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A third-generation hydrogen peroxide biosensor based on horseradish peroxidase immobilized on DNA functionalized carbon nanotubes

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ABSTRACT

In this paper, DNA functionalized SWCNTs were used to immobilize horseradish peroxidase (HRP) on glassy carbon (GC) electrode. Cyclic voltammetry showed that the direct electrochemistry of HRP immobilized on DNA-SWCNTs hybrids was achieved. The DNA interlayer between the SWCNTs and HRP could be used to keep the activity of HRP. Compared with HRP-SWCNTs/GC and HRP-DNA/GC electrodes, the prepared HRP-DNA-SWCNTs/GC electrode exhibited more excellent electrochemical properties. Thus, the prepared HRP-DNA-SWCNTs/GC electrode was proposed as a third-generation H_2O_2 biosensor. The effect of pH and applied potential on the performance of the biosensor was discussed in detail. Under the optimal conditions, a wide linear range of the propose biosensor for the detection of H_2O_2 was observed from 6.0×10^{-7} to 1.8×10^{-3} M. The detection limit was found to be 3.0×10^{-7} M at a signal-to-noise ratio of 3. Furthermore, the proposed biosensor displayed rapid response, high stability, very good reproducibility and high sensitivity for the detection of H_2O_2. Determination H_2O_2 concentration in disinfector sample by the proposed biosensor also showed satisfactory result.

1. Introduction

H_2O_2 is widely used in many fields, including pollution control, textile and paper bleaching, sterilization and so on (Notsu et al., 2002; Tsiafoulis et al., 2005). On the other hand, H_2O_2 is the product of various oxidases in countless biological reactions (Karyakin et al., 1995, Wang and Wang, 2004). Therefore, the determination of H_2O_2 is not only related to the increased awareness of the environmental hazards and the need for safer procedures, but also important for clinical diagnostics. Many methods have been developed for the determination of H_2O_2, such as spectrophotometry (Matsubara et al., 1992; Lobnik and Cajlakovic, 2001), fluorimetry (Genfa et al., 1991), chromatography (Pinkernell et al., 1997), chemiluminescence (Navas et al., 1999; Rocha et al., 2005), and titrimetry (Hurdis and Romeyn, 1954). Compared with these methods, amperometric biosensors based on horseradish peroxidase (HRP) have emerged as the most convenient tools for H_2O_2 determination because of their low detection limit, high selectivity, and high sensitivity (Zhao et al., 2004; Yu et al., 2005). In recent years, the mediator-free third-generation biosensors based on the direct electron transfer between enzymes and the conjoint electrodes have received considerable attentions (Cao et al., 2008; Wang et al., 2009). However, the direct electron transfer between the redox center of the immobilized enzymes and the electrodes is often shielded by the insulating outer protein shell. On the other hand, direct adsorption of enzymes onto the electrode surface may frequently result in their denaturation and the loss of bioactivity. Therefore, it is important to discover appropriate materials that can retain the bioactivity of enzymes and can connect the active center of redox enzymes with the electrode surface.

Since produced in 1991, much attention has been paid to carbon nanotubes (CNTs) for the development of biosensors in the past decades, because of their superior properties, such as high surface area, rich electronic properties and excellent chemical and thermal stability (Ajayan, 1999; Chen et al., 2007; Zhang et al., 2007). However, the applications of CNTs for fabricating biosensors have been severely hindered by two main issues. First, CNTs are not readily soluble in usual solvent media. The as-synthesized CNTs are often bundled together due to strong van der Waals interactions between the nanotubes. This made the dispersion and stabilization of CNTs in solvent media very poor. On the other hand, how to keep the activity of enzymes immobilized on CNTs should also be considered. Recently, Karajanagi et al. reported that the activity of several enzymes decreased seriously after their adsorption onto the surface of CNTs (Karajanagi et al., 2004). To resolve these problems, CNTs have been functionalized through the integration of CNTs with various materials such as bio-macromolecules, conductive polymers and metal nanoparticles (Chen et al., 2007; Daniel et al., 2007). By this means, the nanocomposite materials are endowed

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with unique features for the immobilization of enzymes. Many of these nanocomposite materials have been developed and applied to construct biosensors (Shobha Jeykumari and Narayanan, 2008a,b; Li et al., 2009; Shobha Jeykumari and Narayanan, 2009; Zhao et al., 2009).

DNA is a biological macromolecule with great importance in life sciences. Due to its double helical structure, it can conduct electrons well. The π-stacked DNA base pairs can mediate electrons transfer over long molecular distances. Electron transfer of redox proteins could be addressed to be promoted in DNA film (Nassar and Rusling, 1996; Lisdat et al., 2001). DNA can be attached onto CNTs through the π–π interactions between the nanotube sidewalls and the nucleic acid bases. Since the dispersion of single-wall carbon nanotubes (SWCNTs) in aqueous DNA solutions with the aid of sonication first reported by Nakashima et al. in 2003 (Nakashima et al., 2003), many research has shown DNA to be an excellent dispersant of SWCNTs with many exciting possible applications. Guo et al. described the electrochemical characteristics on the immobilization of both double-stranded and single stranded calf thymus DNA molecules on the surface of MWCNTs (Guo et al., 2004). Noguchi et al. found that SWCNTs/DNA hybrids are highly stable without desorption for at least 1 month (Noguchi et al., 2008). Karachevtsev et al. fabricated a glucose biosensor based on NIR fluorescence of DNA-SWCNTs hybrids (Karachevtsev et al., 2007). It was found that the DNA interlayer between the CNTs and enzyme could be used to keep the activity of enzyme. However, to our best knowledge, there are no reports on the direct electrochemistry of HRP immobilized on DNA functionalized CNTs and no third-generation hydrogen peroxide biosensors have been fabricated based on these nanocomposites.

In this study, DNA functionalized SWCNTs (DNA-SWCNTs) was proposed to immobilize HRP. The direct electrochemistry of HRP immobilized on DNA-CNTs hybrids was observed. A third-generation H_2O_2 biosensor was thus fabricated.

2. Experimental

2.1. Reagents

Double strand DNA (from calf thymus) was purchased from Sigma. HRP (E.C.1.11.1.7, 250 U mg^{-1}) was purchased from Amerisco. Short SWCNTs were purchased from Chengdu Organic Chemicals Co., Ltd., Chinese Academy of Sciences with sizes ranging from 1 to 2 nm in diameter and 1–3 μm long. All these were used as received without further purification. All other chemicals used were analytical grade. Double-distilled water was used for preparation of buffer and standard solutions. H_2O_2 solution was diluted daily before the electrochemical measurements.

2.2. Apparatus and procedures

All the electrochemical measurements were performed with a CHI 660a electrochemical workstation (Shanghai Chenhua Instrument Company, China). A conventional three-electrode system was used with a modified glassy carbon (GC) electrode as working electrode, a platinum wire as counter electrode, and an Ag/AgCl (3 M KCl) electrode as reference electrode. A 0.05 M phosphate buffer solution (PBS) was used as the electrolyte. The PBS was purged with high-purity nitrogen for at least 30 min and a nitrogen environment was then kept over the solutions in the cell. All experiments were done at room temperature (~25 °C).

2.3. Preparation of DNA-SWCNTs hybrids

The DNA-SWCNTs hybrids suspension was obtained as described in the literature (Cathcart et al., 2007): 0.5 mg of SWCNTs was added to 2 mL of DNA (0.5 mg/mL) solution and then was sonicated in an ice-water bath for 2 h. Then, the suspension was gently centrifuged at 3300 × g for 1 h to remove the undispersed SWCNTs.

2.4. Fabrication of the H_2O_2 biosensor

Prior to modification, GC electrode (diameter 3 mm) was polished with 0.3 and 0.05 μm alumina, then ultrasonicated in ethanol and twice-distilled water, respectively. To obtain good cyclic voltammetric responses of the HRP-DNA-SWCNTs/GC electrode, the concentrations and the mass ratios of HRP and DNA-SWCNTs hybrids were optimized in control experiments. Typically, a homogeneous solution was prepared by mixed equal volume of 10 mg/ml HRP and DNA-SWCNTs hybrids solution prepared above. Then 5 μL of mixed solution was cast onto the electrode surface by using a syringe. A beaker was covered over the electrode so that water can evaporate slowly in air and a uniform film can be formed. The dried HRP-DNA-SWCNTs/GC electrode was stored at 4 °C in a refrigerator when not in use. For comparison, HRP-DNA/GC and HRP-SWCNTs/GC electrodes were prepared in the same way as described above.

3. Results and discussion

3.1. Electrochemical characteristic of HRP-DNA-SWCNTs/GC electrode

The electrochemical characteristic of HRP-DNA-SWCNTs/GC electrode was investigated by cyclic voltammetry in deoxygenized PBS (pH 7.0), and the result was shown in Fig. 1 (curves a). For comparison, the cyclic voltammograms (CVs) of HRP-DNA/GC and HRP-SWCNTs/GC electrodes were also shown.

As can be seen, a pair of stable, well-defined and quasi-reversible redox peaks were observed at both HRP-DNA-SWCNTs/GC and HRP-DNA/GC electrodes. These results indicated that direct electron transfer between HRP and GC electrode could be achieved at both electrodes. However, the CV response of HRP-DNA/GC electrode was much smaller than that of HRP-DNA-SWCNTs/GC electrode. Integrating the CV reduction peak of the modified electrode, the amount of reduction charge (Q) passed through the electrode can be obtained. According to Faraday's laws (Murray, 1986), $\Gamma^* = Q/(nFA)$, where n is the number of electron transferred, F is the Faraday's constant, and A is the effective area of the GC electrode, the surface concentration of electroactive

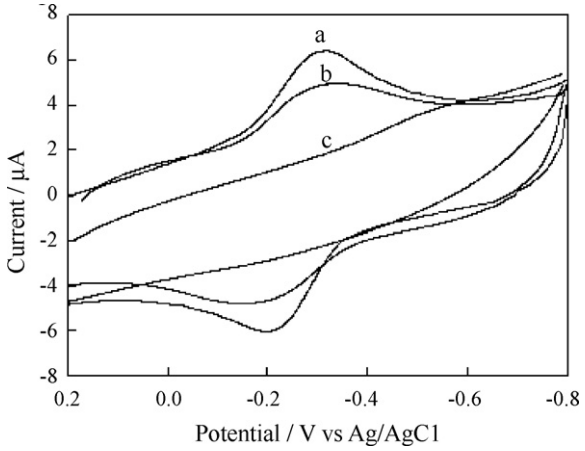

Fig. 1. Cyclic voltammograms of HRP-DNA-SWCNTs/GC electrode (a), HRP-DNA/GC electrode (b) and HRP-SWCNTs/GC electrode (c) in 0.05 M PBS (pH 7.0). The potential scan rate was 0.1 V s^{-1}.

HRP (Γ^*) was estimated to be 1.2×10^{-10} mol cm^{-2} at the HRP-DNA-SWCNTs/GC electrode, which was about 2.3 times of the HRP-DNA/GC electrode (5.2×10^{-11}). The higher concentration of electroactive at the HRP-DNA-SWCNTs electrode would be attributed to the exciting electronic properties and high surface area of SWCNTs. Different from HRP-DNA-SWCNTs/GC and HRP-DNA/GC electrodes, no obvious redox peaks was observed at HRP- SWCNTs/GC electrode. This result indicated that SWCNTs alone did not provide a favorable microenvironment for HRP to transfer electrons directly with underlying GC electrode. This result would be caused by two reasons. One possibility is that the poor dispersion of SWCNTs hampered their ability to promote the electron transfer between HRP and the electrode. On the other hand, the activity of HRP would decrease after direct adsorption onto the surface of SWCNTs (Karajanagi et al., 2004).

The CVs of HRP-DNA-SWCNTs/GC electrode at different potential scan rates were shown in supplemental Figure S.1. With the increase of the scan rates from 0.05 to 0.6 V s^{-1}, both cathodic and anodic peak currents increased linearly. And the anodic and cathodic peak potentials of HRP shifted in positive and negative directions, respectively. All these results indicated that electron transfer between HRP and the electrode was a surface-controlled and quasi-reversible process.

The influence of buffers pH on the electrochemical characteristic of HRP-DNA-SWCNTs/GC electrode was also studied. Both reduction and oxidation peaks shifted negatively as pH increased. The formal potential $E^{\circ\prime}$ of the heme Fe(III)/Fe(II) redox couple for the HRP-DNA-SWCNTs/GC electrode had a linear relationship with pH from 5.0 to 9.0 (see supplemental Figure S.2). The slope of $E^{\circ\prime}$ versus pH was -55.7 mV pH^{-1}, which was approximately close to the theoretical value of -59.0 mV pH^{-1} at 25 °C for a reversible one proton and one electron transfer reaction (Meites, 1965; Bond, 1980). This result indicated that the electron transfer between HRP in HRP-DNA-SWCNTs film and the electrode was coupled by proton transportation. The reaction could be expressed as:

$$\text{HRPFe(III)} + e^- + H^+ \rightleftharpoons \text{HRPFe(II)}$$

3.2. Optimization of the H_2O_2 determination conditions

To improve the performance of the biosensor, the effect of the determination conditions such as the pH value and the applied potential on the response of the HRP-DNA-SWCNTs/GC electrode to H_2O_2 was investigated in detail.

The effect of the applied potential on the response current of the HRP-DNA-SWCNTs/GC electrode is illustrated in Fig. 2A. When the applied potential was changed from 0 to -0.30 V, the response current increased obviously. The maximum response current was achieved at around -0.30 V. When the applied potential became more negative, there may be interfering reactions from other electroactive species in the solution. Therefore, an applied potential of -0.30 V was selected to give a high detection sensitivity and good signal/noise ratio.

The effect of the pH value on the response current of the HRP-DNA-SWCNTs/GC electrode was studied between 5.0 and 8.0 in 0.05 M PBS. As shown in Fig. 2B, the response current increased from 5.0 to 7.0 and decreased from 7.0 to 8.0, and the maximum current response can be obtained at pH 7.0. Therefore, the suitable pH with the maximum activity of the immobilized HRP was at pH 7.0, which was in agreement with that reported for soluble HRP (Wang and Zhang, 2006). The immobilization of HRP on DNA-SWCNTs hybrids did not alter the optimal pH value for the catalytic behavior of HRP.

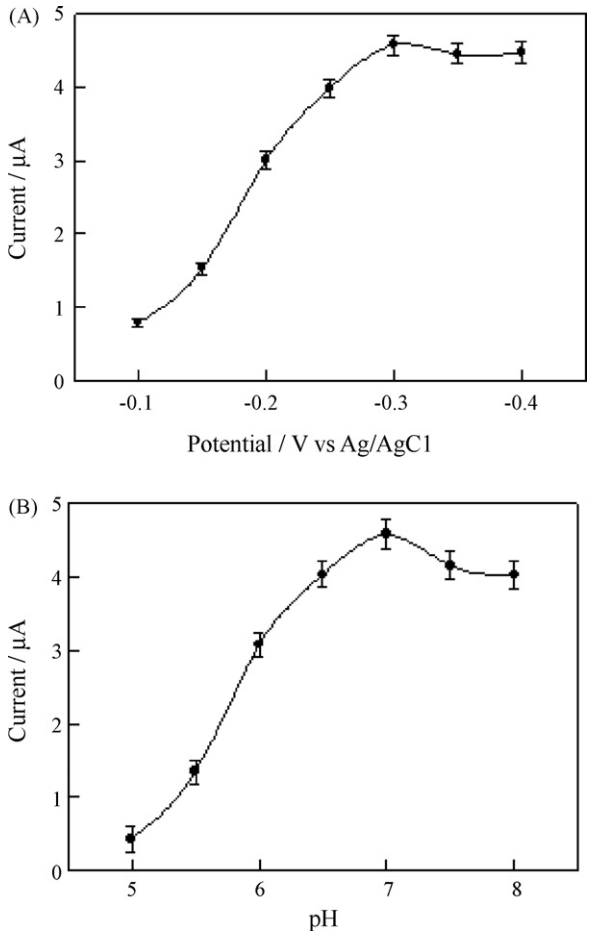

Fig. 2. Effects of applied potential (A) and the solution pH (B) on steady-state response current of HRP-DNA-SWCNTs/GC electrode to 0.1 mM H_2O_2.

3.3. Amperometric response of the proposed H_2O_2 biosensor

Fig. 3A illustrates current–time plot for the HRP-DNA-SWCNTs/GC electrode under the optimized experimental conditions with successive additions of 0.1 mM H_2O_2. As the H_2O_2 was injected into the stirring PBS, the steady-state currents reached another steady-state value (95% of the maximum) in less than 4 s. The calibration curve of the HRP-DNA-SWCNTs/GC electrode is shown in Fig. 3B. For comparison, the current–time plot for the HRP-DNA/GC electrode and the relative calibration curve are also shown in Fig. 3. As can be seen, the HRP-DNA/GC electrode displayed only a linear H_2O_2 concentration range from 6.0×10^{-5} to 1.0×10^{-3} M. The current sensitivity was calculated to be 13.58 μA mM^{-1}. At a signal-to-noise ratio of 3 (S/N = 3), the detection limit was found to be 2.5×10^{-5} M. However, a much wider linear range from 6.0×10^{-7} to 1.8×10^{-3} was obtained at the HRP-DNA-SWCNTs/GC electrode. Furthermore, a much lower detection limit of 3.0×10^{-7} M and a much higher sensitivity of 43.82 μA mM^{-1} were also obtained at the HRP-DNA-SWCNTs/GC electrode.

The high sensitivity and low detection limit may attribute the exciting electronic properties of DNA-SWCNTs hybrids and the excellent bioactivity of HRP immobilized on DNA-SWCNTs hybrids.

As presented in Fig. 3, when the concentration of H_2O_2 was higher than 2.0 mM, the current response of the HRP-DNA-SWCNTs/GC electrode showed a level-off tendency, demonstrating the typical Michaelis–Menten kinetic characteristic of enzyme-based electrode. According to the Lineweaver–Burk equation (Kamin and Willson, 1980), the apparent Michaelis–Menten con-

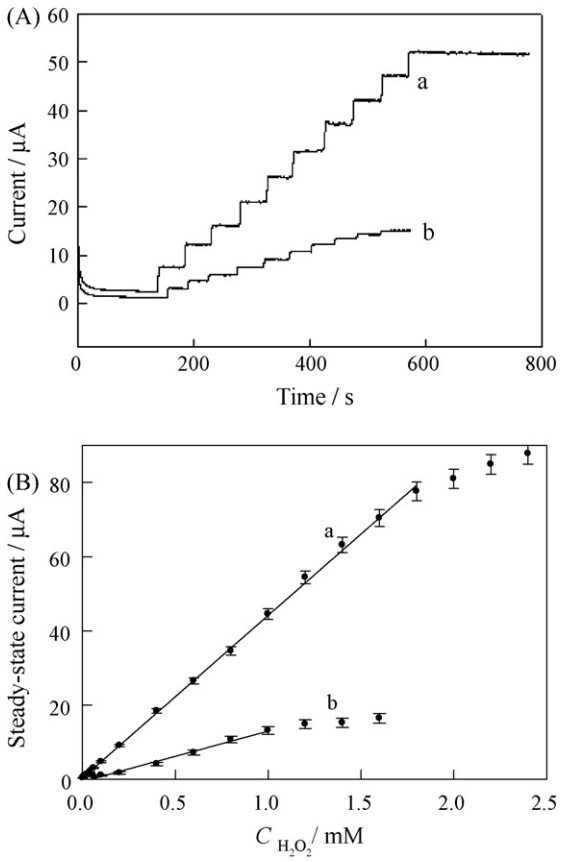

Fig. 3. (A) Typical amperometric response of (a) HRP-DNA-SWCNTs/GC electrode and (b) HRP-DNA/GC electrode upon the successive addition of 0.1 mM H_2O_2 into stirred 0.05 M PBS (pH 7.0). The applied potential was −0.30 V. (B) The calibration curves of (a) HRP-DNA-SWCNTs/GC electrode and (b) HRP-DNA/GC electrode.

stant (K_m) for HRP-DNA-SWCNTs/GC electrode was calculated to be 1.13 mM, which was close to 0.97 mM for HRP-DNA/GC electrode. These results indicated that the immobilized HRP on both DNA and DNA-SWCNTs hybrids possessed higher enzymatic activity, and the two electrodes exhibited almost the same affinity for H_2O_2.

The reproducibility and stability of the sensor were also evaluated. Five electrodes modified identically were investigated in the current response for 1×10^{-4} M H_2O_2 at −0.30 V. The relative standard deviation (R.S.D.) was 5.8%. For 10 replicate measurements of one electrode yielded at 1×10^{-4} M H_2O_2, the R.S.D. was 2.4%. These experimental results confirmed that the results were highly reproducible. The stability of the enzyme electrode under storage conditions (0.05 M PBS, pH 7.0, 4 °C) was investigated. The current response for 1×10^{-4} M H_2O_2 at −0.30 V was measured everyday up to 30 days. The current response did not change in a noticeable way during the 1st week, and only 7% of the current response was lost after 2 weeks. After 30 days, the current response still remained above 85% of its initial response. The relatively good storage stability implies that the HRP-DNA-SWCNTs complex is very stable and HRP immobilized on DNA-SWCNTs hybrids can retain its bioactivity for a long time.

Table 1
Effect of possible interferents on the detection of H_2O_2 with HRP-DNA-SWCNTs/GC.

Interferents	Current ratio[a]
Glucose	1.03
Ascorbic acid	0.94
Uric acid	0.93
Citric acid	0.97
Cysteine	0.78

[a] Current ratio = $I_{H+I}/I_H \cdot I_{H+I}$, response current to H_2O_2 (1.0×10^{-4} M) in the presence of interferents (1.0×10^{-3} M); I_H, response current to H_2O_2 (1.0×10^{-4} M).

3.4. Effect of electroactive interferents

Selectivity is also important in practical use of the biosensors. The influence of possible interfering species on the current response of the sensor was examined and is given in Table 1. The current response obtained with 1:10 concentration ratio of hydrogen peroxide and the interfering species was compared with the results obtained with that of pure H_2O_2 alone. As shown, the enzyme biosensor showed excellent selectivity to H_2O_2 in the presence of glucose, ascorbic acid, uric acid and citric acid. However, cysteine caused interference in the quantification of H_2O_2 with a decrease of about 22% in the response current. This would be caused by the inhibition of HRP activity by thiol-containing compounds such as cysteine (Sariri and Jafarian, 2006).

3.5. Comparison with other hydrogen peroxide biosensors based on HRP

The analytical performances of the proposed biosensor were compared with other HRP based biosensors reported in the literatures. Characteristics such as the applied potential, linear range, and the limit of detection of the biosensors were all summarized in Table 2. As can be observed, the proposed biosensor (HRP-DNA-SWCNTs/GC electrode) exhibited excellent performance in terms of wide linear range and low limit of detection. These results indicated that the proposed HRP-DNA-SWCNTs/GC electrode is an excellent platform for the detection of H_2O_2 detection.

3.6. Real sample analysis

To demonstrate the analytical applicability of the proposed biosensor, the concentration of H_2O_2 in a disinfector sample was determined. The disinfector sample was diluted 50 times with pH 7.0 PBS, and the concentration of H_2O_2 contained in the diluted disinfector sample was tested using the proposed biosensor. The result was compared with that obtained by the potassium permanganate titration (see Table 3). The recovery experiments were also performed. As shown, the result obtained by the proposed biosensor was in good agreement with this measured by the

Table 2
Comparison of the performances of H_2O_2 biosensors based on HRP.

Electrode	Applied potential (V)	Linear range (mM)	Limit of detection (μM)	Reference
HRP-DNA-SWCNTs/GC	−0.30	0.0006–1.8	0.3	This work
HRP-SiO_2-MB-gelation-GC	−0.30	0.01–1.2	0.4	Yao et al. (2005)
HRP-GNPs-SF/GC	−0.60	0.01–1.8	5.0	Yin et al. (2009)
HRP-ADA-pCDSH-Au	−0.30	0.028–5.5	7.0	Camacho et al. (2009)
HRP-ZnO-GNPs-Nafion/GC	−0.30	0.015–1.1	9	Xiang et al. (2009)

MB: methylene blue; GNPs: gold nanoparticles; SF: silk fibroin; ADA: adamantane; pCDSH: polythiolated-β-cyclodextrin polymer.

Table 3
Determination of H_2O_2 and the recovery test in a disinfector sample.

Determined by potassium permanganate titration method (mM)[a]	Glucose added (mM)	Determined by the proposed biosensor (mM)[a]	Recovery (%)
28.18 ± 0.48	0	27.83 ± 0.62	–
	10	37.95 ± 0.58	101
	20	47.24 ± 0.86	97
	30	56.98 ± 1.38	97

[a] Average of five determinations ± standard deviation.

oxidation–reduction titration. The recovery rate was in the range 97–101%. These results indicated that it is feasible to apply the proposed electrode to determine H_2O_2 in real samples.

4. Conclusion

In this work, we have developed and characterized a new third-generation H_2O_2 biosensor based on the immobilization of HRP on GC electrode with DNA functionalized SWCNTs. The DNA-SWCNTs hybrids can effectively maintain the bioactivity of HRP. The direct electron transfer between HRP and electrode surface was achieved. Furthermore, the resulted HRP-DNA-SWCNTs/GC electrode showed an electrochemical activity to the reduction of H_2O_2 without the aid of any electron mediator. The proposed biosensor exhibited fast amperometric response, wide linear range, high sensitivity and stability, good reproducibility and low detection limit. The proposed biosensor also exhibited good selectivity to H_2O_2, which implied that the proposed biosensor could be applied to real sample analysis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2009.09.003.

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