Bilirubin Oxidase and $[Fe(CN)_6]^{3-/4-}$ Modified Electrode Allowing Diffusion-controlled Reduction of O_2 to Water at pH 7.0

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An enzyme-modified electrode was prepared producing a diffusion-limited bioelectrocatalytic current for the reduction of O_2 to water at neutral pH and at ambient temperature. The electrode used bilirubin oxidase as an enzyme and $[Fe(CN)_6]^{3-/4-}$ as a mediator, both of which were immobilized on the surface of a glassy carbon electrode by electrostatic entrapment with poly-L-lysine.

We have previously shown that bilirubin oxidase (BOD) is a remarkable enzyme exhibiting a high catalytic activity at neutral pH to produce a large bioelectrocatalytic current for the reduction of O_2 to water.[1] This is a significant property of the enzyme allowing the four electron reduction of O_2 in a biofuel cell operating at neutral pH[2] and is contrasted to the catalytic property of laccases that are active in acidic pH and accordingly produce appreciable bioelectrocatalytic currents only under acidic conditions.[3–5] BOD is a multi-copper oxidase of a molecular mass 60 kDa with a redox potential 373 mV vs Ag/AgCl (pH 7.8) catalyzing the oxidation of bilirubin to biliverdin[6] and can use 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) as an electron donor in place of bilirubin.[7] The bioelectrocatalytic behavior of BOD has been studied in detail using ABTS as an electron transfer mediator.[1] The electrocatalytic reduction of O_2 to water occurs at the potential at which ABTS is electrochemically generated from the oxidized form, the redox potential of ABTS being 0.505 V vs Ag/AgCl at pH 7.0. The BOD reaction has a high catalytic constant, $k_{cat} = 2.3 \times 10^2\ s^{-1}$, with the Michaelis constant $K_{ABTS} = 11\ \mu M$ for ABTS. The large catalytic constant and the small Michaelis constant are favorable properties for the enzyme to be used in a biocathode reaction of a biofuel cell. However, there is a problem that it is difficult to immobilize ABTS on an electrode surface for obtaining a higher current density. Very recently, Heller et al. have used BODs to realize the bioelectrocatalytic reduction of O_2 at pH 7.4 and at 37.5 °C using a redox polymer as a mediator, in which BOD has been cross-linked with the polymer on carbon felt.[8]

Here we report on the use of $[Fe(CN)_6]^{3-}$ as a mediator, which is easily immobilized on an electrode surface by an electrostatic entrapment with a cationic polymer.[9] Both BOD and $[Fe(CN)_6]^{3-}$ were entrapped with poly-L-lysine (PLL) on a glassy carbon electrode. A stock solution (0.2 ml) was prepared by dissolving 6 mg of BOD (EC 1.3.3.5, from *Myrothecium verrucaria*, a gift from Amano Pharmaceutical Co. Japan) and 4.4 mg of PLL (molecular weight 8000, purchased from Peptide Institute INC. Osaka) in a phosphate buffer (0.0465 M, pH 7). 10 μL of the solution was syringed on the surface of a glassy carbon electrode (GCE) ($\phi = 3$ mm). After allowing evaporation of the solvent, the electrode was immersed in 5 mM potassium hexacyano ferrate (III) for 5 min. Then the electrode was rinsed with a distilled water and 5 μL of 2.2%(w/v) PLL solution was further syringed to cover the BOD-$[Fe(CN)_6]^{3-}$-PLL layer.

As shown in Figure 1, the BOD-$[Fe(CN)_6]^{3-/4-}$-PLL GCE produced peak-shaped cyclic voltammograms (CVs) in a deaerated solution. The peak current increased linearly with the increase in the scan rate, which is typical of the current due to a surface-confined redox species, and the waves are attributable to the redox reaction of $[Fe(CN)_6]^{3-/4-}$ electrostatically entrapped in the BOD-$[Fe(CN)_6]^{3-}$-PLL layer on the GCE surface. The peak potentials of the anodic and cathodic waves shifted positive and negative directions, respectively, with increasing scan rate, which reflects less reversible nature of the electrode reaction of $[Fe(CN)_6]^{3-/4-}$. The amount of the $[Fe(CN)_6]^{3-/4-}$ confined on the GCE was calculated as 0.85 nmol from the areas of the peak-shaped waves, which leads to 1.2×10^{-8} mol/cm^2 with $\phi = 3$ mm of the GCE. It is noted that the mid-potential (formal potential) of $[Fe(CN)_6]^{3-/4-}$, 240 mV vs Ag/AgCl, was 35 mV more positive than the formal potential of $[Fe(CN)_6]^{3-/4-}$ in solution (data not shown). This is attributable to the electrostatic interaction between $[Fe(CN)_6]^{3-/4-}$ and PLL; simple calculation reveals that the shift of 35 mV corresponds to 52 times stabilization of $[Fe(CN)_6]^{4-}$ compared with $[Fe(CN)_6]^{3-}$. The positive shift is a favorable direction allowing the occurrence of a bioelectrocatalytic current at a less negative potential.

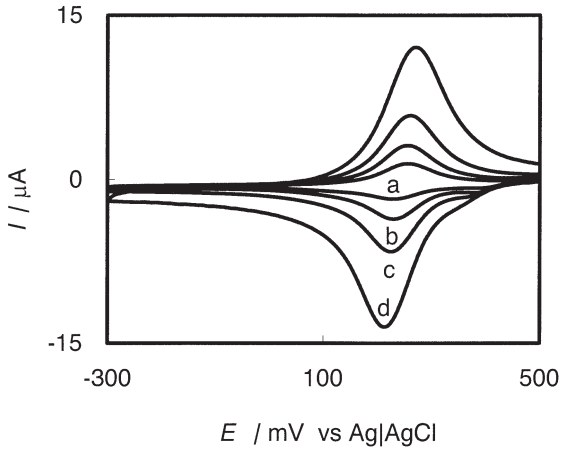

Figure 1. Cyclic voltammograms of a BOD-$[Fe(CN)_6]^{3-/4-}$-PLL GCE in a deaerated phosphate buffer of pH 7.0 at the scan rates: a, 5; b, 10; c, 20; d, 50 mV/s.

When the same solution as in Figure 1 was air-saturated, the BOD-$[Fe(CN)_6]^{3-/4-}$-PLL GCE produces large cathodic currents as illustrated in Figure 2. The CVs had an irreversible character with no anodic currents, and the peak current increased linearly with the square root of the scan rate. This is typical of an

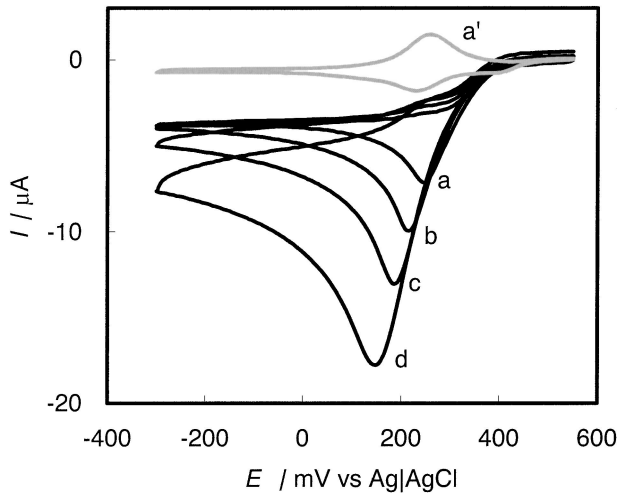

Figure 2. CVs of a BOD-$[Fe(CN)_6]^{3-/4-}$-PLL GCE in an air-saturated phosphate buffer of pH 7.0 at the scan rates: a, 5; b, 10; c, 20; d, 50 mV/s. a′: CV in a deaerated solution at the scan rate 5 mV/s.

irreversible voltammogram of a redox species in solution, and the voltammograms are attributable to the reduction of O_2. Applying the theory of a totally irreversible voltammogram:[10]

$$i_p = n(2.99 \times 10^5)\alpha^{1/2} A c_j^* D^{1/2} v^{1/2} \quad (1)$$

(where i_p, n, α, A, c^*, D, and v are the peak current, number of electrons, transfer coefficient, electrode surface area, bulk concentration of the species j, diffusion coefficient of j and the scan rate, respectively) we obtained the D value of $5.2 \times 10^{-5}\,cm^2\,s^{-1}$ from the dependence of i_p on $v^{1/2}$ with $n = 4$ (for O_2 reduction to water), $A = 0.071\,cm^2$ (the area of the GCE with $\phi = 3$ mm), $c_j = 0.25$ mM (the concentration of O_2) and $\alpha = 0.38$. The α value was estimated from the CVs by the equation:[10]

$$|E_p - E_{p/2}| = 47.7/\alpha\,mV \quad (2)$$

(where E_p and $E_{p/2}$ are the peak potential and the potential where the current is at half the peak value, respectively). Almost the same D value, $4.4 \times 10^{-5}\,cm^2\,s^{-1}$, was obtained from the chronoamperometry at the BOD-$[Fe(CN)_6]^{3-/4-}$-PLL GCE at -0.1 V, the current being corrected for the current measured at the same electrode in a deaerated solution.

The value of $5.2 - 4.4 \times 10^{-5}\,cm^2\,s^{-1}$ is somewhat larger than the reported D value of O_2, $2.0–2.5 \times 10^{-5}\,cm^2\,s^{-1}$,[11] This result is interpretable by an idea that the effective area of diffusion-controlled biocatalytic conversion from O_2 to $[Fe(CN)_6]^{3-}$ is larger than A. In any event, Figure 2 is, as far as we aware, the first demonstration of the diffusion-controlled CVs for the four-electron reduction of O_2 to water at neutral pH. It should be noted, however, that the electrochemical reduction of O_2 proceeds indirectly by the BOD-catalyzed reduction through the mediation of $[Fe(CN)_6]^{3-/4-}$ in the BOD-$[Fe(CN)_6]^{3-/4-}$-PLL layer. The α value is attributed to the electrode reaction of $[Fe(CN)_6]^{4-}$, though the overall reaction is the reduction of O_2. The validity of eq. 1 and eq. 2 for the analysis of the CVs in Figure 2 is not self-evident, but the use of the equations seems to be appropriate. This is supported by the fact that the D values determined from the CVs and the chronoamperogram agree well as mentioned above.

We have confirmed by the measurement of O_2 consumption in the BOD reaction in a solution (data not shown) that four moles of $[Fe(CN)_6]^{4-}$ are consumed for the reduction of one mole of O_2. Measurements of the enzyme kinetics of the BOD reaction revealed that the catalytic constant was $802\,s^{-1}$ and the Michaelis constants for O_2 and $[Fe(CN)_6]^{4-}$ were $50\,\mu M$ and $2.7\,\mu M$, respectively. The large catalytic constant meets the occurrence of the mass transfer-controlled bioelectrocatalytic current. The small Michaelis constants are also in favor of the mass transfer-controlled overall reaction. This is because the rate of the BOD reaction is almost independent of the concentrations of both O_2 and $[Fe(CN)_6]^{4-}$ down to the values as low as the Michaelis constants and is kept a high value even at a much lower O_2 concentration at the electrode surface owing to the mass-transfer limited depletion.

We have observed a steady-state limiting current as large as $150\,\mu A$ ($2.1\,mA/cm^2$ by the calculation using $A = 0.071\,cm^2$) at the BOD-$[Fe(CN)_6]^{3-/4-}$-PLL GCE in a dioxygen-saturated phosphate buffer (pH 7.0) when the solution was gently stirred with a stirring bar. A higher current density per projected surface area will be expected with the use of carbon materials of a large surface to volume ratio as a basal electrode, and the O_2 reduction at more positive potentials would be expected with the use of other kinds of cyano-metal complexes. Extension of this study is now under way in these directions.

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