Layer by Layer Immobilized Horseradish Peroxidase on Zinc Oxide Nanorods for Biosensing

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Using zinc powders as source material, ZnO nanorods (ZnONR) were fabricated on gold wire by a hydrothermal reaction without any other surfactant. The gold wire end was coated by a thin layer of Zn−Au alloy to improve the nucleation for growth of ZnO nanostructures and to further improve the performance of the biosensor, which was constructed by alternatively immobilizing poly(sodium 4-styrenesulfonate) (PSS) and horseradish peroxidase (HRP) on the ZnONR. Electrochemical measurement, ultraviolet−visible spectrum, ζ-potential, and scanning electron microscopic analysis demonstrated that PSS and HRP were stably adsorbed layer by layer on the ZnONR surface, and the HRP kept bioactivity for H2O2 detection without an electron transfer mediator. The multilayered HRP sensors exhibited a wide linear range and low detection limit. The sensitivity of the biosensor increased with the immobilized HRP layers from the lowest value of 36.28 µA mM−1 for a monolayer.

1. Introduction

In recent years, nanostructural ZnO has attracted considerable interest due to its wide direct band gap, strong exciton binding energy, aesthetic morphologies, and multifunctional applications. Besides the optoelectronic properties, nanostructural ZnO also possesses many advantages for biosensing, such as high aspect ratio, polar surface along the c-axis, good electron communication, nontoxic and safe for living organisms. In particular, the isoelectric point (IEP) of ZnO is as high as about 9.5, which is suitable for immobilization of molecules with low IEP, such as poly(sodium 4-styrenesulfonate) (PSS) and some proteins, assisted by electrostatic attraction in proper pH value.1 So far, ZnO nanoparticles, porous films, nanocombs, and nanorods have been developed into biosensors to detect cytochrome c,1−3 protein,4 uric acid,5 glucose,6,7 and phenolic compounds,8 respectively.

Horseradish peroxidase (HRP) is an important enzyme consisting of a heme catalytic center and the surrounding protein. The heme group contains an Fe(III)/Fe(II) redox couple. Since the direct electron transfer of HRP adsorbed on a carbon electrode was first reported by Yaropolov et al.5 in 1978, various materials such as TiO2 nanotubes10 and gold nanoparticles11 have revealed similar function. To improve the performance of such biosensors, a multilayer biomolecular immobilization technique has been developed in recent years; for example, layer by layer modified HRP/protein has been employed to investigate the damage of protein by H2O2.12 Especially in immunosensors, the multilayer immobilization technique has improved the detection limits for antigen or DNA as low as 7 pg/mL or 40 fM through.13 It provides a quick and effective method for early diagnosis of disease.

In the present work, the ZnONR were hydrothermally grown directly on the gold electrode which was pretreated to coat a layer of the Zn−Au alloy in order to improve the molecular immobilization efficiency and the biosensing stability. The electrenegative polymer PSS and HRP with IEP 8.914 were immobilized layer by layer on ZnONR to investigate the electrochemical reaction process and the biosensing behaviors of H2O2. We shall present the construct method of the biosensor and the improved sensing characteristics in detail.

2. Experimental Section

Chemicals. Gold wire (99.99%), zinc powders (99.95%), HRP (200−300 units mg−1, Sigma), and PSS (MW 70000, 30% solution, Aldrich) are commercial. Before the measurements, 0.05 M phosphate buffer solution (PBS) was prepared by mixing the stock standard solutions of Na2HPO4 and the NaH2PO4 was deoxygenated by bubbling with pure nitrogen at least 30 min to obtain the PBS without oxygen (WO-PBS).

Fabrication of the ZnONR/Au Electrode. The hydrothermal reaction has been widely employed to fabricate nanomaterials due to its simple process, low temperature, and mass product. The hydrothermal growth of ZnO nanostructures generally involves chemical reaction between a zinc salt and an amine compound in water or water−ethanol assisted with some surfactant.15,16 Here, we just used Zn powders as the source material and a gold wire as the collection substrate to synthesize...
ZnONR in deionized water. There is not any other ion or molecule in the reaction system, so the defects generated from the heterogeneous ion or molecule were avoided in the product.

Similar to our previous report, the gold electrode was pretreated in order to grow ZnONR on it stably and effectively. One top of the gold wire with a 0.5 mm diameter was heated into a sphere, coated with a thin layer of zinc on the ball surface by thermal evaporation, then put into a heated tube furnace at 300 °C for 30 min to form a Zn–Au alloy layer on the gold wire, and finally immersed into boiling potassium hydroxide solution (2 M) for 2 h, and washed with deionized water.

Zinc powders (0.5 g) were put into deionized water (80 mL, >17 MΩ cm−2) in a Teflon-inner stainless steel autoclave. The sphere end of the gold wire was vertically hung in water and sealed in the autoclave, and then, the autoclave was put into an electric oven kept at 85 °C for hydrothermal reaction to form ZnONR. After reaction for 20 h, the sample was taken out, washed with deionized water, and finally dried in air. The morphology of the product was characterized by scanning electron microscopy (SEM) (JSM-6360 LV).

Construction of the Biosensor. The construction procedure of the biosensor is shown in Figure 1. The ZnONR on the gold sphere were immersed into a PSS solution (1 mg mL−1) containing 0.5 M NaCl for 0.5 h to immobilize the PSS on the ZnONR surface based on the electrostatic attraction between the positively charged ZnONR and the negatively charged PSS at pH 7.0. Then, the electrode was taken out and rinsed with deionized water to remove the free PSS. The modified PSS/ZnONR/Au was immersed into a HRP solution (2 mg mL−1) for 5 h to immobilize the HRP on the PSS layer by attraction between the positively charged HRP and the negatively charged PSS. Then, the electrode was taken out and washed with deionized water to remove the free HRP. To improve the performance, the PSS and HRP were alternatively immobilized on ZnONR repeatedly as mentioned in the above steps. The multilayered immobilization results were examined by SEM images, ultraviolet–visible (UV–vis) absorption (Hitachi UV-3600), and ζ-potential (Zetasizer 4, Malvern Instruments).

Electrochemical Measurements. The electrochemical measurements were performed with a CHI 660C (CH Instrument Co. Shanghai) electrochemical workstation. In a typical three-electrode electrochemical system, the (PSS/HRP)n modified ZnONR/Au wire was employed as the working electrode, a platinum wire acted as the auxiliary electrode, and a saturated calomel electrode (SCE) acted as the reference electrode. Cyclic voltammetric (CV) and amperometric experiments were carried out in WO-PBS in a static electrochemical cell at 25 ± 0.2 °C. The solution pH value was selected as 7.0 according to the previous optimized reports for HRP-catalyzed reduction of H2O2. The HRP-catalyzed electrochemical process is generally assisted by a proper electron transfer mediator to improve the detected signal. In the present case, no electron transfer mediator was employed.

3. Results and Discussion

The SEM images of the as-grown and (PSS/HRP)n modified ZnONR are shown in Figure 2. It can be seen that the smooth surface of ZnONR become rough after the multilayered immobilization. This indicated that the PSS/HRP has been modified on the ZnONR surface successfully.

The UV absorption spectra for the standard solutions of HRP (0.05 mg mL−1) and PSS (0.125 mg mL−1) are shown in Figure 3a. They exhibit two absorption peaks at about 410 and 275 nm from HRP20 and another two peaks at about 258 and 224 nm from PSS. Figure 3b compares the UV absorption behaviors of the modified samples with different PSS/HRP layers on the ZnONR. Besides an invariable peak at 374 nm from the intrinsic absorption of ZnONR for all spectra, each curve hunches at about 415 nm and the inflected pitch increases with the modified
obtained. The alternative variation of the \( \zeta \)-potential in Figure 4 is examined to reveal the effective multilayered assembly of the PSS and HRP on the ZnONR.

Figure 5 shows the CV curves of unmodified, PSS modified, monolayer, and three-layer HRP immobilized sensors in WO-PBS solution with pH 7.0. No reaction peak was observed on the curves of the naked ZnONR and the PSS modified sample. After HRP was immobilized, the direct electrochemistry of HRP on the ZnONR was obtained obviously. As shown in Figure 5, two outstanding redox peaks appear at \(-0.25 \) and \(-0.1 \) V on the curves of the electrodes immobilized with one layer and three layers of PSS/HRP, and the peak values were increasing, allowing the increase of the HRP modified layer. The cathodic and anodic peaks result from the direct electrochemistry of HRP.21-23

The surface concentration of electroactive HRP (\( \Gamma^\ast \)) for various modified layers on electrodes were deduced from the following equation:20

\[
\Gamma^\ast = \frac{Q}{nFA}
\] (1)

where \( Q \) is the charge, the electron transfer number is \( n = 2 \), \( F \) is the Faraday constant, and \( A \) denotes the effective surface area of the working electrode. In order to obtain \( A \), the CV experiments of naked ZnONR electrode were performed in a probe solution of 10 mM K\(_3\)Fe(CN)\(_6\) at various scan rates.25 The slope of the straight line (adjusted \( r \)-square = 0.9981) of \( I_{pc} \) versus the square root of scan rates was \( 1.02 \times 10^{-4} \) A s\(^{1/2}\) V\(^{-1/2}\). For a reversible process,

\[
I_{pc} = (2.69 \times 10^{5})n^{2/3}AD^{1/2}C_0
\] (2)

for K\(_3\)Fe(CN)\(_6\), the electron transfer number \( n \) is 1, the diffusion constant \( D \) is 5.9 \times 10^{-5} \) cm s\(^{-1}\), \( v \) is the scan rate, and \( C_0 \) is the concentration of solution. From eq 2, the effective surface area \( A \) of the ZnONR/Au electrode can be calculated out as 0.49 cm\(^2\). From the integration of the anodic peak of the sensor, following equation:

\[
C = \frac{\Delta I}{nFA}D^{1/2}V^{1/2}C_0^{1/2}
\] (3)

for \( C_0 \) obtained. The alternative variation of the \( \zeta \)-potentials clearly revealed the effective multilayered assembly of the PSS and HRP on the ZnONR.

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\] (3)
the modified-layer increase, and it is the reason that the direct electrochemistry response signal increased along with the modified-layer increase. The multilayer modified electrodes collected a higher concentration of the electroactive HRP on the surface and the monolayer HRP modified electrode presented a similar value compared with another report.26 It substantiates that HRP was immobilized on the ZnONR layer by layer successfully.

As shown in Figure 6a, the cathodic and anodic peak current ($I_{pc}$) increased gradually when the scan rate was accelerated from 25 to 500 mV s$^{-1}$. Figure 6b plots the $I_{pc}$ versus the scan rate and reveals their linear relationship. The linear fitting to the experimental dots gives an adjusted $r^2$-square as high as 0.9929. This indicates that the redox reaction is a surface process and the electrons easily transfer between HRP and ZnONR/Au electrode.25 It proved that the HRP was immobilized on the PSS/ZnONR successfully and retained its bioactivity perfectly.

The average apparent heterogeneous electron transfer rate constant $k_s$ of the immobilized HRP on the electrode surface can be estimated by the Laviron equation.27

$$k_s = \frac{mnFV}{RT}$$  \hspace{1cm} (3)

where $R$ and $T$ are the gas constant and the temperature in kelvins and $m$ is correlative with the difference between the cathodic and anodic potential peak value. According to eq 3, $k_s$ is estimated as 1.15 s$^{-1}$, which is close to the reported value of some better biosensors constructed by carbon nanotubes.28,29 It is demonstrated that the HRP immobilized on ZnONR maintained high activity and catalyzed H$_2$O$_2$ reduction effectively. Figure 7b plots the cathodic $I_{pc}$ difference for naked ZnONR and HRP modified ZnONR electrodes before and after adding 2 mM H$_2$O$_2$. It illustrated that the cathodic $I_{pc}$ difference for ZnONR is far less than that for the HRP modified one. The cathodic $I_{pc}$ difference increased obviously when the HRP was immobilized from one layer to three layers on the ZnONR, and the difference trend to saturation over three layers. It indicated that the catalysis effect was suppressed when there were more than three HRP layers. The catalytic mechanism is exemplified as follows:23

$$\text{HRP (Fe}^{3+}) + \text{H}_2\text{O}_2 \rightarrow \text{compound I (Fe}^{4+}=\text{O}) + \text{H}_2\text{O}$$  \hspace{1cm} (a)

$$\text{compound I (Fe}^{4+}=\text{O}) + e + \text{H}^+ \rightarrow \text{compound II}$$  \hspace{1cm} (b)

$$\text{compound II} + e + \text{H}^+ \rightarrow \text{HRP (Fe}^{3+}) + \text{H}_2\text{O}$$  \hspace{1cm} (c)

As shown in Figure 8a, the typical current–time responses of the sensors with one to five layer(s) of HRP were obtained at a working potential of $-0.25$ V by successively adding 5 μM (inset) and 100 μM H$_2$O$_2$ in pH 7.0 WO-PBS. Figure 8b shows the calibration plots of the current versus the concentration of H$_2$O$_2$. It demonstrates that the HRP modified biosensors exhibit a broad linear response range from 5 μM to 1.7 mM for H$_2$O$_2$ detection. The response time was less than 5 s when the current reached 95% of the steady-state value. The sensitivity, detection limit at a signal-to-noise ratio of 3, and apparent Michaelis–Menten constant ($K_{Mapp}$) according to the Lineweaver–Burk

![Figure 6. CV curve variation of a one-layer HRP modified electrode with a change of the scan rate from 25 to 500 mV with each step of 25 mV in pH 7.0 WO-PBS (a) and the plots of the cathodic and anodic peak vs the scan rate (b).](image)

![Figure 7. CV curves of the HRP modified electrode (a) and the difference between the cathodic peak current in the WO-PBS solution with and without 2 mM H$_2$O$_2$ at pH 7.0 at a scan rate of 100 mV s$^{-1}$ (b). The inset presents the CV curves of the unmodified electrode in the same solutions.](image)
effect of enzyme to catalyzed target; thus, the result $K_{app}$ for H$_2$O$_2$ detection distribute in the range 1.9 to 10.72 µM. As illustrated in Table 1, the detection limit of one to five layer(s) of HRP sensors equation were calculated and listed in Table 1. As illustrated in Table 1, the detection limit of one to five layer(s) of HRP sensors for H$_2$O$_2$ detection distribute in the range 1.9–2.5 µM, which is lower than the other report. The sensitivity increased from one layer to five layers of HRP. That is in accordance with the trend of $\Gamma^*$. The monolayer HRP modified biosensor presents the lowest sensitivity of 36.28 µA mM$^{-1}$ which is higher than the other report. The apparent $K_{app}^*$ constants are in the range of 10.72–26.3 µM, which are lower than the reported value. $K_{app}^*$ decreases with the immobilized HRP layers increasing from one to three, and then increases with the further immobilization; correspondingly, the detection limit displays a similar trend. As is well-known, the lower apparent $K_{app}^*$ reflects the better affinity effect of enzyme to catalyzed target; thus, the result demonstrates that the three-layer immobilized HRP had the maximal catalysis to H$_2$O$_2$ reaction.

4. Conclusions

In summary, a gold electrode was treated with a specific procedure that made the ZnONR grow easily and stably. The hydrothermal fabrication of ZnONR was carried out in water using Zn powders only as a source. The multilayer biomolecular immobilization technique was applied on the ZnONR/Au electrode to construct a biosensor for H$_2$O$_2$ detection. SEM, UV, ζ-potential, and electrochemical measurements demonstrated that the electronegative PSS and HRP with high IEP were alternately immobilized on the ZnONR successfully. The immobilized HRP on the ZnONR retained its bioactivity adequately. The multilayer biosensors exhibit a good performance. The response time is less than 5 s upon adding H$_2$O$_2$ to the buffer solution. $K_{app}^*$ was decreasing from 26.13 to 10.72 µM when the immobilized HRP grew from one layer to three layers, and then increased to 14.57 µM at five layers. The three-layer HRP modified sensor has the lowest $K_{app}^*$ value of 10.72 µM. Along with the immobilized HRP that grew from one layer to five layers, the $\Gamma^*$ was increased from 7.24 $\times$ 10$^{-11}$ to 2.466 $\times$ 10$^{-11}$ mol cm$^{-2}$, and the sensitivity was increased from 36.28 to 58.15 µA mM$^{-1}$. It is demonstrated that multilayer HRP was successfully immobilized on the ZnONR/Au electrode, and kept the bioactivity preferably for efficient catalysis to H$_2$O$_2$.

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References and Notes