Immobilization of Myoglobin on NiO Nanoparticles Matrix for Preparation of Novel Biosensor

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Abstract: Direct electrochemistry that immobilized myoglobin (Mb) on a nanometer-sized NiO nanoparticles matrix modified graphite electrode (GE) and preparation of novel hydrogen peroxide biosensor were studied. The immobilized Mb displayed a couple of stable and well-defined redox peaks with an electron transfer rate constant of 6.48 s⁻¹ and a formal potential of −0.340 V (vs saturated calomel electrode [SCE]) in 0.1 M pH 7.0 PBS. The total surface concentration was 8.06×10⁻¹⁰ mol cm⁻². Dimethyl sulfoxide (DMSO) could play an important role in the electron transfer between Mb and the electrode. Spectroscopy analysis of the Mb/NiO/DMSO film showed that the immobilized Mb could retain its natural structure. The electrocatalytic response showed a linear dependence on the H₂O₂ concentration ranging from 0.8 to 24 μM with a detection limit of 0.039 μM at 3σ. The apparent Michaelis–Menten constant Kₘ for H₂O₂ sensor was estimated to be 0.21 mM, sensitivity was 417 mA/M·cm⁻², showing a high affinity.

Key Words: NiO nanoparticles; Biosensors; Hydrogen peroxide; Myoglobin; Direct electron transfer

1 Introduction

The study of protein and enzyme direct electrochemistry and electrocatalysis, for understanding the life of electron transfer mechanism and enzyme-catalyzed mechanism, as well as key life-substance in the life of important redox process is very important[1]. Recent studies show that the direct electron transfer can be realized between heme proteins and the electrode surface by using the surfactants[2,3], sol-gel film[4], ion polymer[5], nanomaterials[6–11] by embedding, cross-linking, assembling, and so on. People have more interest in the immobilization of protein (enzyme) molecules in nanoparticle with the in-depth research on nanomaterials[6,12,13]. Myoglobin (Mb) is the ideal model of direct electrochemistry on heme proteins, biosensing, and electrocatalytic because it involves the electron delivery in physiological function and the process of metabolism. Nickel oxide as a functional material, many scholars are very much aware on its development and the study of application[14].

This thesis work is to immobilize myoglobin molecule to nickel oxide nanoparticles (NiO) modified graphite electrode (GE) surface, study the direct electrochemistry behavior of protein (enzyme) in the NiO nanoparticles modified GE for the first time, and design an accurate, reliable, convenient, sensitive new third-generation biosensor with good selectivity, stability, and reproducibility. This research takes on the great theoretical and practical significance in the life sciences, environmental science, energy science, and analytical chemistry.

2 Experimental procedures
2.1 Apparatus and reagents

Myoglobin (Mb) was purchased from Beijing Shu Bo Wei Chemicals Instruments Co., Ltd, (Shanghai Branch. No. M1882). Other reagents were of analytical reagent grade. All solutions were prepared with double-distilled water. A saturated calomel electrode (SCE) and GE were purchased from Institute of Soil Science, Chinese Academy of Sciences. Electrochemical workstation (CHI660C) was purchased from Shanghai Chenhua Instrument Company. TU-1901 double-beam UV spectrophotometer was purchased from Beijing Purking General Instruments Co. Ltd., China. Fourier Transform Infrared (Vector 22 FT-IR) spectra spectrometer was purchased from Bruker Company. Scanning Electron Microscope (LEO. Electron Microscopy Ltd) was made in Germany.

2.2 Electrode preparation

Nickel oxide preparation was carried out according to the method described in literature\[15\]. The solution preparation was as follows: A, 2.0 g L–1 Mb water solution; B, 4.0 g L–1 NiO dimethyl sulfoxide (DMSO) suspension; and C, 4.0 g L–1 NiO water solution.

The modified GEs (geometric area: 0.2641 cm2) were polished before experiment with abrasive paper for metallograph (WAW7, 05) and 0.05-μm α-alumina slurry (Beuhler), respectively, rinsed thoroughly with double-distilled water between each polishing step, sonicated in acetone and rinsed with double-distilled water successively and allowed to dry at room temperature.

Six prepared electrodes were as follows: (1) Bare electrodes, no analogs; (2) NiO/DMSO/GE, 5 μL B and 10 μL water; (3) Mb/GE, 10 μL A and 5 μL water; (4) Mb/NiO/GE, 10 μL A and 5 μL C; (5) Mb/DMSO/GE, 10 μL A and 5 μL DMSO; (6) Mb/NiO/DMSO/GE, 10 μL A and 5 μL B. A small bottle was fit tightly over the electrode for 2 h to ensure the slow evaporation of water and the formation of more uniform film. The film was then dried and aged overnight in a sealed flask kept at a constant temperature of 18 ºC. Before the electrochemical experiments, the six electrodes were rinsed thoroughly with double-distilled water and kept in pH 7.0 PBS at 4 ºC.

2.3 Measurements

Electrochemical measurements were performed on a CHI 660C electrochemical workstation (CHI Co., China) at (18 ± 0.2) ºC with a conventional three-electrode system with the modified GE as working electrode, a platinum wire as auxiliary electrode, and an SCE as reference against which all potentials were measured. The stability of Mb/NiO/DMSO/GE was tested by increasing the temperature electrolysis pool, keeping the corresponding temperature 20 min and recording the cyclic voltammetry figure of methods. All experimental solutions were deoxygenated by bubbling highly pure nitrogen for 15 min and maintained under nitrogen atmosphere during measurements. The amperometric experiments were carried out by applying a potential of –350 mV on a stirred cell. The biosensor response was measured as the difference between total and residual currents.

UV-Vis absorbance spectroscopy was performed using TU-1901 double-beam UV spectrophotometer. Fourier transform infrared (FT-IR) spectra were recorded on a Vector 22 FT-IR spectrometer (Bruker). Mb solution, Mb/DMSO solution, Mb/NiO/water, or Mb/NiO/DMSO suspension were cast on a Teflon chip respectively. After the membrane on the chip was dried in air, it was stripped off and tabletted with KBr powder for FT-IR measurement. Twenty scans were collected and averaged for each spectrum. After modifying NiO, Mb and Mb/NiO/DMSO on a glassy slice respectively, these films were examined under a scanning electron microscope.

3 Results and discussion

3.1 Spectroscopic analysis of Mb/NiO/DMSO film

Figure 1 shows the UV-Vis spectra of different solution systems. The research indicates that Soret absorption band of myoglobin in solutions is in 409 nm\[16\], so it is very significant that the absorption peak in Fig.1 is myoglobin’s Soret absorption band in solution. The migration or disappearance of absorption band suggests changes in protein structure\[17,18\]. Thus, the Mb mixed in the suspensions of NiO/DMSO, NiO/water, pure DMSO, and water could retain its native structure.

As well known, the shapes of infrared absorption bands of amide I and amide II in Mb molecule can provide detailed information on the secondary structure of the polypeptide chain. The absorption band of 1600–1700 cm–1 for amide I is caused by C=O stretching vibration of peptide linkages in the protein’s backbone. The absorption band around 1500–1620 cm–1 for
amide II is resulted from a combination of N–H bending and C–N stretching. The loss of activity in Mb will eliminate the distinctive absorption bands of amide I and amide II\(^{[19,20]}\). Figure 2 shows FT-IR spectra. The absorption bands for amide I and amide II in the Mb/NiO/DMSO film were nearly the same as those obtained for the protein itself. Both FT-IR and UV-Vis spectroscopic experiments suggested that the Mb in Mb/NiO/DMSO film was not grossly denatured and retained its native structure.

### 3.2 SEM characterization

Figure 3 shows the SEM images of NiO, Mb, and Mb/NiO/DMSO. It can be seen that the Mb molecules mixed with NiO particles are smaller and dispersed, and the electron transfer more fully between Mb and electrodes because of the dispersion of NiO nano-particles and reduction in the accumulation of Mb serious agglomeration.

### 3.3 Direct electrochemistry of Mb/NiO/DMSO-modified electrode

Figure 4 shows the cyclic voltammograms of different electrodes in 0.1 M pH 7.0 phosphate buffer solution. The Mb/NiO/DMSO/GE gave a couple of stable and well-defined redox peaks at –0.3677 and –0.3127 V at 100 mV s\(^{-1}\) (curve f), while no redox peak was observable in curves a and b. Obviously, the response of the Mb/NiO/DMSO/GE was attributed to the redox of the electroactive centers in the immobilized Mb. However, the peak current of curve f was much bigger than curves c and d. Thus, the presence of DMSO played an important role in accelerating the electron transfer between Mb and the electrode. It was because of the decrease of the dielectric constant of the microenvironment around protein molecules, which decreased the reorganization energy of biological electron transfer, thus accelerated the electron transfer in protein\(^{[19]}\). It was also because of the electronic exchange of the active site relatively exposed, promoting direct electron transfer. However, the peak current curve f was 2 times higher than curve e, thus, NiO nanoparticles were more important for facilitating the electron exchange. NiO nanoparticles provided a three-dimensional stage and some of the restricted orientations also favored the direct electron transfer between the protein molecules and the conductor surface. However, NiO nanoparticles could be strong adsorption, so the electrode had more Mb, and during the electrode processing, it was difficult to get rid of Mb, so the signal became large.

The formal potential \(E^\theta\) of the heme Fe(III)/Fe(II) couple in Mb/NiO/DMSO/GE, estimated as the midpoint of anodic and cathodic peak potentials, was –0.34 V at pH 7.0 PBS. With an increasing scan rate, the anodic and cathodic peak potentials of the Mb showed small shift and the redox peak currents increased linearly, which indicated a surface-controlled electrode process. The electron transfer rate constant \(k_s\) was estimated according to model of Laviron formula \(k_s = mnFv/RT\) to be 6.48 s\(^{-1}\)\(^{[20]}\), where \(m\) is a parameter related to the peak-to-peak separation. From the integration of the reduction peaks of the Mb/NiO/DMSO/GE at different scan rates, an average surface coverage of Mb \((\gamma)\) was calculated to be \(8.06 \times 10^{-10}\) mol cm\(^{-2}\), which indicated a high load of enzyme molecules.

### 3.4 Effect of solution pH on direct electron transfer of immobilized Mb

In pH 5–10, the formal potential and solution pH is a linear relationship, with a slope of –42.3 Mv per pH \((R = 0.9993)\), which was close to 43.9 mV pH\(^{-1}\)\(^{[21]}\) and the expected value of –57.8 mV pH\(^{-1}\) at 291 K, indicating that one proton participated in the electron transfer process\(^{[22]}\). The pH 7.0 PBS buffer solution was the experiment electrolyte because the highest peak current value appeared in pH 7.0 PBS solution.
3.5 Electrocatalysis of Mb/NiO/DMSO/GE to H$_2$O$_2$

Upon addition of H$_2$O$_2$ to the electrochemical cell in pH 7.0 PBS, the reduction peak current increased and the anodic peak current dramatically decreased. This phenomenon was not observed at a bare GE or a NiO/DMSO/GE electrode. Thus, the catalytic reduction of H$_2$O$_2$ was due to the presence of Mb, and the catalytic effect was very obvious. Figure 5 shows the amperometric response of the Mb/NiO/DMSO/GE at an applied potential of ~350 mV on successive additions of H$_2$O$_2$ to a stirring 0.1 M pH 7.0 PBS. Upon addition of an aliquot of H$_2$O$_2$ to the buffer solution, the reduction current increased steeply to reach a stable value. The modified electrode achieved 95% of the maximum steady-state current in less than 5 s. This demonstrated clearly that the electrocatalytic response was very fast. So it can be used for rapid detection of H$_2$O$_2$. The linear response range of the sensor to H$_2$O$_2$ concentration was from 0.8 to 24 μM under the optimum conditions. The limit of detection was estimated to be 0.039 μM at a signal-to-noise ratio of 3σ from the slope. The enzymatic saturation response was observed (not be expressed) when the concentration of H$_2$O$_2$ was higher than 24 μM, which showed a characteristic of the Michaelis-Menten kinetic mechanism. The apparent Michaelis-Menten constant ($K_M$) of Mb/NiO/DMSO-modified electrode for H$_2$O$_2$ was obtained to be 0.21 mM from the electrochemical version of the Lineweaver-Burk equation. The $K_M$ value was much smaller, thus, Mb modified by NiO had a high affinity to H$_2$O$_2$. The Mb/NiO/DMSO-modified electrode showed a sensitivity of 417 mA M$^{-1}$ cm$^{-2}$ to H$_2$O$_2$, which was much higher than 97 mA M$^{-1}$ cm$^{-2}$ for Mb in zirconia nanoparticles enhanced grafted collagen (ZrO$_2$-grafted collagen) hybrid composite$^{[16]}$.

3.6 Thermal stability of Mb/NiO/DMSO/GE

The immobilization of proteins and enzymes onto transducer surfaces can lead to a change of their behavior compared with that observed in homogeneous solution$^{[21,23]}$. Thermal stability is a measure of the ability of the biosensor to withstand elevations in temperature$^{[24]}$. Both the anodic and cathodic peak currents of the Mb/NiO/DMSO/GE increased with increasing temperature from 15 to 85 ºC, displaying an expected Arrhenius-type temperature dependence. Meanwhile, no peak was observed at the Mb/GE up to 50 ºC, indicating the loss of Mb$^{[25]}$. The increase in the thermal stability of the Mb/NiO/DMSO/GE could be attributed to the presence of NiO nanoparticles. The immobilized Mb on hydrophobic NiO nanoparticles could greatly enhance the thermal stability because of the unusual conformational rigidity in this nonpolar binding environment.

3.7 Stability and reproducibility of Mb/NiO/DMSO-modified electrode

When it was cyclically swept between ~0.8 and +0.2 V in 0.1 M pH 7.0 PBS at for 100 times, the peak current measurements corresponding to the direct electrochemistry of the immobilized Mb gave a relative standard deviation of 0.887%. So NiO was very efficient for retaining the oxidation and reduction activity of immobilized Mb and preventing it from leaking out of the biosensor, indicating a good repeatability. This sensor could retain 94.0% of its initial current response after 60-day storage in 0.1 M pH 7.0 PBS at 4 ºC, showing better stability.

References


研究了在氧化镍纳米粒子改性石墨电极上肌红蛋白的直接电化学以及生物反应。肌红蛋白有稳定而明确的氧化还原峰，电子转移对认识生命体内的电子传递以及参与的代谢过程具有重要意义。对于肌红蛋白及其衍生蛋白的直接电化学以及生化反应的研究，国内外都非常活跃。这对于开发新的生物传感器和生物电子设备具有重要的理论意义和实际应用价值。对于肌红蛋白的直接电化学行为，国内外的研究很多，但是直接电化学以及生物反应的研究还不多。本文研究了在氧化镍纳米粒子改性石墨电极上肌红蛋白的直接电化学以及生物反应。肌红蛋白有稳定而明确的氧化还原峰，电子转移对认识生命体内的电子传递以及参与的代谢过程具有重要意义。对于肌红蛋白及其衍生蛋白的直接电化学以及生化反应的研究，国内外都非常活跃。这对于开发新的生物传感器和生物电子设备具有重要的理论意义和实际应用价值。本文研究了在氧化镍纳米粒子改性石墨电极上肌红蛋白的直接电化学以及生物反应。肌红蛋白有稳定而明确的氧化还原峰，电子转移对认识生命体内的电子传递以及参与的代谢过程具有重要意义。对于肌红蛋白及其衍生蛋白的直接电化学以及生化反应的研究，国内外都非常活跃。这对于开发新的生物传感器和生物电子设备具有重要的理论意义和实际应用价值。本文研究了在氧化镍纳米粒子改性石墨电极上肌红蛋白的直接电化学以及生物反应。肌红蛋白有稳定而明确的氧化还原峰，电子转移对认识生命体内的电子传递以及参与的代谢过程具有重要意义。对于肌红蛋白及其衍生蛋白的直接电化学以及生化反应的研究，国内外都非常活跃。这对于开发新的生物传感器和生物电子设备具有重要的理论意义和实际应用价值。本文研究了在氧化镍纳米粒子改性石墨电极上肌红蛋白的直接电化学以及生物反应。肌红蛋白有稳定而明确的氧化还原峰，电子转移对认识生命体内的电子传递以及参与的代谢过程具有重要意义。对于肌红蛋白及其衍生蛋白的直接电化学以及生化反应的研究，国内外都非常活跃。这对于开发新的生物传感器和生物电子设备具有重要的理论意义和实际应用价值。
2.2

改变电极表面的微结构

\( \frac{0.2641 \text{ cm}^2}{5 \mu \text{m}} \) WAW7(05) \( \frac{0.05 \mu \text{m}}{5 \text{ min}} \)

\[ \text{SEM} \]

2.3

\[ \text{CHI600C} \]

NiO/DMSO/GE \( 2.0 \) \( 5 \mu \text{L} \) B + 10 \( \mu \text{L} \) H2O; (3) Mb/GE \( 10 \mu \text{LA} + 5 \mu \text{L} \) H2O; (4) Mb/NiO/GE \( 10 \mu \text{L} \) A + 5 \( \mu \text{L} \) C; (5) Mb/DMSO/GE \( 10 \mu \text{L} \) A + 5 \( \mu \text{L} \) DMSO; (6) Mb/NiO/DMSO/GE \( 10 \mu \text{L} \) A + 5 \( \mu \text{L} \) B

\[ \text{PBS(pH} 7.0) \]

3

\[ \text{Mb/NiO/DMSO} \]

Soret 409 nm \( [16] \)

\[ \text{NiO/DMSO, NiO/GE, DMSO} \]

C=O \( [18, 19] \)

C=N \( [18, 19] \)

N---H \( [18, 19] \)

3.2

\[ \text{Mb/NiO/DMSO} \]

\[ \text{Mb} \]

\[ \text{NiO} \]

\[ \text{Mb/NiO/DMSO} \]

3.3

\[ \text{Mb/NiO/DMSO/GE} \]

\[ \text{DMSO} \]

\[ \text{PBS(pH} 7.0) \]
Fig. 1 Uv-vis spectra of Mb/NI0/DMSO (a) Mb/DMSO (b) Mb/NI0 (c) Mb (d) Ni0/DMSO (e) DMSO (f) solutions

Mb: Myoglobin; DMSO: Dimethylsulfoxide.

Fig. 3 Scanning electron micrographs of Ni0(a) Mb(b) and Mb/NI0/DMSO (c) films on a glass slice

Fig. 2 FT-IR spectra of Ni0(a) Mb/NI0/DMSO (b) Mb (c) Mb/DMSO (d) and Mb/NI0/DMSO (e) films

3.4 pH

- pH 5 - 10
- pH 7.0
- pH 7.0

3.5 Mb/NI0/DMSO/GE

Fig. 4  Cyclic voltammograms of graphite electrode (GE) (a) NiO/DM/GE (b) Hb/GE (c) Mb/NiO/GE (d) Hb/DM/GE (e) Mb/NiO/DM/GE (f) 0.1 mol/L PBS (pH 7.0) at 100 mV/s

3.6 Mb/NiO/DM/GE

Fig. 5  Amperometric response of sensor at −350 mV upon successive additions of H2O2 in pH 7.0 PBS

3.7 Mb/NiO/DM/GE - 0.8 ~ 0.2 V  Mb/NiO/DM/GE  PBS (pH 7.0) 100  4℃  PBS (pH 7.0)  60 d  94%  Mb/NiO/DM/GE

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Abstract  Direct electrochemistry of myoglobin (Mb) immobilized on a nanometer-sized NiO nanoparticles matrix modified graphite electrode (GE) and preparation of novel hydrogen peroxide biosensor were studied. The immobilized Mb displayed a couple of stable and well-defined redox peaks with an electron transfer rate constant of 6.48 s⁻¹ and a formal potential of −0.340 V (vs·SCE) in 0.1 mol/L PBS (pH 7.0). The total surface concentration was 8.06 × 10⁻¹⁰ mol/cm². Dimethyl sulfoxide (DMSO) could play an important role in the electron transfer between Mb and the electrode. Spectroscopy analysis of the Mb/NiO/DMSO film showed that the immobilized Mb could retain its natural structure. The electrocatalytic response showed a linear dependence on the H₂O₂ concentration ranging from 0.8 to 24 μmol/L with a detection limit of 0.039 μmol/L (3σ). The apparent Michaelis Menten constant Kₘₐₓ for H₂O₂ sensor was estimated to be 0.21 mmol/L and the sensitivity was 417 mA cm⁻² L/mol which showed a high affinity.

Keywords Nickelous oxide nanoparticles; Biosensors; Hydrogen peroxide; Myoglobin; Electron transfer

(Received 18 March 2010; accepted 11 June 2010)