Bioelectrocatalytic Reduction of Oxygen by Hemoglobin-Adsorbed Carbon-Felt, and its Inhibition by Azide

Yasushi HASEBEa,* and Yue WANGb

a Department of Life Science and Green Chemistry, Saitama Institute of Technology, 1690, Fusaiji, Fukaya, Saitama 369-0293, Japan
b School of Chemical Engineering, University of Science and Technology Liaoning, 185 Qianshan Middle Road, High-tech zone, Anshan, Liaoning, 114501, China

* Corresponding author: hasebe@sit.ac.jp

ABSTRACT

Hemoglobin (Hb) was adsorbed onto a carbon-felt (CF), which is a microelectrode ensemble of microcarbon fiber (ca. 7 μm diameter) and possesses a random three-dimensional structure. Direct electron transfer between Hb and the CF was achieved without any electron mediating species and special materials. The Hb-adsorbed CF (Hb-CF) showed a pair of well-defined cyclic voltammetric peaks with the formal potential of −0.221 V (vs. Ag/AgCl) at pH 5.0 (0.1 M phosphate/citrate buffer), which is attributed to Hb Fe(III)/Fe(II) redox couple. The apparent heterogeneous electron-transfer rate constant ($k_a$) was estimated to be 14.6 s$^{-1}$. Furthermore, the Hb-CF exhibited an excellent bioelectrocatalytic activity for the reduction of O$_2$. This bioelectrocatalytic activity was inhibited by azide, which binds to active heme center of Hb.

Keywords: Hemoglobin, Carbon-felt, Direct Electron Transfer, Bioelectrocatalytic O$_2$ Reduction

1. Introduction

Hemoglobin (Hb) is a heme-protein which transports molecular oxygen in mammalian blood. Hb consists of two $\alpha$ and $\beta$ subunits, each of which has one electro-active iron heme as prosthetic groups. It has been demonstrated that on conventional solid electrodes (i.e., glassy carbon, gold and platinum electrodes), the fast electron transfer between Hb and electrode is difficult because electro-active centers are buried in the polypeptide. Therefore, great efforts have been devoted to facilitate electron transfer by using mediators, promoters, special electrode materials and appropriate films.1-12

Carbon-felt (CF) is a micro-electrode ensemble of micro-carbon fiber (ca. 7 μm diameter) and possesses a random three-dimensional structure. The CF has high conductivity and a large effective surface area (estimated to be 0.1–20 m$^2$ g$^{-1}$),13 which allows large measurable current density and high electrocatalytic efficiency. In addition, high porosity of the CF (>90%) permits low diffusion barrier of solution-flow. On the basis of these features, the CF has been widely used for redox flow battery,14 electrochemical synthesis,15 rapid coulometry,16 and electrochemical waste-water purification.17 In general, porous three dimensional macro-electrode is useful for a working electrode unit of electrochemical flow-detectors. Therefore, the effective biomolecule-immobilization protocol and novel signal transduction principle are both important for the development of highly functional CF-based electrochemical flow-biosensors.18-24

In this study, Hb was physically adsorbed on the CF surface, and the direct electron transfer between Hb and CF was achieved without any special reagents and materials. The resulting Hb-adsorbed CF (Hb-CF) exhibited an excellent bioelectrocatalytic activity for the O$_2$ reduction, and this catalytic activity was inhibited by azide, suggesting a future application of the Hb-CF for electrochemical flow-sensing of respiratory toxins.

2. Experimental

2.1 Reagents and materials

Hemoglobin (Hb, from bovine) was purchased from Sigma Co., and purified by dialysis against deionized water and isolated by lyophilization. Hemin [iron(III)protoporphyrin IX chloride] was obtained from Tokyo Kasei Co., and used as received. Sodium azide (NaN$_3$) was purchased from Wako Pure Chemicals (Osaka, Japan) and used as received. All of the other chemicals were of the highest grade available, and were used without further purifications. Millipore Mill-Q water (resistivity > 18 MΩ cm, TOC, 3 ppb) was used for preparation of all solutions throughout the experiments. 0.1 M phosphate/citrate buffer solution (pH 5.0) was made up from K$_2$HPO$_4$ and citric acid monohydrate, and used as received. A carbon-felt (CF) sheet was obtained from Nippon Carbon Ltd. (Japan). Effective surface area of the CF is 0.29 m$^2$ g$^{-1}$ (BET method). Hb dissolving aqueous solution was prepared just before the adsorption experiment. Standard solutions of azide were prepared just before each experiment daily by dissolving NaN$_3$ in 0.1 M phosphate/citrate buffer (pH 5.0). All the experiments were performed at ambient temperature (22 ± 2°C).

2.2 Preparation of Hb-adsorbed CF

The CF sheet was cut into 10 × 3 × 3 mm in size (weight, ca. 12 mg). Hb was adsorbed onto the CF surface by immersing the bare-CF into Hb-dissolving aqueous solution (0.5 mg mL$^{-1}$, 2 mL) for 5 min at 25°C. To remove weakly adsorbed species, the Hb-adsorbed CF (roughly rinsed with Milli-Q water) was placed on hand-made flow-cell and 0.1 phosphate/citrate buffer (pH 5.0) was constantly flowed at 3 mL min$^{-1}$ for 1000 sec with a double-plunger Intelligent pump (AI-12, FLOM Co., Japan). The resulting Hb-adsorbed CF is denoted here as Hb-CF.

2.3 Preparation of hemin-adsorbed CF

As a control experiment, a hemin-adsorbed CF (hemin-CF) was prepared by essentially similar manner to the Hb-CF, except for the...
solvent of hemin solution. Because of the low solubility of hemin in water, 0.3 mM hemin solution was prepared by using 0.1 M NaOH solution. The CF was immersed in 0.3 mM hemin/0.1 M NaOH solution for 5 min at room temperature to adsorb hemin onto the CF surface. Other treatments are same as the Hb-CF.

2.4 Electrochemical measurements

Cyclic voltammetry (CV) was carried out to evaluate electron transfer characteristics and bioelectrocatalytic activity of the Hb-CF. The CV measurements were performed with ALS 611B electrochemical workstation (ALS Instrument Co.). An one-compartment electrochemical glass cell with a electrolyte volume of 15 mL was used. The Hb-CF (or bare-CF) with platinum lead wire (0.5 mm diameter) was used as a working electrode. A platinum wire (1 mm diameter, 50 mm length) and Ag/AgCl (BAS, RE-1B, 3 M NaCl) electrode were used as the counter and the reference electrode, respectively. All the potentials reported in this work were versus Ag/AgCl. Air-saturated, oxygen-introduced and deoxygenated 0.1 M phosphate/citrate buffers (pH 5.0, 15 mL) were used as electrolyte solutions. Oxygen-introduced buffer was prepared by purging oxygen gas into the buffer for 20 min. Deoxygenated buffer was prepared by bubbling of high-purity nitrogen gas into the buffer at least for 30 min, and the nitrogen atmosphere was then kept over the solutions during the CV measurements to prevent oxygen from reaching the solution. The Hb-CF was stored at 4°C in 0.1 M phosphate/citrate buffer (pH 5.0) in refrigerator when not in use.

To compare interfacial properties of the Hb-CF, the hemin-CF and bare-CF surfaces, electrochemical impedance spectroscopy (EIS) was carried out with ALS 6122A workstation by using deoxygenated 0.1 M phosphate/citrate buffers (pH 5.0, 15 mL) containing 0.25 mM hydroquinone. The applied potential was set at the formal potential of hydroquinone/p-quinone redox system (i.e., 0.19 V vs. Ag/AgCl at pH 5.0). The frequency ranged from 0.01 Hz to 10 kHz.

3. Results and Discussion

3.1 Electron transfer characteristics of Hb-CF

Figure 1A shows cyclic voltammograms (CVs) of the Hb-CF (curve a) and bare-CF (curve b) in N2-saturated (deoxygenated) 0.1 M phosphate/citrate buffer (pH 5.0) solutions. The Hb-CF exhibited a pair of well-defined nearly reversible redox peaks at a formal potential ($E^\circ$) of about $-0.221$ V vs. Ag/AgCl at pH 5.0 with the peak-to-peak separation of 51 mV. The observed formal potential for the Hb-CF is close to the reported value for other Hb-modified electrodes; $-0.22$ V vs. Ag/AgCl at pH 5.0 for Hb-modified carbon ionic liquid electrode; $-0.208$ V vs. SCE at pH 5.5 for Hb-entrapped montmorillonite membrane-modified pyrolytic graphite electrode; $-0.290$ V vs. SCE at pH 5.5 for Hb-entrapped SP sephadex membrane-modified pyrolytic graphite electrode; $-0.298$ vs. Ag/AgCl at pH 6.0 for DNA-Hb-carbon nanotube electrode.

It is known that heme tends to be released from the active center of hemoglobin during the denaturation process. We prepared hemin-adsorbed CF (hemin-CF) and measured CV under the same condition (see Fig. 1B). Adsorbed hemin on the CF exhibited a couple of well-defined redox peaks at a formal potential ($E^\circ$) of about $-0.229$ V with the peak-to-peak separation of 9 mV, indicating a fast electron transfer. The differences in the electrochemical parameters between the Hb-CF (panel A) and hemin-CF (panel B) may be due to the difference in the microenvironment of the heme between the Hb-CF and the hemin-CF. Namely polyep-tide moieties of adsorbed Hb disturb the direct electron transfer between the heme and the CF.

Figure 1C shows Nyquist plot of EIS taken for bare-CF (curve e), hemin-CF (curve f) and Hb-CF (curve g). It is clear that the Hb-CF showed much larger resistance as compared with hemin-CF and bare, indicating that protein covered area on the Hb-CF makes barrier to approach of redox marker (in this case, hydroquinone) at the electrode surface. These results imply that the Hb is strongly adsorbed onto the CF surface by just 5 min adsorption period, and that the adsorbed Hb was not released from the CF surface even after the subsequent 1000 s flashing (washing).
 treatment. These results are similar with our recent studies concerning the enzyme-adsorbed flow-CF reactors and flowdetectors, in which uricase and horseradish peroxidase were strongly adsorbed on the CF surface, probably due to the hydrophobic interactions between these proteins and the CF surface.21–23 These observations suggest that the Hb molecule is also strongly adsorbed onto the CF surface and the adsorbed Hb on the CF undergoes direct electron transfer between active heme center and the CF surface, and the observed redox peaks are attributed to the Hb heme Fe(III)/Fe(II) redox couple, as follows1,6

$$\text{Hb-Fe}^\text{III} + H^+ + e^- \rightleftharpoons \text{Hb-Fe}^\text{II}$$

(1)

Differing from other Hb-modified electrodes which employ special materials, films, and electrodes to facilitate direct electron transfer,14–23 this Hb-CF system is very simple (just adsorption for 5 min).

Figures 2A and 2B show CVs of Hb-CF in deoxygenated buffer (pH 5.0) at various scan rates (panel A, low scan rate region; and panel B, higher scan rate region). As shown in Fig. 2C, peak currents linearly increased with increasing scan rates, indicating a surface-controlled electrode process. The slope obtained by the linear regression of log \(I_{pc}\) versus log \(v\) is 1.04 (Fig. 2D), which is close to the theoretical slope of 1 for thin-layer voltammetry.1,6

$$y = -59.585x - 0.270 \quad R^2 = 0.999$$

$$y = 60.075x - 0.152 \quad R^2 = 1.00$$

In general, protein adsorption is a complex process involving van der Waals, hydrogen bonding, hydrophobic interaction and electrostatic interaction. In some cases, surface-attached protein try to relax, i.e., to optimize its interaction with the surface. This relaxation process usually leads to some degree of spreading of protein molecules over the surface, involving structural rearrangements or conformational changes in the proteins.26 Therefore, it would be safe to assume that some types of structural change are likely to occur upon adsorption of Hb on the CF surface, which provides suitable microenvironment of heme to transfer the electron to the CF surface.

The surface coverage (\(I'\)) of the electro-active Hb molecule on the CF surface was estimated from integration of the reduction peak charge (8.26 µC), \(Q\) is the charge (8.26 µC), \(n\) is the number of electron transferred (in this case, 1), \(F\) is faraday’s constant and \(A\) is the electrode surface area (37.7 cm²). The estimated \(I'\) value of adsorbed Hb on the CF was 2.27 \(\times\) \(10^{-12}\) mol cm⁻². This \(I'\) value is about one eighth of the theoretical value for monolayer coverage 1.89 \(\times\) \(10^{-11}\) mol cm⁻², (which is estimated taking into account the crystallographic dimensions of 6.4 \(\times\) 5.5 \(\times\) 5.0 nm, assuming the long axis parallel to the electrode surface).7 This result may lead to the following assumptions: (1) the amount of adsorbed Hb on the CF is sub-monolayer; (2) only the Hb molecules being preferable conformation on the CF surface can undergo the direct electron transfer reaction. Scanning probe microscope experiment may reveal the surface morphology of the Hb-CF, and this is important future work.

According to the Laviron theory, the electron transfer coefficient (\(\alpha\)) and the electron transfer rate constant (\(k_e\)) can be estimated by measuring the variation of peak potential with potential scan rate.27 The \(\alpha\) and \(k_e\) values for the Hb-CF were estimated to be 0.42 and 14.6 s⁻¹, respectively. This \(k_e\) value of the Hb-CF is higher than other Hb-modified electrodes: e.g., Hb immobilized on a glassy carbon electrode modified with nickel oxide nanoparticles, ca. 5.2 s⁻¹.28 Hb immobilized on a Au colloid-cysteamine-modified gold electrode, 0.49 s⁻¹.29 Hb immobilized in synthetic hydrophilic polymer-modified glassy carbon electrode, 3.45 s⁻¹.30 Hb immobi-
lized on nanometer-sized ZrO$_2$ modified pyrolytic graphite electrode, ca. 7.9 s$^{-1}$;\textsuperscript{11} Hb immobilized in carbonic liquid electrode 0.75 s$^{-1}$;\textsuperscript{12} and smaller than those at Hb incorporated in a montmorillonite membrane-modified pyrolytic graphite electrode, 58.1 s$^{-1}$;\textsuperscript{3} and Hb entrapped in SP Sephadex membrane-modified pyrolytic graphite electrode, 102.1 s$^{-1}$;\textsuperscript{4} The observed CV peaks did not change during 600 continuous cyclic scans at 5 mV s$^{-1}$ for at least about 5 h under nitrogen atmosphere. This result implies that the rate of direct electron transfer of Hb is not changed during this repetitive CV measurement period, suggesting that the Hb is tightly adsorbed on the CF surface.

### 3.2 Electrocatalytic activity of Hb-CF for O$_2$ reduction and its inhibition by azide

The electrocatalytic reaction of Hb-modified electrodes based on direct electron transfer is extensively reported.\textsuperscript{1,12} For example, the electrocatalytic reduction of hydrogen peroxide,\textsuperscript{2,6-8,12} oxygen,\textsuperscript{12} trichloroacetic acid,\textsuperscript{7,12} and nitric oxide\textsuperscript{2,12} have been reported. It is known that Hb-Fe(II) reacts with oxygen and the produced oxyhemoglobin [Hb-Fe(II)-O$_2$] can be electrochemically reduced back to Hb-Fe(II) at the electrode surface, according to the following schemes:\textsuperscript{2}

\[ \text{Hb-Fe(II)} + O_2 \rightarrow \text{Hb-Fe(II)-O}_2 \]  \hspace{1cm} (2)

\[ \text{Hb-Fe(II)-O}_2 + 2H^+ + 2e^- \rightarrow \text{Hb-Fe(II)} + H_2O_2 \]  \hspace{1cm} (3)

Figure 3 shows comparison of CV responses of Hb-CF and bare-CF in air-saturated 0.1 M phosphate/citrate buffer (pH 5.0). Potential scan rate is 5 mV s$^{-1}$. Starting potential is 0.45 V vs. Ag/AgCl. Inset figure is CVs of Hb-CF in O$_2$-saturated, air-saturated and N$_2$-saturated (deoxygenated) 0.1 M phosphate/citrate buffer (pH 5.0).

![Figure 3: CVs of Hb-CF and bare-CF in air-saturated 0.1 M phosphate/citrate buffer (pH 5.0). Potential scan rate is 5 mV s$^{-1}$. Starting potential is 0.45 V vs. Ag/AgCl. Inset figure is CVs of Hb-CF in O$_2$-saturated, air-saturated and N$_2$-saturated (deoxygenated) 0.1 M phosphate/citrate buffer (pH 5.0).](image)

Figure 3. CVs of Hb-CF and bare-CF in air-saturated 0.1 M phosphate/citrate buffer (pH 5.0). Potential scan rate is 5 mV s$^{-1}$. Starting potential is 0.45 V vs. Ag/AgCl. Inset figure is CVs of Hb-CF in O$_2$-saturated, air-saturated and N$_2$-saturated (deoxygenated) 0.1 M phosphate/citrate buffer (pH 5.0).

Observed O$_2$ reduction current of the Hb-CF is originated from the catalytic activity of Hb-heme that adsorbed on the CF surface. The storage stability of Hb-CF was investigated. When not in use, the Hb-CF was stored in 0.1 M phosphate/citrate buffer (pH 5.0) at 4°C, and the same CV measurements were repeated every 4 days during 2 weeks. The Hb-CF kept almost same activity for O$_2$ reduction at least 2 weeks.

It is well known that Hb can bind a variety of ligands such as azide. Sodium azide is a toxic chemical widely used in hospitals and laboratories as a chemical preservative, in agriculture for pest control, detonators, and other explosives. Therefore, the quantitative determination of azide is important. Then, next, the inhibitory effect of azide on the Hb-CF-catalyzed O$_2$ reduction was investigated. Figure 4 shows the CVs of Hb-CF in the absence and the presence of various concentrations of azide in air-saturated 0.1 M phosphate/citrate buffer (pH 5.0). It is clear that the catalytic current for the O$_2$ reduction by Hb-CF decreased with increasing concentration of azide in electrolyte buffer. Because clear inhibitory effect can be observed even in the concentration of 1 µM, the present Hb-CF-based biosensor (in future work) would have sufficient sensitivity to detect azide ion (sub µM order) in fruit juice.\textsuperscript{29}

Insert graph of Fig. 4 shows the relationship between % inhibition (% In) and total azide concentration. % In was calculated with the current value at $-0.2$ V by the following equation.

\[ \% \text{ inhibition} = \frac{(I_0 - I)/I_0 \times 100}{(4)} \]

where $I_0$ is a current value at $-0.2$ V without azide, $I$ is current value in the presence of azide.

The apparent inhibition constant, $K'_{i}$ was evaluated using the Hughes-Klotz equation.\textsuperscript{30}

\[ 1/\% In = 1/\% In_{\text{max}} + K'_{i}/\% In_{\text{max}} [\text{azide}] \]  \hspace{1cm} (5)

$K'_{i}$ was found to be 5.45 µM. $y = 0.0354x + 0.0065$; correlation coefficient, $r^2 = 0.9719$.

Not only azide but also other respiratory toxins such as cyanide and thiocyanide also bind to heme center of Hb. Therefore, if the binding of these respiratory toxins to active heme center of Hb is reversible and the bioelectrocatalytic activity of Hb for the O$_2$-reduction is reversibly changed, we can expect the possibility of developing Hb-CF-based electrochemical flow-biosensor for respiratory toxins, which detects toxins as peak current responses. This aspect is now under way in our laboratory.
4. Conclusion

Hb-adsorbed CF exhibited an excellent bioelectrocatalytic activity for O₂ reduction based on the direct electrochemistry. This bioelectrocatalytic activity was inhibited by azide, suggesting a future application of the Hb-CF for electrochemical flow-biosensing of respiratory toxins.

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References