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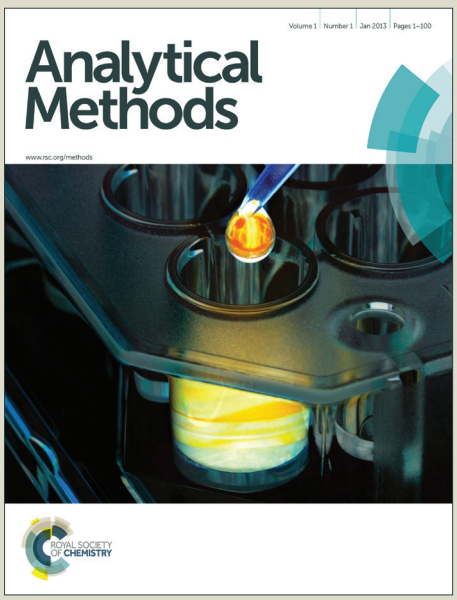

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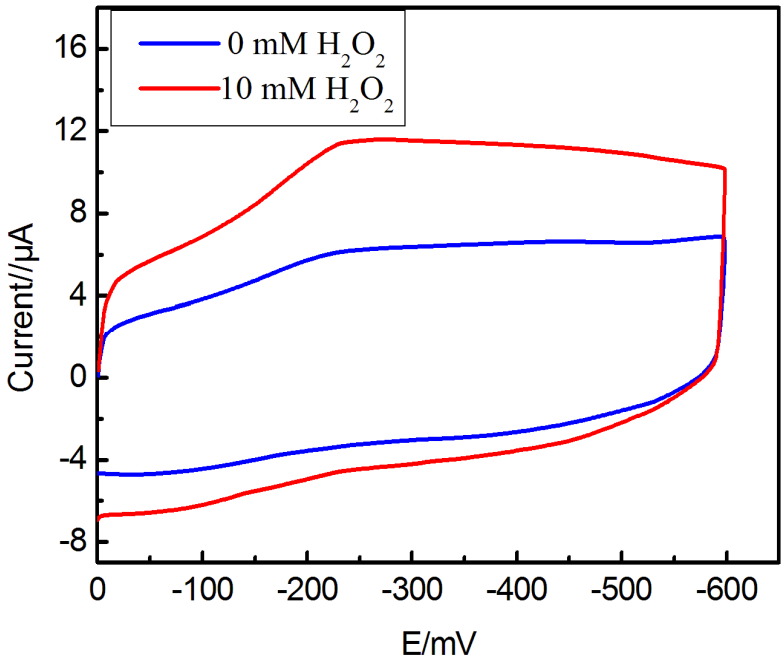
Graphical Abstract

A third-generation H_2O_2 biosensor was developed by using tetraethoxy-silicone sol-gel film for the horseradish peroxidase on a multi-walled carbon nanotubes modified glass carbon electrode. The sol-gel film provided a favorable biocompatible microenvironment for HRP and the special structure of MWNTs promoted the direct electron transfer between the HRP and electrode.



A third-generation hydrogen peroxide biosensor based on horseradish peroxidase immobilized by sol-gel thin film on muti-wall carbon nanotubes modified electrode

Shu-Xia Xu[*ab], Jia-Ling Li[a], Zhi-Ling Zhou[a], Cheng-Xiao Zhang[b]

ABSTRACT

A third-generation H_2O_2 biosensor was developed by using tetraethoxy-silicone (TEOS) sol-gel film for the immobilization of horseradish peroxidase (HRP) on a multi-walled carbon nanotubes (MWNTs) modified glass carbon electrode (GCE). MWNTs have good promotion effects on the direct electron transfer offering between HRP and electrode surface. The sol-gel film provided a favorable biocompatible microenvironment for HRP. The performance and factors influencing the performance of the resulting biosensor were studied in detail. The developed biosensor was applied for fabrication of a sensitive and selective measurement of H_2O_2 biosensor with the low operation potential (-300 mV versus Ag/AgCl). The amperometric response was proportional to H_2O_2 concentration in the range of 70 μmol L^{-1} - 3 mmol L^{-1} and the detection limit was 14 μmol L^{-1} at a signal-to-noise ratio of 3. The biosensor exhibited good sensor-to-sensor reproducibility (similar to 5%) and long-term stability (95% of its original activity retained after 60 days) when stored in 0.10 mol L^{-1} Phosphate buffer solution at pH 7.0 and 4 °C.

[*a]*College of Material and Chemistry & Chemical Engineering, Chengdu University of Technology, Chengdu 610059, China. E-mail: xushux2013@163.com; Fax: +86-28-84079012; Tel.: +86 28 84078957*

[b]*School of Chemistry & Chemical Engineering, Shaanxi Normal University, Xi'an 710062, China*

Introduction

The rapid and accurate determination of H_2O_2 is very important, as H_2O_2 is the product of the reactions catalyzed by a great deal of oxidases, and it is employed in various fields such as food, clinical, industrial and environmental areas. [1] Among in many analytical techniques that were developed for determination of H_2O_2, amperometric biosensor based on enzyme/protein horseradish peroxidase (HRP) is quite unique, because it combines the specificity of enzyme/protein with the sensitivity and simplicity of electroanalytical techniques.[2] Much attention has been paid to the third generation amperometric biosensor on the basis of the direct electron transfer between electrode and immobilized peroxidase/protein.[3-5] The third generation biosensor can detect H_2O_2 at relatively low applied potentials, and features the advantages of operation simplicity, no mediator, easy to fabrication and high sensitivity. However, it is usually difficult to achieve the direct electron transfer between the enzyme and bare electrode because large electrochemical prosthetic groups is deeply embedded into the structure of the enzyme. Besides, the enzyme absorbed on the bare electrode surface is apt to be irreversibly denatured.[6] Therefore, to improve the performance and long-term stability of the enzyme electrode, it is essential to find suitable material to modify bare electrode and develop effective method to immobilize enzymes on the electrode surface.

Recently, carbon nanotubes (CNTs) are promising as modifying substrates for the construction of sensors and biosensors owing to their significant mechanical strength, high surface area, excellent electrical conductivity and good chemical stability.[7-9] Owing

to small sizes of CNTs are same as protein/enzyme, CNTs allow good communication with redox centers which buried deep within protein shells. It has been proved that CNTs could shorten the electron transfer distance between the active center of the enzyme and the electrode, thus promoting electron transfer reaction. Due to improved conductive property, the direct electron transfer of horseradish peroxidase (HRP),[10,11] glucose oxidase (GOD),[12] tyrosinase,[13] glucose dehydrogenase,[14] alcohol oxidase,[15] fructose dehydrogenase,[16] bilirubin oxidase,[17] microperoxidase,[18] oxalate oxidase,[19] cellobiose dehydrogenase,[20] superoxide dismutase, hemoglobin (Hb),[21] myoglobin (Mb),[22] and cytochrome *c*[23] have achieved on CNTs modified electrode.

Above-mentioned proteins/enzymes were immobilized on the CNTs modified electrode surface by various immobilization protocols include adsorption, physical entrapment, cross-linking and covalent bonding. While some of these procedures were tedious, resulted in poor stability, or required environmentally unfriendly solvents such as glutaraldehyde. Glutaraldehyde contains complicated chemical species of documented cytotoxic nature and damages the bioactivity of protein/enzyme.[24] Although adsorption is the simplest way and involves minimal preparation. The adsorbed enzymes were easy to fall off from CNTs especially in a flow system. Thus a desired immobilization scheme is necessary to provide a simple means for attaching the protein/enzyme, so that it retains its affinity and stability over prolonged periods.[25]

Sol-gel has been emerged as a promising biosensor material since Zusman[26] and Broun[27] firstly reported on the attempt of protein encapsulation within silica glasses in 1990. The sol-gel process can be carried out at low temperature, and it features

chemically inert, porous structure, high-thermal stability, wide potential window, negligible swelling in aqueous solution and the capability of forming films.[28,29]. There have a few reports about combining the unique properties of the CNTs with the advantages of sol-gel technology to obtain the direct electron transfer of HRP. For examples, Dong et al. propose a H_2O_2 sensor based on HRP and CNTs simultaneously embedded in methyltrimethoxysilane sol.[30] Lin et al. integrated sol-gel, carbon nanotubes and HRP within one layer for the immobilization of HRP;[31] Di et al. developed a HRP-sol-gel/CNTs/GCE by spread the mixed suspension containing HRP silica sol and polyvinyl alcohol solution on CNTs/GCE.[32] These works showed that HRP maintained good enzymatic and electrochemical activities when immobilized on MWNTs modified electrode by sol-gel.

In this paper, we described a new, simple and stable method to immobilize HRP on the MWNTs-modified GCE by sol-gel method and developed a third-generation H_2O_2 biosensor. The sensor architecture was designed by immobilizing CNTs, HRP and tetraethoxy-silicone on electrode surface layer by layer respectively. Compared with co-immobilizing of silica sol-gel and HRP, it might reduce the leakage of enzymes from electrode and thus improved the stability of the biosensors. The performance and factors influencing the performance of the resulted biosensor have been studied in detail.

Experimental

Reagents

HRP (250 U mg^{-1}) was purchased from Sino-American Biotechnology Company (Luoyang, China). MWNTs were purchased from Shenzhen Nanotech Port Co. Ltd (Shenzhen, China). Phosphate buffer solutions (0.10 mol L^{-1}) with various pH values were prepared by mixing stock standard solutions of K_2HPO_4 and KH_2PO_4 and adjusting the pH with HCl or NaOH. All double-distilled water was used in all experiments. H_2O_2 solutions were prepared daily from 35% H_2O_2 solution. All other chemicals were of analytical grade and used without further purifications.

Apparatus

All electrochemical experiments were performed with a Bioanalytical Systems BAS-100B/W electrochemical analyzer (BAS Co, U.S.A) in conjunction with a standard three electrode voltammetric system consisted of a chemically modified glassy carbon (GC) electrode as working electrode (3 mm in diameter), a platinum wire counter electrode and a Ag/AgCl (3 mol L^{-1} NaCl solution) reference electrode. All potentials were reported with respect to the reference electrode. All measurements were carried out in isothermal reactor (10 mL, single electrolyte compartment) at constant temperature (30 ± 0.2 ^{o}C) with 0.10 mol L^{-1} Phosphate buffer solution as background electrolyte. All experimental solutions were deaerated by nitrogen gas for at least 25 min, and a nitrogen atmosphere was kept over the solutions in the cell to protect the solution from oxygen during the measurements.

Preparation of silica sol solution

To prepare of silica sol, 2 mL tetraethoxy-silicone (TEOS), 1.0 mL water, and 0.2 mL 0.01 mol L^{-1} hydrochloric acid were mixed, and then the solution was sonicated for 30 min until a clear and homogeneous solution resulted. The resulted sol was subsequently stored at 4 ^{o}C before the fabrication of the enzyme electrode.

Preparation of MWNTs modified GCE

The GCE was polished with 0.3 μm alumina slurry and then ultrasonic in ethanol and double distilled water for several minutes. MWNTs were pretreated as described previously.[33] The MWNTs were immobilized by casting 25 μL of treated MWNTs solution onto the GCE and then evaporating the N, N-dimethylformamide solvent in air to form MWNTs modified electrode (MWNTs/GCE).

Preparation of HRP/sol-gel/MWNTs modified GCE

The HRP/MWNTs/GCE was obtained by casting 5 μL of the HRP solutions (20 mg mL^{-1}) on MWNTs/GCE for 1 h. Then the HRP/MWNTs/GCE was dipped in the silica sol for 2 h. The resulted HRP/sol-gel/MWNTs modified GCE was rinsed with double distilled water and stored in Phosphate buffer solution with pH 7.0 at 4 ^{o}C when not in use.

Result and discussion

Study of direct electrochemistry of HRP by cyclic voltammetry

The electrochemical behavior of the HRP imbedded in the sol-gel film was studied using cyclic voltammetry (CV). Fig. 1 shows the CVs of the HRP electrode in 0.10 mol L^{-1} Phosphate buffer solution (pH =7.0) at different scan rates. With increasing the scan rate from 20 to 200 mV s^{-1}, redox peak currents increased, and the separation of the anodic and cathodic peak potential also increased. Both the cathodic and anodic peak currents were linearly proportional to the scan rates when the scan rate was lower than 200 mV s^{-1}. Thus the electrode reaction is typical of surface-controlled quasi-reversible process. When the scan rate was higher than 200 mV s^{-1}, the wave shape was distorted severely, indicating that the electrode reaction became electrochemical irreversibly at higher scan rate. The formal potential ($E^{0'}$) of the Fe^{III}/Fe^{II} redox couple, which was calculated according to average of anodic and cathodic peak potentials, is -290 mV versus Ag/AgCl. This potential is close to the -220 mV of native HRP in solution, suggesting that most HRP molecules kept their native structure after being immobilized in sol-gel film.

After successive scanning, no noticeable change in CVs of the HRP/sol-gel/ MWNTs/GCE was observed. This also suggested that the silicon sol film on the surface of MWNTs provided a favorable and suitable microenvironment for the HRP. The enzyme was stably embedded in the silica sol-gel network, so HRP could be effectively

immobilized on the surface of the MWNTs/GCE.

According to the Laviron's equation, the average surface coverage of HRP immobilized on MWNTs-GCE was calculated to be 2.82×10^{-10} mol cm^{-2}, indicating that HRP maybe has a high saturated coverage on MWNTs surface.

Eelectrocatalytic reduction of H_2O_2

The CVs behavior of the proposed HRP electrode in 0.10 mol L^{-1} Phosphate buffer solution of pH 7.0 were investigated at a scan rate of 20 mV s^{-1} (Fig. 2). Upon addition of 0.10 mmol L^{-1} H_2O_2 to electrolyte, an obvious electrocatalytic characteristics appeared with the fast increasement of reduction peak current and the great decreasement of oxidation peak current (Fig. 2 c and d). These phenomena indicated that HRP embedded in sol-gel film had good catalytic activity toward H_2O_2. As expected, only a weak current response to H_2O_2 could be observed at the MWNTs/GCE without HRP (Fig.2 b). It demonstrated that catalytically active HRP is an essential component of the biosensor. Furthermore, the proposed HRP electrode hardly show the direct electron communication between HRP and GCE in the absence of MWNTs(Fig.2 a). From these results, we can confirm that the catalytic current was mainly due to the direct electron transfer from the HRP molecules to the MWNTs/GCE. The high surface area and the excellent electric conductivity of MWNTs made the electron transfer from the bulk electrode surface to the redox center of HRP easier.

Influence of pH and applied potential on biosensor response

The dependence of the biosensor response on pH of the measurement solution was explored between 5.8 and 8.5 in 0.10 mmol L^{-1} Phosphate buffer solution. As shown in Fig. 3, the current response increased from pH 5.8 to 7.0 and reached the maximum at pH 7.0. A further increase of buffer pH from 7.0 to 8.5 led to decrease in the response. This may be due to the influence of pH on protein metamorphosis. At low and high pH the bioactivity of HRP will decline which may be caused by denaturing of the enzymes, leading to an obvious decrease in the response current. Therefore, pH 7.0 was used to obtain the maximum electrocatalytic activity of the immobilized HRP, which is in agreement with that reported optimum pH for soluble HRP.[34] This demonstrated that the sol-gel matrix did not alter the optimal pH value for the bioelectrocatalytic reaction.

The effect of applied potential on the steady-state current of the biosensor at different potentials was investigated and shown in Fig. 4. Results showed that applied potential possessed significant effect on the response of the biosensor. Little current was seen at -50 mV, and a flat response was observed as the applied potential shifts negatively from -50 to -200 mV. Then the current response began to increase when the potential changed from -200 to -350 mV, which might due to the increased driving force for the fast reduction of HRP at low potential. A further increase of the negative potential resulted in slow increase in current response because the limiting potential has been reached. It is preferable to control the lower working potential to decrease or avoid the interference from some electroactive species. Nevertheless, when applied potential was more and more negative, serious interference problem appeared. Therefore, considering both the

selectivity and the sensitivity, the operating potential of -300 mV was chosen as the optimum condition.

Amperometric response of the proposed H_2O_2 sensor

The amperometric response of the HRP electrode resulted from increasing concentrations of H_2O_2 was investigated using chronoamoerometry mode. As the H_2O_2 was added into the buffer solution, the biosensor responded rapidly and 95% of the steady-state current could be obtained within 5 s, which was similar to the reported results of HRP immobilized on polysaccharide-incorporated sol-gel,[35] which indicated a fast process. This is mainly attributed to two factors. On the one hand, the bioactivity of HRP immobilized in sol-gel is high, and the MWNTs are favorable to the orientation of the HRP molecule on the electrode in the process of bioelectrocatalysis. On the other hand, the sol-gel film is very thin due to large volume shrinkage during the drying process,[36] resulting in a small diffusion barrier.

Fig. 5 displayed the calibration plot of the biosensor. The biosensor responded to H_2O_2 in the linear range from 70 μmol L^{-1} to 3 mmol L^{-1}, and the linear regression equation was I (μA) = 5.17 C (mmol L^{-1}) + 5.27, with a correlation coefficient of 0.9951. The limit of detection was 14 μmol L^{-1} estimated at a signal-to-noise ratio of 3. Table 1 showed the parameter of the proposed HRP/sol-gel/MWNTs film in terms of analytical performance are compared with earlier reported third generation H_2O_2 biosensors.

Stability and repeatability

Stability and reproducibility are two important parameters for the evaluation of biosensor. The stability of the HRP/sol-gel/MWNTs/GCE was investigated by amperometric measurements in the presence of 0.10 mmol L^{-1} H_2O_2. No obvious decrease of current was observed after the electrode was tested 15 times continuously. Without in use, it was stored in 0.10 mol L^{-1} pH 7.0 Phosphate buffer solution solution at 4 ^oC in a refrigerator. The biosensor retained about 95% of its original response after two month. The stability of the biosensor was better than those of HRP adsorbed on CNTs,[9] immobilized to Au/self-doped ipolyaniline nanofibers,[41] and embedded in a silica sol-gel film.[30-32]

The good stability of the enzyme electrode could be due to three aspects: First, the sol-gel process does not involve the chemical modification of the enzyme molecule. It provides HRP molecules a biocompatible microenvironment, so the enzyme can maintain its biological activity to a large extent. Second, sol-gel is a porous network and large quantities of hydrogen bonds form during the sol-gel course, which could prevent the enzyme leaking out of thin film. Third, the sensor architecture was designed by immobilizing CNTs, HRP and tetraethoxy-silicone on electrode surface layer by layer respectively. Compared with co-immobilizing of silica sol-gel and HRP, it might reduce the leakage of enzymes from electrode and thus improved the stability of the biosensor.

The reproducibility of the sensor was examined at a H_2O_2 concentration of 0.10 mol L^{-1} with the same enzyme electrode. The relative standard deviation (RSD) was < 4.7% for 10 successive assays. The fabrication reproducibility of four independently prepared

electrodes, displaying an acceptable reproducibility with a relative standard deviation of < 6% for the response to the same concentration of H_2O_2.

Conclusions

A third-generation H_2O_2 biosensor was constructed based on the combination of sol-gel technology and nanomaterial. The HRP has been embedded in silica sol-gel on GCE surface modified by MWNTs, and the direct electron transfer of HRP was realized. The porous network structure of sol-gel film provided a favorable microenvironment for HRP to retain its native structure and bioelectrocatalytic activity. Meanwhile, the special structure of MWNTs can promote the direct electron transfer of HRP, so the proposed biosensor exhibited good electrochemical characteristics with good stability and fast response to H_2O_2. Furthermore, the proposed H_2O_2 biosensor is not only suitable for the immobilization of HRP, but also can be extended to the fabrication of other enzyme/bienzyme-based biosensor.

Acknowledgements

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Figure captions

Fig.1 CVs of the biosensor in 0.10 mol L^{-1} Phosphate buffer solution (pH =7.0) at 20, 50, 60, 100 and 200 mV s^{-1}, respectively. (From lowest to highest peak currents).

Fig. 2 CVs of HRP/CE(a), MWNTs/CE (b), and HRP/sol-gel/MWNTs/GCE (c, d) in the absence of H_2O_2 (c) and in the presence of 0.10 mmol L^{-1} H_2O_2 (d) at 20 mV s^{-1}.

Fig. 3 Effect of pH on the response of biosensor. Applied potential, -300 mV and concentration of H_2O_2, 0.10 mmol L^{-1}.

Fig. 4 Effect of applied potential on the response of biosensor in 0.10 mol L^{-1} Phosphate buffer solution (pH =7.0) and containing 0.10 mmol L^{-1} H_2O_2.

Fig. 5 Calibration plot of the H_2O_2 sensor in 0.10 mol L^{-1} phosphate buffer solution (pH =7.0) at a applied potential of -300 mV.

Table 1 Comparison between the proposed sensor and other H_2O_2 sensors based on HRP

Electrode material	Linear range (mM)	LOD (μM)	Reference
HRP/SGCCN/GCE	0.495-10.6	12.89	26
HRP/chitosan/sol–gel/CNT/GCE	0.0048-50	1.4	27
HRP–SG/CNT/GCE	0.0005–0.3	0.1	28
HRP-BSA-MWNTs-GCE	0.00095-9.5	0.4	29
Au/SPAN-HRP-CS/GCE	0.01-2	1.6	33
HRP-TTF-TCNQ/MWNTs	0.005-1.05	0.5	36
CHIT/HRP/KNs/Au electrode	0.04-6	12	37
Sol–gel/chitosan/HRP/CPE	0.25–3.4	3	38
NF/HRP/Bi_2O_3–MWCNT/GCE	8.34–28.88	Not available	39
HRP/sol-gel/MWNTs/GCE	0.07-3	14	This work

SGCCN: sol–gel-derived ceramic–carbon nanotube; SG: sol–gel; BSA: bovine serum albumin; SPAN: self-doped polyaniline nanofibers; CS: chitosan; TIF-TCNQ: tetrathiafulvalene- tetracyanoquinodimethane; CHIT: chitosan; KNs: $KNbO_3$ nanoneedles; CPE: carbon paste electrode; NF: nafion

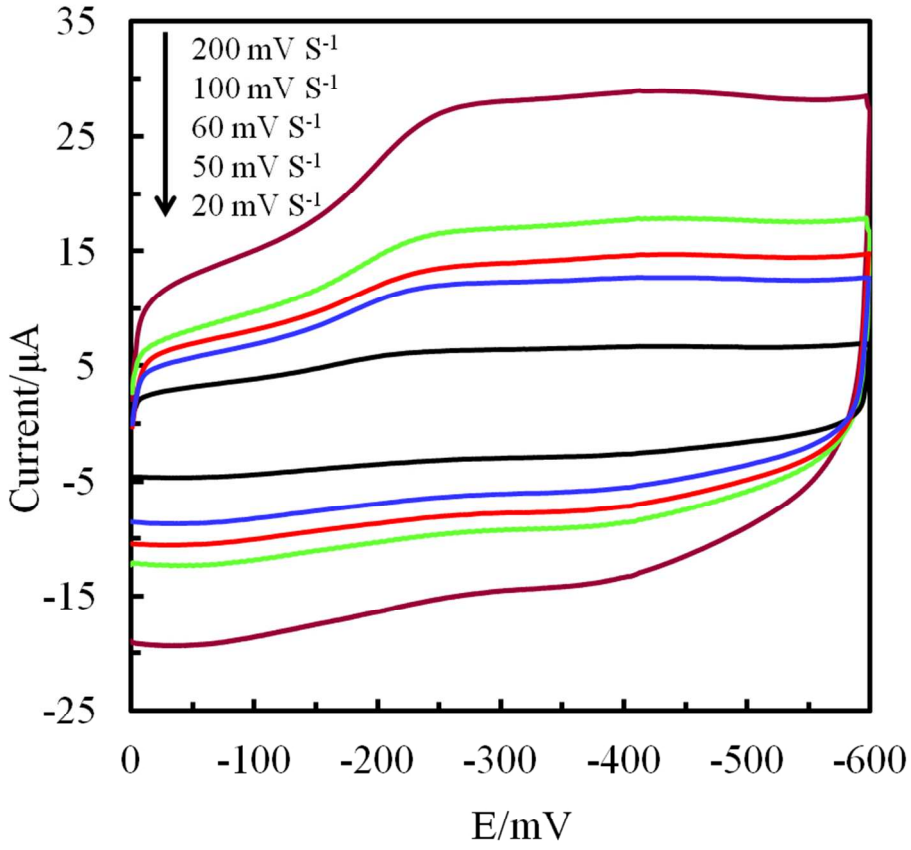

Fig.1 CVs of the biosensor in 0.10 mol L-1 Phosphate buffer solution (pH =7.0) at 20, 50, 60, 100 and 200 mV s-1, respectively. (From lowest to highest peak currents).
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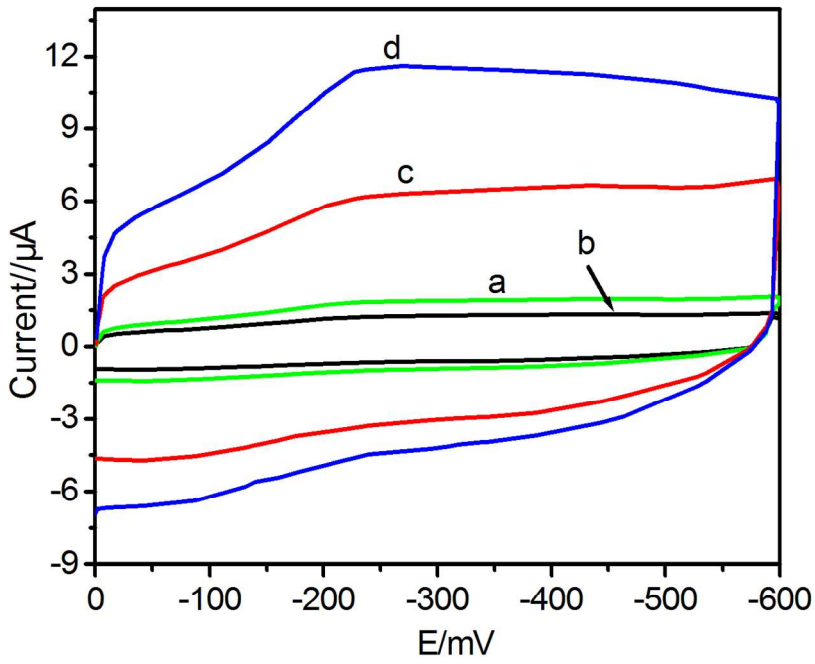

Fig. 2 CVs of HRP/CE(a), MWNTs/CE (b), and HRP/sol-gel/MWNTs/GCE (c, d) in the absence of H2O2 (c) and in the presence of 0.10 mmol L-1 H2O2 (d) at 20 mV s-1.
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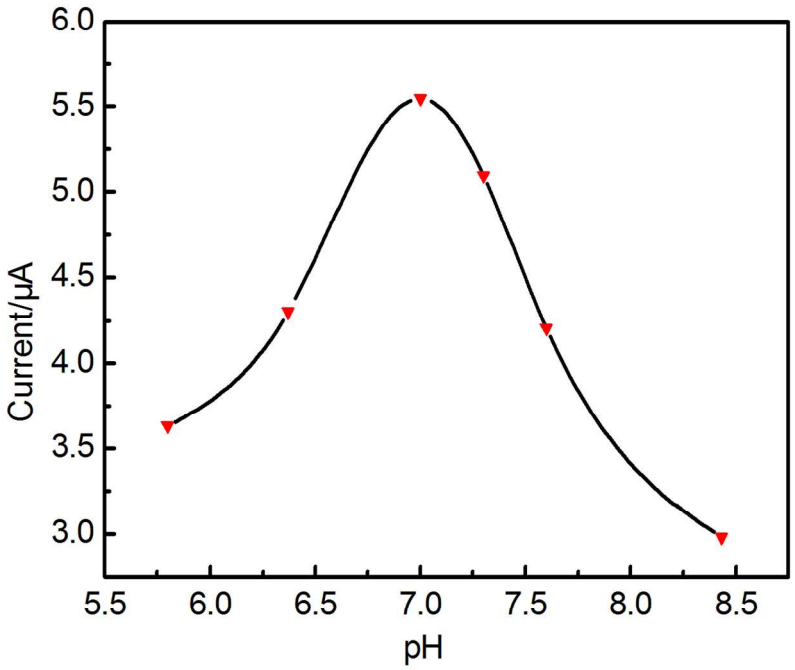

Fig. 3 Effect of pH on the response of biosensor. Applied potential, -300 mV and concentration of H2O2, 0.10 mmol L-1.
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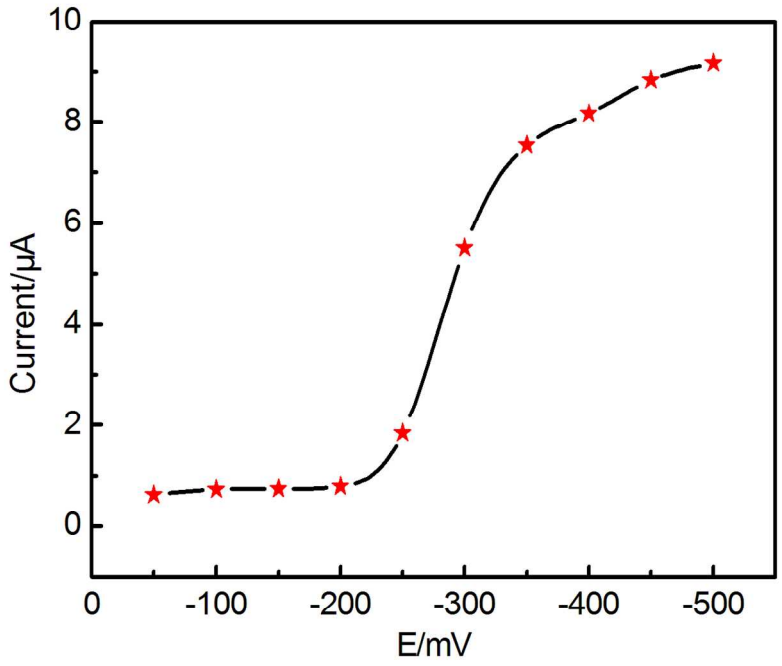

Fig. 4 Effect of applied potential on the response of biosensor in 0.10 mol L-1 Phosphate buffer solution (pH =7.0) and containing 0.10 mmol L-1 H2O2.
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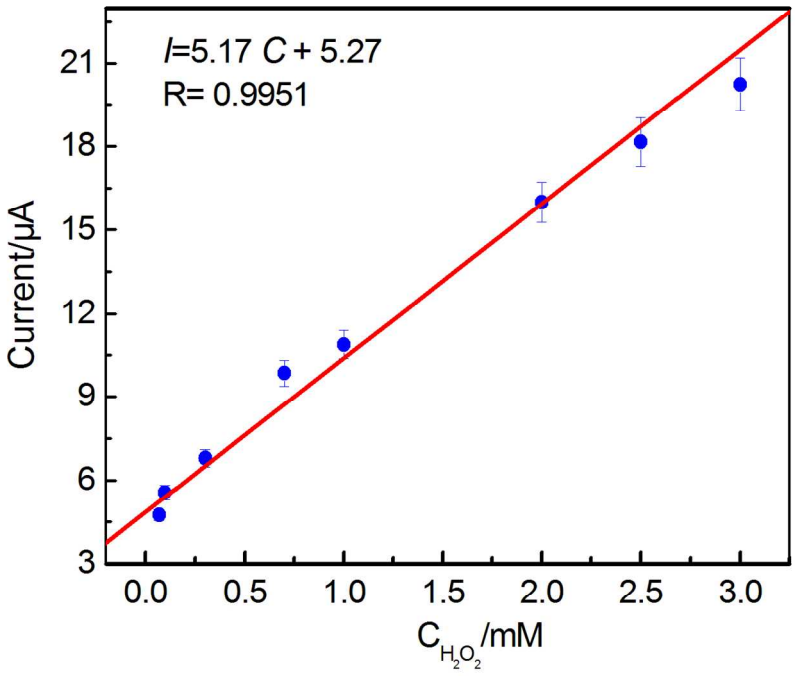

Fig. 5 Calibration plot of the H2O2 sensor in 0.10 mol L-1 phosphate buffer solution (pH =7.0) at a applied potential of -300 mV.
279x215mm (150 x 150 DPI)