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PAPER

A novel hydrogen peroxide biosensor based on sol–gel poly (vinyl alcohol) (PVA)/(titanium dioxide)TiO₂ hybrid material

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A novel hydrogen peroxide biosensor has been fabricated based on Hb entrapped poly(vinyl alcohol) (PVA)/Titanium dioxide (TiO₂) hybrid material. Multi-walled carbon nanotubes (MWCNTs) were then dispersed into the composite matrix. It was found that such hybrid material could retain the native biocatalytic activity of the entrapped Hb by electrochemical experiments. In addition, MWCNTs enhanced catalytic performance of hydrogen peroxide and promoted electronic transfer. Effects of some experimental variables such as the amount of MWCNTs, concentration of enzyme, amounts of modifier on the current response of the biosensor were investigated. A linear calibration graph was obtained in the concentration range of H₂O₂ from 0.5 to 2.7 μ M (linear regression coefficient = 0.997) with a detection limit of 0.01 μ M ($S/N = 3$). The apparent Michaelis–Menten constant K_m was 0.997 μ M. The biosensor displayed excellent repeatability, high sensitivity, long-term stability, and good selectivity. The recovery of H₂O₂ in samples was testified with satisfactory results.

1. Introduction

Detection of H₂O₂ is important in chemical, biological, clinical, environmental, food and industrial analysis. Most of the analytical techniques for measuring hydrogen peroxide (H₂O₂) such as chromatographic, colorimetric and photometric methods are time-consuming and difficult to adapt to automatic detection.¹ Among these, electrochemical method is especially promising because of its simplicity, selectivity, high sensitivity and accurate determination of hydrogen peroxide (H₂O₂).^{2–7}

Heme proteins have important physiological functions in living cells which contain the porphyrin complex of iron(II)-heme or iron(III)-heme as a prosthetic group. Recently, direct electron transfer studies of these proteins have attracted considerable attention,^{8–14} because fundamental studies of the redox behaviors of these proteins may be of great help to better understand the relationship between their structures and biological functions.¹⁵ Haemoglobin (Hb) is regarded as an ideal model molecule for the study of electron transfer between redox proteins and the underlying electrode owing to its known structure, commercial availability and modest cost.¹⁶ However, either proteins or enzymes all exhibit rather slow electron transfer rates at conventional electrodes, which may result from the deep burying of electroactive prosthetic groups, unfavorable orientation of proteins on the electrode surface, the adsorption of impurities and denatured protein onto an electrode surface.^{17,18} In order to achieve the direct electron transfer between the proteins or

enzymes and the underlying electrodes, many methods were used to fabricate the third-generation biosensor.^{8,11,19,20}

The immobilization of enzymes is a key step for the preparation of the biosensor. Sol–gel and organic polymer hybrid material can effectively eliminate the brittleness of pure inorganic sol–gel and the swelling property of some pure polymer or hydrogel. Sol–gel physical rigidity, high stability and biocompatibility of organic polymer materials was retained, respectively.²¹ The polyvinyl alcohol (PVA) is a non-toxic and biocompatible synthetic polymer with good thermal stability as well as good film-forming ability.^{22–24} PVA is indeed well known for its wide range of potential applications in pharmaceutical, medical, and membrane fields.²⁵ PVA is a polymer composed of a carbon chain backbone attached with hydroxyl groups, which can provide a biocompatible micro-environment for proteins or enzyme immobilization and improve the stability of the modified electrode.²⁶

Meanwhile, many nanosized materials were used to fabricate available protein electrodes and enhance electron transfer^{10,19,27–30} due to good biocompatibility, high surface activity and perfect uniformity. Carbon nanotubes (CNTs) have unusual electronic properties and many potential applications,^{31,32} there have been a series of investigations to use CNTs for biosensor interface fabrication.^{33,34} For example, it can be used as a reaction promoter in biosensors for the electrochemical detection of dopamine,³⁵ hydroquinone and catechol,³⁶ and the electrocatalysis of oxygen.³⁷ Recent studies demonstrated that CNTs could promote the electron transfer reactions of proteins including cytochrome c,³⁸ myoglobin,³⁹ catalase,⁴⁰ hemoglobin,⁴¹ glucose oxidase,^{42,43} and horseradish peroxidase (HRP).^{44–49}

TiO₂ material is a widely used inorganic material, it shows good biocompatibility, stability and environmental safety, it was

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used for the immobilization of heme protein and biomaterials in investigating spectroelectrochemical properties or bioanalytical application.^{12,17,50–53} Xiao *et al.* reported direct electron transfer and electrocatalysis of hemoglobin on chitosan-TiO₂ nanorods-Glass carbon electrode.⁵⁴ Zheng and his co-workers studied direct electrochemistry and electro-catalysis of hemoglobin immobilized in TiO₂ nanotube films.⁵⁵ Zhang *et al.* fabricated hydrogen peroxide biosensor based on Mb-TiO₂/MWCNTs. The resultant Mb-TiO₂/MWCNTs modified glassy carbon electrode exhibited fast amperometric response to hydrogen peroxide reduction, long term life and excellent stability.⁵⁶

In this study, hybrid material (PVA/TiO₂) was prepared by sol-gel process. Furthermore, Hb immobilized in the matrix of multi-walled and PVA/TiO₂ film still kept its electrocatalytic reduction to H₂O₂. The PVA/TiO₂ film provided a favorable microenvironment for Hb to exchange electron with the electrode. The experiment results showed that carbon nanotubes enhanced catalytic performance of hydrogen peroxide and promoted electronic transfer. The electrochemical and analytical characteristics of the biosensor were studied. The results showed that the biosensor exhibited a variety of good characteristics including high sensitivity, repeatability, good selectivity, and long-term stability.

2. Experimental

2.1. Apparatus

Electrochemical measurements were performed with CHI660A and CHI440A Electrochemical Station (Shanghai Chenhua Instrument Co., China). The three electrode system consisted of a platinum wire counter electrode, a saturated calomel reference electrode, and the modified glassy carbon electrode (2.0 mm diameter) as working electrode. All electrochemical experiments were carried out in a conventional electrochemical cell holding 6.0 mL of PBS at room temperature. All the electrochemical processes were in aerated solutions.

2.2. Reagents

Hb was obtained from Fluka. MWCNTs (>95% purity) was bought from Cheng du Organic Chemicals Co. Ltd. Chinese Academy of Sciences. Poly (vinyl alcohol) (*M_w* 89000~98000) were from Sigma-Aldrich. 30% H₂O₂ was purchased from Guangzhou Chemical (Guangzhou, China). Butyltitanate (TBT) was obtained from TianJin Kermel Chemical Reagents Development Centre. Other reagents were of analytical reagent grade. All solutions were prepared with doubly distilled water. The exact concentration of H₂O₂ was determined by titration against a standard potassium permanganate solution.

2.3. Preparation of PVA/TiO₂ hybrid materials by the sol-gel process

TBT and normal butylalcohol (*v/v* = 1 : 4) were mixed together in a cuvette. Then the pH of the above solution was adjusted to 2.0 below by adding HCl solution. Uniform and transparent titanium dioxide sol was obtained by stirring for three hours at room temperature. A stock mixture sol-gel titanium dioxide and PVA solution was prepared by mixing 990 μ L (PVA aqueous

solution of 6%), 10 μ L titanium dioxide sol, and in a 2 mL PVC tube. The mixture was sonicated for 30 min until a clear and homogeneous solution resulted. This stock solution was freshly prepared daily just before the fabrication of biosensors.

2.4. Fabrication of PVA/TiO₂-MWCNTs-Hb/GCE

A glassy carbon electrode (GCE, 2 mm in diameter) was polished with 1.0, 0.3, and 0.05 mm Al₂O₃ slurry successively followed by rinsing thoroughly with doubly deionised water until a mirror-like surface was obtained. Then it was sonicated in 1 : 1 nitric acid, absolute ethanol and doubly distilled water, each for 5 min, and allowed to dry at room temperature. A sample of Hb (15 mg) was dissolved in 1 mL of 0.12 M PBS (pH = 6.2). The Hb stock solution was stored at 4 °C in a refrigerator when not in use. A 2.5 mg MWCNTs dispersion was prepared by dissolving MWCNTs in 1 mL doubly distilled water with the help of 30 min ultrasonic agitation. A 70 μ L PVA/TiO₂ hybrid materials, 30 μ L MWCNTs dispersion and Hb (15 mg mL⁻¹) with the volume of 25 μ L in a 2.0 mL PVC tube was mixed until a clear and homogeneous solution resulted. A drop of this dispersion with the volume of 4 μ L was casted onto the surface of GCE, then it was dried in air for 3 h.

3. Results and discussion

3.1. Electrochemical characteristics of the biosensors

Fig. 1 showed the typical cyclic voltammograms of different electrodes in pH 6.2 PBS at scan rate 50 mV s⁻¹. No electrochemical responses were observed at (a) PVA/GCE (c) PVA/TiO₂/GCE, (e) PVA/TiO₂-MWCNTs/GCE, which indicated no electroactive substances existed on the electrode surface. While on the PVA/Hb/GCE only an irreversible reduction peak was observed (curve b), which can be explained as only the few Hb molecules closest to the electrode could exchange electrons with the electrode and contribute to the observed redox reaction. For PVA/TiO₂-Hb/GCE electrode (curve d), only a pair of small, unsymmetric redox peaks were observed, which indicated that

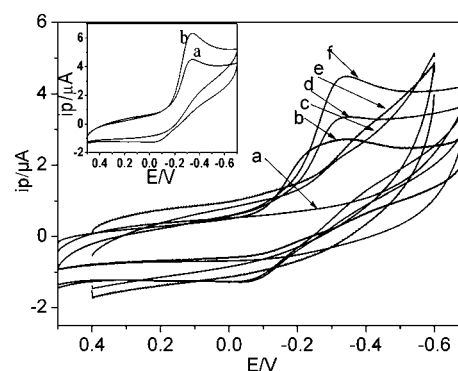


Fig. 1 Cyclic voltammograms of (a) PVA/GCE, (b) PVA/Hb/GCE, (c) PVA/TiO₂/GCE, (d) PVA/TiO₂-Hb/GCE, (e) PVA/TiO₂-MWCNTs/GCE and (f) PVA/TiO₂-Hb-MWCNTs/GCE in 0.12 M pH 6.2 PBS scan rate 50 mV s⁻¹. Inset: cyclic voltammogram of (a) PVA/TiO₂-Hb-MWCNTs/GCE in 0.12 M pH 6.2 PBS at scan rate 50 mV s⁻¹, (b) PVA/TiO₂-Hb-MWCNTs/GCE in 0.12 M pH 6.2 PBS with 1.0 μ M H₂O₂ at scan rate 50 mV s⁻¹.

the direct electron transfer of Hb with GCE had taken place with slow electron transfer rate. The peak current of PVA/TiO₂-Hb/GCE (curve d) was much higher than that of PVA/Hb/GCE (curve b), that is to say, more electrons of Hb entrapped in PVA/TiO₂ film participated in the electrochemical reaction. The PVA/TiO₂ film provided a favorable microenvironment for Hb to exchange electron with the electrode. While on the (f) PVA/TiO₂-Hb-MWCNTs/GCE, the electrochemical response of Hb was greatly enhanced and a pair of well-defined quasi-reversible cyclic voltammetric peaks were observed (curve f), which revealed that MWCNTs played an important role in facilitating the direct electron transfer of Hb by the synergism with PVA/TiO₂ film.

To explore the potential application of PVA/TiO₂-Hb-MWCNTs/GCE, the electrocatalytic reduction of H₂O₂ on the electrode had been studied. The PVA/TiO₂-Hb-MWCNTs/GCE showed good electrocatalytic ability to H₂O₂. As shown in Fig. 1 Inset, with the addition of H₂O₂ into 0.12 M pH 6.2 PBS an obvious increase of the cathodic peak current with the decrease of the anodic peak current (curves a, b), which indicated the Hb incorporated in the composite film could act as an effective catalyst to the reduction of H₂O₂.

The CVs at different scan rates can be seen from Fig. 2 both anodic and cathodic peak currents increased linearly with the square root of the scan rates in the range from 10 to 600 mV s⁻¹, which indicated that a diffusion-controlled process occurred at the PVA/TiO₂-MWCNTs-Hb/GCE.

3.2. Electrochemical impedance spectroscopy characterization

EIS is a powerful tool for studying the interface properties of surface modified electrodes. The electron transfer resistance (RCT) at the electrode surface is an important parameter. Fig. 3 illustrated the typical results of AC impedance spectra of the (a) bare GCE, (b) PVA/TiO₂-Hb/GCE, (c) PVA/TiO₂-MWCNTs-Hb/GCE, respectively. Obviously, the whole profile for (b) PVA/TiO₂-Hb/GCE exhibited the greatest semicircle, which indicated the PVA/TiO₂ hybrid material impeded electron transfer of the electrochemical probe. However, the curve (c) PVA/TiO₂-MWCNTs-Hb/GCE exhibited much smaller semicircle compared with (a) bare GCE and (b) PVA/TiO₂-Hb/GCE, which

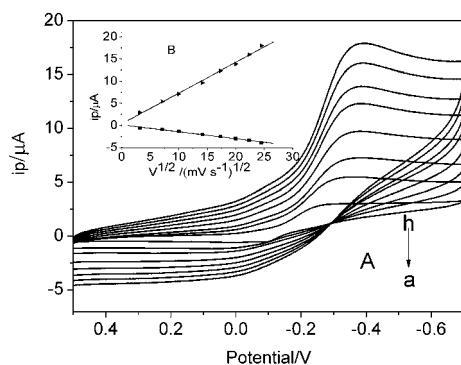


Fig. 2 The cyclic voltammograms of PVA/TiO₂-MWCNTs-Hb/GCE with different scan rates: a) 10, b) 50, c) 100, d) 200, e) 300, f) 400, g) 500, h) 600, mV s⁻¹ in 0.12 M pH 6.2 PBS containing 1.0 μM H₂O₂, (inset B) the linear relation between peak current and $v^{1/2}$.

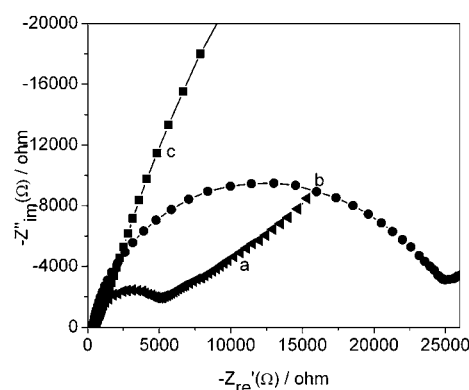


Fig. 3 Electrochemical impedance spectroscopy (a) bare GCE (b) PVA/TiO₂-Hb/GCE, (c) PVA/TiO₂-MWCNTs-Hb/GCE in the presence of a solution 5.0×10^{-3} M K₃Fe(CN)₆/K₄Fe(CN)₆ (1 : 1) containing 0.1 M KCl.

showed that the presence of MWCNTs could accelerate the electron transfer.

3.3. Optimization of experimental conditions

Carbon nanotubes can largely promote the electron transfer of Hb, so the volume of MWCNTs solution is an important factor of the performance of the biosensor. In order to determine the optimal volume of MWCNTs casted on the electrode, a plot of cathodic currents *versus* amounts of MWCNTs was made as shown in Fig. 4. The cathodic peak current achieved its maximum value when the amount of MWCNTs was 0.5 mg. Because too much amounts of MWCNTs will agglomerate on the electrode surface and decrease the stability of the sensing surface. Moreover, the electron transfer will become difficult if amounts of MWCNTs was 0.5% below. Obviously, the optimal amount of MWCNTs was 0.5 mg. These results indicated that the conductivity of MWCNTs might not be able to extend to the others especially when the nanotubes were packed randomly and disorderly, thus limiting the effect of MWCNTs in improving the protein electron transport.

The concentration of enzyme in composite is a vital factor affecting the biosensor response. Fig. 5 displayed the cathodic

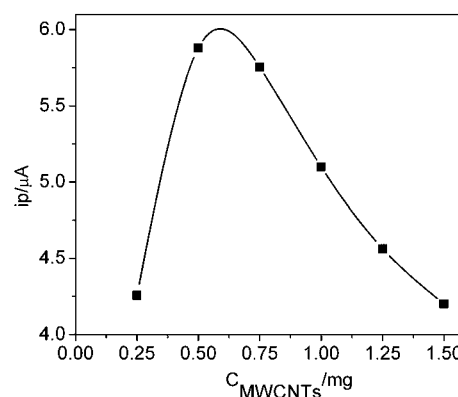


Fig. 4 Effect of MWCNTs amount on the biosensor responses at 50 mV s⁻¹ in 0.12 M PBS (6.2) containing 1.0 μM H₂O₂.

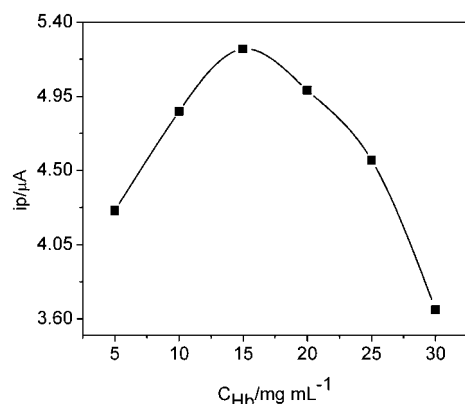


Fig. 5 Effect of Hb concentration on cathodic peak current of the enzyme electrode in 0.12 M PBS (6.2) at 50 mV s⁻¹ containing 1.0 μM H₂O₂.

peak current response of the biosensor with different concentrations of Hb-loading. The cathodic peak current arrived at its peak value, when the Hb concentration on electrode was 15 mg mL⁻¹. So an optimal loading of 15 mg mL⁻¹ Hb was used for subsequent experiment.

The effect of amounts of modifier on the catalytic current for H₂O₂ oxidation was also studied. Fig. 6 displayed the response

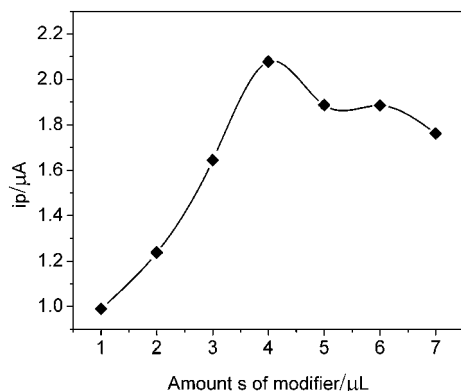


Fig. 6 Effect of amounts of modifier on the electrode response in 0.12 M pH 6.2 PBS containing 1.0 μM H₂O₂.

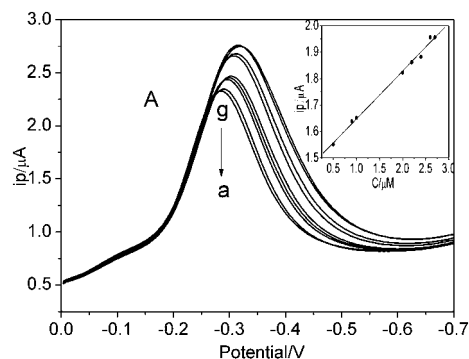


Fig. 7 (A) Differential pulse voltammograms of biosensor (a) in the absence and (b–g) successive addition of 1.0×10^{-4} M H₂O₂, (inset) calibration curve of biosensor response with H₂O₂ concentration.

of the electrode containing different amounts of modifier. The response current increased along with increased amounts of modifier. The maximum response was obtained with 4 μL. The electron transfer will become difficult if modifier was casted on the electrode surface too much. So amounts of modifier of 4 μL was used for the subsequent experiments to obtain the greatest sensitivity.

3.4. The linear range and the detection limit of electrode

Fig. 7 showed the typical response of the biosensor for successive additions of H₂O₂ under the optimized experimental conditions. The linear range spans the concentration of H₂O₂ from 0.5 to 2.7 μM with a correlation coefficient of 0.997. The regression equation was $i(\mu\text{A}) = 1.47 + 0.18 C(\mu\text{M})$. The detection limit of this biosensor was estimated as 0.01 μM at a signal to noise ratio of 3.

The apparent Michaelis–Menten constant (K_m) is a reflection of the enzymatic affinity. The apparent Michaelis–Menten constant of immobilized enzyme was obtained according to (Eq. 1)⁵⁷

$$\frac{1}{I_{ss}} = \frac{1}{I_{max}} + \frac{K_m}{I_{max}} C$$

where I_{ss} = steady state current after the addition of substrate, C = bulk concentration of the substrate, I_{max} = maximum current measured under saturated substrate condition. According to this equation, K_m was calculated to be 0.161 μM. This value was lower than the literature^{58–61} reported (2.21 μM, 0.68 mM, 0.75 mM, 0.898 mM), which indicated the higher enzymatic activity of immobilized Hb. Thus the above result further showed that the PVA/TiO₂-MWCNTs-Hb modified biosensor possessed a high affinity to H₂O₂. This is because polyvinyl alcohol and titanium dioxide hybrid materials can well maintain the catalytic activity of the enzyme. This film was an excellent carrier for immobilized enzyme (see Table 1).

3.5. Biosensor repeatability and stability

The repeatability of response current of the Hb electrode was investigated at H₂O₂ concentration of 1.0 μM. The variation coefficient (DPV) was 3.68% for eight successive assays. Five Hb electrodes, made independently, showed an acceptable reproducibility with a variation coefficient of 6.92% ($n = 5$) for the current determination at 1.0 μM H₂O₂.

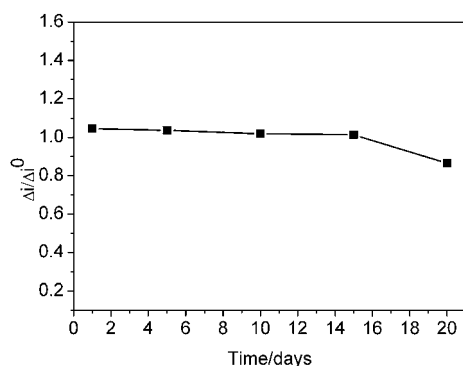
The biosensor was stored in a refrigerator at 4 °C when not used. The stability of the biosensor under storage was investigated by measuring the biosensor response with 1.0 μM of H₂O₂ everyday. The corresponding result was shown in Fig. 8. Δi is the different current of the enzyme electrode after storage. In the figure, only 2% loss of the response current could be observed within 5 days. After 20 days, the biosensor retained about 90% of its original response. The stability of the biosensor is much better than that of the previous biosensor.⁶¹ Good stability can be attributed to the following factors: the Hb was physically embedded in the hydrogel; large quantities of hydroxyl and amino groups in the PVA, which were favorable in maintaining the activity of the Hb, could form strong interactions between the Hb and the hydrogel; the enzymes were embedded temperately in

Table 1 Peroxidase-like activity of some Hb films^a

Film	Linear range/ μM	Detection limit/ μM	$K_m/\mu\text{M}$
Hb/carbon nanotube ⁶²	210–900	9	675
Hb/ZrO ₂ /DMSO ⁶³	1.5–30.2	0.14	310
Hb/ α -ZrP ⁶⁴	0.2–10.8	0.07	123
SA/Hb/CILE ⁶⁵	1.0–100.0	1.0	—
PVA/Hb/MWCNTs/CILE ⁶⁶	1.2–30.0	0.5	0.928
PVA/TiO ₂ -Hb-MWCNTs/GCE	0.5–2.7	0.01	0.161

This paper

^a DMSO: dimethyl sulfoxide; CILE: carbon ionic liquid electrode; SA: sodium alginate.

**Fig. 8** Stability of enzyme electrode stored at 4 °C in 0.12 M pH 6.2 PBS containing 1.0 μM H₂O₂.

the sol-gel network, which could more effectively immobilize Hb.

3.6. Interference

Selectivity is an important factor in the performance of sensor. Several substances were measured to examine whether they interfered the determination of H₂O₂. Table 2 displays minimum concentration of the interferent that provides an error $\pm 5\%$ to the response current of 1.0 μM H₂O₂. In our experiments, the tested substances did not interfere significantly with the resulting biosensor.

3.7. Analytical application of the biosensor

The accuracy of the biosensor was evaluated by determining the recoveries of hydrogen peroxide in a disinfectant using

Table 2 Possible interference tested with the biosensor

Interferences	Minimum concentration/mM of the interferents that provides an error $\pm 5\%$ to the response current of 1.0 μM H ₂ O ₂
Fructose	1.14
L-Cysteine	1.64
L-Lysine	1.20
Tyrosine	1.32
Cl ⁻	1.21
NH ₄ ⁺	1.21
Ca ⁺	1.18

Table 3 Determination results of recovery using the present biosensor

S.NO.	Added H ₂ O ₂ / μM	Found H ₂ O ₂ / μM ^a	Recovery (%)
1	1.0	1.115	115
2	1.5	1.591	106
3	2.0	1.935	96.75

^a Average of five measurements.

a standard addition method with the recovery ranging from 96.75% to 115% (see Table 3).

4. Conclusions

A Novel PVA/TiO₂ organic-inorganic hybrid material has been prepared by the sol-gel process with biocompatibility and network structure. Such a hybrid material could retain the native biocatalytic activity of the entrapped Hb. Carbon nanotubes can promote direct electron transfer of Hb immobilized onto the surface of GCE. We have successfully developed a mediator-free, third-generation H₂O₂ biosensor, which exhibited a variety of good electrochemical characteristics including reproducibility, high sensitivity, good long-term stability. The method presented here not only can be easily extended to immobilized and obtain direct electrochemistry of other proteins, but also can be used for two-enzyme systems. It showed that the films might be useful for practical application in fabricating biosensors or bioreactors based on the direct electron transfer of protein without using mediators.

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