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Bio- and bioelectro-catalytic properties of polyaniline/poly(acrylic acid) composite films bearing covalently-immobilized acid phosphatase

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ABSTRACT

Conducting films composed of polyaniline (PANI) and poly(acrylic acid) (PAA) were prepared by electrochemical polymerization of aniline in the presence of various concentrations of PAA. The content of PAA moiety on the surface of the composite films (PANI/PAA films) was estimated by determination of carboxyl groups and found to be controlled by the concentration of PAA in polymerization solution. Acid phosphatase (ACP) was immobilized covalently on the PANI/PAA films by the condensation reaction with the carboxyl groups on the films. It was confirmed that the enzyme activity of the ACP-immobilized PANI/PAA film increased with increasing content of PAA moiety on the surface of the film, accompanying an increase in the amount of the immobilized ACP. The activity of the covalently immobilized ACP was significantly higher than that of the ACP adsorbed on the PANI/PAA film. By use of the ACP-immobilized PANI/PAA film as an enzyme electrode, bioelectrocatalytic oxidation of L-ascorbic acid 2-phosphate (ASA2P) was examined. The enzyme electrode gave the current due to the oxidation of ASA2P in proportion to the content of PAA moiety on the surface of the PANI/PAA film used, which was relevant to the activity of the covalently immobilized ACP.

1. Introduction

In recent years, a variety of conducting materials have been modified with enzymes for fabricating enzyme electrodes which are applied to bioelectrochemical devices such as biosensors and biocatalytic fuel cells [1–4]. Conducting polymers have attracted much attention as the component of enzyme electrodes because they are readily synthesized by electrochemical polymerization of such compounds as pyrroles, thiophenes and anilines, and can be modified with enzymes by use of their functional groups as binding sites for enzyme immobilization [5–7]. If the conducting polymers are substituted with carboxyl groups, for example, they can be subjected to covalent immobilization of enzymes through amide linkages formed by the condensation reaction with amino groups of the enzymes. However, the conductivity of the polymers is lowered by such substituents as carboxyl groups [8–11], which is unfavorable for the use of them as electrode materials.

It is known that the composite film composed of polyaniline (PANI) and poly(acrylic acid) (PAA) can be prepared by electrochemical polymerization of aniline in the presence of PAA in a one-step manner [12–18]. The composite film (PANI/PAA film) has a part of carboxyl groups of the PAA on its surface and, therefore, enzymes can be immobilized covalently on the surface through amide linkages. In addition, as reported in our previous paper [15], the PANI/PAA film has the conductivity in the same order of magnitude as the PANI film without PAA. For such reasons, the PANI/PAA film has been used as a conducting component of enzyme electrodes and applied to bioelectrochemical devices [16–18]. Nevertheless, little investigation has been conducted into the relation between the content of PAA moiety on the PANI/PAA film and the performance of the enzyme electrode based on the film. From the viewpoint that the carboxyl groups on the PANI/PAA film are used as binding sites for enzyme immobilization [19,20], the content of PAA moiety on the PANI/PAA film must be an important factor to determine the bio- and bioelectro-catalytic properties of the enzyme electrodes. As for fabrication of enzyme electrodes, in addition to the covalent immobilization, the non-covalent immobilization of enzymes due to adsorption should be also taken into account. Because of a fibrous network structure [15], the PANI/PAA film has so large surface area that adsorption of enzyme molecules onto the surface of the film cannot be neglected. In a previous study, it was confirmed that acid phosphatase (ACP) was immobilized not only covalently but also non-covalently on the PANI/PAA film by adsorption [21]. The adsorbed ACP participates in the catalytic reaction on the PANI/PAA film as a matter of course though the amount of adsorbed ACP may not be affected by the PAA moiety on the film.

We have investigated the properties of the PANI/PAA film directing our attention to the role of the PAA moiety on the surface

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of the film. In the present study, the PANI/PAA films were prepared by electrochemical polymerization of aniline in the presence of PAA, and the PAA moiety on the surface of the films was determined quantitatively. ACP was immobilized covalently on the surface of the PANI/PAA films by the condensation reaction with the carboxyl groups of the PAA moiety on the surface. The amount and activity of the immobilized ACP were evaluated as functions of the content of PAA moiety on the surface of the films and compared with those of the ACP immobilized non-covalently by adsorption. The PANI/PAA films bearing immobilized ACP (PANI/PAA–ACP films) were applied to bioelectrocatalytic oxidation of L-ascorbic acid 2-phosphate (ASA2P).

2. Experimental

2.1. Materials

Aniline, ACP (EC 3. 1. 3. 2, from wheat germ) and *N*-hydroxysuccinimide (NHS) were obtained from Nacalai Tesque, Inc. The aniline was distilled under reduced pressure and stored under nitrogen at −20 °C until use. PAA (molecular weight 250,000) and a trisodium salt of ASA2P were purchased from Wako Pure Chemical Ind. The ASA2P was purified by recrystallization from water and methanol at room temperature prior to use. *N*-Cyclohexyl-*N*-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate (CMC, used as a condensing reagent), toluidine blue O (TB) and 4-nitrophenyl phosphate disodium salt hexahydrate were purchased from Sigma–Aldrich, Inc. Other chemicals were of analytical grade, which were used without further purification. All aqueous solutions were prepared with distilled water.

2.2. Preparation of PANI/PAA films

PANI/PAA films were prepared by the electrochemical polymerization of aniline in 0.5 M H_2SO_4 solutions containing 0.5 M aniline and various concentrations of PAA as described elsewhere [15]. The solutions were purged with nitrogen prior to the polymerization. The polymerization was carried out in a conventional three-electrode cell equipped with a potentiostat/galvanostat (μAutolab Type III, Eco Chemie). A gold film deposited on a quartz plate was used as a working electrode (0.25 cm^2). Prior to the polymerization, the working electrode was cleaned with piranha solution (H_2SO_4:30% H_2O_2 = 3:1). A platinum plate and a saturated calomel electrode (SCE) were used as a counter electrode and a reference electrode, respectively. The polymerization was conducted by means of cyclic voltammetry from −0.4 to + 0.9 V vs. SCE at a scan rate of 0.05 V/s. The potential scan was repeated until the amount of passed charge reached to 140 mC. The resulting PANI/PAA films were washed with 0.5 M H_2SO_4 and then with distilled water.

2.3. Quantitative determination of PAA moiety on the PANI/PAA film

The content of PAA moiety on the surface of the PANI/PAA film was estimated by determination of carboxyl groups [21]. The PANI/PAA film was immersed in 2.0 ml of 0.5 mM aqueous solution of TB adjusted to pH 10.0 for 5.0 h to form ionic complexes between TB and the carboxyl groups of PAA on the surface of the film. Then the PANI/PAA film was rinsed with NaOH solution (pH 10.0) to remove residual TB. The PANI/PAA film complexed with TB was immersed in 50 wt% acetic acid solution for 1.0 h and then the solution involving dissociated TB was subjected to measurement of absorbance at 633 nm on a Shimadzu UV-3100PC spectrometer. The content of PAA moiety on the PANI/PAA film was evaluated on the basis of the amount of carboxyl groups on the film calculated from the difference in the absorbance between the cases of the PANI/PAA and PANI films.

2.4. Covalent immobilization of ACP on the PANI/PAA film

ACP was immobilized covalently on the surface of the PANI/PAA film in the following manner. In advance, the PANI/PAA film was immersed in 2.0 ml of aqueous solution containing 50 mg/ml CMC and 7.5 mg/ml NHS for 20 min to activate the carboxyl groups of PAA moiety on the film by esterification with NHS. After washing with distilled water, 15 μl of 5.0 mg/ml aqueous solution of ACP was dropped onto the PANI/PAA film, and the film was left at room temperature for 30 min being covered with a Petri dish for prevention of water evaporation. Then the PANI/PAA film treated thus was washed with 1.0 ml of 0.5 M acetate buffer solution (pH 5.0) and then with distilled water. The buffer solution containing unbound ACP was recovered, and the amount of immobilized ACP was estimated from the difference in enzyme activity between the ACP solution recovered and that prepared for the immobilization.

For a comparison, ACP was immobilized non-covalently by adsorption on the surface of the PANI/PAA film in the same manner as described above except that the esterification with NHS was omitted.

2.5. Biochemical measurement

Kinetic parameters were evaluated for dephosphorylation of 4-nitrophenyl phosphate (4NPP) with the ACP immobilized on the surface of the PANI/PAA film. The dephosphorylation of 4NPP was followed by measuring the rate of liberation of 4-nitrophenol (4NP) spectrophotometrically. The dephosphorylation was carried out in 2.0 ml of 0.1 M acetate buffer solution (pH 5.0) containing a given concentration of 4NPP at 25 °C. A 100 μl aliquot of the solution was taken out at intervals and mixed with 1.0 ml of 1.0 M NaOH solution for coloring the solution of the liberated 4NP. The concentration of the 4NP was determined on the basis of absorbance of the solution at 405 nm.

The enzymatic activity of the immobilized ACP was evaluated with respect to dephosphorylation of ASA2P by a spectrophotometric method involving the reaction of phosphate ions produced by ACP-catalyzed dephosphorylation of ASA2P with molybdate ions to yield molybdophosphate ions [22]. The dephosphorylation with ACP was conducted in 0.1 M acetate buffer solution (pH 5.0) containing 10 mM ASA2P at 25 °C. The activity was determined from absorbance of the solution at 700 nm due to reduction of the molybdophosphate ions.

2.6. Electrochemical measurement

The PANI/PAA–ACP films fabricated as described in Section 2.4 were applied as enzyme electrodes to bioelectrocatalytic oxidation of ASA2P. Oxidation currents were measured with the same apparatus as described in Section 2.2 except that an Ag/AgCl electrode was used as a reference electrode. The measurement was carried out by applying a constant potential of +0.2 V vs. Ag/AgCl in 0.1 M acetate buffer solution (pH 5.0) containing 10 mM ASA2P. In advance, the buffer solution was purged with N_2.

3. Results and discussion

3.1. Composition of the surface of the PANI/PAA films

Fig. 1 shows the content of PAA moiety on the surface of PANI/PAA films prepared by the electrochemical polymerization of aniline in the presence of various concentrations of PAA. As can