Electrocatalytic Reduction of Hemoglobin at a Self-Assembled Monolayer Electrode Containing Redox Dye, Nile Blue as an Electron-Transfer Mediator

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Keywords Hemoglobin, Nile Blue, electrocatalytic reduction

The investigation of the oxidation-reduction characteristics of biological compounds is important for understanding biological processes and developing various biosensor devices on the basis of the use of enzyme or receptor functions. Although there are many biocomponents having a redox center, it is difficult to transfer electrons directly between biocomponent and electrode in many cases, particularly if the redox center is surrounded by a protein matrix.^{1,2} Many studies have been performed to activate various biocomponents to obtain the electrochemical information.³⁻⁷ One of these approaches includes the use of a mediator as an electron transfer intermediate which carries out the chemical oxidation or reduction of the biocomponent.^{1,2,7-13}

Hemoglobin (molecular weight 64500), the main component in red blood cells, is a protein responsible for the transport of O₂ and CO₂ in primarily mammalian body tissues. Any abnormalities in Hemoglobin composition or content result in clinical diseases such as anemia or excessive loss of blood. Therefore, the detection of hemoglobin is very important in clinical medicine. Generally, it has been carried out by spectrophotometry using cyanidation with KCN. However, these methods often require highly purified reagents and are also time-consuming. In addition, the toxicity of KCN is a major problem in handling and disposal. On the other hand, the electrochemical behavior of hemoglobin has been reported using the polymer-modified^{10,11}, chemically modified^{7,13} and Nafion modified electrodes.¹² Some dyes such as Methylene Blue^{7,12}, Azure A¹¹, Brilliant Cresyl Blue¹⁴, Nile Blue¹⁰ or riboflavin¹³ have been used as a mediator for hemoglobin. However, few studies on introducing these mediators into a self-assembled monolayer on an electrodes have been reported.

In this study, we investigated the electrochemical behavior and the detection of hemoglobin on the basis

of an electrocatalytic reduction by a mediator on SAM electrode. Redox dye, Nile Blue was selected as a mediator and was immobilized at an electrode by the self-assembly technique. In order to examine the features of an NB-SAM electrode, the surface density of the mediator at the electrode was estimated, and the electrocatalytic activity for hemoglobin was demonstrated.

Experimental

Reagents

Hemoglobin (from bovine blood) was obtained from Wako Pure Chemicals Co. (Osaka, Japan). Nile Blue chloride, *N*-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) were purchased from Sigma. 3,3'-Dithiodipropionic acid was supplied from Aldrich Chemical Co. Phosphate buffer used was prepared with 0.1 M KH₂PO₄ and 0.1 M NaOH. Prior to each voltammetric measurement, a sample solution was deoxygenated by deaeration for 15 min at least with high quality nitrogen gas. All other reagents were of analytical reagent grade and were used without further purification. Deionized and twice distilled water was used in all experiments.

Apparatus

All voltammetric measurements were carried out by a CV-50W Voltammetric Analyzer (Bioanalytical Systems Inc. (BAS), USA). A three-electrode system was used in all electrochemical experiments. A gold disk electrode with a diameter of 1.6 mm was purchased from BAS (Model NO. 11-2014). A platinum wire was used as a counter electrode, and a Ag/AgCl electrode (Model NO. 11-2020, BAS) as a reference electrode. All potentials were measured against the Ag/AgCl electrode. The pH of buffer solutions were measured by a Horiba pH METER M-13.

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Fig. 1 Scheme for stepwise organization of the NB-SAM electrode.

Fig. 2 Progressive cyclic voltammograms at A) NB-modified electrode, B) NB-SAM electrode in phosphate buffer solution (pH 5.5). Scan rate: 100 mV/s.

Preparation of the NB-SAM electrode

A gold electrode was polished sequentially with 0.3 and 0.05 μm alumina and washed with concentrated nitric acid for 15 min. After rinsing with water, the potential scan between -600 mV and 1400 mV (*vs.* Ag/AgCl) was repeated until a reproducible voltammogram was obtained. The cleaned electrode was immersed for 2 h at room temperature in a 10 mM 3,3'-dithiodipropionic acid ethanolic solution, followed by rinsing with water. Then a mixed solution of 100 mg/ml NHS and EDC was placed on the electrode surface for 30 min in a 100% humidity environment to prevent the solution from evaporating. The surface was then rinsed with water and immersed in ethanol:phosphate buffer solution (pH 8.0)=3:2(v/v)% including 1 mg/ml Nile Blue for 5 h at room temperature and then rinsed with water. This stepwise organization of a NB-SAM electrode was illustrated in Fig. 1.

Results and Discussion

Characterization of the NB-SAM electrode

The stability of the NB-SAM electrode was compared with that of the modified with NB electrode based on adsorption of NB. The NB-modified electrode was prepared by immersing a bare Au electrode into 1 mg/ml NB solution for 5 h at room temperature. The NB-modified electrode was then rinsed thoroughly with water. In this case, NB is absorbed only by a hydrophobic interaction between NB and the surface of a bare Au electrode. Figure 2 shows the progressive cyclic voltammograms obtained with the NB-SAM electrode and the NB-modified electrode in the buffer solution by repeated potential scanning within the range of 0 to -600 mV for 20 times at a scan rate of 100 mV/s. The cyclic voltammograms obtained at a NB-modified electrode indicated that both oxidative

Fig. 3 A) Cyclic voltammograms of NB-SAM electrode in phosphate buffer solution (pH 5.5) at scan rates (from inner to outer): 30, 50, 70, 90, 120, 150 mV/s. B) Relationship between the cathodic peak current and scan rate.

and reductive peak currents decreased with the repetitive sweeps (Fig. 2A) because of the elution of NB from the electrode. However, the decrease in the peak currents at the NB-SAM electrode was much smaller than that at the NB-modified electrode (Fig. 2B). This is because the leakage of NB molecules from the electrode into the aqueous solution is suppressed through the construction of NB-SAM.

Figure 3A shows the cyclic voltammograms of an NB-SAM electrode in a phosphate buffer solution (pH 5.5) with various scan rates. The peak current is directly proportional to the scan rate (Fig. 3B). It indicates that the electrode process of NB at the SAM electrode was controlled by NB assembled at the electrode surface, but not by NB which diffused from the solution. Moreover, the cathodic peak potential (E_{pc}) shifts toward more negative values and the anodic peak potential (E_{pa}) to more positive values with increasing scan rate. The surface density (Γ) of NB at the SAM electrode was estimated by integrating the peak area under the cathodic peak according to the following equation:

$$Q = nF\Gamma$$

The density of NB in the SAM was 5.54×10^{-11} mol/cm². This value is within the range of the values reported for the surface density of thionine and pyrroloquinoline quinone (PQQ) at the SAM electrodes, 7.4×10^{-12} mol/cm² and 1.2×10^{-10} mol/cm², respectively.^{2,8} Consequently, we conclude that the monolayer of NB molecules is formed on the surface of the SAM electrode.

Electrocatalytic reduction of hemoglobin at the NB-SAM electrode

Figure 4A shows the cyclic voltammograms of the NB-SAM electrode in the absence and presence of 0.3 mM hemoglobin. An obvious increase in the cathodic current and a decrease in the anodic current (curve b)

were observed, compared with the current curves obtained in the buffer solution without hemoglobin (curve a). This suggests that the NB-SAM electrode has a catalytic activity for the reduction of hemoglobin. The electrode process can be expressed by the following EC catalytic mechanism.

The NB_{ox} in a SAM was reduced at the electrode to the NB_{red}. Then the NB_{red} was oxidized to NB_{ox} by the HbFe³⁺ diffusing up to the electrode surface. The cathodic current enhancement is attributed to the regeneration of the NB_{ox} by the reduction of hemoglobin during the sweep.

The optimal pH of the catalytic activity at the NB-SAM electrode for the reduction of hemoglobin was about 5. At higher pH than the isoelectric point of hemoglobin (pI=6.8), the catalytic currents decreased because of the electrostatic repulsion between hemoglobin and carboxyl group (–COO[–]) of the electrode surface, which was formed by the hydrolysis of unreacted succinimide ester on the electrode surface. On the other hand, the catalytic currents also decreased at lower pH. Although this seems to be due to a shift of the redox potential of NB in SAM from the potential adequate for reduction of hemoglobin by pH, the detailed reason is not clear.

In order to clarify one of the features of the NB-SAM electrode, the absorption behavior of hemoglobin at a bare Au electrode was investigated. Figure 5 (curve a) shows the cyclic voltammograms of 1.0 mM potassium ferrocyanide at a bare Au electrode in a phosphate buffer solution (pH 8.0). When 20 μM hemoglobin was added in the solution, a decrease in the response current of potassium ferrocyanide was observed (Fig. 5 curve b). This showed the adsorption of hemoglobin

Fig. 4 A) Cyclic voltammograms of the NB-SAM electrode in a) the background solution, b) the presence of 0.3 mM Hb in buffer. Scan rate: 100 mV/s. B) Response curve for Hb. Data pointing at -390 mV in A).

Fig. 5 Cyclic voltammograms of ferrocyanide/ferricyanide redox couple obtained at a bare Au electrode for a) 1 mM $K_4Fe(CN)_6$, b) 20 μM Hb + 1 mM $K_4Fe(CN)_6$ in phosphate buffer solution (pH 8.0). Scan rate: 50 mV/s. E_a : 0 V, t_a : 30 s.

on the electrode. That is, the negative charges of hemoglobin adsorbing on the electrode prevent an approach of ferrocyanide to the electrode. On the other hand, the catalytic peak currents for the reduction of hemoglobin are proportional to the square root of the scan rates in the case of the NB-SAM. This means that hemoglobin is transferred on the NB-SAM electrode surface by diffusion but not by absorption. That is, the NB-SAM electrode has advantages that it can suppress the strong absorption of hemoglobin on the electrode as well as that it can catalyze the reduction of hemoglobin.

The resulting catalytic peak currents are proportional to the hemoglobin concentrations over the range tested from 50 to 300 μM (Fig. 4B). The relative standard

deviation at an identical electrode was 5.6% for five successive determinations at 100 μM hemoglobin. It is known that the amount of hemoglobin in normal human blood samples is 2.2 – 2.6 mM. Therefore, this method using a NB-SAM electrode has enough sensitivity to show the possibility of the detection for hemoglobin in clinical blood samples.

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(Received December 24, 1998)

(Accepted March 1, 1999)