

Improvement in Sensing Performance of H₂O₂ Biosensor Electrodes through Modification of Anatase TiO₂ Nanorods and Pretreatment of Electrochemical Reduction

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(Received June 17, 2016; accepted September 8, 2016)

Keywords: TiO₂ nanorods, electrochemical reduction treatment, biosensor electrode, hydrogen peroxide, sensitivity

Electrochemical biosensors are essential health monitors that aid in the detection and diagnosis of diseases. In this research, anatase titanium dioxide (TiO₂) nanorods (TNR-A) were synthesized on a titanium (Ti) substrate in three stages, namely, hydrothermal, alkali, and heat treatments, and utilized as a modified electrode (TNR-A/Ti). The pretreatment of electrochemical reduction was adopted to increase the Ti³⁺ amount in TiO₂ nanorod surfaces of the electrodes, thus improving electron transfer. The electrodes were characterized by X-ray diffractometry (XRD), field-emission scanning electron microscopy (FESEM), and X-ray photoelectron spectroscopy (XPS). The electrodes after pretreatment (Ti³⁺-TNR-A/Ti) showed a better electrochemical response to hydrogen peroxide (H₂O₂), indicating that pretreatment improves the performance of electrodes. The assembled biosensor electrode (Nafion/HRP/Ti³⁺-TNR-A/Ti) exhibited a sensitivity of 6096.4 μA·mM⁻¹·cm⁻², a detection limit of 0.008 μM, a linearity of 0.04–700 μM, and an apparent Michaelis–Menten constant K_M^{app} of 0.0027 μM, which are higher than those in previous research studies on TiO₂ or similarly nanostructured modified biosensor electrodes. This research could provide a potential competent method that can be used to modify electrodes for high-performance amperometric biosensors.

1. Introduction

Electrochemical biosensors are one of the essential health monitors used to help detect and diagnose diseases, among which hydrogen peroxide (H₂O₂) biosensors are deemed important.^(1–4) This is because H₂O₂ is the main component of reactive oxygen species (ROS) in living organisms, and increasing levels of H₂O₂ in cells could indicate oxidative stress and cellular damage.⁽⁵⁾ This damage could result in many diseases such as cancer, neurodegenerative disorders, diabetes, and atherosclerosis.⁽⁶⁾ Thus, H₂O₂ biosensors with high accuracy, high sensitivity, good selectivity, and good anti-interference ability play vital roles in detecting and monitoring the H₂O₂ concentration under these conditions.

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<http://dx.doi.org/10.18494/SAM.2017.1418>

To increase sensing capacity, enzymes are utilized. The enzyme adsorption on electrodes and direct electron transfer between them have been recognized as key factors that affect biosensor performance.⁽⁷⁾ Horseradish peroxidase (HRP) is one such enzyme, which belongs to the superfamily of peroxidases and heme-containing glycoproteins.⁽⁸⁾ Therefore, HRP is always used to determine H_2O_2 .⁽⁹⁾ If a mediator with nanostructures is built on the electrodes, it could increase the surface area and facilitate more enzyme immobilization to improve biosensor performance.^(9,10)

Nanostructured TiO_2 has a strong protein adsorption ability and great biocompatibility.⁽¹¹⁾ Moreover, TiO_2 with an isoelectric point of 8.9 is very appropriate for HRP enzyme immobilization.⁽¹²⁾ In our previous work, TiO_2 nanodot film and rutile TiO_2 nanorods were reported as a mediator to construct a H_2O_2 biosensor electrode and showed good performance.^(13,14) However, nanostructured anatase TiO_2 was reported to well retain the biological activity of the enzyme and it can be used to load an increased enzyme concentration.⁽¹⁵⁾

The electrical conductance of TiO_2 is closely related to the Ti^{3+} amount. If there are more Ti^{3+} ions in the nanostructured TiO_2 surface, it should favour electron transfer within the electrode. An electrochemical reduction or self-doing approach was reported to be effective for producing Ti^{3+} ions on a TiO_2 surface and demonstrated to have the capability of improving the performance of TiO_2 -nanotube-based biosensor electrodes.^(16,17)

The main problem with H_2O_2 biosensors is their dependence on the dissolved oxygen concentration. This problem can be overcome by utilizing 'mediators' that transfer the electrons directly to the electrode bypassing the reduction of the oxygen cosubstrate.⁽¹³⁾ The advantage of these biosensors is that they react rapidly with the reduced form of the enzymes. Moreover, they are sufficiently soluble, in both oxidized and reduced forms, to be able to rapidly diffuse between the electrode surface and the active site of the enzyme.^(13,18) The purpose of this work is to develop a highly sensitive electrode by intensifying direct electron transfer between the electrode substrate and the functional enzyme through TiO_2 nanorod modification.

In this work, anatase TiO_2 nanorods on Ti substrates were prepared via three stages, hydrothermal, alkali, and heat treatments, following a pretreatment of electrochemical reduction. The modified electrodes were then characterized and their electrochemical behaviour and performance were measured. The effect of pretreatment on the performance of the electrodes was also evaluated.

2. Experimental Methods

2.1 Materials and reagents

Titanium (Ti) foils with a purity of 99.99% and a thickness of 0.1 mm were used as a conductive material for the construction of anatase TiO_2 nanorod biosensor electrodes. The structure with dimensions of $1 \times 2 \text{ cm}^2$ (length \times width) was then cleaned in a mixture of ethanol, distilled water, and acetone at a ratio of 1:1:1. 250 $\text{U} \cdot \text{mg}^{-1}$ HRP (Aladdin Chemistry Co., Ltd.) was used. Nafion (5 wt%) was purchased from Sigma-Aldrich. All the chemicals used in the experiments that were of analytical reagent grade were purchased from Sinopharm Chemical Reagent Co., Ltd. The 0.1 M phosphate buffer solution (PBS) used in the experiments was prepared with the constituents of NaH_2PO_4 (AR, >99.0), Na_2HPO_4 (AR, >99.0), NaCl (AR, >99.5), and H_2O . The HCl (37 wt%) and NaOH (AR, >96.0) used to adjust the pH of the PBS were prepared with various pH values. The PBS solution was deoxygenated by bubbling pure N_2 gas for 30 min, prior to use.

2.2 Electrode construction and modification

Anatase TiO₂ nanorods were obtained through three steps. The first step was a hydrothermal process described as follows: The hydrothermal solution was prepared by mixing 0.45 g of picric acid (AR, >99.8%), 15 mL of ethanol, 60 mL of H₂O, 40 mL of HCl, and 220 mL of titanium tetrabutoxide (TBOT, CP, >98%). After stirring the chemicals sufficiently, the Ti substrate and mixture were placed in a Teflon vessel of a hydrothermal autoclave and then into an oven at 160 °C for 4 h. After the hydrothermal treatment, the autoclave vessel was taken out and allowed to cool to room temperature. Rutile TiO₂ nanorod films were obtained by rinsing with deionized water and ethanol. In the second step, the rutile TiO₂ nanorod films were hydrothermally alkali-treated in a 100 mL Teflon vessel with a solution of hydroxides (KOH/NaOH = 1:1) at 200 °C for 2 h, then washed with deionized water and soaked in 0.1 M HCl for 4 h. In the third step, the films with acidic treatment were heat-treated at 500 °C for 2 h, after which anatase TiO₂ nanorod films on Ti substrates (TNR-A/Ti) were obtained. According to the literature,⁽¹⁶⁾ the electrochemical reduction method was performed by applying -1.5 V to the electrodes in 1 M (NH₄)₂SO₄ (AR, >99.0%) to obtain a Ti³⁺-TNR-A/Ti electrode. Field-emission scanning electron microscopy (FESEM) (Hitachi, S-4800) was performed to observe the morphology of the anatase TiO₂ nanorods, and X-ray diffractometry (XRD; PANalytical, X'pert PRO) was carried out to analyse the phase and crystal of TiO₂ nanostructure films. Also, X-ray photoelectron spectroscopy (XPS, ESCALAB 250Xi) was performed to analyse the role of pretreatment in changing the surficial Ti valence for the electrochemical reduction pretreatment. The anatase TiO₂ nanorod biosensor electrodes were assembled via the same procedure using the same enzyme and protective membrane, and the steps of biosensor electrode construction were started by sealing the electrodes with epoxy resin except for an area of 2.5 × 2.5 mm², which was left as the measuring area. The physical adsorption method was performed to immobilize the HRP enzyme on the electrode surface, where 10 μL of HRP solution was dropped on the electrode surface and left to dry at room temperature. The HRP solution was prepared by dissolving HRP in 0.01 M PBS (pH = 7.4^(13,19)) in order to obtain a 0.01 g mL⁻¹ solution. Routinely, Nafion has been used as a biosensor and as an immobilisation matrix for the enzyme, which helps in maintaining the stability of the biosensor.⁽²⁰⁾

Hence, as the final step, 4 μL of a 0.5 wt% solution of Nafion was dropped onto the biosensor surface to protect the enzyme and make the biosensor biocompatible, and then was left to dry at room temperature (Fig. 1). The Nafion/HRP/Ti³⁺-TNR-A/Ti and Nafion/HRP/TNR-A/Ti biosensor electrodes were washed after storing at 4 °C for 1 d.⁽⁹⁾ When not in use, the modified anatase TiO₂ nanorod biosensor electrodes were stored at 4 °C.

2.3 Measurement of performance characteristics of the biosensor electrodes

An electrochemical workstation device (CHI 660D, from Shanghai Instrument Co., Ltd.) was used to characterize the modified biosensor electrodes. The Pt foil and saturated calomel electrodes (SCEs) served as the counter and reference electrodes, respectively. The modified biosensor electrodes were used as the working electrode. The cyclic voltammetry technique was used to test the modified electrodes in 20 mL of PBS (PBS, 0.1 M at 25 °C and purged with pure nitrogen for 30 min in order to remove oxygen) with an optimum pH. Through this technique, we can investigate and study the electrochemical behaviour of the modified electrodes and the effect of electrocatalytic

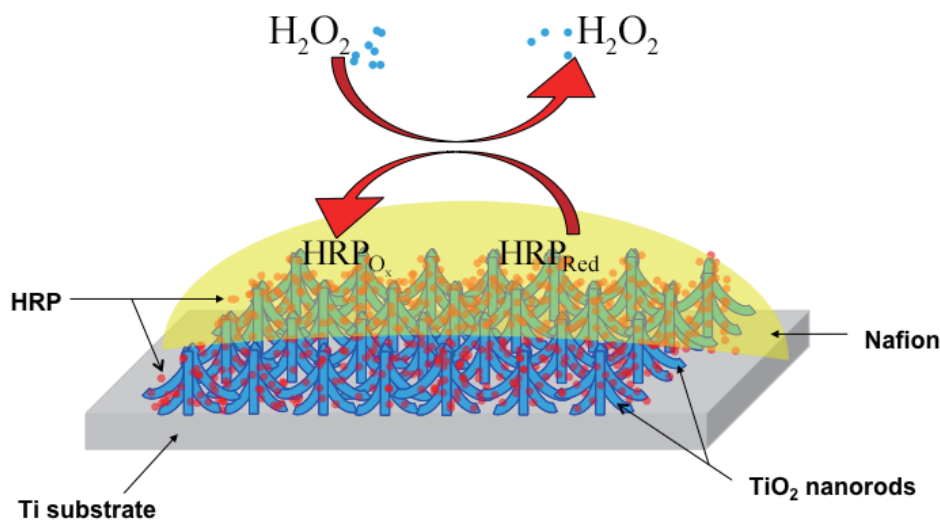


Fig. 1. (Color online) Schematic representation of the H_2O_2 biosensor based on Nafion/HRP/TNR-A/Ti electrode.

activity towards the redox reaction of H_2O_2 . The scan rate effect on the electrochemical behaviour of the electrodes was also studied by cyclic voltammetry. The amperometric technique was carried out on the biosensors in order to study the performance characteristics of the modified biosensor electrodes, such as sensitivity, limit of detection (LOD), linearity, selectivity, and stability, by applying an optimum voltage. The optimum applied voltage was selected via the amperometric technique by applying a voltage in the range between -0.3 and -0.9 V; the voltage with high current response was selected as the optimum applied voltage. The optimum pH of the PBS was selected depending on the best performance of the modified electrodes.

3. Results and Discussion

3.1 Characterization of anatase TiO_2 nanorod modified electrodes

After alkali, acidic, and heat treatments of hydrothermally grown rutile TiO_2 nanorods, the XRD pattern (Fig. 2) of the nanorods on Ti substrate fits well with the anatase TiO_2 phase (JCPDS No. 21-1272). The formation of the anatase TiO_2 phase starts with breaking in the Ti–O–Ti building units in rutile TiO_2 to form Ti–O–Na (Ti–O–K). After the Na^+ and K^+ ions are exchanged with H^+ ions in acid wash, Ti–OH bonds are formed during alkali treatment. The resulting hydrogen titanate is finally transformed to anatase TiO_2 after heat treatment at 500 °C (Fig. 3).

3.2 Effect of electrochemical reduction on the electrodes

After the electrochemical reduction treatment was performed, the Ti^{3+} contribution in the XPS spectra (Fig. 4) increased, and the calculated Ti^{3+} molar percentage on the surface also increased from 19 to 40.8%. This indicated that the electrochemical reduction treatment is really an effective surface reduction procedure.

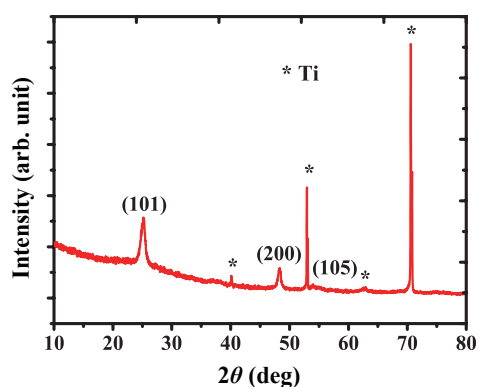


Fig. 2. (Color online) XRD of TNR-A/Ti electrode.

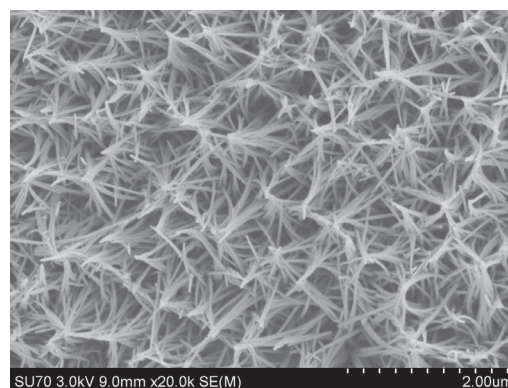
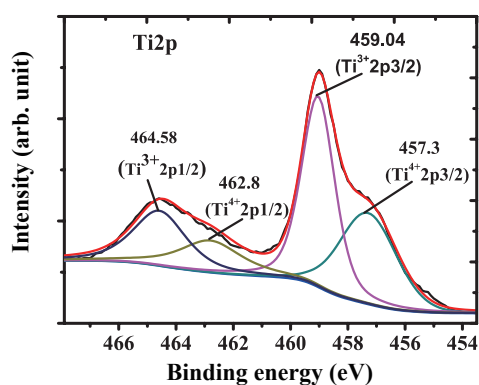
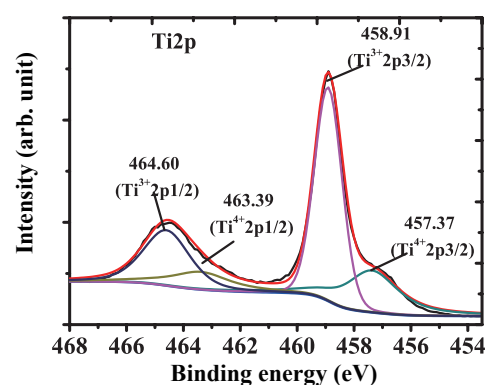


Fig. 3. SEM image of TNR-A/Ti substrate.



(a)



(b)

Fig. 4. (Color online) XPS spectra of the two electrodes: (a) TNR/Ti and (b) Ti³⁺-TNR/Ti.

3.3 Effects of electrochemical reduction of the electrodes on electrochemical behaviours and sensing performance characteristics

3.3.1 Electrochemical behaviours

When a biosensor electrode was assembled, its cycle voltammograms (CVs) [Fig. 5(a)] showed that the electrochemical activity of the electrode (Nafion/HRP/Ti³⁺-TNR-A/Ti) enhanced after the pretreatment, and the biosensor electrode had a higher oxidation peak current when 50 μ M H₂O₂ was added, as observed in the CV curve [Fig. 5(b)].

The CVs [Fig. 6(a)] of the Nafion/HRP/Ti³⁺-TNR-A/Ti electrode at different scan rates showed that the current response increased proportionally with the scan rate, and that the reduction current peak (I_{pc}) was linear as a function of the scan rate [Fig. 6(b)], implying that the behaviour followed a surface-controlled process with direct electron transfer.^(21,22) Hence, the improvement in the electrochemical response of the biosensor electrode could be attributed to enhancing direct electron transfer within the electrode by electrode electrochemical reduction pretreatment.

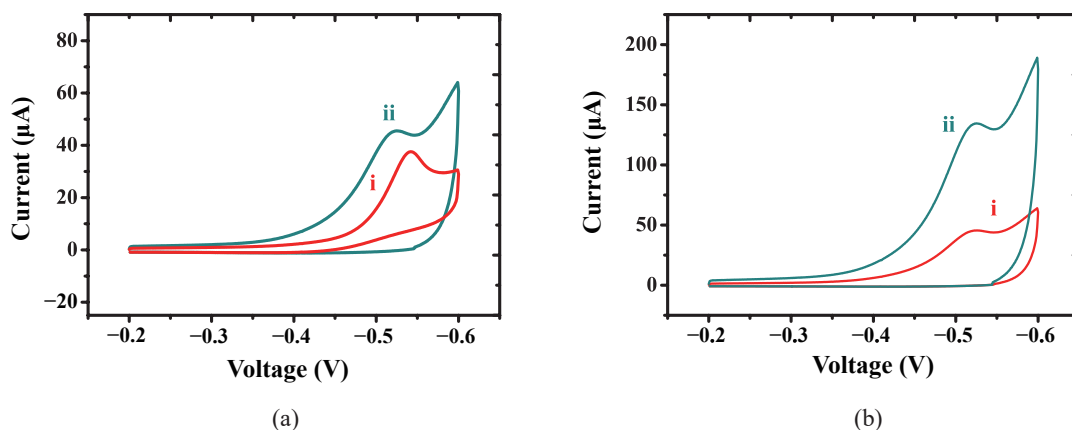


Fig. 5. (Color online) (a) CVs of anatase TiO_2 nanorods modified on electrode before (i) and after (ii) electrochemical reduction pretreatment in 0.1 M PBS at a scan rate of $0.1 \text{ V}\cdot\text{s}^{-1}$. (b) CVs of Nafion/HRP/ Ti^{3+} -TNR-A/Ti in 0.1 M PBS in absence (i) and presence (ii) of H_2O_2 .

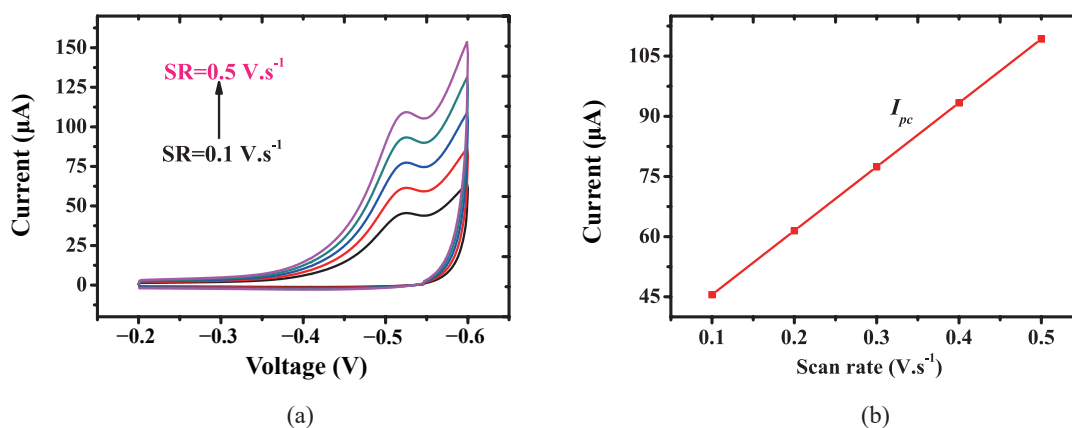


Fig. 6. (Color online) (a) CVs of Nafion/HRP/ Ti^{3+} -TNR-A/Ti electrode in phosphate buffer solution (PBS 0.1 M, 6.5 pH) under different scan rates (0.1 to $0.5 \text{ V}\cdot\text{s}^{-1}$). (b) Reduction current peaks vs scan rates.

3.3.2 Sensing performance of the biosensor electrodes

After the optimization of the applied voltage [Fig. 7(a)] and the pH of the PBS [Fig. 7(b)] for the electrode with the highest response towards $20 \mu\text{M H}_2\text{O}_2$, the amperometric response of the Nafion/HRP/ Ti^{3+} -TNR-A/Ti electrode for the successive addition of H_2O_2 was measured under the applied voltage of -0.55 V and 0.1 M PBS with pH 6.5.

The amperometric technique was used to test the sensing performance of the biosensor electrodes (Fig. 8) and the sensitivity of Nafion/HRP/ Ti^{3+} -TNR-A/Ti with $6096.4 \mu\text{A}\cdot\text{mM}^{-1}\cdot\text{cm}^{-2}$ (curve i). The limit of detection (LOD) was found to be $0.008 \mu\text{M}$, the linearity ranged from 0.04 to $700 \mu\text{M}$ with the correlation coefficient $R = 0.999$ ($n = 28$) in the inset, and the required time to reach 95% of the steady-state current was less than 3 s. Compared with Fig. 8 curve ii, the electrode with pretreatment had a 1.15-fold increase in the sensitivity. Table 1 shows a comparison of the TiO_2 nanorod electrode before and after the electrochemical reduction pretreatment and other previously reported electrodes.

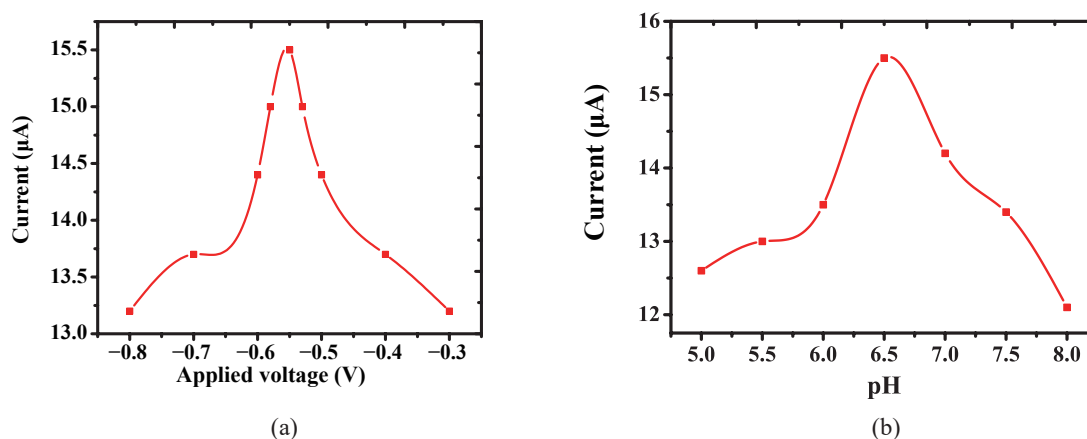


Fig. 7. (Color online) (a) Current response of Nafion/HRP/Ti³⁺-TNR-A/Ti electrode to 20 μM H₂O₂ with different applied voltages. (b) Current response of Nafion/HRP/Ti³⁺-TNR-A/Ti electrode to 20 μM H₂O₂ with different pH values at -0.55 V.

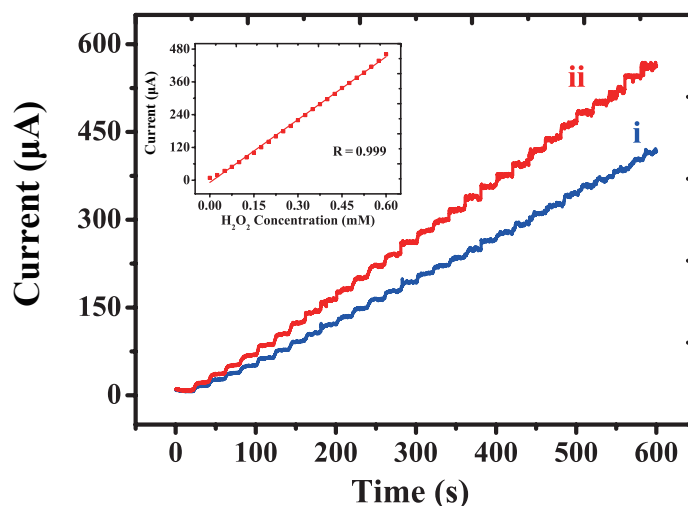


Fig. 8. (Color online) Typical amperometric technique ($I-t$) curve of TiO₂ nanorod electrode before (i) and after electrochemical reduction process (ii), after adding H₂O₂ in stirred solution of 0.1 M PBS. Inset plot: calibration plot of response current of Nafion/HRP/Ti³⁺-TNR-A/Ti electrode vs H₂O₂ concentration for modified electrode.

Table 1

Comparison of the performance characteristics of Nafion/HRP/Ti³⁺-TNR-A/Ti with different biosensors.

Electrode biosensor	LOD (μM)	Linearity range (μM)	Sensitivity ($\mu\text{A} \cdot \mu\text{M}^{-1} \text{cm}^{-2}$)	Reference
Pt/TiO ₂ /SWCNT	—	1–150	571.7	23
HRP/TiO ₂ -microspheres/Nafion/GCE	0.05	0.4–140	282.94	19
Nafion/HRP/SnO ₂ nanorods/alloy	0.2	0.01–3.2	379	24
Nafion/HRP/TiO ₂ nanodots/ITO	0.18	1–780	1176	13
PtRu/NPs	1.7	0.25–3.25	539.01	25
Nafion/HRP/TNR/Ti	0.012	0.025–460	4632.22	14
Nafion/HRP/Mg-TND-2/Ti	0.027	6–640	1377.64	26
Nafion/HRP/TNR-A/Ti	0.01	0.05–700	5332.11	This work
Nafion/HRP/Ti ³⁺ -TNR-A/Ti	0.008	0.04–700	6096.4	

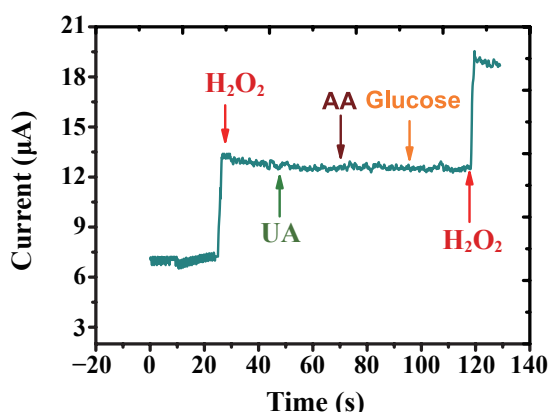


Fig. 9. (Color online) Interfering effect of the electroactive species on the Nafion/HRP/Ti³⁺-TNR-A/Ti electrode in 0.1 M PBS (pH = 6.5).

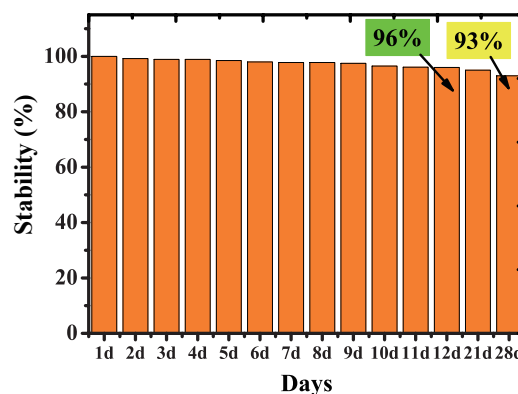


Fig. 10. (Color online) Stability of the Nafion/HRP/Ti³⁺-TNR-A/Ti electrode during 28 d.

This indicates that both the present nanostructure and the pretreatment provide an effective way of enhancing the sensing performance. Moreover, the apparent Michaelis–Menten constant K_M^{app} could reflect the enzyme affinity.⁽²⁷⁾ The K_M^{app} of the electrodes before and after the electrochemical reduction pretreatment was calculated to be 0.029 and 0.027 mM, respectively. The results show that the value decreases after the reduction pretreatment, indicating that the enzyme achieves a higher catalytic efficiency at a low H₂O₂ concentration owing to the higher affinity of the enzyme with the mediator.

3.3.3 Selectivity and stability of Nafion/HRP/Ti³⁺-TNR-A/Ti biosensor electrode

Selectivity is very important for the biosensor electrode with anti-interference towards other species. The amperometric technique was used to study the selectivity. The electrode was examined in the presence of 10 μM H₂O₂, where the current response of the electrode was clearly rapid and strong. In contrast, when each 100 μM of uric acid (UA), ascorbic acid (AA), and glucose were successively injected into the PBS, no notable current response was observed, as indicated in Fig. 9. However, when 10 μM H₂O₂ was again injected into the same supporting electrolyte solution, an immediate current response was observed (Fig. 9). The obtained results suggest that the biosensor electrode had good anti-interference and a high selectivity. Thus, it could be used for the determination of H₂O₂ from real species.

Owing to its stability, the amperometric technique was also used. About 96% of its initial current response to H₂O₂ still remained after the electrode was stored for 12 d at 4 °C and 93% of it remained after the electrode was stored for 28 d (Fig. 10). This indicates that the Nafion/HRP/Ti³⁺-TNR-A/Ti electrode behaved as a biosensor in an acceptable manner for applications.

4. Conclusions

Anatase TiO₂ nanorods for modifying the electrode surface and electrochemical reduction pretreatment for increasing the amount of Ti³⁺ on the surfaces of the nanorods were adopted to improve the sensing performance of the biosensor electrodes. The nanostructure gave a high

sensitivity and the pretreatment resulted in further improvement in sensing performance. The Nafion/HRP/Ti³⁺-TNR-A/Ti electrode showed a sensitivity as high as 6096.4 $\mu\text{A}\cdot\text{mM}^{-1}\cdot\text{cm}^{-2}$. The nanostructure could increase the affinity of the enzyme and the pretreatment could increase the direct electron transfer within the electrodes. The present approach is a facile and effective manner to improve the amperometric biosensor performance.

Acknowledgements

This work was financially supported by The Key Science Technology Innovation Team of Zhejiang Province (2013TD02), the National Natural Science Foundation of China (51272228, 51372217, 51472216, and 31570962), the National Basic Research Program of China (973 Program, 2012CB933600), the Zhejiang Provincial Natural Science Foundation (LY15E020004), and Fundamental Research Funds for the Central Universities (2014XZZX005).

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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