Electrochemical Oxidation of NADH Catalyzed by Diaphorase Conjugated with Poly-1-vinylimidazole Complexed with Os(2,2′-dipyridylamine)$_2$Cl

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A new redox polymer containing Os ($E^{\circ\prime} = -0.15$ V vs Ag|AgCl) was designed and synthesized as an efficient mediator of diaphorase-catalyzed electrochemical oxidation of NADH and as a support to immobilize enzyme(s) on electrode surfaces. The electrochemical characteristics of the polymer and its application to biosensors and bioanodes of biofuel cells are briefed.

Electrochemical oxidation of NADH has been a subject receiving great attention in views of biosensors and biofuel cells,[1–3] since NAD-dependent enzymes constitute the largest group of redox enzymes. Because the direct electrochemical oxidation of NADH requires large overpotential,[4] organic or inorganic compounds may be used as nonenzymatic catalysts.[1] More efficient catalytic systems are mediated bioelectrocatalysis consisting of suitable enzymes and mediators. Diaphorase is frequently used for this purpose, and several metal complexes,[5] quinones[6] and also viologens[3] have been utilized as mediators. The formal potential of mediator ($E^{\circ\prime}_M$) and the rate constant between diaphorase and mediator (k_M) are important factors; the more negative in $E^{\circ\prime}_M$ and the larger in k_M, the better the mediator is. Considering the fact that k_M increases exponentially with $E^{\circ\prime}_M$ and reaches the diffusion-controlled limiting value at increased $E^{\circ\prime}_M$,[6] vitamin K_3 is one of the most promising mediators with more negative $E^{\circ\prime}_M$ among the mediators with diffusion-controlled value of k_M.[6,7] However, vitamin K_3 has serious drawbacks in O_2-sensitive property of the reduced form[8] and difficulty in the immobilization on electrode surfaces.

One of promising immobilization methods of mediators as well as enzymes on electrode surface is utilization of redox polymers.[9] Especially, Os polymers have drawn attention. The $E^{\circ\prime}_M$ value of Os polymers can be tuned in a wide range by changing the ligands. Since the ligand effect has been parameterized,[10] $E^{\circ\prime}_M$ of Os redox polymers can be predicted.

In this work, we designed and synthesized a new Os redox polymer with $E^{\circ\prime}_M$ close to that of vitamin K_3. The polymer was used as a support to immobilize diaphorase on electrode surfaces. The bioelectrocatalytic oxidation of NADH at the modified electrode is documented. Future aspects of the NADH-oxidation coupled with several NAD-dependent dehydrogenases are also briefed.

Considering the ligand effects on $E^{\circ\prime}_M$, we focused our attention to an Os complex coordinated with 2,2′-dipyridylamine (dpa). *cis*-[$OsCl_2(dpa)_2$] was synthesized by the method in the literature[11] with some modifications. $(NH_4)_2[OsCl_6]$ (1 mmol) and 2.0 equivalents of dpa (2 mmol) were refluxed in 1,2-ethandiol (18 mL (L = dm^3)) for 1 h under Ar atmosphere. After cooling, the reaction mixture was treated with 30 mL of 1 M (M=mol L^{-1}) $Na_2S_2O_4$. The reaction mixture was cooled for 30 min in an ice bath to precipitate the Os complex. The precipitate was thoroughly washed with cold water and diethyl ether, and dried in vacuum. This was used as *cis*-[$OsCl_2(dpa)_2$] without further purification. The poly-1-vinylimidazole (PVI) was prepared according to the literature,[12] and complexed with *cis*-[$OsCl_2(dpa)_2$] to yield a water-soluble Os polymer (PVI-Os(dpa)$_2$Cl) in the following procedure. *cis*-[Os(dpa)$_2$Cl$_2$] (132 mg, 0.21 mmol) was refluxed with PVI (200 mg, 2.1 mmol) in 200 mL of absolute ethanol for 2 days. After filtration, the solution was poured into 1.5 L of diethyl ether under rigorous stirring. The precipitate was used as PVI-Os(dpa)$_2$Cl. The expected structure of the polymer is depicted in the inset of Figure 1.

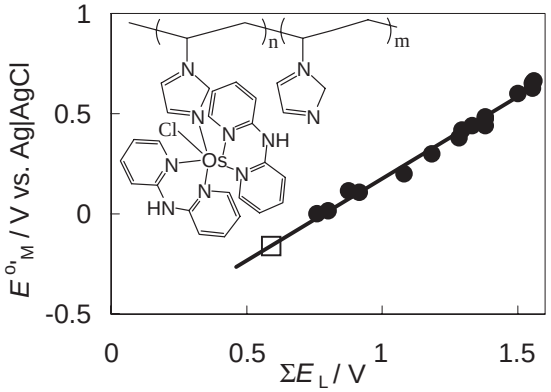

Figure 1. Relation between the formal potential of water-soluble Os complexes at pH 7 and the sum of the ligand parameters (ΣE_L). The inset shows the structure of PVI-Os(dpa)$_2$Cl.

The composite electrode was assembled on a glassy carbon (GC) disk electrode ($\phi = 3$ mm). On the electrode surface, 5 μL of PVI-Os(dpa)$_2$Cl aqueous solution (20 mg mL^{-1}) and 2 μL of a diaphorase solution (2 mg mL^{-1} in 50 mM phosphate buffer, pH 7.0; [EC: 1.6.99.-] from *B. stearothermophilus*, Unitika) were syringed and mixed well. In order to immobilize diaphorase on the polymer, 1.2 μL of poly(ethylene glycol) diglycidyl ether solution (2.5 mg mL^{-1}) was added to the mixture on the electrode surface. The electrode was dried overnight at room temperature to make the polymer water-insoluble. The electrode was used as a diaphorase/Os-modified electrode. All potentials are referred to the Ag|AgCl|KCl(sat.) electrode.

The $E^{\circ\prime}_M$ values of several Os complexes at pH 7 reported so far[13] are in a good linear relation with the sum of the ligand parameters (E_L) for octahedral metal complexes,[10] as shown in Figure 1, closed circles. Judging from the linear relation, the $E^{\circ\prime}_M$ of PVI-[Os(dpa)$_2$Cl]$^{2+/+}$ can be expected to be about -0.16 V (open square in Figure 1), which is comparable with that of

vitamin K_3 at pH 7 ($E^{\circ\prime}_M = -0.19$ V[14]). The synthesized polymer PVI-Os(dpa)$_2$Cl gave a reversible cyclic voltammogram in the soluble state with $E^{\circ\prime}_M$ of -0.15 V, the peak separation being 60 mV at scan rates (v) at least up to 0.2 V s^{-1} (data not shown). The $E^{\circ\prime}_M$ value is very close to the predicted one. This result supports the expected structure of the synthesized polymer.

The diaphorase/Os-modified electrode gave also a couple of redox waves in cyclic voltammetry under NADH-free condition, as shown by curve A in Figure 2. This signal is assigned to the Os(II/III) redox couple in the polymer. Considering surface-confined characteristics of the polymer and the peak separation (60 mV at $v = 5$ mV s^{-1}), the rate of the electron transfer (including the electron exchange in the polymer) was not so large, probably because of rather rigid (or compact) structure of the immobilized polymer compared with other insoluble Os polymers. The midpoint potential (or $E^{\circ\prime}_M$) was -0.13 V, which is more negative than or comparable with those of Os polymers reported so far ($E^{\circ\prime}_M = -0.025$[15] and -0.13 V[16] for 4,4′-dimethoxy-2,2′-bipyridine and 4,4′-diamino-2,2′-bipyridine ligands,[17] respectively), and the synthesis of PVI-Os(dpa)$_2$Cl is more easier than those of the reported ones. The positive shift in $E^{\circ\prime}_M$ by the immobilization might be due to the electrostatic interaction in the cationic polymer.

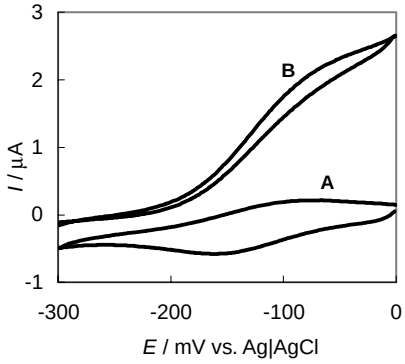

Figure 2. Cyclic voltammogram of a diaphorase/Os-modified GC electrode in phosphate buffer in the absence (A) and the presence (B) of NADH (1 mM) at $v = 5$ mV s^{-1} and at pH 7.0.

In the presence of NADH, the voltammograms changed to a typical catalytic wave (Figure 2, B). Such a catalytic wave was not observed at the Os-modified electrode without diaphorase. Diaphorase was successfully immobilized on the polymer, and the Os redox polymer acted as an efficient electron-transfer mediator between diaphorase and the electrode.

$$\mathrm{NADH} + 2\mathrm{Os(III)} \rightarrow \mathrm{NAD^+} + 2\mathrm{Os(II)} + \mathrm{H^+} \quad \text{(with diaphorase)}$$

$$\mathrm{Os(II)} \rightleftarrows \mathrm{Os(III)} \quad \text{(at electrode)}$$

This is the first example of the catalytic oxidation of NADH at potentials as negative as -0.1 V with diaphorase/mediator co-immobilized electrodes. The steady-state catalytic current, however, did not reach the limiting one, rather increased gradually with the electrode potential at potentials more positive than $E^{\circ\prime}_M$ of the Os polymer. This is due to the kinetic effects in the heterogeneous electron transfer from the Os complex to the electrode. In order to increase the electron transfer rate, some improvements will be needed in the insolubilizing process.

The electrochemical oxidation of NADH can be coupled with a variety of NAD-dependent enzymes. Note here that PVI-[Os(II)(dpa)$_2$Cl]$^+$ was insensitive to O_2 and that the presence of O_2 did not affect the catalytic current. This property is valuable in the application of the diaphorase/Os-modified electrode to biosensors and biofuel cells.

Since NAD-dependent enzyme reactions are O_2-insensitive, coimmobilization of any NAD-dependent enzyme and diaphorase allows the detection of the NAD-enzyme substrate under aerobic conditions at potentials around -0.1 V, where there is practically no interference in biological samples.

Such NAD-enzyme/diaphorase-coimmobilized Os polymer electrodes can be utilized as an anode of biofuel cells. Since diaphorase functions in neutral or slightly alkaline conditions, bilirubin oxidase is superior to laccase as the catalyst of 4e$^-$-reduction of O_2 at a cathode.[18] The former works under neutral conditions using an Os polymer (with 2,2′-bipyridine as a ligand; $E^{\circ\prime}_M = 0.35$ V) as mediator.[19] The electromotive force is expected to be about 0.6 V.

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