Diaphorase/Naphthoquinone Derivative-modified Electrode as an Anode for Diffusion-controlled Oxidation of NADH in Electrochemical Cells

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Diaphorase and 2-amino-3-carboxy-1,4-naphthoquinone as an enzyme and a mediator for NADH oxidation, respectively, were successfully co-immobilized on a glassy carbon electrode with poly-L-lysine and glutaraldehyde as a polymer support and a cross-linking reagent, respectively. The diffusion-controlled oxidation of NADH (10 mM) was achieved around −0.25 V vs Ag|AgCl|KCl(sat.).

Increasing attention has been paid to biofuel cells, in which enzymes and redox couples are utilized as catalysts and electron transfer mediators, respectively.[1,2] To date, glucose and hydrogen have been utilized as biofuel, where the enzymes used have redox cofactors such as flavin, quinone, and metal.[1,2] Nicotinamide-adenine dinucleotide (NAD) is an important coenzyme participating in various biochemical redox reactions, and NAD-dependent enzymes constitute the largest group of redox enzymes. Therefore, NAD-dependent dehydrogenases are catalysts of great promise in biofuel cell anodes.[3] However, direct electrochemical oxidation of NADH requires large overpotentials. The most efficient catalytic system for NADH oxidation would be mediated bioelectrocatalysis, in which diaphorase (DI; EC: 1.6.99.-) has been frequently utilized as an enzyme and metal complexes or quinones work as mediators.[4,5] For biofuel anodes, mediators are required that have negative formal potential ($E^{\circ\prime}_{M}$) and large rate constant between enzyme and mediator (k_M). 2-Methyl-1,4-naphthoquinone (Vitamin K_3; VK_3) is one of the most promising mediators of DI.[6,7] Unfortunately, it is difficult to immobilize VK_3 on electrode surfaces. 2-Amino-3-carboxy-1,4-naphthoquinone (ACNQ) has $E^{\circ\prime}_{M}$ (= −0.27 V vs Ag|AgCl|KCl(sat.) at pH 7.0) slightly more negative than that of VK_3 and k_M close to the diffusion-controlled limiting value.[8] Furthermore, ACNQ may be immobilized via its amino or carboxyl group. Here we report on the co-immobilization of DI and ACNQ on the surface of a glassy carbon (GC) electrode with poly-L-lysine (PLL) and glutaraldehyde (GA) as a polymer support and a cross-linking reagent, respectively, and on the electrochemical behavior of catalytic oxidation of NADH at the modified electrode.

DI and ACNQ were co-immobilized on a GC electrode using the following procedure. On the bare GC electrode surface ($\phi = 3.0$ mm), 2 μL of a DI (from *Bacillus stearothermophilus*, Unitika Ltd., Kyoto) solution (47 μM in phosphate buffer), 2.8 μL of an ACNQ solution (10 mM in ethanol), 3 μL of a GA aqueous solution (0.125%) and 3 μL of a PLL (Peptide Institute Inc., Osaka) aqueous solution (1%) were successively syringed and well-mixed. After being dried at room temperature, the electrode was rinsed with distilled water. The electrode is referred to as a DI/ACNQ-modified electrode. An ACNQ-modified electrode without DI was also prepared using the same procedure without a DI solution syringing. A platinum disk and Ag|AgCl|KCl(sat.) were used as the counter electrode and reference electrodes, respectively. A deaerated phosphate buffer solution of pH 8.0 (25 °C) was used in this work as the electrolyte solution.

As shown in Figure 1, the DI/ACNQ-modified electrode produced a couple of redox waves. The waves are reasonably assigned to the two-electron redox reaction of ACNQ immobilized on the electrode surface, while the anodic peak current was larger than the cathodic one. The peak currents were proportional to the square root of the scan rate (ν) and the peak separation was 56 mV at $\nu = 50\,\mathrm{mV\,s^{-1}}$, while the separation increased slightly with ν. The behavior suggests an almost reversible electron transfer of ACNQ under (pseudo) infinite diffusion within the immobilized membrane.

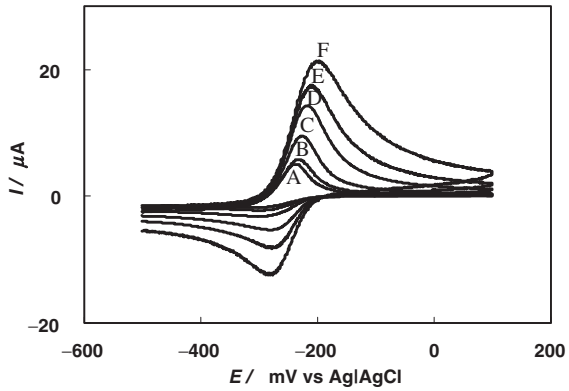

Figure 1. Cyclic voltammograms of a DI/ACNQ-modified GC electrode at ν = (A) 5, (B) 10, (C) 20, (D) 50, (E) 100, and (F) 200 mV s^{-1} (pH 8.0).

In the presence of NADH (10 mM), the DI/ACNQ-modified electrode exhibited a large irreversible anodic wave around the formal potential of immobilized ACNQ, as shown by curve A in Figure 2. A voltammogram at the ACNQ-modified electrode without DI exhibited a direct oxidation wave of NADH around 0.7 V, as well as an oxidation wave of ACNQ that remained unchanged in the presence of NADH (curve B). This suggests that DI is essential for the generation of the irreversible wave of curve A and that the irreversible wave is the DI-catalyzed oxidation wave of NADH, where immobilized ACNQ functions as a good mediator. It is noteworthy that no direct oxidation wave of NADH was observed around 0.7 V, supporting the attainment of a diffusion-controlled oxidation of NADH at the DI/ACNQ-modified electrode. This may be the first case of the diffusion-controlled oxidation of NADH at relatively high concentrations and at negative potentials. The catalytic peak currents (i_p) were proportional to $\nu^{1/2}$ (Figure 2, inset). This is in marked contrast with the situation observed in the catalytic process-limited electrode process. Rather, it is typical of an irreversible electrode process. Similar behavior has been reported for a four-electron reduction of dioxygen at a bilirubin oxidase (BOD) and $[Fe(CN)_6]^{3-/4-}$-modified electrode.[9] We attempted to evaluate a diffusion coefficient (D) of NADH based on the theory of the totally irreversible cyclic voltammogram:[10]

$$i_p = 2.99 \times 10^5 n(\alpha n_\alpha)^{1/2} ACD^{1/2} \nu^{1/2} \quad (1)$$

where $n = 2$ (the number of the electrons for NADH), α: transfer coefficient, n_α: the number of electrons involved in the rate-determining step, A: the electrode surface area, and C: the concentration of NADH. The physical meaning of αn_α is complicated in this case and should reflect the electron transfer property of ACNQ immobilized in the membrane. However, the value was evaluated as 0.5 from the peak width according to the theory of (noncatalytic) irreversible process:[10]

$$|E_p - E_{p/2}| = 47.7/(\alpha n_\alpha) \quad [\text{mV}] \quad (2)$$

where E_p and $E_{p/2}$ are the anodic peak potential and the half-peak potential, respectively. Thus the D value was evaluated as 4.1×10^{-7} cm^{2}s^{-1} from the dependence of i_p on $\nu^{1/2}$. This D value is smaller than that in aqueous state (2.4–6.7×10^{-6} cm^2 s^{-1}).[11,12] This result suggests that the diffusion process of NADH is characterized not by the mass transfer from the bulk phase to the membrane, but by that in the membrane or by the penetration process into the membrane. The thickness of the membrane (d) was evaluated as 15 μm with microscopic method. The d value is comparable with the thickness of the diffusion layer generated during the potential scan up to E_p at $\nu = 100$ mV s^{-1}. Then the D value evaluated here can be reasonably assigned to that in the membrane.

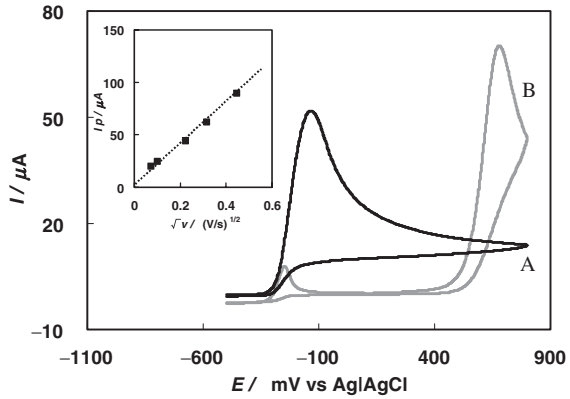

Figure 2. Cyclic voltammograms of NADH (10 mM) at (A) a DI/ACNQ-modified electrode and (B) an ACNQ-modified electrode at $\nu = 20$ mV s^{-1} (pH 8.0). The inset shows the linear relation between i_p and $\nu^{1/2}$.

Figure 3 shows rotating disk voltammograms of NADH at the DI/ACNQ-modified electrode. At low rotation rates (ω) up to 50 rpm, the catalytic oxidation of NADH produced a peak wave. After the peak, the current reached a steady state. The steady-state current (i_s) (measured at 0.1 V) seemed to increase with $\omega^{1/2}$ at low ω, suggesting the diffusion-controlled catalytic oxidation of NADH. At $\omega = 200$ rpm, the catalytic wave became typical sigmoidal shape. However, the i_s value was little affected by ω at increased ω, as shown in the inset of Figure 3. Under these conditions, there is no clear wave due to the direct oxidation of NADH around 0.7 V, as shown by curve G in Figure 3. These results indicate that NADH penetrated in the immobilized membrane is completely oxidized enzymatically to generate the reduced ACNQ which is oxidized at the electrode, and that the limiting steady-state current ($i_{s,lim}$) is governed by the permeability of NADH in the membrane (P_M):

$$i_{s,lim} = nFAP_M C \quad (3)$$

where P_M is independent of ω and given by $P_M = \beta D/d$, β being the partition coefficient. The P_M value was evaluated to be 4×10^{-4} cm s^{-1} from $i_{s,lim}$, and d/β was calculated to be 10 μm using the D value evaluated above. Since the value is close to d evaluated microscopically, β would be close to unity. At decreased ω, however, i_s is defined by the mass transfer from the bulk phase to the membrane surface. Therefore, i_s may be written as:

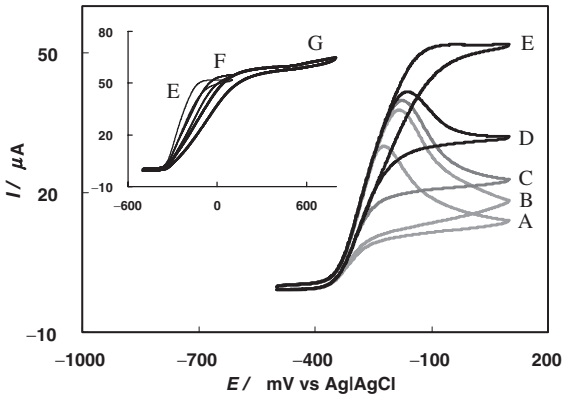

Figure 3. Rotating disk voltammograms of NADH (10 mM) at a DI/ACNQ-modified electrode at $\omega =$ (A) 0, (B) 10, (C) 20, (D) 50, (E) 200, (F) 300, and (G) 500 rpm ($\nu = 20$ mV s^{-1}, pH 8.0).

$$1/i_s = 1/i_{s,lim} + 1/i_L \quad (4)$$

where i_L is given by the Levich equation ($i_L = i'_L \omega^{1/2}$). The ω dependence of i_s seems to be explained by Eq 4. The important point here is that no kinetic term appears in Eq 4. This means that the oxidation of NADH must be diffusion-controlled.

The DI/ACNQ-modified GC electrode showed a novel performance for NADH oxidation. The maximum current density was found to be 0.78 mA cm^{-2} using rotating disk voltammetry. This current density was governed not by the enzyme kinetics but by the permeability of NADH in the membrane. The softening and/or thinning of immobilized membranes would be important to increase in the current density. Utilization of carbon-felt electrodes easily increases the current density by one order or more. Therefore, DI/ACNQ-modified electrodes show great promise as anodes in NAD-dependent biofuel cells. Incorporation of NAD-dependent dehydrogenases into DI/ACNQ-modified electrodes is now under way.

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