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CONSTRUCTION OF PQQ-ENZYME MULTI-IMMOBILIZED ELECTRODES FOR ELECTROCATALTYIC REDUCTION OF CARBONYL COMPOUNDS

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Abstract – A thin poly(arylamine) (PAA) and poly(acrylic acid) (PAAc) layers-coated graphite felt (GF) electrode immobilizing all mediation components of pyrroloquinolinequinone (PQQ), diaphorase (Dp), oxidized nicotinamide adenine dinucleotide (NAD^+) and alcohol dehydrogenase (ADH) to construct a complete bioelectrochemical reactor was prepared and applied to electrocatalytic reduction of carbonyl compounds in a phosphate buffer at constant potential of –0.65 V *vs.* Ag/AgCl. The carbonyl compounds were reduced to the corresponding alcohols with high current efficiency (97.2 – 100%) and high yield (97.4 – 100%), respectively.

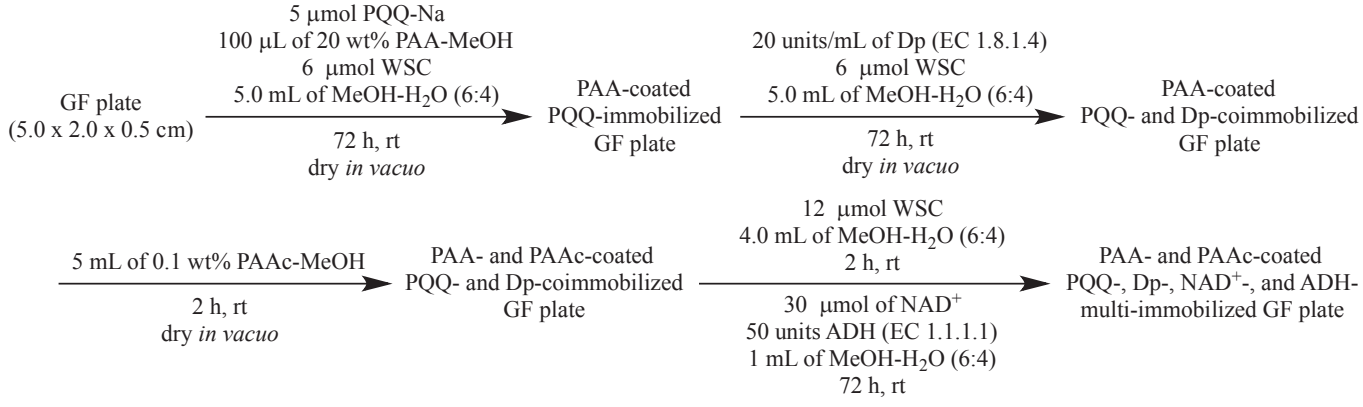
INTRODUCTION

A mediator-modified electrode that can be widely used in advanced technologies should have the following features.[1] First, the amount of mediators required for modification, such as expensive catalysts and enzymes, should be minimized to assure an easy, economical process for electrochemical reactions. Second, the product converted from the substrate should be easily isolable from the electrolyte solution by the use of electric energy. The system should provide a simple, clean process. Third, appropriate design of the mediator and its environment at the electrode surface is necessary to enhance the reactivity and selectivity of intended reactions, and to change the reaction paths from those in the homogeneous system. The use of enzymes in organic synthesis is highly promising,[2-4] as they show

remarkable chemo-, substrate-, region- and stereoselectivities. In particular, NAD(P)(H)-requiring oxidoreductases are the most useful and widely investigated enzymes.[5] In order to apply those enzymes to organic synthesis, much effort has been devoted to the development of an efficient coenzyme-recycling system by means of chemical, electrochemical, and enzymatic strategies.[6-8] As an electrochemical method for ketone reduction, we have already reported the preparation of a thin cation-exchange polymer (Nafion)- and poly(acrylic acid) (PAAc)- layers-coated graphite felt (GF) electrode that immobilized methyl viologen (MV^{2+}) (electron transfer mediator), diaphorase (Dp) (flavoprotein), NAD^+ (coenzyme) and alcohol dehydrogenase (ADH) (enzyme).[9,10] However, this GF electrode fixed MV^{2+} in the Nafion layer of the electrode by ion-pair formation and therefore MV^{2+} that was fixed on the electrode would come off during electrolysis, making the electrode unstable. This paper deals with electrocatalytic reduction of carbonyl compounds on a polymer layer-coated GF electrode on which electron transfer mediator pyrroloquinolinequinone (PQQ) was immobilized by chemical bonds. Yoneyama *et al*. have reported electroenzymatic reduction of formate with methanol dehydrogenase (MDH) and PQQ.[11,12] However, the electrolysis reaction they reported occurred without immobilizing MDH and PQQ on the electrode.

RESULTS AND DISCUSSION

The detailed preparation procedure of the poly(allylamine) (PAA)- and PAAc-coated GF electrode multi-immobilizing PQQ, Dp, NAD^+ and ADH is demonstrated in Scheme 1. The oxidation potential of the PQQ-immobilized GF electrode was –0.10 to –0.25 V *vs.* Ag/AgCl and the peak split between the anodic and cathodic peak potentials was widened with the increasing repeated cyclic voltammetry scans (Figure 1). A linear relationship between the peak current and the square root of the scan rate was observed.[13] It means that the reduction species of PQQ can be effectively used as reduction mediator. The amounts of electroactive PQQ on the electrode surface was determined by integrating PQQ reduction



Scheme 1. Preparation of PAA- and PAAc-coated GF electrode multi-immobilizing PQQ, Dp, NAD^+ and ADH

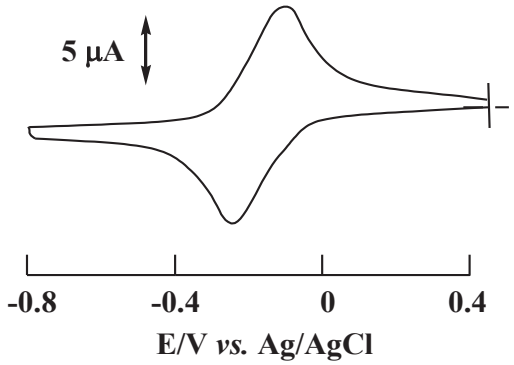


Figure 1. Cyclic voltammogram of PQQ-immobilized GF electrode (1.0 x 1.0 x 0.5 cm^3) in 100 mM phosphate buffer solution (pH 7.4) at the scan rate 50 mV s^{-1}

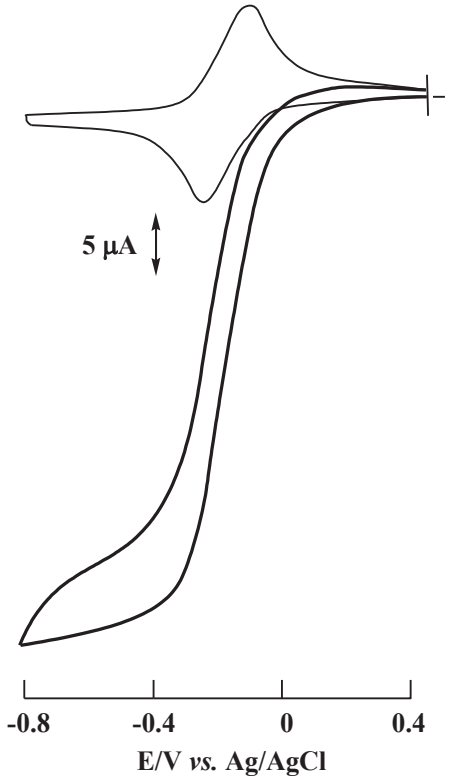


Figure 2. Cyclic voltammograms on PQQ- and Dp-coimmobilized GF electrode in the presence (———) and absence (——) of 50 mM NAD^+. 100 mM phosphate buffer solution: pH 7.4. Electrode size: 1.0 x 1.0 x 0.5 cm^3. Scan rate: 50 mV s^{-1}.

current peak of the cyclic voltammogram (CV) and applying Faraday's law of *ca.* 1.76 x 10^{-8} mol cm^{-2}. The CVs revealed that the PQQ- and Dp-coimmobilized GF electrode thus prepared afforded an electrocatalytic current required for the reduction of NAD^+ while the PQQ-immobilized GF electrode scarcely showed the similar values (Figure 2). The electrocatalytic reduction of NAD^+ took place on the PQQ-immobilized GF electrode and the PQQ- and Dp-coimmobilized GF electrode in 20 mL of 50 mM NAD^+/100 mM phosphate buffer solution (pH 7.4) at –0.30 V. The consumption of NAD^+ in electrolysis solutions was analyzed by the absorbance increase at 340 nm.[14] For the electrolysis on the

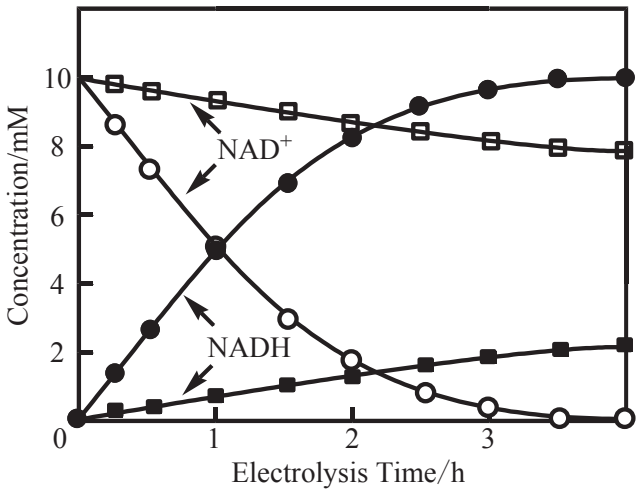


Figure 3. Macroelectrolysis of NAD^+ on PQQ- and Dp-coimmobilized GF electrode (○, ●) and PQQ-immobilized GF electrode in the presence of 10 U/mL Dp (□, ■). Electrode size: 1.0 x 1.0 x 0.5 cm^3. Reduction potential: –0.30 V *vs.* Ag/AgCl.

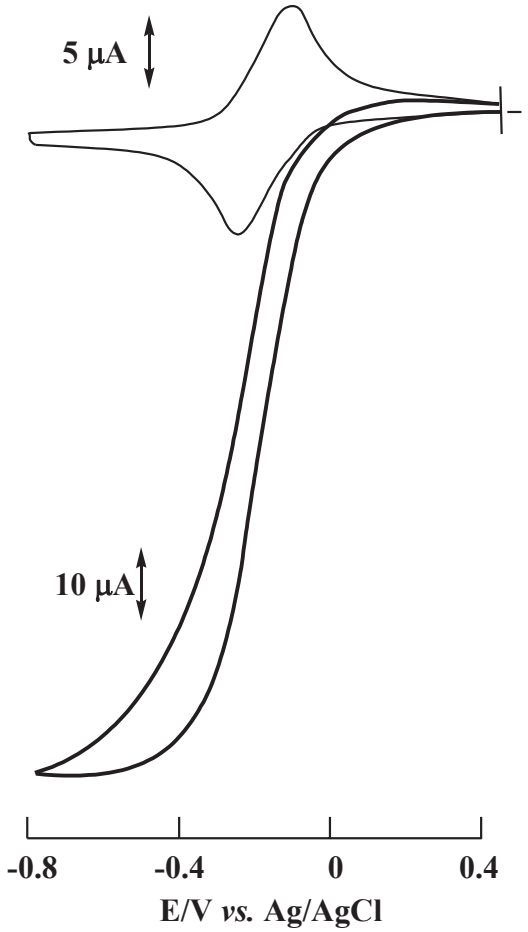


Figure 4. Cyclic voltammograms on PAA- and PAAc-coated GF electrode multimmobilizing PQQ, Dp, NAD^+ and ADH in the presence (——) and absence (——) of 10 mM formate. 100 mM Phosphate buffer solution: pH 7.4. Electrode size: 1.0 x 1.0 x 0.5 cm^3. Scan rate: 50 mV s^{-1}.

PQQ-immobilized GF electrode, 10 U/mL of Dp (EC 1.8.1.4) was added. The results are shown in Figure 3. The PQQ- and Dp-coimmobilized GF electrode afforded complete conversion and quantitative reduction of NAD^+ to NADH with 3.5 h electrolysis. The current efficiency and turnover number of PQQ for NADH production were 98.5% and 18, respectively at that stage. On the other hand, the PQQ-immobilized GF electrode with a Dp-containing solution achieved 20% conversion of NAD^+ to NADH with 4 h electrolysis. From these results, it is obvious that the combination of the constituents in the thin PAA layer on the GF electrode allows electron transfer between PQQ and Dp and diffusion of substrates, such as NAD^+.

The PAA- and PAAc-coated GF electrode multi-immobilizing PQQ, Dp, NAD^+ and ADH showed a high electrocatalytic current for the reduction of formate with the peak potential of –0.65 V (Figure 4), which suggests the potential utility of this GF electrode for macroelectrolysis.

The time course of the reaction is shown in Figure 5. Formate was reduced selectively to methanol and was consumed almost completely in approximately 1 h. When the PQQ- and Dp-coimmobilized GF electrode was used in the presence of 30 μmol of NAD^+ and 50 units of ADH in a 20 mL of 100 mM phosphate buffer solution (pH 7.4), the electrochemical reduction rate was slower, but no product other than methanol was formed. This suggests that an all-mediation-constituents-immobilized electrode offers a more efficient electroenzymatic reduction. The results of formate reduction on the PAA- and PAAc-coated GF electrode multi-immobilizing PQQ, Dp, NAD^+ and ADH were as follows; conversion: 100%, current efficiency: 99.97%, and selectivity for methanol: 100%. The turnover number of PQQ for methanol production was 180 at that stage. The peak current of PQQ of a used electrode was almost equal to that of a new one, and a used electrode showed nearly the same electrocatalytic current for the reduction of formate as a new electrode. Therefore, it can be concluded that the electrode is not deactivated during macroelectrolysis and can be used repeatedly.

We have reported that a thin cation exchange polymer (Nafion)- and PAAc-coated GF electrode immobilizing MV^{2+}, NAD^+, Dp and ADH was prepared and applied to electroenzymatic reduction of ketones.[8] The electrocatalytic reduction of formate with this type of GF electrode was tested at the constant potential of –0.80 V *vs.* Ag/AgCl. The time course of the reaction is shown in Figure 6. Formate was first reduced to formaldehyde, and when formaldehyde accumulated, methanol production began. The current efficiency was 42.5% in the formaldehyde production and 48.8% in the methanol production. The turnover number of MV^{2+} for the formaldehyde and methanol production was 691 for 20 h electrolysis.[15] On the other hand, when PQQ was used as electron transfer mediator, methanol alone was obtained as reduction product as shown in Figure 5. It is not clear why PQQ produces methanol, while MV^{2+} mainly produces formaldehyde. Presumably, interaction between the enzyme and the electron relays is different.

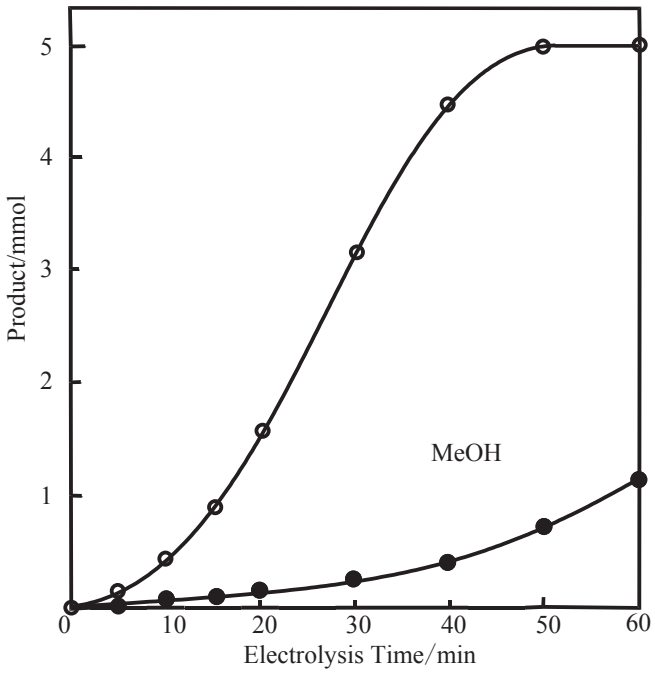


Figure 5. Macroelectrolysis of formate on PAA- and PAAc-coated GF electrode multimmobilizing PQQ, Dp, NAD^+ and ADH (○), and PQQ- and Dp-coimmobilized GF electrode in the presence of 30 μmol of NAD^+ and 50 units of ADH (●). Electrode size: 1.0 x 1.0 x 0.5 cm^3. Reduction potential: –0.65 V *vs.* Ag/AgCl.

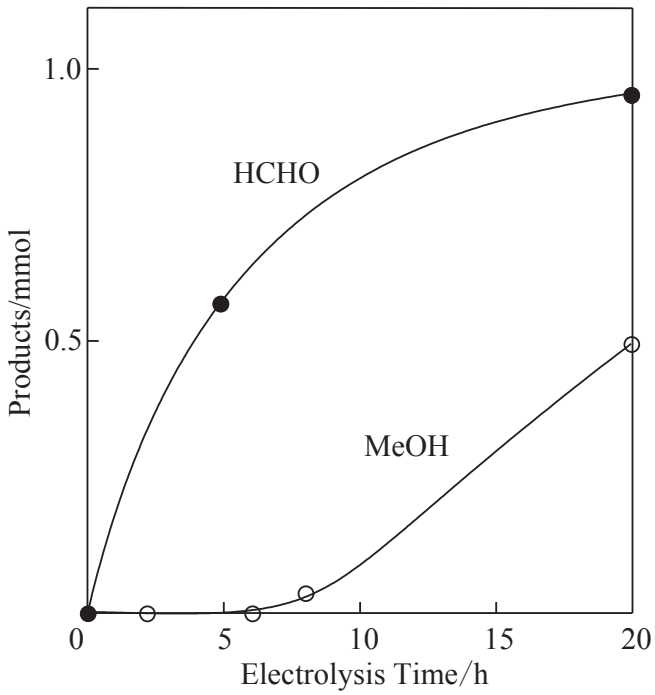
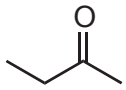
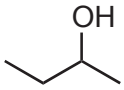
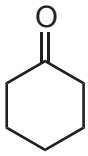
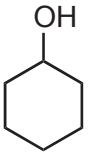
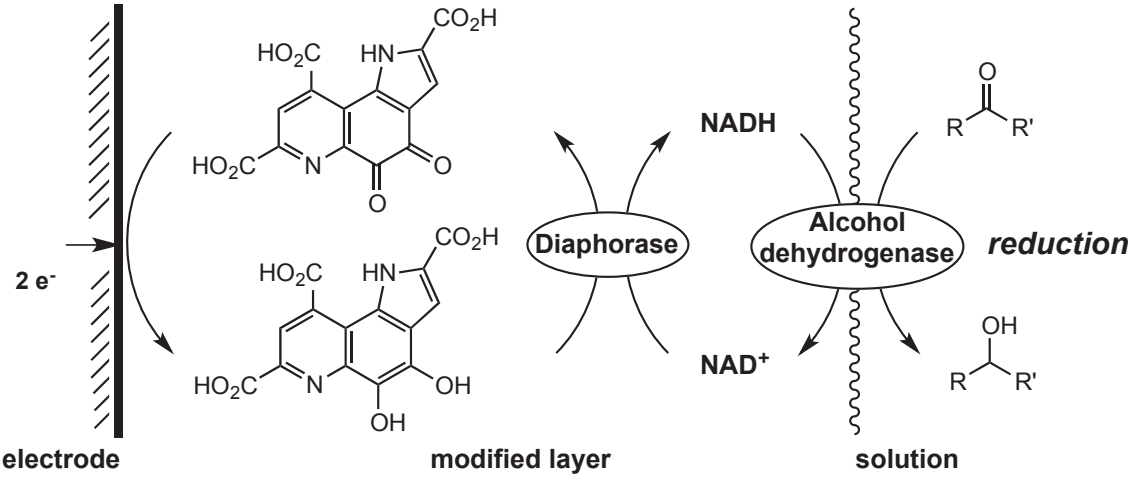


Figure 6. Macroelectrolysis of formate on Nafion- and PAAc-coated GF electrode immobilizing MV^{2+}, NAD^+, Dp and ADH. Electrode size: 1.0 cm x 1.0 cm x 0.5 cm. Reduction potential: –0.80 V *vs.* Ag/AgCl.

Table 1. Electrocatalytic reduction of various carbonyl compounds on the PAA- and PAAc-coated GF electrode multimmobilizing PQQ, Dp, NAD^+ and ADH

Substrate	Product	Currnt efficiency / %	Yield / %
$MeCO_2H$	EtOH	99.5	99.2
$Me(CH_2)_2CO_2H$	$Me(CH_2)_3OH$	98.6	98.5
$Me(CH_2)_4CO_2H$	$Me(CH_2)_5OH$	97.9	98.3
		97.8	97.4
		97.2	97.7



Scheme 2. A depicted mechanism of reduction for carbonyl compounds on PQQ-enzyme multi-immobilized electrode

The results from the electrocatalytic reduction of various carbonyl compounds on the PAA- and PAAc-coated GF electrode multimmobilizing PQQ, Dp, NAD^+ and ADH are shown in Table 1. The carboxylic acids such as formate, acetic acid, butyric acid and hexanoic acid are usually reduced to the corresponding primary alcohols with high current efficiency (97.9 – 99.5%) and high yield (98.3 – 99.2%). The reduction of 2-butanone and cyclohexanone as ketone led to 2-butanol and cyclohexanol as secondary alcohol, respectively, in high current efficiency (97.8% and 97.2%) and high yield (97.4% and 97.7%). A conceptual mechanism is depicted in Scheme 2.

The current density of ca. 1.7 mA GF electrode cm^{-2} in the electrolysis can be evaluated better than those of an enzymatic solution method,[11,12,16] from the viewpoint of productively. This fact also suggests that the enzymes immobilized to polymer layer on the GF electrode carry out the enzymatic reaction efficiently without deactivation.

In summary, the PAA- and PAAc-coated GF electrode immobilizing all the mediation constituents, PQQ, Dp, NAD^+ and ADH, was successfully applied to the electroenzymatic reduction of carbonyl compounds. The carbonyl compounds were reduced to the corresponding alcohols with high current efficiency and high yield. The electrolysis is simple and clean and it is probably easy to separate the reaction product from the unreacted substrate. This method, therefore, will provide a new type of bioelectrochemical reactor.

EXPERIMENTAL

1. General Information

GF electrode was purchased from Nippon Kynol Inc. PAA was purchased from Nittobo Medical Co. LTD. PAAc was purchased from Sigma-Aldrich Co. LLC. PQQ-Na, ADH, Dp and NAD^+ were purchased from Wako Pure Chemical Industries, Ltd. All the other reagents used in this study were of the commercially available reagent grade.

2. Preparation of PQQ-enzyme Multi-immobilized Electrodes

A GF plate (5 cm x 2.0 cm x 0.5 cm) was impregnated with a solution of 5 μmol (1.87 mg) of PQQ-Na, 100 μL of 20 wt% PAA-MeOH solution and 6 μmol (1.15 mg) of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (WSC) in 5.0 mL of MeOH-H_2O (6:4). The GF plate was treated for 72 h at room temperature and dried *in vacuo*. It was then washed three times with MeOH-H_2O (3:7) and dried *in vacuo*. The PQQ-immobilized GF electrode was then treated with a solution of 20 U/mL of Dp (EC 1.8.1.4) and 6 μmol (1.15 mg) of WSC in 5 mL of MeOH-H_2O (6:4) for 72 h at room temperature and dried *in vacuo*. It was washed three times with MeOH-H_2O (3:7) and dried *in vacuo*. The PQQ- and Dp-coimmobilized GF electrode was then coated with PAAc by being immersed the electrode in 5 mL of 0.1 wt% PAAc MeOH solution for 2 h at room temperature and dried *in vacuo*. The PAA- and PAAc-coated GF electrode with immobilized PQQ and Dp was dipped in a solution of 12 μmol (3 mg) of WSC in 4 mL of MeOH-H_2O (6:4) for 2 h. Then, it was put in a solution of 30 μmol (19.9 mg) of NAD^+ and 50 units of ADH (EC 1.1.1.1) in 1 mL of MeOH-H_2O (6:4) and it was treated for 72 h at room temperature. It was washed three times with MeOH-H_2O (3:7) and dried *in vacuo*.

3. Cyclic voltammetry

The PQQ-modified electrode (1.0 x 1.0 x 0.5 cm) and platinum plate ware employed as the working electrode and counter electrode, respectively. An Ag/AgCl electrode was used as the reference electrode. The cyclic voltammetry characterization was conducted on a BAS ALS/Chi420A. All electrochemical measurements were carried out at room temperature under nitrogen atmosphere.

4. Macroelectrolysis of Carbonyl Compounds on PQQ-enzyme Multi-immobilized Electrodes

Macroelectrolysis was conducted on a Hokuto Denko Model HABF-501. Twenty mL of 250 mM formate/100 mM phosphate buffer solution (pH 7.4) was electrolyzed with the PAA- and PAAc-coated GF electrode multi-immobilizing PQQ, Dp, NAD^+ and ADH (1.0 x 1.0 x 0.5 cm) in an H-type two-compartment cell separated by a cation exchange membrane (Nafion 117) at the constant potential of –0.65 V *vs.* Ag/AgCl under a nitrogen atmosphere. During electrolysis, the substrate and products were occasionally analyzed by gas chromatography (GC, CP-cyclodextrin-B-2,3,6-M-19, 0.25 mm ϕ x 25 m / raising temp 3 °C min-1 from 80 to 150 °C, inj. temp 200 °C, detc. temp 240 °C) and high performance liquid chromatography (HPLC, CHRALCEL-OD, 0.46 mm ϕ x 25 cm / column temp 30 °C, flow speed: 0.5 mL min^{-1}, solvent: hexane:isopropanol = 95:5). The carbonyl compound was identified as the reaction products by comparing its retention time of GC and HLPC with an authentic sample.

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