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Influence of Electric Field on the Adsorption and Bioactivity of Hemoglobin on A Macroporous Gold Electrode

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Abstract: The adsorption and bioactivity of proteins at interfaces have been widely studied due to their important role in the construction of biosensors, bioelectronics and biofuel cells. Interfacial electric field is one of the important factors which could affect the adsorption and bioactivity of proteins at materials surfaces. It could dramatically change the adsorption density, molecular conformation and orientation at material surfaces. In this paper, the influence of interfacial electric field on the adsorption kinetics and bioactivity of hemoglobin (Hb) on a three-dimensional (3D) macroporous gold electrode surface has been studied using electrochemical methods and infrared spectroscopy. It was found that the interfacial electric field created excess surface charge which would accelerate the adsorption rate of Hb on the substrate by the enhanced electrostatic interactions between the electrode and protein patches. However, higher interfacial electric field could damage the conformation of the adsorbed Hb molecules, resulting in loss of the catalytic activity towards the reduction of hydrogen peroxide. Only at a surface with zero charge, the conformation and bioactivity of the adsorbed Hb molecules can be well retained. This work would provide fundamentals for the construction of biosensors, bioelectronics and biofuel cells, and assist to understand the interfacial behavior of biomolecules on charged biological interfaces.

Key words: adsorption; protein; hemoglobin; charged surface; electric field; biocatalytic activity

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1 Introduction

Protein molecules, with dual hydrophilic and hydrophobic properties, can easily adsorb onto materials surfaces[1] via the weak supermolecular interactions. Such interactions could cause conformation change and even denaturation of the biomolecules in some cases[2-5]. In certain practical biosystems, the adsorption of proteins will disturb the physiological function of human beings. For example, the adsorption of fibrinogen causes platelets to aggregate and coagulate on the blood vessel, which induces myocardial infarction and apoplexy. For avoiding proteins adsorption, Martins et al. synthesized a kind of polymers which effectively reduced the fibrinogen adsorption and thus prevented the adhesion of platelet, cell and even bacterium[6]. Obviously, investigation of the adsorption behavior of proteins at materials surfaces is one of important issues determining their application in food industry, biomaterials, biosensors and clinical medicine[7-13].

Various factors of radiation, interfacial electric field, magnetic field, ionic strength and solution pH value affect the adsorption behavior of proteins at materials surfaces. They could dramatically change the adsorption density, molecular conformation and molec-

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ular orientation at the surfaces[14-17]. Proteins are neutral molecules at their isoelectric points, but the charges around the molecules are usually not uniform, e.g., positive charges or negative charges could form different domains around the biomolecules. Therefore, as soon as biomolecules in solution contacts with a substrate, they can adsorb on the substrate surface via the electrostatic interactions. This adsorption behavior is obviously determined by the interfacial excess charges. It has been reported that solution ionic strength or solution pH could create excess interfacial charges, which in turn affected the adsorption behavior of biomolecules[18-19].

In electrochemical systems, interfacial electric field is an additional variable factor determining the surface excess charges at a conductive electrode. Recently, we have studied adsorption behavior of hemoglobin (Hb) on a macroporous gold electrode at open-circuit potential and found that the assembled Hb molecules retained their secondary structure and bioactivity[20]. In this paper, the influence of interfacial electric field on the adsorption and conformation of proteins on a gold electrode surface was studied by using Hb as probe. Electrochemical methods were used to characterize the adsorption and bioactivity of the adsorbed Hb. The conformation change of the adsorbed Hb molecules was characterized by using infrared spectroscopy. The present work will shed light on the understanding of the adsorption behavior of biomolecules on charged biological interfaces, and also provide fundamentals for the fabrication of biosensors, bioelectronics and biofuel cells.

2 Experimental

2.1 Instruments and Chemicals

Hemoglobin (bovine blood) was purchased from Tokyo Kasei Co., Ltd and used as received. Hydrogen peroxide (H_2O_2) solutions were prepared using 0.1 mol · L^{-1} phosphate buffer solution (PBS, pH 7.0) which was obtained from KH_2PO_4 and Na_2HPO_4 solutions. All other chemicals were of analytical grade. All the solutions were prepared by de-ionized water (> 18 MΩ, Purelab Classic Co., USA).

Electrochemical experiments were carried out on a CHI 650 electrochemical working station (CH Instrument, USA). Measurements were carried out in a three-electrode system: a macroporous gold electrode was used as the working electrode, a saturated calomel electrode (SCE) as the reference and a platinum sheet as the counter electrode. All the potentials in this paper refer to the SCE.

Infrared spectra averaged over 64 scans (resolution of 4 cm^{-1}) were recorded on a Tensor 27 Fourier transfer infrared spectrometer (Bruker, Germany) equipped with a liquid nitrogen cooled mercury cadmium telluride (MCT) detector.

2.2 Procedure

1) Preparation of Macroporous Au Electrode

The fabrication of 3D gold macroporous film electrodes was the same as that reported previously[20]. In brief, monodispersed SiO_2 spheres (320 nm, synthesized using the Stöber method[21]) were firstly assembled on gold slide, forming the highly ordered colloidal crystal template by the vertical deposition method[22]. Before electrochemical deposition, the silica colloidal crystal was sintered at 200 ℃ under nitrogen atmosphere for 2 h. Then, the silica crystal template was immersed into a 5.0×10^{-3} mol · L^{-1} $HAuCl_4$ solution for 1 h prior to electrolyzing in order to allow the solution to penetrate throughout the template. Gold was then electrodeposited into the interspaces of the silica crystal template at 0.5 V. After chemical removal of the silica template using aqueous HF (5%), a highly ordered macroporous Au film was obtained. The electrodeposited Au film consisted of Au nanoparticles with the average particle size of 4.09 nm calculated from the X-ray diffraction results according to reference 20. Such macroporous gold film can provide good microenvironment for proteins.

2) Characterization of the Adsorbed Hemoglobin

Electrode potential induced adsorption of hemoglobin on a gold electrode was carried out at different potentials in a 50 mmol · L^{-1} Hb solution (prepared with pH 7.2 PBS) under nitrogen atmosphere. The electrode potentials were in the range of 0.7 V to −0.4 V with an interval of 0.1 V. Adsorption isotherms at 0.1 V were obtained by changing adsorption time.

3 Results and Discussion

3.1 Influence of Electrode Potential on the Adsorption Behavior of Hb

The Hb was assembled on the gold electrode surface at different potentials from a PBS solution containing 50 mmol · L^{-1} Hb for 1 h. Then, the electrode was washed subsequently with PBS and water. Electrochemical impedance spectra of the Hb assembled marcroporous gold electrode were collected at 0.19 V in a solution containing 0.1 mol · L^{-1} KCl + 10 mmol · L^{-1} $Fe(CN)_6^{3-}$ + 10 mmol · L^{-1} $Fe(CN)_6^{4-}$ are shown in Fig. 1. The electrode modified with Hb at 0.0 V showed the lowest electrochemical impedance. This impedance increased with the increase of the absolute electrode potential for Hb adsorption. Since the electrochemical impedance reflects the electron transfer resistance of the electrochemical probe (ferricyanide/ferrocyanide couple), larger impedance indicates more Hb molecules adsorbed on the electrode surface. It is clear that the surface excess charge increases with the absolute value of the electrode potential deviated from 0.0 V which is roughly close to the zero charge potential[23]. More positive potentials with respect to the zero charge potential will result in more positive excess charges on the electrode surface, while more negative potentials will result in more negative excess charges at the electrode surface. The increased surface excess charges will certainly induce electrostatic interactions between the electrode surface and the Hb molecules due to the existence of positively and negatively charged amino residuals on the surface of the protein molecules[24-25]. This result demonstrated that the surface excess charge, which can be modulated by the interfacial electric field, plays a determining role in the protein adsorption kinetics.

In order to understand the influence of interfacial electric field on the adsorption of Hb at gold electrode, the adsorption isotherms of Hb on a macroporous gold electrode at 0.1 V from a PBS solution

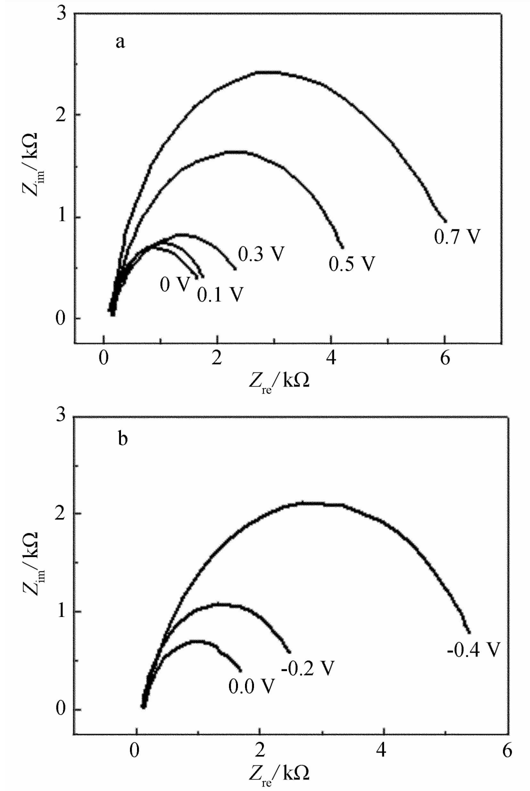

Fig. 1 Nyquist-diagrams of macroporous gold electrodes modified with Hb layers from a solution of 0.1 mol · L^{-1} KCl + 10 mmol · L^{-1} $Fe(CN)_6^{3-}$ + 10 mmol · L^{-1} $Fe(CN)_6^{4-}$ The Hb layers were deposited at different potentials from a PBS solution containing 50 mmol · L^{-1} Hb for 1 h, the electrochemical impedance spectra were collected at 0.19 V in the frequency range of 0.1 Hz to 1.0×10^5 Hz with an amplitude of ± 5 mV

containing 50 mmol · L^{-1} Hb was followed by the electrochemical impedance spectroscopic (EIS) measurements. Since the electrochemical impedance also reflects the surface coverage of Hb adsorbed on the electrode surface[20], we can monitor the adsorption isotherms of Hb on the gold electrode at this potential. The surface coverage (θ) was derived according to the following equation[26-27].

$$\theta = 1 - R_{ct}^{network}/R_{ct}^{Hb} \quad (1)$$

where $R_{ct}^{network}$ denotes the charge transfer resistance of the electrochemical probe at the macroporous gold film electrode and R_{ct}^{Hb} the resistance of the electrochemical probe at the electrode covered by Hb incubating for different time. θ is the surface coverage of Hb on the macroporous gold electrode.

Electrochemical impedance spectra of the macroporous gold electrode adsorbed with Hb for different adsorption time from a solution of 0.1 mol · L^{-1} KCl containing 10 mmol · L^{-1} $Fe(CN)_6^{3-}$ + 10 mmol · L^{-1} $Fe(CN)_6^{4-}$ were collected under potentiostatic control at 0.19 V in the frequency range of 0.1 Hz to 1.0×10^5 Hz. As shown in Fig. 2a, the electrochemical impedance for the electron transfer of ferricyanide/ferrocyanide couple at the gold electrode increased with the deposition time, indicating that the Hb adsorption was a slow process. Based on the equation (1), the coverage of Hb for different adsorption time was estimated and the results are plotted in Fig. 2b. It showed that the surface coverage of Hb on the macroporous gold electrode firstly increased exponentially with adsorption time. Then, it leveled off after 90 min, indicating that a saturation layer of Hb was formed. By comparison with the results for Hb adsorbed at open circuit potential[20], it can be concluded that the Hb adsorption amount increased dramatically when an interfacial electric field was applied. The impedance of the modified electrode with saturation adsorption of Hb at open circuit potential and at external potential of 0.1 V were 3295Ω and 5865Ω, corresponding to a adsorption coverage of 87.4% and 93.1%, respectively. In addition, when the interfacial electric field of 0.1 V was applied, the time for reaching saturation adsorption was 90 min, which was only the half of the saturation adsorption time at open circuit potential. The results clearly showed that the surface electric field accelerated the adsorption rate and adsorption amount of Hb on the gold electrode via the electrostatic interactions between the surface excess charges and the protein.

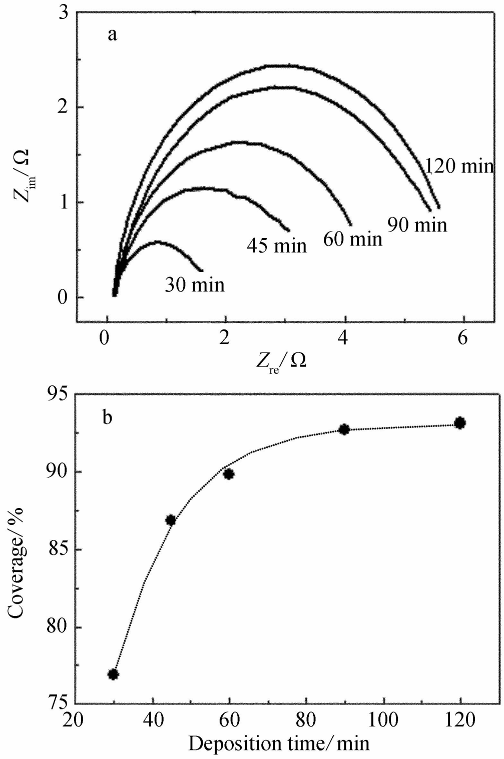

Fig. 2　Nyquist-diagrams of a macroporous gold electrode modified with Hb layers from a solution of 0.1 mol · L^{-1} KCl + 10 mmol · L^{-1} $Fe(CN)_6^{3-}$ + 10 mmol · L^{-1} $Fe(CN)_6^{4-}$ (a) and plots of the surface coverage of Hb as a function of deposition time (b) based on Figure 2a

3.2　IR Characterization of the Secondary Structure of the Absorbed Hb

The secondary structure change of the Hb adsorbed on gold electrode at different potentials was characterized by the infrared spectroscopy (IR). Hb was deposited on macroporous gold electrodes at different potentials from a PBS solution containing 50 mmol · L^{-1} Hb for 1 h. As shown in Fig. 3, when the Hb was deposited in the potential region between −0.3 V and 0.5 V, the amide I and amide II bands located at 1658 cm^{-1} and 1578 cm^{-1}, respectively. The amide I band is attributed to the C—O stretching vibration of peptide linkages in the backbone of protein. The amide II band results from a combination of N—H bending and C—N stretching[27]. Appearance of these two bands demonstrated that the secondary structure of Hb deposited at 0.1 V was retained. The band intensity indicates the coverage of the secondary structure retaining Hb molecules. When the Hb adsorbed on the macroporous gold electrode at 0.0 V and 0.1 V, the intensities for the amide I and amide II bands had the highest values. When the deposition potential for Hb increased from 0.1 V to 0.7 V, the band intensities decreased rapidly, e. g. , at a deposition potential of 0.7 V, these two bands almost disappeared. In addition, when the deposition potential went to the negative potentials with respect to 0.0 V, similar results as in the positive potential region were obtained. These results demonstrated that the Hb coverage with native secondary structure decreased with the increase of deposition potential. However, the result in Fig. 1 showed that the amount of Hb on the macroporous gold electrode increased with the increase of the absolute external potential deviated from 0.0 V. Therefore, we can conclude that although the interfacial electric field could enhance the adsorption amount of Hb on the macroporous gold electrode, the most of the deposited Hb secondary structure were damaged.

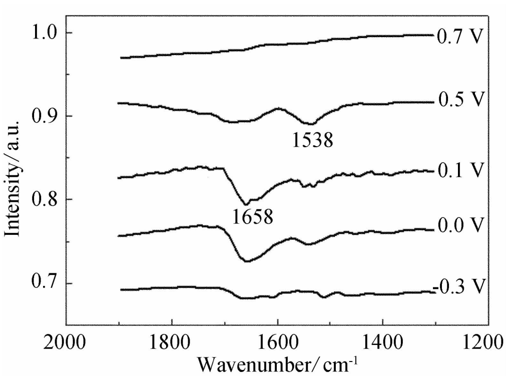

Fig. 3 ATR-FTIR spectra of Hb adsorbed on a macroporous gold film electrode at different potential in a PBS solution containing 50 mmol · L^{-1} Hb for 1 h

3.3 Influence of Deposition Potential on the Catalytic Activity of Hb

Under normal physiological conditions, hemoglobin can catalyze the electrochemical reduction of H_2O_2. The electrocatalytic activity of Hb adsorbed at different potentials towards the electrochemical reduction of hydrogen peroxide was characterized. Fig. 4 shows the cyclic voltammograms (CV) of the macroporous gold electrode adsorbed with Hb in a PBS solution containing 10 mmol · L^{-1} H_2O_2. It is clear that the dotted lines in CV curves of the Hb modified macroporous gold electrode showed the characteristics for the direct electron transfer of deposited Hb on gold electrode when the Hb molecules were deposited at 0.0 V. The oxidation and reduction peaks of Hb appeared at 0.27 V and 0.09 V, respectively, which suggested that the adsorbed Hb molecules had good electrochemical activity, e. g., the electrochemically active center of adsorbed Hb can exchange electron with the gold electrode. However, as the adsorption potential increased, the current peak for the direct electron transfer of Hb decreased significantly. This change demonstrated that the electrochemical activity of the adsorbed Hb would be gradually lost as the adsorption potential increased. This result reminded us to study the biocatalytic activity of the Hb on the electrode under potential control. In this case, we used hydrogen peroxide as a probe to study the influence of interfacial electric field on the biocatalytic activity of the adsorbed Hb. As shown by the solid curves in Fig. 4, the influence of the interfacial electric field on the biocatalytic activity of the adsorbed Hb was similar to the electrochemical activity. Higher interfacial electric field considerably decreased the bioactivity of Hb towards the reduction of hydrogen peroxide. When the Hb adsorbed at 0.0 V, a larger catalytic reduction current was observed upon addition of H_2O_2, indicating that the Hb immobilized at 0.0 V showed good

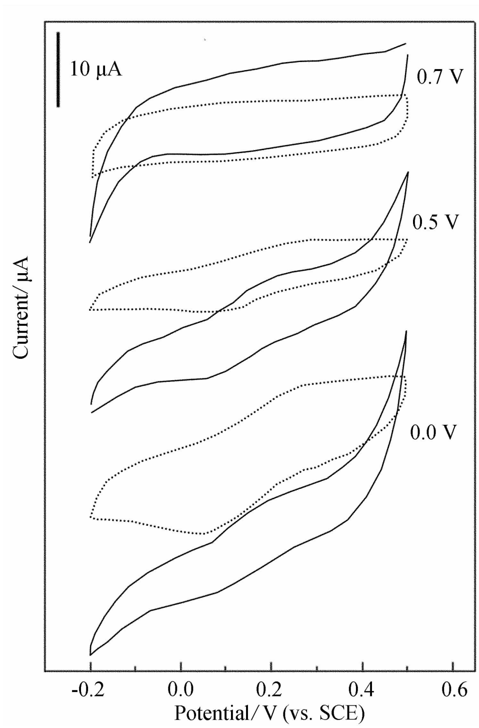

Fig. 4 Cyclic voltammograms (CVs) of Hb adsorbed on a macroporous gold electrode in pH 7.0 PBS containing 10 mmol · L^{-1} H_2O_2, the CVs for the Hb deposited gold electrode in the base electrolyte of PBS were also displayed as dotted curves (scan rate: 0.01 V/s)

biocatalytic activity. When the adsorption potential was increased 0.5 V, an electrochemical catalytic reduction current for hydrogen peroxide could still be seen, but the current was much smaller than that for 0.0 V. If the adsorption was increased to 0.7 V, no biocatalytic activity of the Hb on gold electrode was observed. These results are in good agreement with the IR results in Fig. 3.

These results let us know that although strong interfacial electric field enhances the adsorption amount and adsorption rate of Hb on the macroporous electrode as indicated by the impedance measurements, it will change the conformation of Hb molecules on the electrode surface, resulting in loss of electrochemical activity and biocatalytic activity of the immobilized protein molecules.

4 Conclusion

In summary, we emphasized on the importance of surface excess charges in determining the adsorption of proteins on a gold electrode surface. Although high electric field accelerated the Hb adsorption, the secondary structure and bioactivity of the adsorbed Hb could not be retained due to the strong electrostatic interactions. Since biological interfaces are usually charged, the present work will shed light on the understanding of the adsorption behavior of biomolecules on charged biological interfaces, and also provides fundamentals for the fabrication of biosensors, bioelectronics and biofuel cells.

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界面电场对血红蛋白在多孔金电极上的吸附和生物活性的影响

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摘要： 蛋白质的界面吸附及其生物活性因它在构建生物传感、生物电子器件和生物燃料电池等方面具有重要的作用而倍受关注. 对此，界面电场是吸附的一个重要影响因素，它能明显地影响蛋白质分子在材料界面的吸附量、分子构象以及分子定向. 本文应用电化学方法和红外光谱技术研究了血红蛋白在三维多孔金膜电极上的吸附动力学及其生物活性随界面电场的变化关系. 结果表明，由界面电场产生的过量表面电荷可借助与蛋白质分子之间的静电作用加速蛋白质分子在电极表面的吸附，提高其吸附量；但是，过高的界面电场将破坏吸附蛋白质的构象以及降低它还原过氧化氢的催化活性；只有在零电荷电位下，吸附在电极表面的血红蛋白才能保持其天然的构象和生物催化活性. 本研究将为生物传感器、生物电子器件和生物燃料电池的构建提供理论依据，加深对荷电生物界面上生物分子界面行为的认识.

关键词： 吸附；蛋白质；血红蛋白；荷电界面；界面电场；生物催化活性