



Fabrication of Multilayers Film of Myoglobin and Glutaraldehyde on the Surface of Gold Electrode Based on Covalent Interaction and Its Application

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Abstract

In this work, the multilayers film of myoglobin (Mb) and glutaraldehyde (GA) was assembled on the surface of gold electrode modified with MWCNTs by layer by layer (LBL) assembly technique, and the assembly process was characterized by cyclic voltammetry (CV) and UV-Vis spectroscopy. UV-Vis spectroscopy showed that Mb in the multilayers film retained its native biology activity, and the multilayers film exhibited good electrocatalytic activity toward reduction of H₂O₂ in pH 7.0 phosphate buffer solution (PBS), the currents intensity were linearly with concentrations of H₂O₂ in the range of 6×10⁻⁶ to 8.4×10⁻⁵ M with the detection limit of 5×10⁻⁷ M (S/N=3). The Michaelis-Menten constant was 0.115 mM. In addition, interference experiments showed no interference response obtained for dopamine, ascorbic acid, ethanol, uric acid species et al at this working potential.

Keywords: Myoglobin; Glutaraldehyde; Multiwall carbon nanotubes; Layer-by-layer assembly technique.

1. Introduction

Myoglobin (Mb) is a 16.7 k Da heme protein in muscle tissues which the functions to store and transport oxygen. It contains a single polypeptide chain with an iron heme as its prosthetic group. Studies of electrochemical behavior of heme proteins are essential for a fundamental understanding of their biological activity. Mb is an

ideal molecule for the study of electron –transfer reactions of heme proteins and also for biosensing and electrocatalysis [1]. Although Mb possesses functional groups that can be readily oxidized or reduced by chemical redox agents, it does not easily undergo facile redox reactions in solution with bare or “naked” solid electrodes, this is because unfavorable orientation of the protein on electrode surface may increase the distance between its heme redox center and electrode

surface. Another reason for the difficulty of electron exchange may be the adsorption of impurities in the protein solution on the surface of electrodes. The adsorption of the impurities or denatured protein can block the electron communication between heme and electrode. So facilitation of the electron transfer between Mb and electrode is very challenging, Li and his coworker have studied the kinetics of hemoglobin electron transfer in solution and immobilized on electrode surface with electrochemical and spectroscopy [2,3]. Hence, to explore methods to increase the electron transfer rate between Mb and the electrode, much effort have been devoted to the characterization of the electrochemistry of Mb using various films [4, 5]. Hu's and Rusling's groups have provided a lot of good work in this area [6, 7]. From the publish literature, most of work about the direct electrochemistry of Mb was performed at monolayer film. The film has low Mb content and poor stability for preparing sensor.

In recent years, LBL assembly as a relatively new film-forming technique has been developed rapidly due to its procedure simplicity, wider selectivity and controlled thickness from nanometer to micrometer scale. As a result, LBL assemble technique has attracted much attention in various research field. To date, there are various LBL methods have been reported, including electrostatic interaction [8], hydrogenbonding [9], charge-transfer interaction [10] and coordination bonding [11, 12]. Multilayer films containing protein [13-14] or nanoparticles (NPs) [15] and carbon nanotubes (CNTs) [16-23] via LBL assemble technique have been reported. Such as, Ju and his co-works [22] have fabricated a multilayers film composed of GNPs, MWCNTs, and glucose oxidase (GOD); Dong and his co-works [23] has assembled PDDA , MWCNTs and platinum nanoparticles(Pt NPs) multilayers film. Our groups have fabricated multilayers film composed of Mb, chitosan, and polyelectrolyte-wrapped MWCNTs [24]. All the above mentioned multilayers film is obtained via electrostatic interaction. As we know, electrostatic interaction is a weak interaction force, which the stability of assembled films is poor. To overcome this drawback, the LBL covalent attachment technique provides a platform because covalent interaction is

stronger. So the assembled film has higher stability.

In this work, we want to fabricate multilayers film of Mb based on covalent attachment mode, and investigate the direct electrochemistry of Mb and electrocatalytic activity towards the reduction of H₂O₂, and aim to fabricate a sensitive electrochemical biosensor of H₂O₂. Chitosan (CS), derived from chitin via deacetylation with alkali is a linear copolymer of glucosamine and N-acetylglucosamine units. It displayed an excellent film-forming ability, good adhesion, biocompatibility and low toxicity. It is easy to take Schiff's reaction with glutaraldehyde (GA) because it contains NH₂ groups. The multilayers film of Mb assembled process is follows: First, GA was covalent attachment matrix of MWCNTs-CS composite based on Schiff's reaction. Next, Mb was covalent attachment with GA, and multiple repetition of the assemble process leads to form multilayers of Mb. The assemble process was conformed by CV technique and UV-Vis spectroscopy. Final, we investigated the electrocatalytic activity towards reduction of H₂O₂ by the multilayers film modified electrode.

2. Experimental

2.1. Reagents and apparatus

MWCNTs were purchased from Chengdu Institute of Organic Chemistry, Chinese Academy of Sciences and used without further purification (purity>90%). CS, GA (50%) and Mb were from Sigma and used as received. Other chemicals were of analytical reagent grade. 0.1 M phosphate buffer solution (PBS) was prepared by mixing the stock standard solutions of K₂HPO₄ and KH₂PO₄, all containing 0.1 M NaCl. The pH was adjusted with 0.1 M H₃PO₄ or 0.1 M NaOH.

A 5.0 mg / mL CS was prepared by dissolving 50 mg CS in 10 mL 0.05 M HCl, and the pH of the solution was adjusted to 5.0 with NaOH; Suitable mount of MWCNTs were dispersed in 5.0 mg / mL CS with the help of sonication for 1h. The accurate concentration of H₂O₂ was determined by titration with potassium permanganate solution. All solutions were prepared with double-distilled deionized water.

Cyclic voltammetry (CV) and amperometry experiments were performed using a CHI660A electrochemical workstation (Shanghai Chenhua Instruments Co., China). The three-electrode system consisted of the working electrode of interest, a saturated calomel electrode (SCE) reference electrode, and a platinum wire auxiliary electrode. UV-Vis spectroscopy was performed using a U-3010 spectrophotometer (Hitachi, Tokyo, Japan). All experiments were performed at ambient temperature.

All solutions were deoxygenated by bubbling high-purity nitrogen for 30 min, and nitrogen atmosphere was kept over the solution during measurements. All potentials in this work are reported referenced to the SCE.

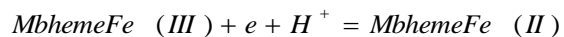
2.2. Multilayers film of Mb assembly process

Prior to use, Au electrode (d=2 mm) was pre-treated according to literature [25]. Then it was modified with 1 μ L of MWCNTs dispersion and to dry at ambient temperature. The MWCNTs film modified electrode was immersed in 10% GA for 6 h and 4 mg/mL Mb for 6 h alternately at room temperature, the process was repeated until demand layers. During the film assembly process, the aldehyde groups of GA and the amine groups of CS or Mb would take Schiff reaction and formed a new bond C=N. Thus, the multilayers film (GA/Mb)_n was successfully assembled on the MWCNTs film. The assemble process was shown in Scheme.1.

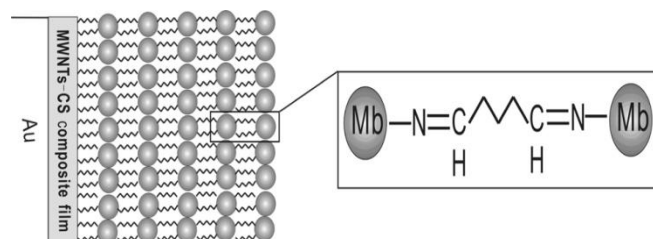
3. Results and Discussion

3.1. The Characterization of the Multilayer Film

In experiments, CVs was used to monitor the growth of (GA/Mb)_n films. Fig. 1 showed the CVs of different film in pH 7.0. No redox peak can be observed at bare Au electrode (curve a) or MWCNTs film modified Au electrode (curve b). However, a pair of well-defined and symmetrical redox peak can be observed at Mb/GA/ MWCNTs -CS film-modified electrode (curve c), whose formal potential was at about -0.3V. The redox peak was from reduction and oxidation of Mb heme (Fe(III)/Fe(II)) in films. The electrode reaction is follow:



Further, the peak currents of the reduction and oxidation of Mb are increased nonlinearly with the number of multilayers (n) increased from 1 to 6, and then it kept a constant when n>6.



Scheme 1. Schematic representation assembly process of the multi-films

The Soret band of heme protein is usually an indicator of the microenvironment where heme center locates. This peak will be diminished if the protein is denatured. Fig. 2 showed UV-Vis spectroscopy of different films, From Fig. 2, we obviously observed an absorption peak of Mb at 410 nm, suggesting that Mb in films has a secondary structure nearly the same as the native state of Mb [26], and the absorb intensity increased linearly with the number of Mb bilayers (inset), indicating (GA/Mb)_n film was successful fabricated on the surface of the glassy carbon electrode. In this work, we select (GA/Mb)₆ film for the next electrochemical experiment.

3.2. Effect of scan rate and pH

Effects of scan rate and pH on voltammetric response of multilayers film have been investigated. It was obviously observed that the peak currents increased linearly with scan rate from 50 to 1000 mV s^{-1} (data is not shown), indicating that the electrode reaction was surface-controlled electrode process. Fig.3 showed the CVs of (GA/Mb)₆ multilayer film at various pH. It was clearly observed that peak potentials of Mb both cathodic and anodic shifted linearly negatively with increasing pH of solution from 5.0 to 9.0, the reason was attributed to proton participate in the electrode reaction, and the formal potential had linear relationship with pH of

solution, the linear regression equation is $E^o(V) = 0.0501 - 0.057 \text{ pH}$ with the correlation coefficient of 0.9940. Additionally, all changes as pH were reversible. For example, the CVs of the $(\text{GA}/\text{Mb})_6$ film modified electrode at pH 8.0 buffer was reproduced after immersion in pH 5.0 buffer and then returned to the pH 8.0 buffer. In this work, pH 7.0 solution was select for the following electrochemical experiments.

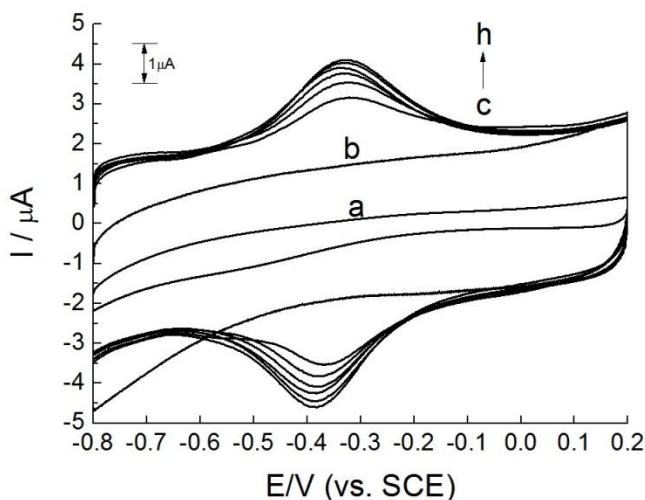


Figure 1. Cyclic voltammograms of different modified electrodes in 0.1 M PBS (pH 7.0) at 100 mV s^{-1} : (a) bare Au, (b) MWCNTs-CS/Au, (c) to (h) represented 1 to 6 bilayers, respectively.

3.3. Electrocatalytic activity towards reduction of H_2O_2

In order to investigate the electrocatalytic activity of Mb in the multilayers film, H_2O_2 was selected for target, and the electrocatalytic activity of H_2O_2 was investigated by CV and amperometry technique. As shown in Fig. 4A, with the addition of H_2O_2 , the reduction peak current was enhanced corresponding with the decrease of the oxidation peak current. The reduction peak current was increased with the concentration of H_2O_2 increased in solution. Fig. 4B displayed a typical hydrodynamic amperometry cure of the multilayer films modified electrode for successive injection of different volume H_2O_2 . From Fig. 4B, we can observe that the magnitude of the current increases with the successive injection of H_2O_2 successive. The response currents were linear with concentration of H_2O_2 from 6.0×10^{-6} to 8.4×10^{-5}

M, with a correlation coefficient of 0.9994 (Fig. 4C). The detection limit of $5.0 \times 10^{-7} \text{ M}$ ($\text{S}/\text{N} = 3$) was obtained.

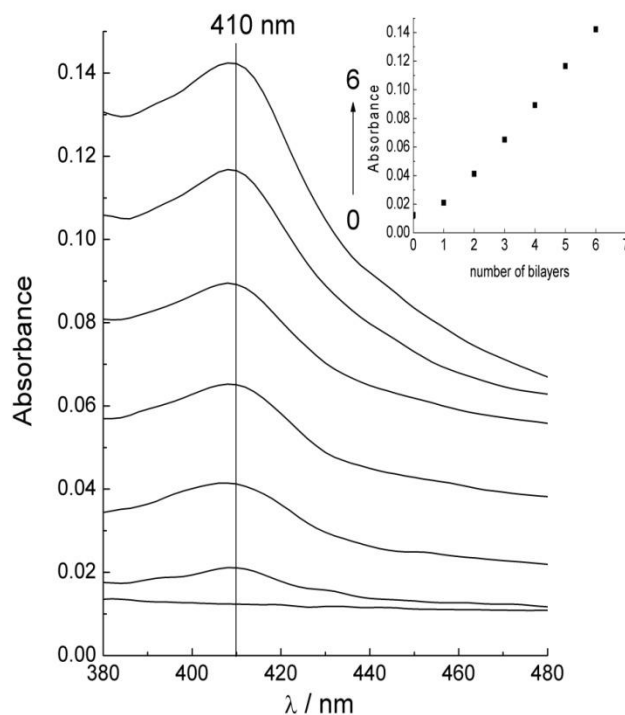


Figure 2. UV-vis spectra for $(\text{GA}/\text{Mb})_n$ films on the quartz slides with varying number from 0 to 6. Inset: Plot of the absorbance vs. the number of bilayers (n)

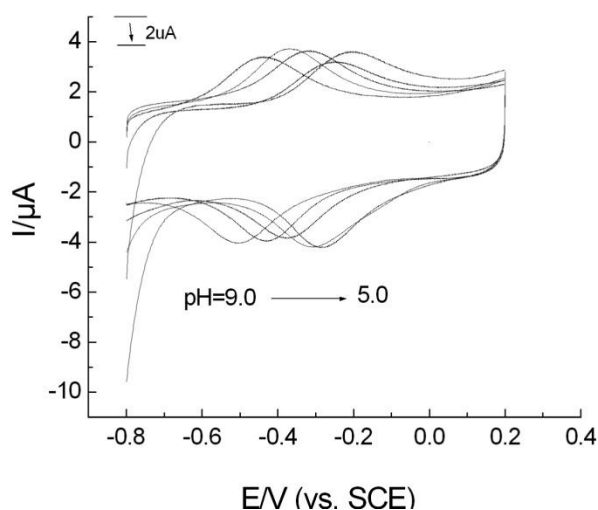


Figure 3. Cyclic voltammograms of the $(\text{GA}/\text{Mb})_6$ film modified electrode at different pH. $v=100 \text{ mV s}^{-1}$.

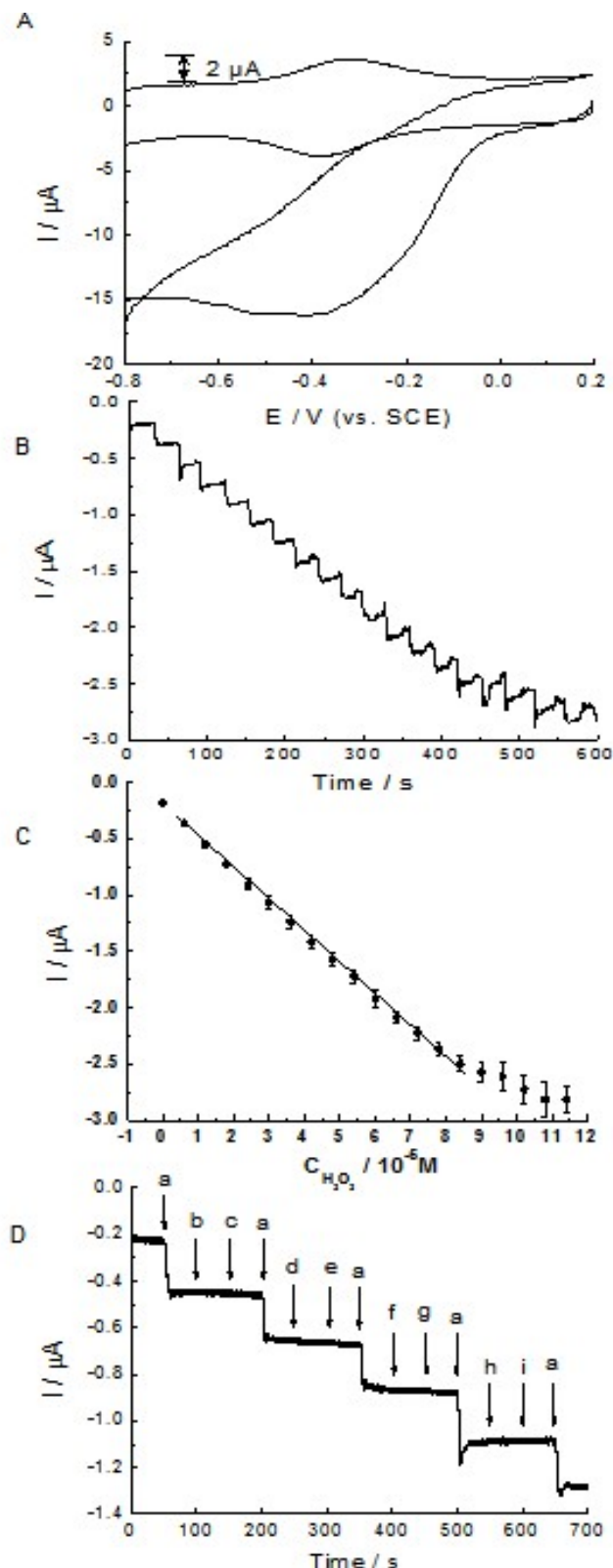


Figure 4. (A) Cyclic voltammograms of the (GA/Mb)₆ film modified electrode in pH 7.0 buffers with (a) 0 M; (b) 8×10^{-5} M H₂O₂ at 100 mV s^{-1} . (B) The hydrodynamic response of the film modified electrode in pH 7.0 PBS upon additions of $6.0 \mu\text{M}$ H₂O₂ every 30 s at potential of -0.350 V .

(C) the plots of current vs concentrations of H₂O₂ (D) The hydrodynamic response of the (GA/Mb)₆ modified electrode in pH 7.0 PBS upon additions of 0.01 mM H₂O₂ (a), 1 mM ascorbic acid (b), 0.1 mM dopamine (c), 0.1 mM uric acid (d), 0.5 mM glucose (e), 0.05 mM L⁻¹ tyrosine (f), 0.1 mM catechol (g), 3.0mM ethanol (h), 10 mM Cd²⁺(i).

3.4. Interference Study

The interference experiments were investigated using amperometry. In this work, the interference species were select as ascorbic acid, dopamine, uric acid, glucose, L-tyrosine, catechol, ethanol, Cd²⁺. Although dopamine, ascorbic acid, ethanol, uric acid and catechol are electroactive substances, their oxidation potential are higher than that of Mb, at the meantime, these substance oxidation signal can be separated from that of Mb, glucose, L-tyrosine have nooxidation signal in this window, so these substance should not be interfered in the determination of H₂O₂. Our judgments were conformed by experiment results and the results were shown in Fig.4D. From Fig. 4D, it was obviously observed that no interference response was obtained for these species at this working potential, which show that the multilayer films modified electrode can be used to detect H₂O₂ in presence of this interfering species.

3.5. Stability

The stability of (GA/Mb)₆ multilayer film was also tested by CV with two different methods. In the solution studies (method I), the modified electrode were stored in a buffer solution all the time, and CVs were measured periodically. Alternatively, with method II, the modified electrode was stored in air, and CVs were run periodically after returning the dried electrodes to a buffer solution. The results of method I showed that the peak currents gradually decreased as the storing time increased. However, the peak currents retained about 95% of the initial response after a two week's storage. With method II, the voltammetric response hardly decreased after 50 cycles. Hence, the multilayer film of (GA/Mb)_n had a better stability. The reason is attributed to a stronger interaction force of GA and Mb, and it is easy for Mb not to lose during experiments.

3.6. Determination of the Michaelis–Menten constant

The determination of Michaelis–Menten constant was following: when the concentration of H₂O₂ further increased, the calibration curve tended to level off. A response plateau was obtained, showing the characteristics of the Michaelis–Menten kinetic mechanism. To obtain K_m, which gives an indication of the enzyme–substrate kinetics, one can use the Lineweaver–Burk equation [27]

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

Here, I_{ss} is the steady-state current after the addition of H₂O₂, I_{max} is the maximum current measured under saturated H₂O₂ solution and C is the H₂O₂ concentration. The K_m in this work was calculated as 0.115 mM. It is well known that a smaller K_m represents a higher catalytic ability. This value was lower than some H₂O₂ sensors based on HRP [28-29]. The low value of K_m indicates that the modified electrode possesses properties of an excellent performance biosensor for the determination of H₂O₂.

4. Conclusions

Multilayers film of (GA/Mb)₆ have been fabricated on the matrix of MWCNTs-CS composite on Au electrode via LBL covalent mode. The assembly process was investigated by CV and UV-Vis spectroscopy. Mb in film kept its biology activity. Moreover, the multilayers film modified electrode exhibited excellently electrocatalytic activity towards the reduction of H₂O₂, indicating the potential application as an H₂O₂ biosensor.

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