

## Electrochemical Behavior of Redox Proteins Immobilized on Nafion-Riboflavin Modified Gold Electrode

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Electron transfer of a redox protein at a bare gold electrode is too slow to observe the redox peaks. A novel Nafion-riboflavin functional membrane was constructed during this study and electron transfer of cytochrome *c*, superoxide dismutase, and hemoglobin were carried out on the functional membrane-modified gold electrode with good stability and repeatability. The immobilized protein-modified electrodes showed quasi-reversible electrochemical redox behaviors with formal potentials of 0.150, 0.175, and 0.202 V versus Ag/AgCl for the cytochrome *c*, superoxide dismutase and hemoglobin, respectively. Whole experiment was carried out in the 50 mM MOPS buffer solution with pH 6.0 at 25 °C. For the immobilized protein, the cathodic transfer coefficients were 0.67, 0.68 and 0.67 and electron transfer-rate constants were evaluated to be 2.25, 2.23 and 2.5 s<sup>-1</sup>, respectively. Hydrogen peroxide concentration was measured by the peroxidase activity of hemoglobin and our experiment revealed that the enzyme was fully functional while immobilized on the Nafion-riboflavin membrane.

**Key Words :** Nafion-riboflavin membrane, Cytochrome *c*, Superoxide dismutase, Hemoglobin, Peroxidase

### Introduction

It is well known that biological macromolecules exhibit a rather slow rate of heterogeneous electron transfer at conventional electrodes, ascribed to either their extended three-dimensional structures or the resulting inaccessibility of the electro-active center or to their adsorption onto and subsequent passivity of the electrode surface.<sup>1</sup> Chemically modified electrodes have been prepared in order to overcome this problem.<sup>1-4</sup> Two kinds of modified electrodes have been applied. One is a mediator-modified electrode while the other is a promoter electrode. The difference is due to the electro-activity of the modifiers. The mediator-modified electrode has a component, which takes part in electron transfer in the potential range of interest, while the promoter electrode does not. Therefore, the materials served as mediator can yield redox waves. Commonly, small organic molecules and some polymeric materials have been used as mediators and promoters.<sup>1,4</sup> It was shown that redox behavior of a protein is related to the type of working electrode, protein solution, fixing and its method, protein concentration, type of mediator or promoter used in experiment.<sup>1,4</sup> The redox behavior of a protein can be used as design a biosensor, measurement of protein concentration in the solution and structural investigation of this protein. The redox potential of a protein depends on many factors. These factors have been found to determine the intrinsic redox potential of heme proteins include the axial ligation pattern, orientation of the ligands, porphyrin conformation, solvent exposure of the heme and thus the polarity and

hydrophobicity of heme pocket, interactions of heme and its ligands with protein environment.<sup>5-12</sup> Recently, different methods are being used to improve electron transfer between electrode and redox proteins. Great progress in this field has shown that the modified films on electrodes may provide a favorable microenvironment for proteins to exchange electrons directly with underlying electrodes, and thus afford a new opportunity for the detailed study of enzyme electrochemistry.<sup>13-15</sup> Successful approaches have included cast films of proteins with insoluble surfactants,<sup>16</sup> biological organic substances,<sup>17</sup> inorganic membranes,<sup>18</sup> and films of proteins and poly ions growing.<sup>19,20</sup> In previous work of our laboratory, a Nafion-cysteine modified electrode was constructed for the electron transfer of proteins.<sup>21</sup> In recent study, we have developed a new kind of functional membrane composed of riboflavin and Nafion. The functional membrane could provide suitable sites for adsorbing biomolecules, thus promoting electron transfer of proteins on the gold electrode. In this report, a Nafion-riboflavin membrane was used not only to facilitate the electron transfer rate but also to offer a biocompatible micro-environment on the electrode surface. The electron transfer of redox proteins, including horse heart cytochrome *c* (Cyt *c*), superoxide dismutase (SOD) and hemoglobin (Hb) was studied. Hydrogen peroxide catalysis was carried out by the hemoglobin, immobilized on the Nafion-riboflavin membrane, for checking the activity of the protein after immobilization.

Nafion is a per-fluorinated anionic polyelectrolyte. The increasing popularity of Nafion for the fabrication of redox polymer-modified electrodes in recent years arises from its

ease of use, good electrical conductivity and the high partition coefficients of many redox compounds in Nafion. In addition, Nafion film has high chemical stability, good biocompatibility and the ability to resist interferences from anions and biological macromolecules.<sup>22-24</sup>

Flavins work as co-factors and form an integral part of the redox active sites of many different enzymes involved in dehydrogenation reactions, dioxygen activation, and electron transfer reactions.<sup>25,26</sup> The importance of flavins in enzymatic catalysis has motivated studies of the electrochemical behavior of monolayers or aggregates containing synthetic flavins.<sup>28</sup> The flavin nucleotide has the unique ability to transfer one or two electrons and to promote easy reduction of molecular oxygen.<sup>28,29</sup> Flavins also work as redox coenzymes in many biological transformations and, hence, they are being used in this study as the electron transfer facilitators between the electrode surface and redox proteins.

### Materials and Methods

**Materials.** Hemoglobin (Hb), superoxide dismutase (SOD) and Nafion® (5 wt % ethanol solution) were purchased from Sigma. cytochrome *c* (Cyt *c*) and other reagents (analytical grade) were purchased from Merck. The aqueous riboflavin solution was freshly prepared for using. The solutions were prepared in deionized double distilled water (18 MΩ cm, Barnstead, Dubuque, USA).

**Preparation of Nafion-riboflavin-protein modified gold electrode.** First the gold electrode was mechanically polished twice with alumina (particle sizes 10 and 0.3 μm) to a mirror finish. Then, it was ultrasonically treated in water for 10 min. Thereafter, it was treated electrochemically in 0.2 M sulfuric acid, cycling between -0.2 and +1.5 V (vs. Ag/AgCl) at a sweep rate of 0.1 Vs<sup>-1</sup> until the appearance of a clean gold electrode was obtained. Finally the electrode was washed with deionized double distilled water. Nafion solution (2 μL, 5%) was dropped onto the surface of freshly prepared gold electrode and dried at room temperature for 20 min. Then the electrode was dipped into a freshly prepared riboflavin solution (1 mM) for 10 min and carefully was washed with deionized double distilled water.

The protein (Cyt *c*, Hb or SOD) was immobilized by dropping 2 μL of 10 mg/mL of the protein solution on the nafion-riboflavin modified gold electrode and dried for about 30 min at room temperature. Then the electrode was gently washed with deionized double distilled water and stored at 4 °C when not in use.

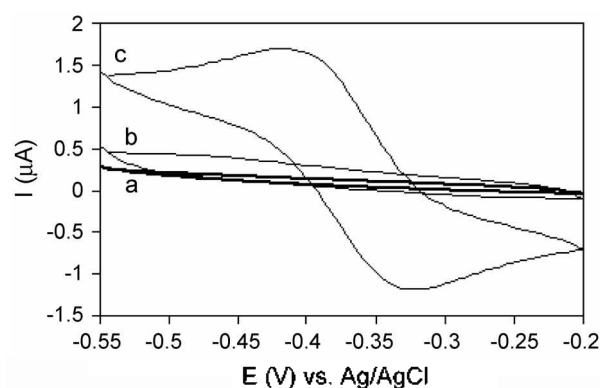
**Apparatus and measurements.** Electrochemical measurements were carried out with a potentiostat/galvanostat (Model 263A, EG&G, USA) using a single-compartment voltammetric cell equipped with a platinum rod auxiliary electrode, an Ag/AgCl reference electrode (Metrohm) and a gold working electrode with a disk diameter of 1 mm (Azar Electrode, Iran). The experimental solutions were deaerated using highly pure nitrogen for 30 min and a nitrogen atmosphere was kept over the solutions during measurements. All

the electrochemical measurements were carried out in 50 mM MOPS buffer solution, pH 6.0 at 25 ± 1 °C.

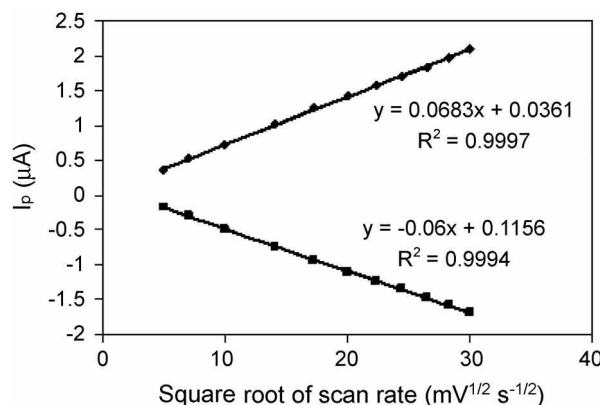
### Results and Discussion

Cyclic voltammograms (CVs) obtained for the gold electrode (Figure 1a) and Nafion modified gold electrode (Figure 1b) did not show any current peak in the potential range of -0.2 to -0.55 V, while for the Nafion-riboflavin modified gold electrode (Figure 1c) a clear redox process can be observed indicating that riboflavin was immobilized on Nafion, presenting a good electrochemical signal. The riboflavin may accept two electrons from the electrode and two protons from the solution to change into RFH2.<sup>30</sup> Formal potential,  $E^0$ , determined using the following equation:  $E^0 = (E_{pc} + E_{pa})/2$ , where  $E_{pc}$  is the cathodic and  $E_{pa}$  the anodic peak potential, was about -0.365 V at pH 6.

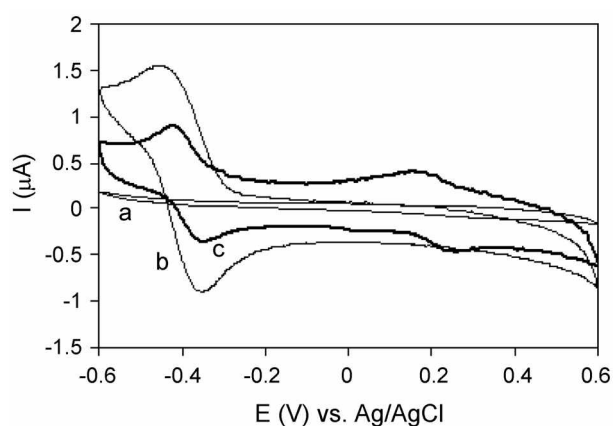
A linear dependence of the anodic and cathodic peak current on the square root of scan rates,  $v^{1/2}$ , is illustrated in Figure 2 for riboflavin. This behavior suggests a diffusion-controlled redox process. However, as no decrease in peak current after many repeated cycles was observed. Our experiments showed this film has very good stability after



**Figure 1.** Cyclic voltammograms obtained in 50 mM MOPS buffer solution (at pH 6.0) for the (a) gold electrode, (b) Nafion-modified gold electrode and (c) Nafion-riboflavin modified gold electrode at a scan rate of 100 mV/s.



**Figure 2.** Dependence of the cathodic and anodic peak currents on the square root of the scan rate obtained for Nafion-riboflavin modified gold electrode in 50 mM MOPS buffer, pH 6.0.

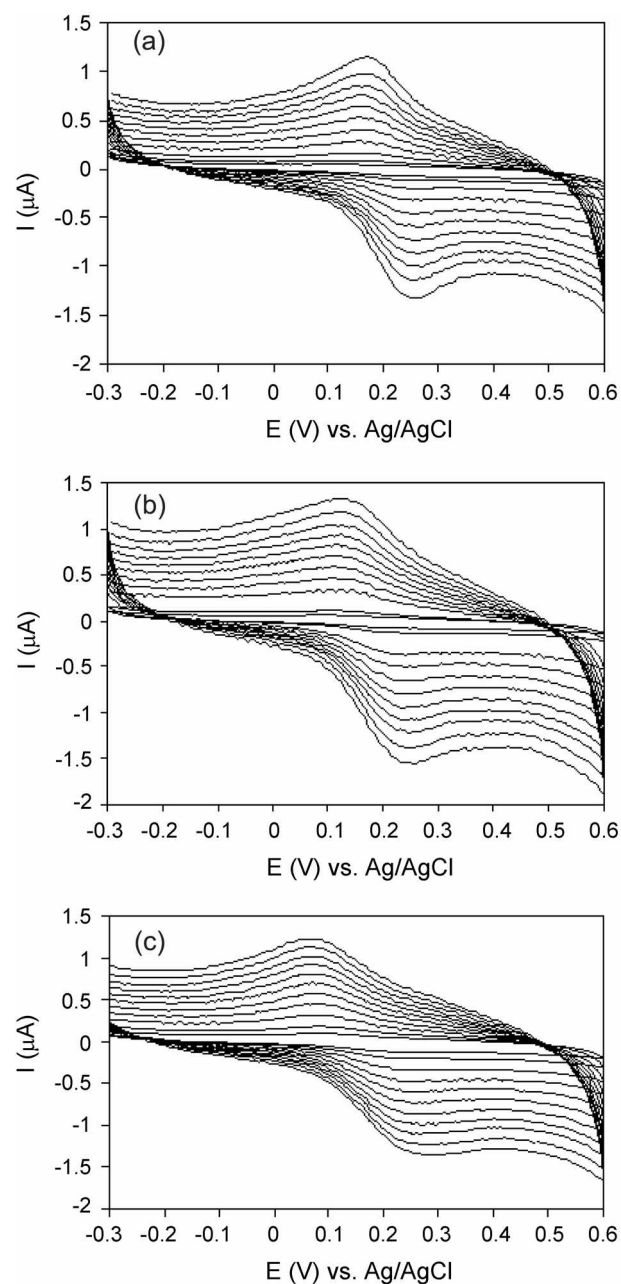


**Figure 3.** Cyclic voltammograms (CVs) obtained in 50 mM MOPS buffer solution at pH 6.0 for (a) Nafion, (b) Nafion-riboflavin and (c) Nafion-riboflavin-protein modified gold electrode at a scan rate of 100 mV/s.

putting it in 4 °C for 20 days (data is not shown), indicating that the riboflavin is strongly adsorbed on the Nafion, the redox process could be controlled by the diffusion of counter ions to keep the electro neutrality on the electrode surface. Other possibilities for this behavior are due to resistance of the material or a mechanism of electron transfer occurring by the electron jumping process. This data is according with previous work in fixing riboflavin in the surface electrode by another way.<sup>30</sup>

Figure 3 represents CVs of Nafion (Figure 3a), Nafion-riboflavin (Figure 3b) and Nafion-riboflavin-protein (Figure 3c) modified gold electrode in the potential range of +0.6 to -0.6 V. As show in this figure, riboflavin can act as a facilitator for existence of redox behavior of proteins.

Figure 4 represents the electron transfer properties of the immobilized redox proteins (Hb, SOD or Cyt c) on the Nafion-riboflavin modified gold electrode at different sweep rates. A pair of well-defined redox peaks was observed in the potential range of -0.3 to +0.6 V, which could be ascribed to the electron transfer of protein at the modified electrode at each sweep rate. The electron transfer of the redox protein at the bare gold electrode is very slow so that the redox peak usually cannot be observed.<sup>31,32</sup> Furthermore, no redox peaks were observed for redox proteins at the Nafion-modified gold electrode (without riboflavin) nor for the Nafion-riboflavin modified gold electrode at -0.3 to +0.6 V range. However, the Nafion-riboflavin functional membrane significantly facilitates the electron-transfer rates of proteins. Nafion improves the stability of the proteins and offers a biocompatible microenvironment on the electrode surface.<sup>27,29</sup> The electrochemical and kinetic parameters of the proteins immobilized on the functional membrane are given in Table 1. The rate constant of the electrode reaction ( $k_s$ ) and cathodic transfer coefficient ( $\alpha_c$ ) for the redox reaction of the proteins confined on the modified gold electrode were evaluated based on Laviron's Equation.<sup>33,34</sup> Based on the observed CVs of the proteins (Fig. 4), the peak currents increased with increasing sweep rate ( $\nu$ ) and they were



**Figure 4.** Cyclic voltammograms of the gold electrode modified with: (a) Nafion-riboflavin-Hb, (b) Nafion-riboflavin-SOD and (c) Nafion-riboflavin-Cyt c in a 50 mM MOPS buffer solution (pH 6.0). Sweep rates (from inner to outer) for (a), (b) and (c) are as follows: 0.025, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 and 0.9 V s<sup>-1</sup>.

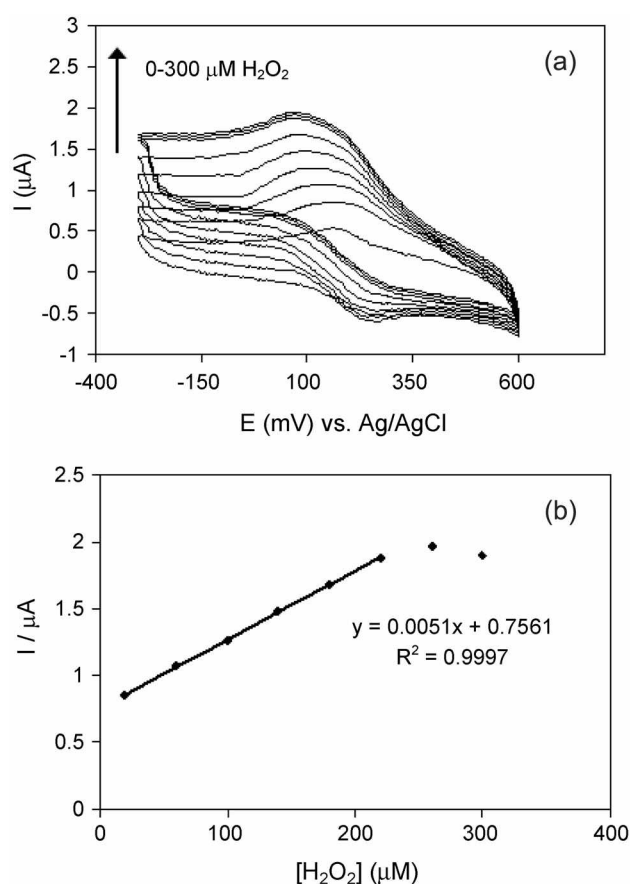
proportional to  $\nu$  (not  $\nu^{1/2}$ ) in the range of 0.025-1.0 V s<sup>-1</sup>. The integration of reduction peaks at different sweep rates gave a nearly constant charge (Q) value. These are characteristics of diffusion less, thin-layer electrochemical behavior.<sup>35</sup> As represented in Table 1, the proteins have nearly the same cathodic transfer coefficients (0.67) in the redox reaction.

Figure 5a represents the incorporation of hemoglobin in the Nafion membrane, which functions as an effective catalyst for the reduction of H<sub>2</sub>O<sub>2</sub>. The calibration curve (figure 5b) shows the linear dependence of the cathodic peak

**Table 1.** Electrochemical parameters of redox proteins on Nafion-riboflavin functional membrane modified gold electrode

Proteins ( <i>s</i> <sup>-1</sup> )	Molecular weight (MW)	Electrode reaction	<i>E</i> <sup>o</sup> (mV)	$\Delta E_p$ (mV)	<i>αc</i>	<i>k</i> <sub>s</sub>
			vs. (Ag/AgCl)			
Horse heart cytochrome c	12,380	Cyt <i>c</i> [heme(Fe <sup>3+</sup> )] + e <sup>-</sup> ⇌ Cyt <i>c</i> [heme (Fe <sup>2+</sup> )]	150	180	0.67	1.7
Bovine erythrocyte superoxide dismutase	32,500	SOD (Cu <sup>2+</sup> ) + e <sup>-</sup> ⇌ SOD (Cu <sup>+</sup> )	175	140	0.68	2.23
Hemoglobin	66,000	Hb [heme(Fe <sup>3+</sup> )] + e <sup>-</sup> ⇌ Hb [heme (Fe <sup>2+</sup> )]	202	105	0.67	2.5

$E^o$  and  $\Delta E_p$  were estimated as  $(E_{pa} + E_{pc})/2$  and  $(E_{pa} - E_{pc})$  in the CVs obtained with the modified electrodes in 20 mM, pH 7.0 potassium phosphate buffer solution at 100 mV/s.  $E_{pa}$  and  $E_{pc}$  were anodic and cathodic peak potentials of the proteins.



**Figure 5.** (a) Cyclic voltammograms obtained at a Nafion-riboflavin-Hb modified gold electrode for a 50 mM MOPS buffer solution (pH 6) for 0–300  $\mu\text{M}$  concentrations of  $\text{H}_2\text{O}_2$  and (b) the relationship between cathodic peak current of hemoglobin and different concentrations of  $\text{H}_2\text{O}_2$ .

current on the  $\text{H}_2\text{O}_2$  concentration in the range of 20–220  $\mu\text{M}$ . The relative standard deviation was 2.9% for 4 successive determinations at 40  $\mu\text{M}$  and the detection limit was 15  $\mu\text{M}$ . The recent experiment has introduced a new biosensor for the sensitive determination of  $\text{H}_2\text{O}_2$  in the solution. The data also shows that the protein, immobilized on the riboflavin-Nafion membrane, did not lose its activity.

In addition, the measured formal potentials of the Cyt c, SOD and Hb on the modified membrane were 150, 175 and 202 mV, respectively. It seems that the combination of Nafion and riboflavin provides suitable sites for the adsorption of biomolecules. However, understanding the property of Nafion-riboflavin functional membrane is much more complicated. We expect that when the Nafion-riboflavin functional membrane acts as the electron transfer mediator at the gold electrode surface, the Nafion offers a biocompatible microenvironment to confine the bio-macromolecules in its ionic cluster region (30–50 nm), based on a stylized, semi-empirical view of a polar/nonpolar micro-phase separation in a hydrated ionomer.<sup>36,37</sup> Moreover, the CVs of the proteins in Figure 4 and the separation between the anodic and cathodic peak potentials ( $\Delta E_p$ ) in Table 1 indicate that the redox peaks of Hb are sharper than those scanned for Cyt c and SOD, suggesting that Hb is more suitable to carry out the electron transfer under the same experimental conditions on the Nafion-riboflavin functional membrane.

### Conclusion

A novel Nafion-riboflavin functional membrane presented that significantly facilitate the electron-transfer rate of proteins. This study has revealed that the Nafion-riboflavin membrane acts as a mediator of electron transfer between the protein molecules and the gold electrode. The similarity of the cathodic transfer coefficients of all the tested proteins (Hb, SOD and Cyt c) is due to the special property of riboflavin where it affects as a facilitator of the electron transfer between a protein and the surface of electrode. In addition, the proportionality of peak currents to sweep rate and the integration constancy of the redox peaks at different scan rates represents the characteristics of diffusion-less and thin-layered electrochemical behavior. The results also determine that the modified membrane did not lose the activity of proteins after immobilization.

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