

Immobilization of Alcohol Dehydrogenase on Films Prepared by the Electrochemical Copolymerization of Pyrrole and 1-(2-Carboxyethyl)pyrrole for Ethanol Sensing

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ABSTRACT: The electrochemical copolymerization of pyrrole and 1-(2-carboxyethyl)pyrrole (Py-COOH) was carried out, and a conducting polymer film with a Py-COOH content of 5% and a conductivity of 6.6×10^{-4} S/cm was obtained. Alcohol dehydrogenase (ADH) was attached through amide linkage onto the surface of the conducting polymer film for the purpose of fabricating an ADH-immobilized electrode applicable to the amperometric sensing of ethanol. The quantity and activity of the immobilized ADH were determined to be $400 \mu\text{g}/\text{cm}^2$ and 0.6 U/mg, respectively. With the ADH-immobilized electrode, amperometric ethanol sensing was attempted in the presence of nicotinamide adenine dinucleotide (a cofactor of ADH) and Meldo-

la's blue (an electron-transferring mediator). The ADH-immobilized electrode made the current response correspond to ethanol concentration, which reached $10 \mu\text{A}/\text{cm}^2$ at a concentration of 14 mM. The selectivity of the sensing was examined with alcohols other than ethanol. It was found that the ADH/copolymer electrode had a considerably larger current response to allyl alcohol. The selectivity was attributed to the specificity of native ADH with respect to the oxidation of alcohols. © 2010 Wiley Periodicals, Inc. *J Appl Polym Sci* 116: 2651–2657, 2010

Key words: conducting polymers; electrochemistry; enzymes

INTRODUCTION

In recent years, conducting polymers have attracted much attention as important materials for the fabrication of enzyme electrodes, which are used as key components in bioelectrochemical sensing systems^{1–8} and biofuel cell systems.^{9,10} Conventionally, enzyme electrodes have been fabricated by the entrapment of enzyme molecules physically within the films of conducting polymers during electrochemical polymerization. The enzyme electrodes prepared by this method have been practically applied to *in vivo* and *in vitro* measurements in clinical analyses.^{11–15} Indeed, the entrapment method may be a simple way of fabricating an enzyme electrode, but it is a matter of concern that the enzyme molecules entrapped deep in the conducting polymer film will contribute little to the sensing ability of the electrode, for the enzyme reaction is considered to occur exclusively on the surface of the electrode. It should be taken into account, in addition, that leakage may be inevitable for the enzyme molecules entrapped in close vicinity to the electrode surface.

As for the efficiency of enzyme utilization, the entrapment method seems to be inferior to the attachment method, which binds enzyme molecules onto the surfaces of conducting polymer films. From such a point of view, we attempted to immobilize enzymes covalently by using the surface functional groups of conducting polymer films as binding reaction sites. In a previous study,⁸ a conducting copolymer of pyrrole (Py) and 1-(2-carboxyethyl)pyrrole (Py-COOH) was synthesized electrochemically in the form of a thin film, and an enzyme electrode was fabricated by the covalent immobilization of glucose oxidase on the film of the copolymer (Py/Py-COOH copolymer). The immobilization was founded on the condensation reaction between the NH_2 groups of glucose oxidase and the COOH groups on the film. According to this study, the amperometric response to glucose, depending on its concentration, was observed successfully by use of the enzyme electrode. The magnitude of the response was found to decrease with increasing content of Py-COOH units in the copolymer because of the decreasing conductivity of the copolymer film, and the appropriate content of Py-COOH units in the copolymer was considered to be 5% or less.

On the basis of these results, an enzyme electrode was fabricated anew with alcohol dehydrogenase (ADH) for ethanol sensing in this study. ADH was immobilized on a film of Py/Py-COOH copolymer

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through amide linkages by a condensation reaction with COOH groups on the film, and the ADH-immobilized copolymer film was applied to the amperometric sensing of ethanol as the enzyme electrode (ADH/copolymer electrode). In this article are reported the results from this investigation of the sensing system, which included an electron-transferring mediator, which combined enzymatic and electrochemical reactions.

EXPERIMENTAL

Materials

Py and 1-(2-cyanoethyl)pyrrole (Py-CN) were purchased from Aldrich Chemical Co. (St. Louis, MO); the former was purified by distillation under reduced pressure before use in polymerization, and the latter was converted to Py-COOH, as described later. Tetra-*n*-butylammonium tetrafluoroborate was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). All of these chemicals were guaranteed reagent grade. ADH (EC 1. 1. 1. 1, from *Saccharomyces cerevisiae*) was supplied by Sigma Chemical Co. (St. Louis, MO), which had a specific activity of 1.8 U/mg. Oxidized nicotinamide adenine dinucleotide (NAD⁺) was obtained from Roche Diagnostics GmbH (grade II, Mannheim, Germany). 1-Cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate (CMC) and Meldola's blue (MB) from Aldrich Chemical were used as a condensing agent and an electron-transferring mediator, respectively. Other chemicals were guaranteed-reagent or analytical grade and were used without further purification.

Synthesis of Py-COOH

Py-COOH was obtained by the hydrolysis of Py-CN in the following manner:¹⁶ a mixture of 25 g of Py-CN and 100 mL of a 15% potassium hydroxide solution was stirred at 50°C for 40 h. Then, the mixture was cooled to room temperature and acidified with hydrochloric acid. After extraction with ether, the crude product (colorless crystals) was collected by the evaporation of ether. The crude product was dissolved in ether and purified by recrystallization from the ether solution. The product was identified as Py-COOH by means of ¹H-NMR and ¹³C-NMR spectroscopy.

Electrochemical copolymerization of Py and Py-COOH

The electrochemical copolymerization of Py and Py-COOH was carried out in a three-electrode cell equipped with an HA-150G potentiostat/galvanostat

(Hokuto Denko Corp., Tokyo, Japan) and an HF-203D coulomb/ampere hour meter (Hokuto Denko). A gold film deposited on an alumina plate was used as the working electrode (0.25 cm²). A platinum plate and a saturated calomel electrode (SCE) were used as a counter electrode and a reference electrode, respectively.

In advance, given amounts of Py and Py-COOH were dissolved in propylene carbonate, and their concentrations were adjusted to 0.45 and 0.05M, respectively. After 20 mL of the solution was placed in the cell, nitrogen was passed through the solution for 20 min. Then, a potential of +1.2 V versus SCE was applied on the working electrode until the amount of passed charge was 240 mC/cm². The copolymer of Py and Py-COOH (Py/Py-COOH copolymer) was deposited as a thin film on the working electrode. The thickness of the film was measured with a needle-touch type thickness meter.

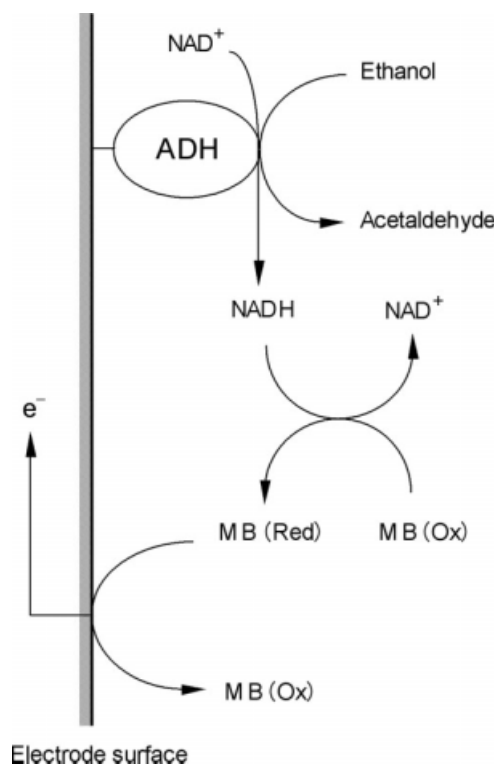
The composition of the Py/Py-COOH copolymer was determined by means of IR spectroscopy (a KBr disk method). The content of Py-COOH units in the copolymer was calculated on the basis of the absorbance in the vicinity of 1700 cm⁻¹; this corresponded to the C=O bond stretching of COOH groups and was measured on a JIR-7000 Fourier transform infrared spectrometer (JEOL, Akishima, Tokyo, Japan). The conductivity of the copolymer film was measured in the thickness direction by a two-probe direct-current method. Before the conductivity measurement, another gold electrode was deposited on the surface (opposite to the alumina plate) of the copolymer film, and ohmic contact of the film with the gold electrodes was confirmed to verify no formation of a rectifying junction.

Immobilization of ADH on the Py/Py-COOH copolymer film

With CMC as a condensing agent, ADH was immobilized covalently on the Py/Py-COOH copolymer film to prepare the ADH/copolymer electrode as follows:¹⁷ 27 mg of ADH and 30 mg of CMC were dissolved in 2.0 mL of distilled water, and the solution was kept at 4°C. The copolymer film was immersed in the solution for 18 h and then rinsed with distilled water.

The quantity of immobilized ADH was determined, according to the method of Lowry et al.,¹⁸ by analysis with a Folin-Ciocalteu phenol reagent after alkaline copper treatment, in which colorimetry was carried out at 750 nm with a UV-3100 PC ultraviolet-visible (UV-vis) spectrometer (Shimadzu Corp., Kyoto, Japan).

The activity of the immobilized ADH was measured by the tracing of reduced nicotinamide adenine dinucleotide (NADH; the reduced form of



Scheme 1 Series of redox reactions on the ADH/copolymer electrode.

NAD⁺) formed by ethanol oxidation with ADH and NAD⁺: in 3.0 mL of a phosphate buffer (0.05M, pH 8.0), 40 μ L of a NAD⁺ solution (7.5 mM) and 100 μ L of an ethanol solution (1.0 M) were added, and then, the ADH/copolymer electrode was immersed in the mixture. The activity was determined on the basis of the NADH formation rate calculated from the change in absorbance of the mixture at 340 nm.

Surface characterization of the copolymer film and the ADH/copolymer electrode

The imaging of the Py/Py-COOH copolymer film and the ADH/copolymer electrode was carried out on a JEOL JSM-6301F scanning electron microscope by the application of an acceleration voltage of 10 kV on sputtered samples.

Electrochemical measurements

Scheme 1 illustrates the series of redox reactions on the ADH/copolymer electrode associated with ethanol sensing, where MB, whose structure is shown in Figure 1, was used as an electron-transferring mediator. In this system, the immobilized ADH catalyzed the oxidation of ethanol with NAD⁺ (a cofactor of ADH). Thus, ethanol was converted into acetaldehyde, and NADH resulting from NAD⁺ reduced MB. The reduced form of MB was then oxidized electrochemically on the ADH/copolymer electrode,

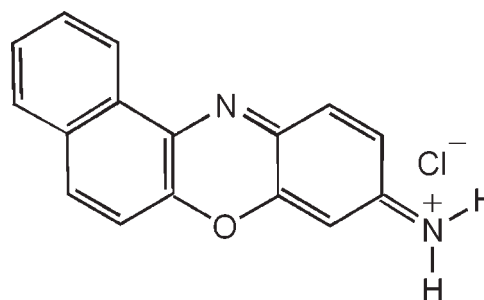


Figure 1 Structure of MB.

and the accompanying electron transfer to the electrode was detected as a current change.

Amperometric ethanol sensing was carried out by use of the ADH/copolymer electrode as the working electrode (0.25 cm²) in the same three-electrode cell as that which served the electrochemical copolymerization, with the current response measured at a constant potential of +100 mV versus SCE in 20 mL of a phosphate buffer solution (0.05M, pH 8.0) in the presence of 10 mM NAD⁺ and 1.0 mM MB. In advance, nitrogen was passed through the buffer solution for 20 min. After the background current was allowed to become constant, a given concentration of ethanol solution was added incrementally to the buffer solution, and the resulting current change was recorded.

The redox behavior of NADH and MB was examined by cyclic voltammetry. A gold film on an alumina plate was used as the working electrode. The measurement was conducted at a scan rate of 20 mV/s in a phosphate buffer solution (0.05M, pH 8.0) with a function generator (Hokuto Denko, HB-105A)

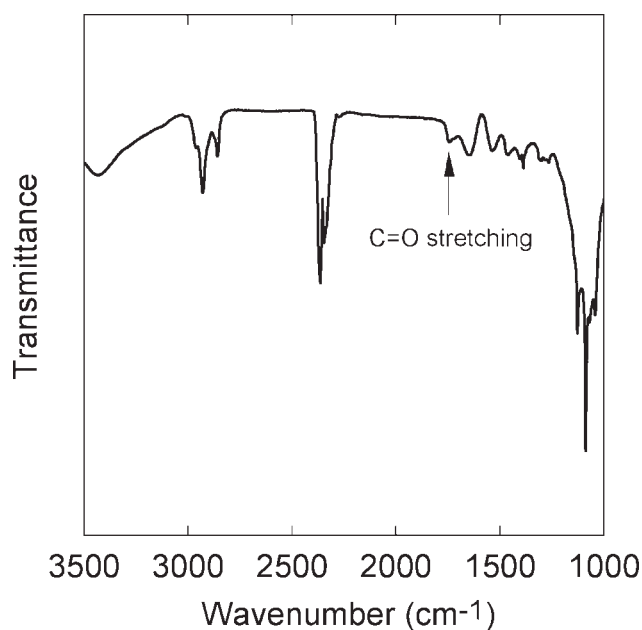


Figure 2 IR spectrum of the Py/Py-COOH copolymer.

TABLE I
Characterization of the ADH/Copolymer Electrode

Item	Specification
Apparent surface area	0.25 cm ²
Thickness	9 μm
Py-COOH content in the copolymer film	5%
Conductivity	6.6 × 10 ⁻⁴ S/cm
Quantity of immobilized ADH	100 μg (400 μg/cm ²)
Activity of immobilized ADH	0.06 U (0.6 U/mg)

after nitrogen was passed through the buffer solution for 20 min.

RESULTS AND DISCUSSION

Characterization of the ADH/copolymer electrode

The IR spectrum of the Py/Py-COOH copolymer is shown in Figure 2, where an absorption corresponding to the C=O bond stretching of the COOH groups is shown. Table I summarizes the characteristics of the ADH/copolymer electrode. The copolymer was obtained as a thin film with a thickness of 9 μm. It had a Py-COOH content of 5% (Fig. 3) and a conductivity of 6.6 × 10⁻⁴ S/cm, which were in accordance with the previous study.⁸ The quantity and specific activity of the immobilized ADH were determined to be 400 μg/cm² and 0.6 U/mg, respectively. This activity corresponded to a third of the native one. Figure 4 shows the scanning electron microscopy images of the Py/Py-COOH copolymer film [Fig. 4(A)] and the ADH/copolymer electrode [Fig. 4(B)]. The actual surface area of the copolymer film was larger than the apparent one because of its grained structure.

Role of the electron-transferring mediator

At the beginning of this study, the ADH/copolymer electrode was expected to have an amperometric response to ethanol in the presence of NAD⁺, for NADH resulting from NAD⁺ was believed to take part in direct electron transfer to the copolymer film. However, no response was observed at ethanol concentrations up to 10 mM and at applied potentials

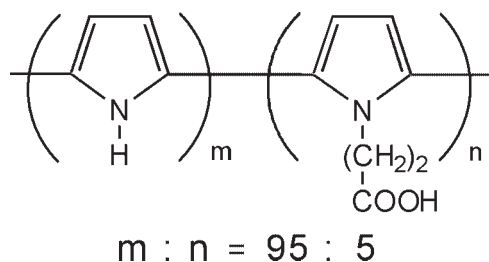


Figure 3 Structure of the Py/Py-COOH copolymer.

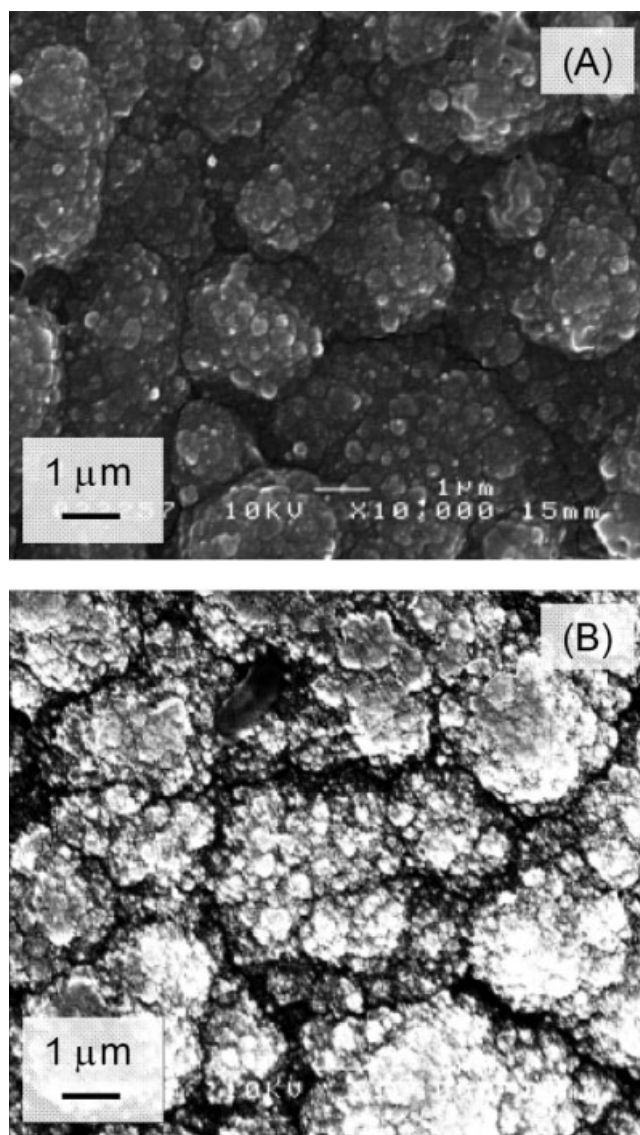


Figure 4 Scanning electron microscopy images of the surfaces of the (A) Py/Py-COOH copolymer film and (B) ADH/copolymer electrode.

up to +850 mV versus SCE, and thus, the amperometric sensing of ethanol met with failure. Furthermore, the ADH/copolymer electrode made no response, even to 10 mM NADH. These results may reflect the difficulty in the electrochemical oxidation of NADH on the electrode or the lowered activity of the immobilized ADH due to the potential applied to the electrode. It was a matter of importance, therefore, to incorporate an effective electron-transferring mediator into the ethanol-sensing system.

A number of compounds, including dyes, have been considered to be promising candidates for electron-transferring mediators.¹⁹⁻²⁴ With the redox behavior taken into account, MB was selected from these as the mediator and used in ethanol sensing. As shown by the cyclic voltammograms in Figure 5, MB gave a higher peak of the oxidation current than

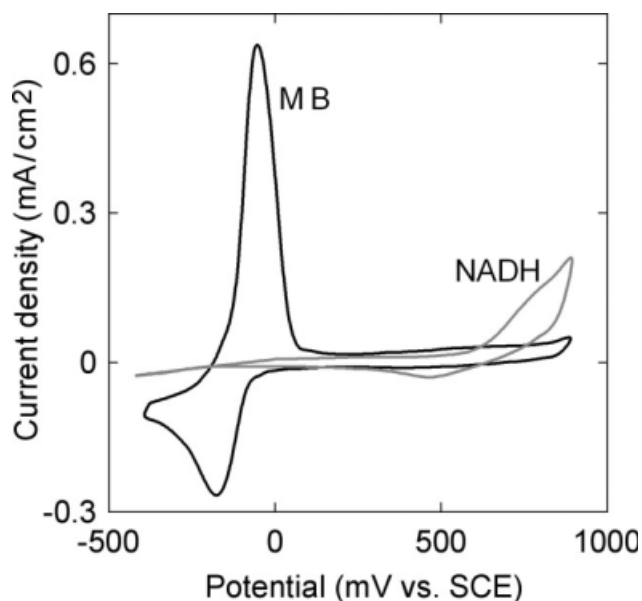


Figure 5 Cyclic voltammograms of NADH and MB. The concentration of NADH and MB was adjusted to 1.0 mM.

NADH. In addition, the onset potential for the MB oxidation was -200 mV versus SCE, which was 800 mV lower than that for the NADH oxidation. This result suggests that, if NADH reduces MB at a high rate, ethanol sensing can be carried out at a considerably low potential. The redox reaction between NADH and MB was examined by means of UV-vis spectroscopy. When equivalent molar amounts of NADH and MB were mixed, the absorption of

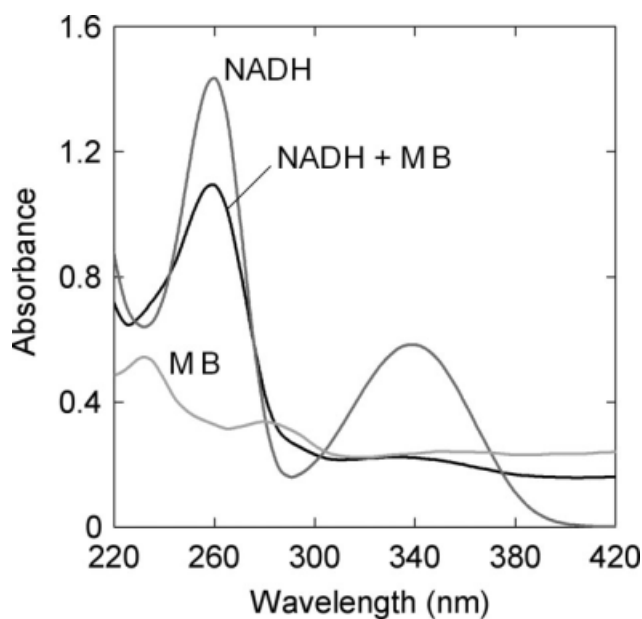


Figure 6 UV-vis spectra of NADH, MB, and their mixture. The spectra were measured with phosphate buffer solutions (0.05M, pH 8.0) containing NADH (0.10 mM), MB (0.10 mM), or an NADH/MB mixture with equivalent molar amounts (0.10 mM in total).

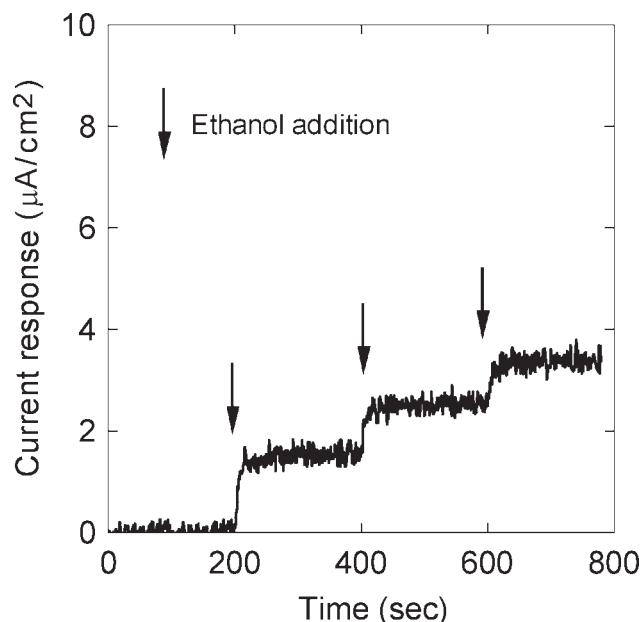


Figure 7 Typical amperometric response to ethanol. An ethanol solution (0.10M, 100 μ L) was added incrementally to 20 mL of a phosphate buffer solution (0.05M, pH 8.0) in which the ADH/copolymer electrode was immersed.

NADH at 340 nm was not observed, as shown Figure 6 (NADH + MB). Because the spectrum of the mixture was found to be similar to that of NAD^+ , we confirmed that NADH readily reduced MB to become NAD^+ . Thus, MB was used as the mediator in the ethanol-sensing system.

Ethanol sensing with the ADH/copolymer electrode

On the basis of the preliminary results described in the previous section, ethanol sensing was attempted in the presence of MB. A potential of +100 mV versus SCE was applied to the ADH/copolymer electrode in consideration of the result shown in Figure 5 that the peak of the MB oxidation current was observed around -60 mV versus SCE. As shown in Figure 7, we confirmed that the ADH/copolymer electrode marked current response to ethanol successfully in the presence of MB. Incremental ethanol addition brought about a stepwise current increase. In addition, the current increased rapidly with ethanol addition and became almost constant within 20 min or less. This result suggests that a considerably high reaction rate was ensured for the reduction of MB with NADH and for the oxidation of the reduced MB on the ADH/copolymer electrode. Thus, the electrode came to function well as an ethanol sensor by the incorporation of MB into the electrochemical redox system.

Figure 8 shows the plots of the current response to various concentrations of ethanol measured with

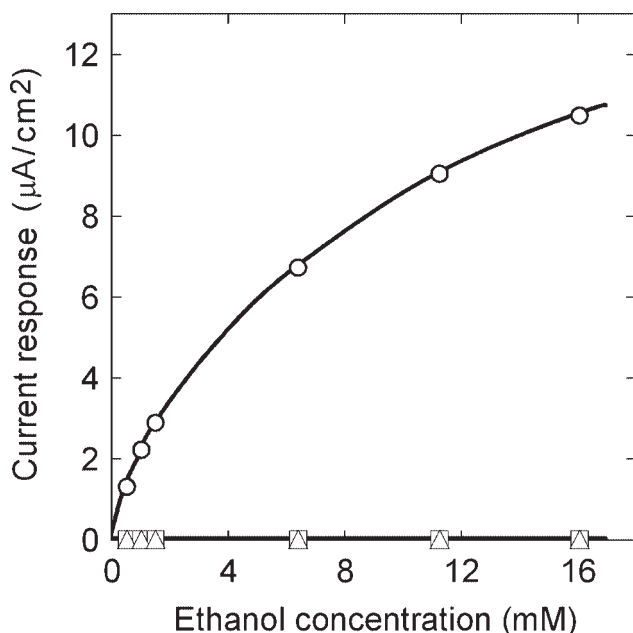


Figure 8 (○) Current response to various concentrations of ethanol measured with the ADH/copolymer electrode. The measurement was also carried out in the absence of (△) NAD^+ or (□) MB.

the ADH/copolymer electrode, where the data obtained without NAD^+ or MB are appended. Clearly, the current response increased with increasing ethanol concentration in the presence of both NAD^+ and MB. This was an important fact that led to the quantitative determination of ethanol. The relative standard deviations of the ethanol-responding currents were within 13% for three samples of the ADH/copolymer electrode. Taking into account the background noise in Figure 7, the ethanol detection range was roughly estimated to be 0.2–30 mM. In the absence of NAD^+ or MB, however, no current response was observed, even at an ethanol concentration higher than 10 mM. These results support the conclusion that amperometric sensing of ethanol was achieved through the series of the redox reactions shown in Scheme 1.

In this sensing system, both NAD^+ and MB were used in a dissolved state. When these were bound onto the ADH/copolymer electrode surface, the ethanol sensing could be carried out without their addition to the sample solutions. With respect to the conditions of NAD^+ and MB in the bound state, their mobility had to be maintained, for example, with a flexible linker, to allow their interaction with NAD^+ and ADH. Thus, the binding of NAD^+ and MB onto the electrode surface was never easy, but it is a very important subject for improving the performance of the electrode. Such modification of the electrode surface will be realized by adequate design of the linker chains and binding reactions. These are under consideration.

Selectivity of the sensing with the ADH/copolymer electrode

In view of practical application, selectivity is one of the essential properties required for sensors. As for the ADH/copolymer electrode, it has been a point of interest to determine what response is made to alcohols other than ethanol. In this study, methanol, 2-propanol, and allyl alcohol were subjected to amperometric sensing. Figure 9 shows the current response observed for various concentrations of these alcohols and ethanol. The ADH/copolymer electrode had little current response to methanol and 2-propanol, but it had a considerably large response to allyl alcohol. Provided that unexpected side reactions in the sensing system were negligible, the difference in current response should have been due to the specificity of native ADH with respect to the oxidation of those alcohols. For example, ADH is commonly known to catalyze the oxidation of allyl alcohol. Thus, the selectivity of the ADH/copolymer electrode will be forecast from the specificity of native ADH.²⁵ However, it goes without saying that in the case of the application to practical samples, the response to coexisting major species must be examined in advance.

CONCLUSIONS

A film of conducting polymer was prepared by the electrochemical copolymerization of Py and Py-COOH, and ADH was immobilized covalently through amide linkages on the surface of the

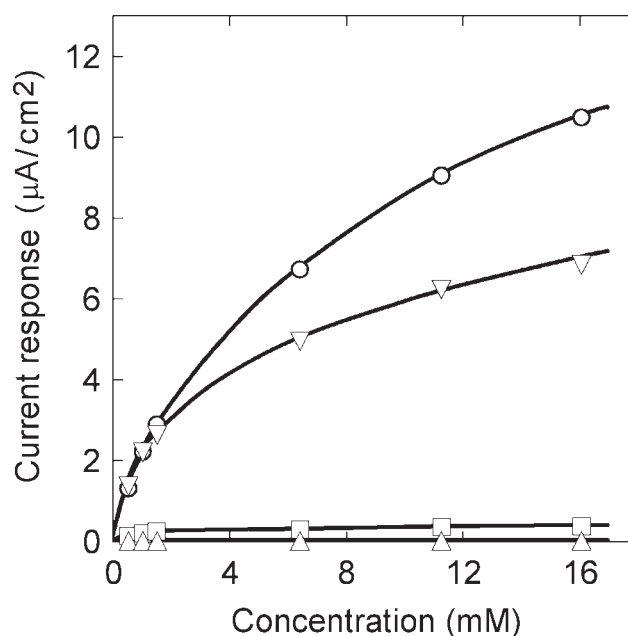


Figure 9 Current response to various concentrations of (○) ethanol, (△) methanol, (□) 2-propanol, and (▽) allyl alcohol.

conducting polymer film for the purpose of fabricating an ADH-immobilized electrode applicable to the amperometric sensing of ethanol. The quantity and activity of the ADH immobilized on the electrode were determined to be $400 \mu\text{g}/\text{cm}^2$ and $0.6 \text{ U}/\text{mg}$, respectively. This activity corresponded to a third that of the native one.

At the beginning of this study, amperometric sensing was attempted without the incorporation of any electron-transferring mediator into the sensing system. As a result, the ethanol sensing met with failure. However, the ADH-immobilized electrode caused a rapid current response to ethanol in the presence of MB, which was selected as the mediator on the basis of its redox behavior. The current response was found to increase with increasing ethanol concentration. Thus, the amperometric sensing with MB gave a result that led to the quantitative determination of ethanol. The selectivity of the sensing was examined with methanol, 2-propanol, and allyl alcohol. The ADH-immobilized electrode had a considerably large current response to allyl alcohol, whereas little response was observed for the others. Such selectivity was attributed to the specificity of native ADH with respect to the oxidation of alcohols.

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