexylpyridinium hexafluorophosphate (HPPF₆, >99%, Lanzhou Yulu Fine Chem. Ltd. Co., China), carbon sphere (CS, Nanjing JCNano. Technology Co., China.), chitosan (CTS, minimum 95% deacetylated, Dalian Xindie Chem. Co., China), graphite powder (Shanghai Colloid Chem. Co., particle size of 30 μm) and methylene blue (MB, Shanghai Chem. Co., China) were used. Various buffers such as 50.0 mmol L⁻¹ pH 7.0 PBS and 50.0 mmol L⁻¹ pH 7.0 Tris-HCl buffer solution were employed.

Adh 1 gene sequences of corn endogenous were purchased from Shanghai Sangon Biological Engineering Tech. Co. (China) with the following sequences.

- Probe ssDNA: 5'-AAT CAG GGC TCA TTT TCT CGC TCC TCA-3';
- Target ssDNA: 5'-TGA GGA GCG AGA AAA TGA GCC CTG ATT-3';
- One-base mismatched ssDNA: 5'- TGA GGA GCG AGC AAA TGA GCC CTG AGT-3';
- Three-base mismatched ssDNA: 5'- TGC GGA GCG AGC AAA TGA GCC CTG AGT-3';
- Non-complementary ssDNA: 5'- AGT CGA CGA ACT TCC A-3'.

The DNA sample extracted from corn endogenous gene was used for PCR, which was performed using the following oligonucleotide primers for adh 1 gene.

- Primer F: 5'-CGT CGT TTC CCA TCT CTT CCT CC-3';
- Primer R: 5'-CCA CTC CG GAC CCT CAG TC-3'.

2.2 Construction of electrochemical DNA sensor

CILE was prepared according to the reported method [24]. 7.0 μL of mixed solution that contained 0.5 mg mL⁻¹ CS and 1.0 mg mL⁻¹ CTS was casted to electrode surface and dried at room temperature. Then 10.0 μL 1.0×10⁻⁶ mol L⁻¹ probe ssDNA solution (in pH 7.0 PBS) was dropped on CTS-CS/CILE. The negatively charged phosphate group of the probe ssDNA sequence could be immobilized on the electrode interface by strong affinity between CTS and the phosphate group. After that the electrode was rinsed by 0.5% sodium dodecyl sulfate (SDS) solution and water for 3 times to wash unbound probe ssDNA sequence.
2.3 Hybridization

Hybridization was carried out by casting 5.0 μL of target ssDNA sequence solution (in pH 7.0 PBS) on the surface of ssDNA/CTS-CS/CILE and reacting for 20 min. After that, the electrode was rinsed by 0.5% SDS solution and water to get rid of non-hybridized target ssDNA sequence to obtain dsDNA/CTS-CS/CILE.

2.4 Electrochemical detection

Subsequently, dsDNA/CTS-CS/CILE was put in a 20.0 μmol L⁻¹ MB solution for 10 min, followed by washing 3 times with pH 7.0 PBS. Then the electrode was put in Tris-HCl buffer for electrochemical investigation and experimental parameters were set as: pulse width of 0.05 s, pulse period of 0.2 s and pulse amplitude of 0.008 V.

2.5 PCR amplification of adh 1 gene

Based on the procedure recommended by DNA extraction kit, 30 mL of edible oil was mixed with same volume of n-hexane and sonicated homogeneously for more than 3 hours, then the corn endogenous gene in the oil was extracted. The reaction solution contained 200.0 nmol L⁻¹ each primer, 10×reaction buffer B, 2.0 mmol L⁻¹ MgCl₂, 200.0 nmol L⁻¹ each of dATP, dCTP, dGTP and dTTP, 1.5 U of Taq DNA polymerase, and 1.0 μL DNA samples was used for PCR amplification, which was performed at 94 °C (30 s), 35 cycles amplification (94 °C, 30 s; 58 °C, 30 s; 72 °C, 30 s) and finally at 72 °C (5 min). The PCR product was stored at 4 °C for the further use.

3. RESULTS AND DISCUSSION

3.1. Characteristics of CS and the modified electrodes

As shown in Fig. 1 A and B, SEM images of CS with different amplitude indicated that CS had a uniform size of 640 nm with well-dispersion ability. CS is benefit for the electrochemical sensing because of the big surface area and versatility for surface functionalization. As shown in Fig. 1C, a pair of well-defined redox peaks appeared on the cycle voltammograms. The redox peak currents increased greatly on CTS-CS/CILE (curve b), as compared with that of CILE (curve a). Due to large surface area and high electron conductivity, CS on the modified electrode is in favor for the increase of effective electrode surface area with the diffusional rate accelerated.
Figure 1. SEM images of CS (A) and (B) at different enlarged magnitude; (C) cyclic voltammograms of (a) CILE and (b) CTS-CS/CILE in a mixture solution of 1.0 mmol L$^{-1}$ K$_3$[Fe(CN)$_6$] and 0.5 mol L$^{-1}$ KCl, scan rate 100 mV s$^{-1}$.

3.2 Electrochemical behaviors of MB

Differential pulse voltammetric (DPV) responses of MB on different ssDNA modified electrodes were studied with the results shown in Fig. 2. A well-defined reduction peak of MB could be observed at -0.275 V, which was ascribed to electrochemical reaction of phenothiazine group in MB molecular structure. The interaction of MB with the guanine group at ssDNA resulted in the accumulation of MB on the electrode surface, then electrochemical reaction of MB could be realized on ssDNA modified electrode [25, 26]. The peak current of MB increased on ssDNA/CS/CILE (curve b) than that of ssDNA/CILE (curve a), demonstrating that CS could adsorb more ssDNA sequence due to its large surface area and excellent conductivity for the electrochemical reaction of MB. Because of the specific affinity between the positively charged CTS and the negatively charged 5’-phosphate group of ssDNA sequence, the reduction current of MB on ssDNA/CTS/CILE (curve c) increased greatly. As shown on ssDNA/CTS-CS/CILE (curve d) the synergistic effects of CS and CTS could result in the largest reduction peak of MB. Also the CTS film can improve the stability of CS modified electrode and inhibit the falling off of CS. These results proved that CTS-CS/CILE was benefit for the ssDNA immobilization with good conductivity and stability.

Figure 2. DPV of different ssDNA modified electrode in a 50.0 mmol L$^{-1}$ pH 7.0 Tris-HCl buffer containing 2.0×10$^{-5}$ mol L$^{-1}$ MB on (a) ssDNA/CILE, (b) ssDNA/CS/CILE, (c) ssDNA/CTS/CILE, (d) ssDNA/CTS-CS/CILE.
3.3 Optimization of the experimental conditions

The experimental conditions were investigated to optimize the performance of electrochemical DNA sensor. The amount of CS on the electrode will affect the electron transfer efficiency of MB and the effective surface area of the modified electrode. When the concentration and the amount of CS solution were selected as 0.5 mg mL\(^{-1}\) and 7.0 μL, the largest response of MB appeared, therefore this concentration was chosen as the best conditions.

The concentration and the accumulation time of MB were further optimized, which could influence the MB concentration accumulated on the probe ssDNA sequence with further influence on sensitivity of DNA sensor. As shown in Fig. 3A, the reduction current increased gradually and reached a platform after 2.0×10\(^{-5}\) mol L\(^{-1}\), therefore 2.0×10\(^{-5}\) mol L\(^{-1}\) of MB was selected as the optimal concentration. Similarly, the accumulation time was tested and the signal increased with the accumulation time from 2 to 10 min and then kept unchanged (Fig. 3B). Therefore 10 min of accumulation was selected as the optimum time.

![Figure 3](image)

**Figure 3.** Effects of concentration (A) and accumulation time (B) of MB on the peak current at ssDNA/CTS-CS/CILE.

3.4 Selectivity of electrochemical DNA sensor

MB has various binding model to ssDNA or dsDNA with good distinguish ability, so it serves as an effective hybridization indicator in electrochemical DNA sensor. To investigate the selectivity of electrochemical DNA biosensor designed, four kinds of DNA sequences were chosen to compare with the results shown in Fig. 4. The response of complementary sequence had the smallest value (curve e) and the DPV signals were enhanced with increase of the amounts of mismatched sequence. However the electrochemical response of blank was the largest one (curve a). MB can bind with the guanine bases on ssDNA sequence, while the guanine groups in dsDNA sequences are wrapped, therefore the formation of hybrid dsDNA structure on the electrode block the specific interaction of MB with guanine. Therefore it can be concluded that this electrochemical DNA biosensor could distinguish mismatched DNA sequences from the complementary sequences with good selectivity.
Figure 4. DPV of 20.0 μmol L⁻¹ MB on different electrodes. (a) probe ssDNA/CTS-CS/CILE, after hybridization with non-complementary (b), three-base mismatched (c), one-base mismatched (d) and the complementary ssDNA sequence (e).

3.5 Sensitivity of the electrochemical DNA sensor

To investigate the sensitivity of this electrochemical DNA sensor, the concentration of complementary adh 1 sequences was changed to hybridize with ssDNA/CTS-CS/CILE. As shown in Fig. 5 the electrochemical responses of blank was the highest (curve a) and the DPV responses of MB decreased gradually with increasing concentration of complementary target ssDNA sequence from 1.0×10⁻¹³ to 1.0×10⁻⁶ mol L⁻¹ (curves b to i). The detection limit was 6.42×10⁻¹⁴ mol L⁻¹ (3σ), and the linear regression equation was ΔI (µA) = 2.27 log[C/(mol L⁻¹)]-6.00 (γ = 0.994) (inset in Fig. 5), where ΔI was the differences of peak current and C was the concentration of the complementary ssDNA sequence.

The stability of DNA modified electrode was investigated at 4 °C for 10 days storage and applied to hybridize with the complementary ssDNA sequence. The result indicated that the peak currents had 95.9% of the initial value. The reproducibility of ssDNA/CTS-CS/CILE hybridized with 1.0×10⁻⁶ mol L⁻¹ target ssDNA sequence for six repeated detection gave the relative standard deviation (RSD) as 3.7%, showing good reproducibility.
3.6 Detection of PCR products of adh 1 gene sequence

The PCR amplification product was dissolved in pH 7.0 PBS. Then it was heated at 100 °C for 10 min with ice cooling in for 2 min to get the sample solution. 10 μL sample solution was casted on different modified electrodes and the DPV results after hybridization were recorded. As shown in Fig. 6, the reduction peak currents of MB on CTS-CS/CILE (curve a), on the electrode of hybridized with sample solution of corn endogenous adh 1 gene (curve b) and ssDNA/CTS-CS/CILE (curve c) increased gradually, indicating that ssDNA modified electrode could adsorb more MB than the hybridized dsDNA modified electrode. Therefore the fabricated electrochemical sensor has the potential application to detect the PCR product of adh 1 gene in samples solution.

![Figure 6. DPV of MB on different electrodes. CTS-CS/CILE (a), hybridized with PCR product of corn endogenous adh 1 gene (b) and ssDNA/CTS-CS/CILE (c).](image)

4. CONCLUSION

By using CTS-CS/CILE as the substrate electrode, probe ssDNA sequence of adh 1 gene from corn endogenous was immobilized on CTS-CS/CILE. The existed CTS and CS resulted in a biocompatible platform with increased surface area, fast conductivity and strong affinity with probe ssDNA sequence. After hybridization with target ssDNA sequence, the decrease of the voltammetric response of MB was in proportion to target ssDNA sequence concentrations. A comparison of this electrochemical DNA sensor with other reported papers was listed in table 1. It can be seen that a relative wider linear range and lower detection limit could be achieved on CTS-CS/CILE with the simple procedure and the usage of only one kind of nanomaterial. Therefore CS has the potential application in the fabrication of electrochemical DNA sensor.

<table>
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<th>Modified electrode</th>
<th>Linear range (M)</th>
<th>Detection limit (M)</th>
<th>Target gene</th>
<th>Ref.</th>
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<tr>
<td>GNRs/AuE</td>
<td>1.0×10^{-11} to 1.0×10^{-7}</td>
<td>2.0×10^{-14}</td>
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<td>Gene / Protein</td>
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<td>rGO/graphene</td>
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<td>1.58×10^{-13}</td>
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<td>3.23×10^{-14}</td>
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<tr>
<td>S2/GOx-Thi–Au</td>
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<td>Au@SiO_2/DNAzyme/S/MH/Si/nano-Au/GCE</td>
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* AB, acetylene; AuE, gold electrode; AQ, anthraquinone; AuNPs, Au nanoparticles; CTS, chitosan; CS, carbon sphere; CILE, carbon ionic liquid electrode; ERGO, electrochemically reduced graphene oxide; GCE, glassy carbon electrode; GNPs, gold nanorods; GOx, graphene oxide; GR, graphene; G-3D Au, graphene-three dimensional nanostructure gold; MH, 6-mercapto-1-hexanol; MWCNTs, multi-walled carbon nanotubes; PNA, peptide nucleic acid; rGO, reduced graphene oxide; S, target gene; S1, capture probe DNA; S2, signal DNA; SPCE, screen-printed carbon electrode; Thi, thionine.

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