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Third Generation Horseradish Peroxidase Biosensor Based on Self-assembling Carbon Nanotubes to Gold Electrode Surface

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Abstract: A third-generation horseradish peroxidase (HRP) biosensor has been developed by adsorbing HRP on multi-wall carbon nanotube (MWNTs) monolayer modified gold electrode surface. The assembly process was investigated by electrochemical and spectroscopic techniques. Results showed that the immobilized HRP exhibited direct electrochemical behavior toward the reduction of H_2O_2. The resulting biosensor shows a fast amperometric response (<2 s) to H_2O_2. The linear response range was from 5.0×10^{-7}~1.0×10^{-5} mol/L with a detection limit of 1.0×10^{-7}mol/L. Moreover, the biosensor has a good reproducibility, and long-term stability.

Keywords: Horseradish peroxidase, MWNT, self-assembly, third-generation biosensor.

Carbon nanotube (CNT) is a novel carbon material discovered by Iijima[1]. It had been found to have the ability to promote electron transfer reactions when it was used to fabricate electrodes for the oxidation of biomolecules[2-4]. Just recently, the interest in demonstrating CNT for biosensing applications is now emerging[5-10]. However, most of these biosensors were based on CNT paste electrode or CNT modified glassy carbon electrode by casting technology. Here, we reported another assembly method to construct a third-generation HRP biosensor based on MWNTs self-assembled monolayer modified gold electrode.

The biosensor was prepared as follows. A clean gold electrode was firstly immersed in a 0.1 mol/L cysteamine solution in dark for 8 h at 4°C. The resulting cysteamine monolayer modified electrode was dipped into a mixture of 0.1 mg/mL MWNTs (purified with nitric acid up to >90%) and 1mg/mL 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride for 24 h at 4°C. The MWNT-modified gold electrode was finally dipped into a 2 mg/mL HRP solution (pH 7.4 PBS) for 24 h at 4°C. In such a way, a HRP biosensor can be achieved. The samples for spectroscopic characterization were prepared on commercial gold foils according to a strict same procedures described above.

Electrochemical measurements were performed on CHI 660 electrochemical workstation (CH Instruments Co., USA.) with a three-electrode system comprising a gold disk electrode with a diameter of 0.5 mm as working electrode, a platinum wire as

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auxiliary electrode, and a saturated calomel electrode (SCE) as reference electrode. The electrochemical impedance spectra (EIS) measurements were performed in 0.1 mol/L KCl containing 5.0×10^{-3} mol/L $K_3[Fe(CN)_6]$ and 5.0×10^{-3} mol/L $K_4[Fe(CN)_6]$. $Fe(CN)_6^{3-}$ and $Fe(CN)_6^{4-}$ were used as redox probe. All impedance measurements were performed at the formal potential of the redox probe with a sinusoidal potential perturbation at amplitude of 5 mV in the frequency range of 0.05 Hz ~ 100 kHz. The results were plotted in the complex plane diagrams (Nyquist plots). UV-Vis reflection and absorbance spectra were measured on a UV-2500 spectrometer (Shimazu, Japan).

Figure 1a shows the complex plane diagram (Z″ *vs.* Z′, Nyquist plot) of the EIS obtained at bare Au electrode. The electron transfer resistance, R_{et}, derived from the semicircle domains of impedance spectra equals to 90Ω. After being immersed in deaerated cysteamine aqueous solution, the resulting cysteamine monolayer showed a higher interfacial electron transfer resistance (50kΩ, **Figure 1b**), indicating that the cysteamine monolayer obstructed the electron transfer of the electrochemical probe. When the electrode was further modified with MWNTs, R_{et} decreased to about 38 kΩ (**Figure 1c**), which was attributed to the good conductivity of the MWNTs. After absorbing HRP, R_{et} increased again (80 kΩ, **Figure 1d**). The increase in R_{et} might be caused by the hindrance of the macromolecular structure of HRP to the electron transfer, and it also confirmed the successful immobilization of HRP.

The UV-Vis spectrum of HRP shows a Soret band at 406 nm in pH 7.4 phosphate buffer solution (PBS) (**Figure 2a**). The location of the Soret absorption band of iron heme can provide the information about the denaturation of heme proteins. When HRP is denatured, the Soret band will shift or disappear. **Figure 2b** shows the Soret band of HRP immobilized on a MWNTs modified electrode. Its peak is very close to that in solution; a difference of only 2 nm was observed. Such a small movement shows that the immobilized HRP molecule retains its biological activity.

Figure 1 EIS of (a) bare gold electrode, (b, c, d) cysteamine, MWNT-cysteamine, HRP- MWNT-cysteamine modified gold electrode, respectively

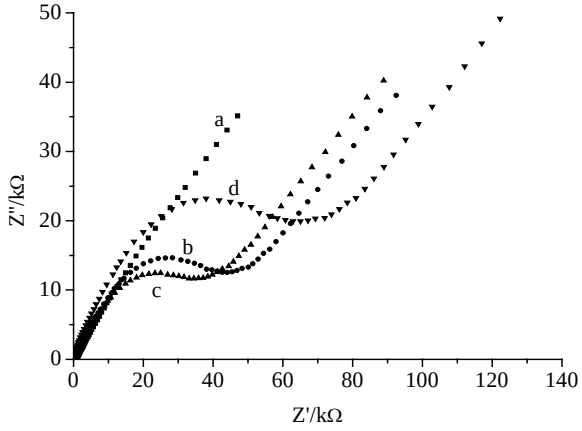


Figure 2 UV-Vis spectra of (A) pH7.4 PBS solution containing 0.1 mg/mL HRP and (B) HRP on the MWNT film modified electrode.

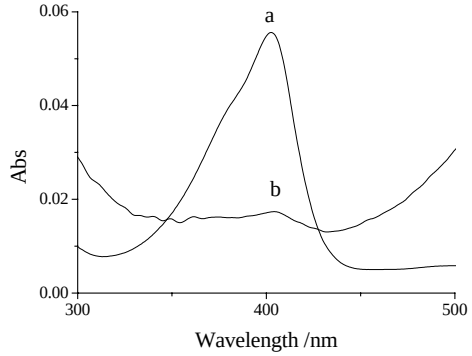
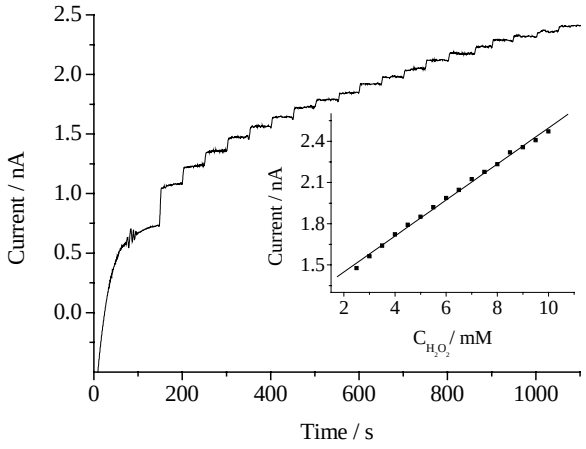


Figure 3 Hydrodynamic response of the biosensor for H_2O_2 in PBS upon the concentration of 5.0 $\times$ 10^{-7} mol/L.Inset: Linear calibration curve for the determination of H_2O_2. Detection potential: 0.2V



The immobilized HRP exhibited direct electrochemical behavior toward the reduction of H_2O_2. **Figure 3** showed the steady-state current response of H_2O_2 at the biosensor. The response was linear in the range from 5.0×10^{-7} mol/L~1.0×10^{-5} mol/L. The detection limit was 1.0×10^{-7} mol/L(S/N=3). The response time was less than 2 s and RSD was 1.7% for eight successive assays at a H_2O_2 concentration of 1.0×10^{-6} mol/L. At higher concentration of H_2O_2, a response plateau was observed, showing the characteristics of the Michaelis-Menten kinetic mechanism. The apparent Michaelis-Menten constant is determined to be 4.0 μmol/L which was little smaller than those reported before[11] for HRP immobilized on the gold colloid modified electrode surface. The smaller apparent Michaelis-Menten constant means that the immobilized HRP shows a higher affinity to H_2O_2.

The effect of temperature on steady-state amperometric response of the biosensor

was investigated in the temperature range 288-348 K. The response gradually increases with increasing temperature (approximate to 0.0145 nA/K) and reaches a maximum at 310K, and then goes down quickly as the temperature is higher than 318K, which indicated that the enzyme is denatured. The dependence of amperometric current on temperature in an initial region can be expressed as an Arrhenius relationship[12]: $i(T) = i_0 \exp\{-E_a / RT\}$, where $\boldsymbol{i_0}$ represents a collection of currents, R is the gas constant, T is the temperature in Kelvin degrees, and E_a is the activation energy. The activation energy for enzymatic reaction was calculated to be 2.6 kJ/mol from the slope of i-1/T in the adoptive region of temperature. This E_a values obtained was smaller than those (13.8 kJ mol^{-1}) reported by Yang *et al.*[13] for HRP immobilized on the polyaniline films. The smaller E_a value also means that the immobilized HRP on MWNTs possesses higher enzymatic activity.

The interference tests were carried in PBS containing 1.0×10^{-6} mol/L H_2O_2 in the presence of 50 times of cysteine, 2500 times of glucose, 2500 times of acetaminophenol, 500 times of uric acid, 500 times of ethanol, 500 times oxalic acid and 500 times of ascorbic acid. It was found that these substances did not cause any observable interference except ascorbic acid.

The biosensor's stability was also examined with intermittent measuring the current response to H_2O_2 standard solution every 2-3 days in the period of two months. The catalytic current response could maintain about 80% in two months.

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