Preparative, Electroenzymatic Reduction of NAD^+ to NADH on a Thin Poly(Acrylic Acid) Layer-Coated Graphite Felt Electrode Coimmobilizing Ion-paired Methyl Viologen-Cation-Exchange Polymer and Diaphorase

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A thin poly(acrylic acid) layer-coated graphite felt electrode coimmobilizing ion-paired methyl viologen — cation-exchange polymer and diaphorase was prepared and applied to preparative electroenzymatic reduction of NAD^+ in phosphate buffer at constant potential of - 0.80 V *vs.* SCE. The electrode was stable for the reaction and yielded NADH quantitatively in 97.8% current efficiency.

Recently much effort has been focused on the electroenzymatic synthesis based on high stereoselectivity and species-selectivity of enzyme reactions involving nicotinamide adenine dinucleotide (oxidized form, NAD(P)$^+$; reduced form NAD(P)H).[1,2] If the regeneration of NAD(P)$^+$/NAD(P)H system is achieved by an electrochemical process, one can construct an electrochemical bioreactor which makes possible to produce optically pure compounds, in combination with NAD(P)H-dependent enzymes.[3-5] The electrochemical regeneration of NAD(P)H has been always performed by indirect electrochemical method, because the direct electrochemical reduction of NAD(P)H leads usually to yield enzymatically inactive NAD-dimers.[6] To avoid this, viologen (VL) as one-electron mediator has been often used in combination with ferredoxin-$NADP^+$-reductase (FNR),[7] lipoamide dehydrogenase (diaphorase, Dp),[1,8-10] enoate reductase[11] or VL accepting pyridine nucleotide oxidoreductases[12] as regeneration enzymes, while the indirect electrochemical NAD(P)H regeneration without regeneration enzyme has succeded by the use of rhodium complexes.[3a, 13-15]

The advantages of electroenzymatic process can be emphasized by means of enzyme- and all other components-modified electrode. Simon *et al.* coimmobilized VL and VL-accepting pyridine nucleotide oxidoreductase at the surface of carbon cathode and showed an electrochemical NADH production rate of 9 nmol cm^{-2} h^{-1} from NAD^+.[12] Beley and Collin electropolymerized pyrrole-linked rhodium complexes on reticulated vitreous carbon electrode and showed an electrochemical NADH production rate of 0.4 μmol cm^{-2} h^{-1} from NAD^+.[14] A reactive polymer-coated electrode swelled in aqueous solution may be a better candidate to remove the defect. For electroenzymatic oxidation of alcohols, we have used a poly(acrylic acid)-coated graphite felt (PAA-GF) electrode whose surface layer was able to not only load sufficient amounts of mediator and enzyme but also penetrate substrates without hindrance.[4,16]

Incorporation of VLs by electrostatic binding to cation-exchange membranes has been reported for the purpose of electrochemical[17-20] and photoelectrochemical[21-23] studies. In this communication, we newly immobilized a cation-exchange polymer layer to fix VL by ion-pair formation together with the PAA layer to fix Dp for preparative electrocatalytic reduction of NAD^+ to NADH.

A typical procedure for the preparation of the VL- and Dp-comodified electrode is shown in Scheme 1. A GF (National Electric Carbon Corp., WDF) plate (5.0 x 2.0 x 0.5 cm^3) was first dipped in a 10 ml of 5 mM(M = mol dm^{-3}) VL and 2.5 wt% Nafion 117 solution (95 wt% lower aliphatic alcohols and 5 wt% water). The GF plate was impregnated with 5.5 ml of the solution and was dried in vacuo, and then coated with 5.5ml of 0.1 wt% PAA (average MW: 1400000) methanol solution and dried in vacuo. The GF thus prepared was treated in a 5.5 ml of 20 μM Dp (*EC. 1.8.1.4*) / phosphate buffer (pH 7.2) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (WSC) (10 equiv. to Dp) at 4 °C for 48 h, washed in 50 ml water three times and dried in vacuo.

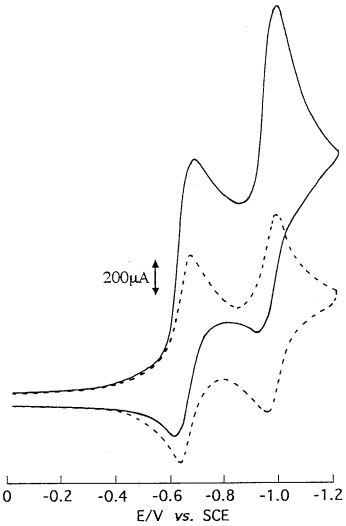
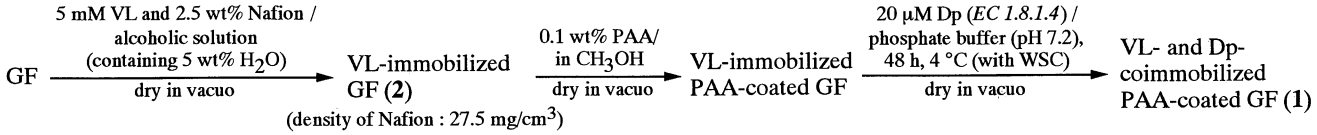


Figure 1. Cyclic voltammograms on **1** in the presence (———) and absence (- - - - -) of 10 mM NAD^+. Phospate buffer: pH 7.0. Electrode size: 1.0 x 1.0 x 0.5 cm^3. Scan rate: 10 mV· s^{-1}.



Scheme 1. Preparation of VL- and Dp-coimmobilized electrode (**1**).

The VL- and Dp-coimmobilized GF electrode (**1**) thus prepared showed two well defined redox waves in the cyclic voltammogram (CV) (Figure 1). Though several investigations have indicated the stable couple ($VL^{+\cdot}$-VL^{2+}) at the first redox wave,[17-23] it should be noted that the second redox couple (VL^{0}-$VL^{+\cdot}$) is also stable in this CV. Therefore, both reduction species ($VL^{+\cdot}$ and VL^{0}) can be effectively used as reduction mediator. In fact, **1** revealed a large electrocatalytic current for the reduction of NAD^+ at the peak potential of -0.70 V (Figure 1), which suggests a possible use of **1** for macroelectrolysis. The characterization data of **1** are summarized in Table 1.

Table 1. Characterization Data of **1**

Ratio of COOH groups of PAA (%)[a]		Density of VL
Dp-immobilized	Free	(μmol / cm ^{3})
16	84	5.5

a) The values were calculated by back titration (an excess amount of 0.01 M NaOH) with 0.01 M HCl solution.

Preparative electrocatalytic reduction of NAD^+ was carried out on **1** or VL-immobilized GF electrode (**2**) (both sizes are 1.0 x 1.0 x 0.5 cm^3) in an undivided cell containing 10 ml of 10 mM NADH / phosphate buffer (pH 7.0) at - 0.80 V or - 1.20 V *vs.* SCE under nitrogen atmosphere. The reaction products were analyzed by UV spectroscopy (340 nm)[24] and high performance liquid chromatography (Biofine RPC-PO, 0.46 mm ϕ x 25 cm / column temp 30 °C, flow speed: 1.0 ml min^{-1}, solvent: phosphate buffer (pH 7.0): CH_3CN = 95:5).

NAD^+ was selectively reduced to NADH on both **1** and **2** at -0.80 V *vs.* SCE, containing 1 μM Dp in phosphate buffer in the case of **2**. The initial reduction rate of NAD^+ was 5.2 times faster on **1** than on **2**. Figure 2-*a* shows that NAD^+ was perfectly converted to NADH on **1** at 4 h in current efficiency of 97.8% and turnover number of 364 based on VL. The reaction rate of 1 μmol cm^{-2} h^{-1} (Figure 2-*a*) in the present work is 2.5 times faster than that in the previously reported works.[14] On the other hand, the NAD^+ reduction at - 1.20 V yielded some amounts of NAD-dimer as a by-product in the final part of electrolysis probably due to the direct electrochemical reduction of NAD^+ (Figure 2-*b*). The NAD-dimer formation was 4 times more significant on **2** than on **1**. These results suggest that the selective reduction of NAD^+ proceeds smoothly at - 0.80 V *vs.* SCE on **1**.

The reusability of **1** for the reduction of NAD^+ was examined by repeating the electrolysis four times. The reduction peak currents in CVs on **1** with and without 10 mM NAD^+ remained unchanged during the repeated electrolysis. This means that **1** is not deactivated during macroelectrolysis and can be used repeatedly. Turnover number of NAD^+ based was 1456 after the four batch electrolysis.

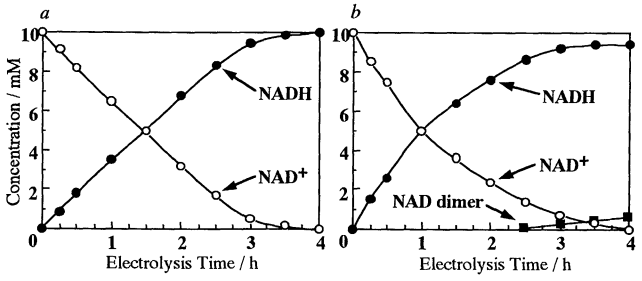


Figure 2. Macroelectrolysis of NAD^+ on **1** at -0.80 V (*a*) and -1.20 V (*b*). Electrode size: 1.0 x 1.0 x 0.5 cm^3.

In conclusion, a thin poly(acrylic acid) layer-coated graphite felt electrode coimmobiling methyl viologen and diaphorase was stable and reduced NAD^+ to NADH quantitatively by electroenzymatic reduction. If the electrode is combined with other NADH-depending enzymes, stereoselective and species-selective electroenzymatic reactions will be realized for preparative synthesis on the electrode.

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