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PAPER

An electrochemical DNA sensor based on a layers–film construction modified electrode

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This work developed a relatively inexpensive and layers–film construction electrochemical sensor for DNA recognition and its performance was investigated. The Fe_3O_4 magnetic nanoparticles-cysteine were immobilized on the carbon paste electrode (CPE) surface using magnetic force. Multiwalled carbon nanotubes (MWCNTs), gold nanoparticles (GNPs), and chitosan (Chi) were used successively to coat on the electrode surface. The thiolated capture probe was assembled and competitively hybridized with the target nucleic acid and biotinylated response probe. The electrochemical behavior was analyzed by cyclic voltammetry and electrochemical impedance spectroscopy. In addition, the sensor performance was also analyzed by introducing the notion of detection efficiency. The experimental results showed that although the electron transfer capability of the CPE is less strong than that of a metal electrode used in the DNA sensor, the materials modified on the CPE could significantly improve the performance. A detection limit of 1 nM of target DNA and a sensitivity of 2.707×10^3 mA M^{-1} cm^{-2} were obtained. Although the resulting detection limit was not remarkable, further experiments could improve it.

Introduction

Electrochemical sensors have received considerable attention due to their fast response, remarkably high sensitivity, good selectivity, and strong operability.[1,2] Compared with sensors based on SPR and QCM techniques, electrochemical sensors need smaller operating costs and relatively simpler equipment and operation.[3] With increasing attention of novel nanomaterials, a kind of electrochemical sensor based on paramagnetic construction has been increasingly reported in recent years.[4–8] When an enzyme and immunoglobulin were applied into the sensor strategy, the sensors had good performance. The sensors based on paramagnetic immobilization of biomolecules could offer a good microenvironment for retaining the bioactivity of the biomolecules, and were regenerated easily.[6–8] How about using this strategy to detect DNA?

The gold electrodes or screen printed electrodes based on gold are commonly used as a working electrode for DNA sensors.[1,9,10] The mercapto gene probe can assemble easily on the electrode by the coupling between gold and the sulfhydryl group. However, the gold electrode is expensive and the total cost of screen printed electrodes is also huge due to its one-off property. In this regard, the strategy using magnetic nanomaterials and an inexpensive carbon paste electrode appeared to be a desirable one. Nevertheless, the electron transfer capability of the carbon paste electrode is less strong than that of the gold electrode.

So how to improve electron transfer capability of the carbon paste electrode? To address this problem, some nanomaterials and biopolymer materials were modified on a carbon paste electrode surface to improve its electron transfer capability. Nanomaterials are widely recognized as excellent carriers to improve the sensitivity of electrochemical sensors such as carbon nanotubes (CNTs), Fe_3O_4 magnetic nanoparticles (MNPs), and gold nanoparticles (GNPs), because they offer high electrical conductivity, provide a large specific surface area, and retain high bioactivitiy.[11,12] In recent years, CNTs have emerged as an excellent candidate for the development of electrochemical sensors. They have a unique ability to promote fast electron transfer kinetics for a wide range of electroactive species, and their length-to-diameter ratio offers a broad specific surface area.[13] The high electrical conductivity of Fe_3O_4 MNPs is ascribed to the Fe^{2+} and Fe^{3+} arranged in a disordered manner in an octahedral structure of Fe_3O_4. Their electrons can shuttle rapidly between the two oxidation states of iron. In addition, owing to their inherent magnetic properties, MNPs attract more attention as special biomolecule-immobilizing carriers. GNPs can couple the sulfhydryl group to easily and directly immobilize mercapto biomolecules with no modifying materials. In addition to nanomaterials, a polysaccharide biopolymer, chitosan (Chi) has also been extensively used in biosensors due to

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its non-toxicity, biocompatibility, and good film-forming ability.[14] Further, many sensors have been developed using combined forms of these materials. The familiar combined forms include CNTs-NPs, CNTs-Chi, and NPs-Chi.[13,15–18] Herein, the proposed sensor was constructed based on the MNPs-cysteine/MWCNTs-GNPs/chitosan modified electrode.

To investigate the sensor performance, the lignin peroxidase (LiP) gene was used as the analyte. Lignin is a universal and recalcitrant biomaterial in agricultural and municipal solid waste.[19] White-rot basidiomycetes are believed to trigger lignin biodegradation by secreting various types of non-specific and oxidative extracellular enzymes, such as lignin peroxidase, manganese peroxidase, and laccase. LiP is regarded as a key to lignin degradation by these fungi.[20] In *Phanerochaete chrysosporium*, the best-studied white-rot fungus, LiP is encoded by a family of 10 closely related genes, labeled *lipA* to *lipJ*, the amino acid sequence identities of which range from 70 to 99%.[21]

The aim of this work is to use some novel materials to improve the kind of electrochemical sensor based on paramagnetic construction to develop a layers–film construction sensor for DNA detection and to investigate its performance. This study considered the characteristics of Fe_3O_4 MNPs, multiwalled carbon nanotubes (MWCNTs), GNPs and chitosan and used all the materials simultaneously to construct a highly sensitive electrochemical DNA sensor for recognition of the target sequence of *P. chrysosporium lip* genes. The target DNA and the biotinylated response probe competitively hybridized with the capture probe, and the signal was amplified using an HRP-SA conjugate, resulting in a low detection limit and high sensitivity. The concept for detecting DNA using such a sensor is relatively new. Compared with the common DNA sensor based on expensive metal electrodes, the proposed sensor was more economical with similar sensitivity and reproducibility; due to the strong electronic conductivity of the modified materials on the electrode, the sensor demonstrated excellent electrochemical response capability with high sensitivity and appropriate response range, and there was still room for improvement; this kind of sensor was more thorough in sensor regeneration to guarantee the duplicate detection accuracy. The conformation of the proposed sensor should have great development potential.

Experimental

Apparatus

Electrochemical measurements were carried out on a CHI660B electrochemistry system (Chenhua Instrument, Shanghai, China). The three-electrode system used in this work consists of a carbon paste electrode (8 mm in diameter) as working electrode, a saturated calomel electrode (SCE) as reference electrode and a Pt foil auxiliary electrode. Scanning electron micrographs (SEM) of the morphology of the CPE surface were obtained with a JSM-6700F field emission scanning electron microscope, and transmission electron micrographs (TEM) of MWCNTs-GNPs were obtained with a JEM-3010 transmission electron microscope (JEOL Ltd., Japan). A Sigma 4K15 laboratory centrifuge, a Sigma 1-14 Microcentrifuge (Sigma, Germany), and a Model CS501-SP thermostat (Huida Instrument, Chongqing, China) were used in the assay. All work was performed at room temperature (25 °C) unless otherwise mentioned.

Reagents

MWCNTs were supplied by Applied Nanotechnologies, Inc. (Shanghai, China). HRP-SA was purchased from Dingguo Biotechnology Co., Ltd. (Beijing, China). Cysteine (Cys), chitosan and tris(hydroxymethyl)aminomethane (Tris) were from Sigma-Aldrich. Hydrochloroauric acid was from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All chemicals were of analytical grade and used as received. Sodium chloride–sodium citrate buffer (SSC, 0.3 M NaCl and 0.03 M sodium citrate, pH 8.0) was prepared as the hybridization solution. This work used a Tris-HCl buffer (0.1 M Tris adjusted to pH 8.0 with 0.1 M HCl) and phosphate buffered saline (PBS, 0.07 M KH_2PO_4 and 0.07 M Na_2HPO_4). All solutions were prepared in deionized water of 18 MΩ purified using a Milli-Q purification system.

The DNA target-specific probes used for competitive hybridization in our experiment were synthesized by Sangon (Shanghai, China). They were designed by Primer Premier 5.0 in our lab using ClustalX to run a sequence alignment of 10 *lip* sequences from *P. chrysosporium* available in GenBank.[21] The sequences of the oligonucleotides include: 5′-HS$(CH_2)_6$TTGTTGACGAAG GACTGCCA-3′ (T1, Capture probe), 5′-Biotin-TGGCAGT CCTTCGTCAACAA-3′ (T2, Response probe), 5′-Biotin-TGC CAGTCGTTCGTCTACAA-3′ (T3, Two-base-mismatched response probe), 5′-TGGCAGTCCTTCGTCAACAA-3′ (T4, Target oligonucleotide) and 5′-TGCCAGTCGTTCGTCTA CAA-3′ (T5, Two-base-mismatched oligonucleotide).

Sensor fabrication

As explained in Zhang *et al.*,[6] the self-made carbon paste electrodes (CPEs) contained a cylinder magnet (diameter of 6 mm and height of 1 mm). First, the Fe_3O_4 MNPs were prepared. After amino-functionalization, they were mixed with Cys for 6 h under stirring. The resulting products, MNPs-Cys, were washed with PBS (pH 4.8), then vacuum filtered for collection.

Next, MWCNTs-GNPs were prepared. The MWCNTs were purified by ultrasonic treatment in a mixture of hydrogen peroxide and sulfuric acid (1 : 3, v/v) for 2.5 h at 50 °C. Afterwards, the MWCNTs were rinsed with water and ethanol several times until the pH value reached neutral, and then they were filtered and dried in a vacuum at 60 °C.[22] This procedure shortened the nanotubes and produced carboxylic acid groups, mainly on the open ends and sidewalls.[23] The MWCNTs were then mixed with Cys solution and ultrasonically treated for 1.5 h, washed, and filtered to yield the MWCNTs-Cys.

Meanwhile, the GNPs (4 nm in diameter) were prepared.[24] 36.8 mL water, 1 mL 0.01 M $HAuCl_4$ solution, and 1 mL 0.01 M trisodium citrate solution were mixed, then 1.2 mL of ice-cold aqueous sodium borohydride solution (0.1 M) was added all at once with stirring. The solution turned wine red immediately after the addition of the borohydride, indicating gold nanoparticle formation. The GNP solution was placed in darkness for 3 h to degrade any remaining borohydride by reaction with water. The MWCNTs-Cys suspension was then added to GNP

solution under vigorous stirring under darkness for 8 h, producing the black precipitates, MWCNTs-GNPs.

Finally, the MNPs-Cys were suspended in 1 mL PBS (pH 4.8), and 20 μL MNPs-Cys was placed on the CPE surface for 1 h to immobilize the MNPs-Cys with paramagnetism, before washing with PBS to remove the excess MNPs-Cys. At this point, 20 μL MWCNTs-GNPs and 10 μL 0.7% Chi solution were modified in sequence on the electrode. After natural drying, a 20 μL aliquot of the thiolated capture probe (T1, 16.1 μM) was pipetted onto the electrode surface and kept at 4 °C for self-assembly through thiol–gold bonding. After adsorption, the electrode was copiously rinsed with Tris-HCl buffer and water to remove any non-specifically adsorbed materials. When not in use, the CPE was stored in a moist state at 4 °C.

Detection process

As shown in Fig. 1, the assembly and hydridization of the DNA biosensor are based on competitive hybridization and electrochemical detection. First, 20 μL hybridization solutions containing the biotinylated response probe (T2, 6 μM) and target nucleic acid (T4) of various concentrations were pipetted onto the electrode surface at 37 °C for 1 h, then thoroughly washed with Tris-HCl buffer and water. Next, 20 μL PBS (pH 6.98) containing 2 μg mL^{-1} HRP-SA was pipetted onto the electrode surface at 37 °C for 30 min. The electrochemical redox current catalyzed by HRP was measured amperometrically by the addition of 0.5 mM H_2O_2 into PBS (pH 7.38) containing 1 mM of hydroquinone under constant stirring at a working potential of −0.252 V (*vs.* SCE). The reduction current was recorded as I_x. When no T4 existed in the competitive hybridization, the reduction current was recorded as I_0. The decreased percentage (DP) of current is given by

$$DP = \frac{I_0 - I_x}{I_0} \times 100\%. \quad (1)$$

Regeneration of sensor

To regenerate the biosensor, we turned the nut at the end of the CPE to extrude carbon paste about 1 mm and polished the surface with 0.5-μm diamond paper. The CPE was cleaned by sonication in water for 2 min. The addition of MNPs, MWCNTs, GNPs, chitosan, and gene probes onto the electrode surface was the same as described in the 'Sensor fabrication' section. This process took about 15 h. The time, however, could reduce to 90 min if gene probes had been preassembled with MWCNTs-GNPs.

Results and discussions

Electrode surface morphology

The morphology of the CPE surface was investigated by SEM. Fig. 2A shows that the granular MNPs-Cys evenly paved on the

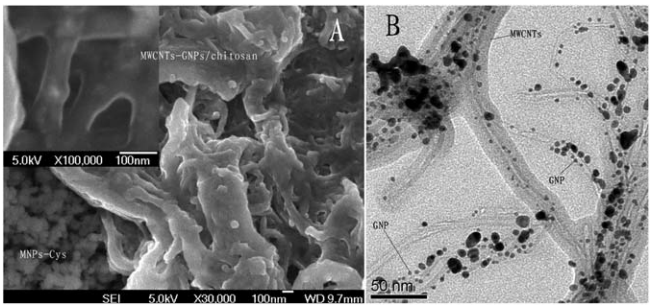

Fig. 2 Morphology of the modified electrode surface: (A) the SEM images of electrode surface, (inset) the SEM image of MWCNTs-GNPs/Chi on the electrode surface; (B) the TEM images of MWCNTs-GNPs.

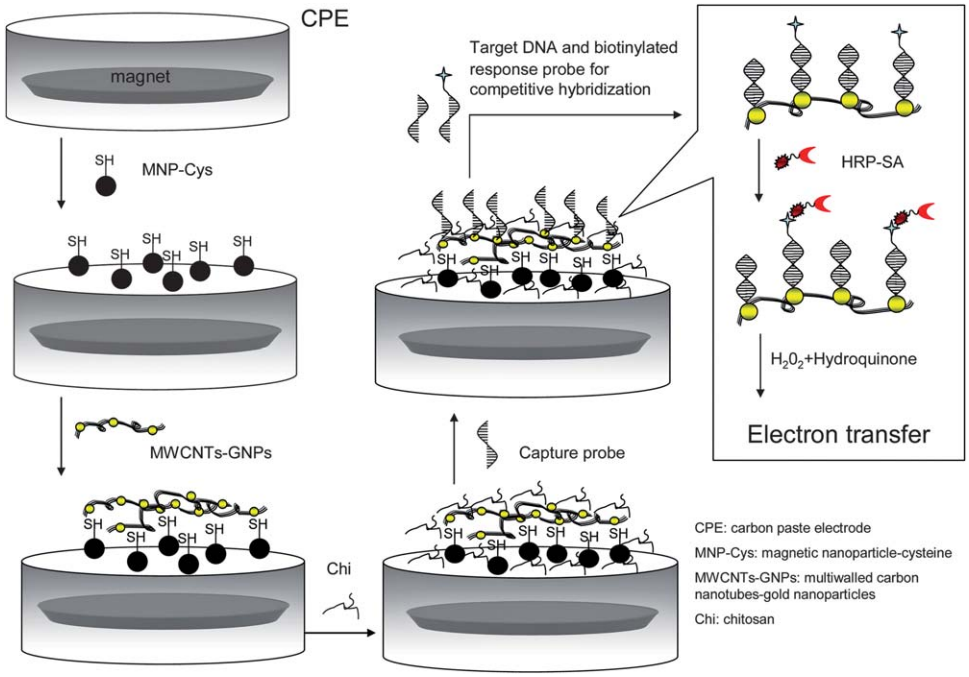

Fig. 1 Assembly and hydridization of the DNA sensor.

electrode surface to form a basement layer and that the twisted MWCNTs-GNPs/Chi were distributed on this layer. The spatial structure of the MWCNTs-GNPs offered many reaction sites and spaces for the hybridization process. However, perhaps owing to Chi enwrapping, the morphology of the GNPs modified on the MWCNTs cannot be clearly discriminated by SEM, even when amplified 100 000 times. Thus, TEM was used to confirm the structure of the MWCNTs-GNPs. The GNPs were bound on the MWCNT wall, as shown in Fig. 2B.

Biosensor electrochemical behavior

To test the performance of the modified electrode, cyclic voltammetry and electrochemical impedance spectroscopy were carried out in a 5 mM $[Fe(CN)_6]^{4-/3-}$ phosphate buffer (pH 7.0) containing 10 mM KCl. As shown in the cyclic voltammogram in Fig. 3, the bare electrode exhibited the maximum potential difference between the reduction peak and oxidation peak. After modification with MNP-Cys, the peak current increased, and the peak potential difference decreased slightly. When MWCNTs-GNP/Chi was modified, the peak current increased and the peak potential difference reached the minimum. Following self-assembly of the DNA probe, the peak potential difference grew appreciably, and the peak current decreased slightly, indicating that the modified electrode had a good current response capability. Correspondingly, electrochemical impedance spectroscopy showed that the impedance of the bare, self-made CPE, the MNP-Cys modified electrode, and the MNP-Cys/MWCNTs-GNP/Chi modified electrode decreased. Especially after MWCNTs-GNP/Chi were applied to the electrode surface, the impedance decreased sharply. After self-assembly of the DNA probe, the impedance had an acceptable increase (Fig. 4). The electron transfer ability reflected by changes in the impedance of the modified electrode was in accordance with the current response reflected by cyclic voltammetry.

All these tests demonstrated that the modified electrode had high sensitivity, which may be ascribed to the following three factors: First, the MNPs-Cys immobilized on the electrode surface with the help of magnetic force could weaken the electron barrier effect[25] of direct absorption of Cys on the electrode or the indirect linking of Cys using other materials. Further, it could reduce impedance and improve the electron transfer capability. Second, when MWCNTs-GNPs were adsorbed on the electrode surface by linking the –SH of Cys, a powerful electron tunnel was built on the electrode surface, markedly improving the electric conductivity and reducing the impedance of the electrode. Furthermore, the spatial structure of the MWCNTs-GNPs offered more reaction sites and space for self-assembly of the DNA probe, which is important for sensor performance. Third, the use of low concentration chitosan protected the link between MWCNTs-GNPs and MNPs-Cys by forming a film. The cancellate film structure of chitosan on the electrode surface was similar to the reported function of mercaptan,[1] weakening unspecific adsorptions, the orientation of the thiolated DNA probe, and the hybridization process. Though chitosan reduced

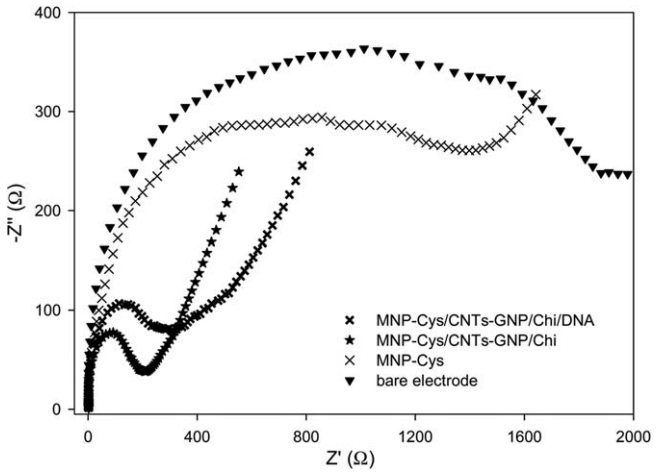

Fig. 4 Electrochemical impedance spectra (Nyquist plots) of different modified carbon paste electrodes in 0.1 M KCl solution containing 5.0 mM ferricyanide with frequency range of 0.01–10^5 Hz.

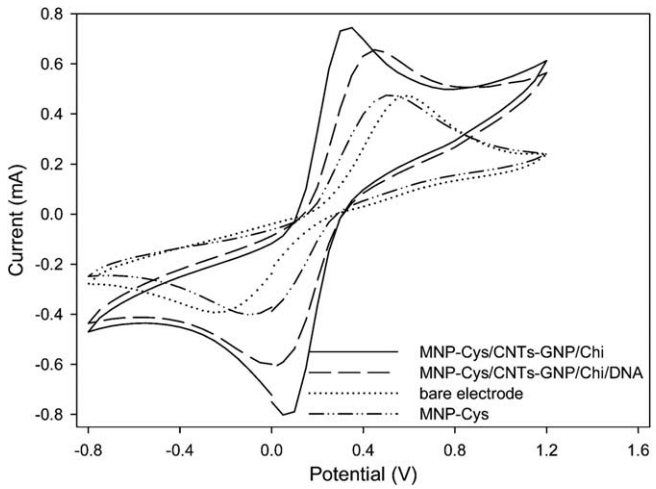

Fig. 3 Cyclic voltammograms in 0.1 M KCl solution containing 5.0 mM ferricyanide at a scan rate of 100 mV s^{-1} of different modified carbon paste electrodes.

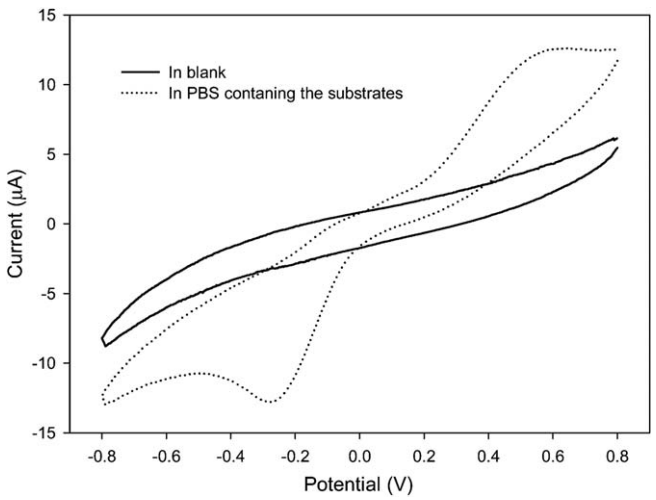

Fig. 5 Cyclic voltammograms of the electrode at a scan rate of 100 mV s^{-1} in blank and in PBS (pH 7.38) containing 0.5 mM H_2O_2 and 1 mM of hydroquinone.

electric conductivity, its advantages outweighed its disadvantages here. The GNP did not directly coat the MNP because, in its natural state, Fe_3O_4 can be oxidized to Fe_2O_3 with poor conductivity, which is unfavorable for long-term storage. Therefore, if Fe_3O_4 MNPs were coated with GNPs, their performance would decline due to oxidation. Synthesis of Fe_3O_4 MNPs is relatively cost-effective and can be easily re-prepared after deactivation. Further, compared with Fe_3O_4 MNPs which can be stored for about 35 days at 4 °C, MWCNTs-GNP can be stored for more than 90 days under the same conditions.

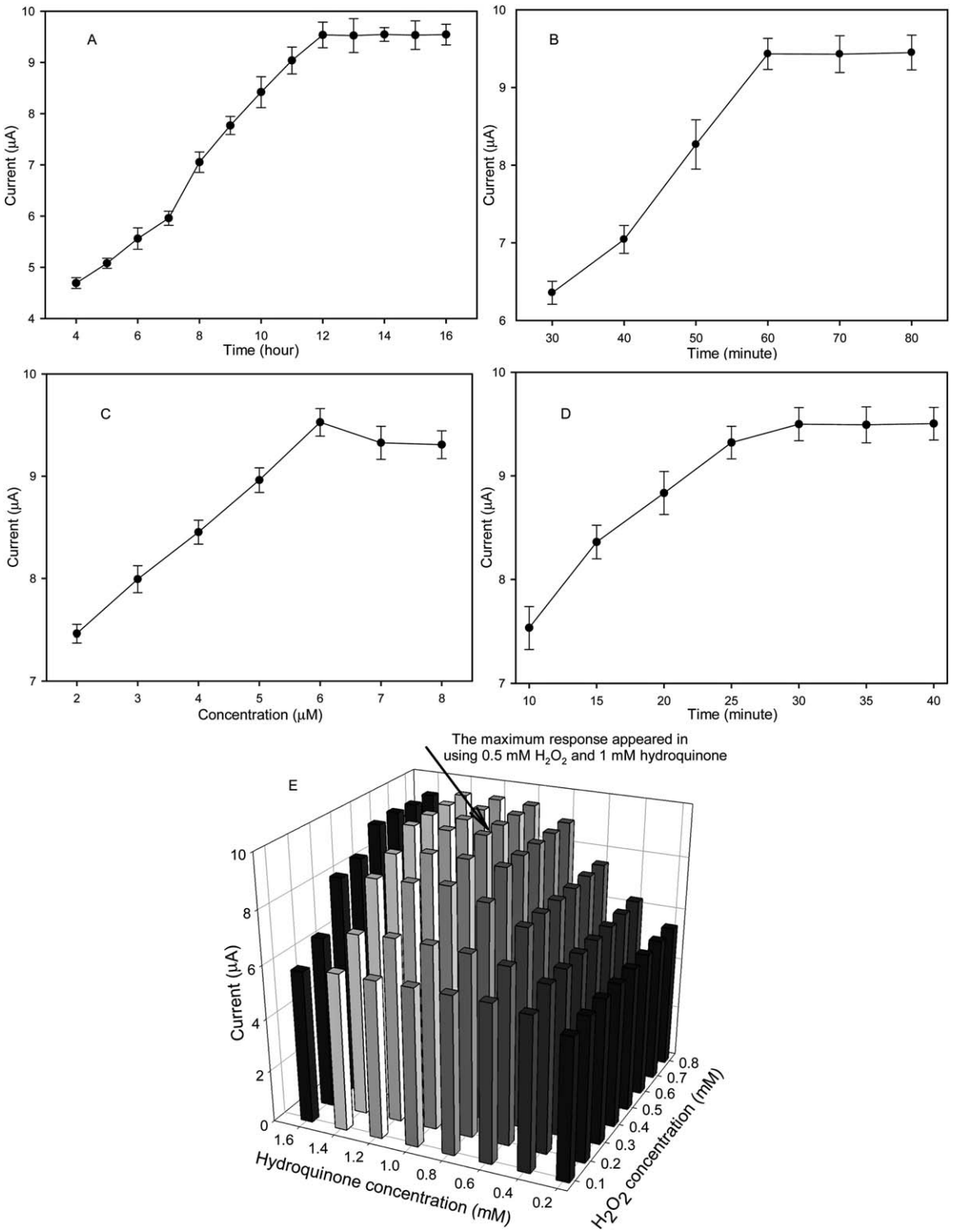

Fig. 6 Optimization of experimental conditions: (A) effect of self-assembly time; (B) effect of hybridization time; (C) amount of response probe for competitive reaction; (D) effect of incubation time for the HRP enzymatic reaction; (E) substrates concentration of H_2O_2 and hydroquinone. All tested electrodes were fabricated by modified 16.1 μM T1 probe on electrodes surfaces at 4 °C, and providing adequate conditions for thorough reaction.

Detection efficiency

Sensor performance could be further analyzed according to the notion of detection efficiency. The gain γ defined as the quotient of the signal input X and the signal output Y was quoted to represent the detection efficiency of the sensor herein, *i.e.*[26]

$$\gamma = Y/X. \quad (2)$$

In the presence of an energy barrier of height U the actual input X^* is

$$X^* = Xe^{-U/KT} = Xb \ (0 \le b \le 1) \quad (3)$$

and Y is

$$Y = yN = (1 - e^{-X^*/N})N \quad (4)$$

where N is the number of receptors covered on the sensor surface. Hence the gain is

$$\gamma = \frac{(1 - e^{-Xb/N})N}{X}. \quad (5)$$

Therefore, reducing b, *i.e.* the energy barrier U can raise the gain, *i.e.* the detection efficiency.

To obtain a good detection efficiency, the nanomaterials and biopolymer materials were modified onto the proposed sensor surface to reduce the energy barrier. Due to offering high electrical conductivity, the materials could reduce the height of the energy barrier, and further reduce the energy consumption used to surmount that energy barrier. Hence a high detection efficiency was obtained. This result could also explain the aforementioned electrochemical behaviors.

Experiment condition optimization

The experimental conditions were optimized before the quantitative analysis of LiP. Fig. 5 shows the cyclic voltammograms of the sensor in PBS (0.07 M, pH 7.38). It could be observed that there is a low background current in the absence of hydroquinone and H_2O_2. Upon the addition of 0.5 mM H_2O_2 and 1 mM hydroquinone to the buffer solution, a reduction peak appeared according to the reduction of quinone species liberated from the enzyme reaction catalyzed by the HRP on the surface of the electrode. In order to achieve the maximum sensitivity, the peak potential of −0.252 V (*vs.* SCE) was chosen for use in subsequent experiments.

Next, chronoamperometry was carried out to investigate other condition variables, including self-assembly, hybridization, and detection conditions. Fig. 6A demonstrated the effect of self-assembly time of capture probe T1 on the electrode surface. The response current increased with the self-assembly time, and then reached a plateau at 12 h. Therefore, the self-assembly time of 12 h was used in the subsequent measurements.

The optimization of hybridization conditions includes hybridization time and the amount of response probe T2 for competitive reaction. The hybridization time is an important factor to ensure the adequacy of a contact reaction. The response current increased sharply with the hybridization time, increasing from 30 to 60 min, and then leveling off (Fig. 6B). The amount of T2 for competitive reaction was also investigated (Fig. 6C). The current responses first increased, and then decreased as the probe concentration varied from 2 to 8 μM. When the T2 concentration was over 6 μM, the current decreased. Accordingly, a 6 μM T2 concentration was used in further detection.

Finally, the incubation time for the HRP enzymatic reaction and the dosage of substrates were optimized (Fig. 6D and Fig. 6E). The current response increased with the incubation time for the HRP enzymatic reaction, and reached the maximum at 30 min. The appropriate dosage of substrates could promote the sensitivity of the detecting system. The maximum response appeared in using 0.5 mM H_2O_2 and 1 mM hydroquinone.

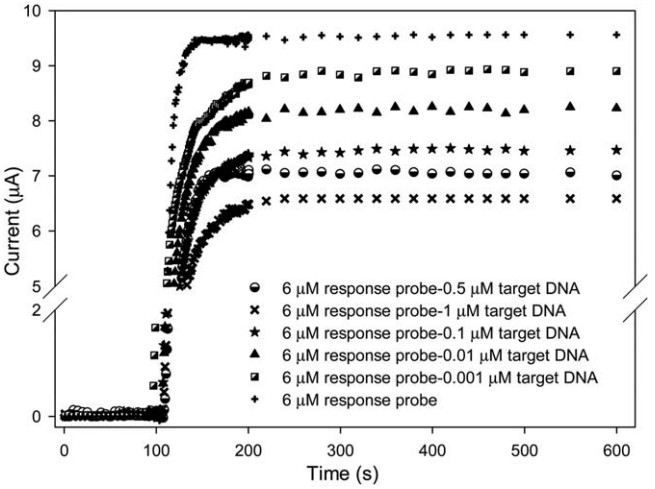

Fig. 7 Amperometric curves of different target sequence of *lip* genes in PBS (30mL, 0.07 M, pH 7.38) containing 0.5 mM H_2O_2 and 1 mM hydroquinone at an applied potential of –0.252 V *vs.* SCE.

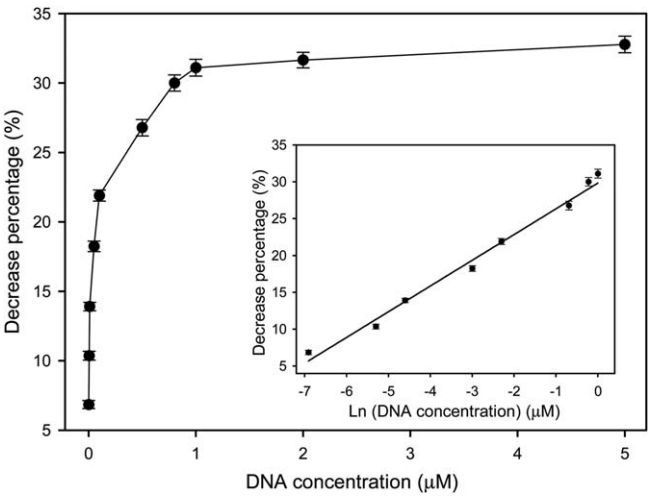

Fig. 8 The plot of average decrease percentage *vs.* target sequence of *lip* gene concentration in PBS (30 mL, 0.07 M, pH 7.38) at an applied potential of −0.252 V *vs.* SCE. The vertical bars designate the standard deviation for the mean of three replicate tests. (Inset) Calibration plot of average decrease percentage *vs.* natural logarithm of target sequence of *lip* gene concentration between 0.001 and 1 μM.

Table 1 Detection results of interference test

Capture probe	Response probe	Detecting gene	Response current/μA
T1	T2	T4	7.468
T1	T2	T5	0.542
T1	T3	T4	0.697

Detection

Under the optimal experimental conditions, the results of chronoamperometry showed a typical current response with a low background, and the sensitivity was estimated to be 2.707×10^3 mA M^{-1} cm^{-2} (Fig. 7). The decrease percentage of amperometric current response was attributed to competitive hybridization increase with the increased T4 concentration. As shown in Fig. 8, the average decrease percentage DP% was linearly related to the natural logarithm of T4 concentration, ranging from 0.001 to 1 μM with the following regression equation:

$$\mathrm{DP}\ (\%) = (3.4908 \pm 0.1725)x + (29.8172 \pm 0.6892). \quad (6)$$

The correlation coefficient is 0.994. Each of the calibrations was done three times, and the average relative standard deviation was 4.93%, which confirmed the precision of the DNA sensor. The detection limit was 1 nM. The results reflected an interesting problem: the response signals were very sensitive, but the detection limit was not remarkable. It may be attributed the carbon paste electrode itself, the distribution of nanomaterials *etc*. Further experiments could improve the problem.

Interference test

In order to verify the selectivity of the biosensor, the two-base-mismatched response probe (T3) and oligonucleotide (T5) were used for the interference test. Under the same optimized hybridization and electrochemical conditions, 6 μM T3 and 0.1 μM T5 were used to replace the T2 and T4 in competitive hybridization, respectively. The response current was faint, which was even lower than the tenth response current of using the same concentration T2 and T4 (Table 1). So, the biosensor could readily discriminate between the complementary and mismatched oligonucleotides.

Conclusion

This work developed a relatively inexpensive electrochemical sensor for recognition of the target sequence of LiP genes. The sensor was based on a MNPs-Cys/MWCNTs-GNPs/Chi-modified electrode and enzyme-amplified amperometric measurement with a competitive hybridization process. This project considered the properties of the MNPs-Cys/MWCNTs-GNPs/Chi membrane for construction of the modified electrode to improve sensor sensitivity. The average decrease percentage of the response current was linearly related to the natural logarithm of the target nucleic acid concentration, ranging from 0.001 to 1 μM, with a detection limit of 1 nM. And the sensitivity was estimated to be 2.707×10^3 mA M^{-1} cm^{-2}. In our opinions, the conformation of the proposed sensor is interesting. Although the resulting detection limit was not remarkable, further experiments such as the ratio of carbon paste, the distribution of nanomaterials, *etc.*, could improve it.

Acknowledgements

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