Novel Hydrogen Peroxide Sensors Based on Peroxidase-Carrying Poly{pyrrole-co-[4-(3-pyrrolyl)butanesulfonate]} Copolymer Films

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Amperometric hydrogen peroxide biosensors were fabricated by incorporating horseradish peroxidase (HRP) into poly{pyrrole-co-[4-(3-pyrrolyl)butanesulfonate]} (Py-PS) copolymer films deposited on an SnO₂ electrode surface by electropolymerization. The HRP/Py-PS electrodes exhibited an extended dynamic range and a markedly improved operational and storage stability, compared with HRP-incorporated polypyrrole (PPy) electrodes prepared under similar conditions. The linear range was expanded from 10⁻⁷ – 10⁻⁴ M to 10⁻⁷ – 10⁻³ M H₂O₂. In about 80 measurements over three weeks, the HRP/Py-PS electrode retained 60% of its initial response, while the HRP/PPy electrode almost completely lost activity. The influence of the electrodeposition solution pH on the sensor response was also investigated. Our results suggest that an expansion of the linear range and an enhancement of lifetime are due to electrostatic interactions of HRP with a negatively-charged Py-PS copolymer.

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Introduction

Electropolymerized conducting polymers have been widely used as an immobilization matrix for constructing enzyme electrodes. Among the conducting polymers, polypyrrole is of most particular interest because it is stable under ambient conditions, and can be easily electrodeposited onto an electrode surface from aqueous solutions, which would be preferable with respect to the compatibility with most of biological elements.¹⁻⁷

The entrapment of enzyme molecules in electropolymerized conducting polymer films provides several advantages for constructing enzyme electrodes. One advantage is that an enzyme electrode can be easily prepared in a rapid one-step procedure. Furthermore, this method enables one to control the amount of enzyme molecules immobilized in the polymer because the thickness of the polymer film can be controlled by an electric charge passed during electropolymerization. Particularly, this procedure allows precise control of the spatial localization of enzyme molecules to specified areas on the electrode surfaces. This is of importance for the miniaturization of biosensors.

In designing biosensors for on-line monitoring or for multiple measurements, the stability of the sensor is one of the critical parameters. It is of considerable importance to study the rate of loss in activity of the enzyme electrodes, especially if the enzyme molecules are physically entrapped within the polymer films. The loss in activity may arise from a loss of enzymatic activity of the entrapped enzyme and/or leaching out of the enzyme from the electropolymerized films. The latter can be caused by washing of the films and by agitation during measurements of the sensor response.

In previous work, we fabricated hydrogen peroxide biosensors based on HRP entrapped within conducting polypyrrole ultra-thin films, HRP/PPy electrodes.⁵ In the present work, to

improve the stability of such enzyme electrodes, a self-doped conjugated copolymer with fixed charge, in which anionic dopants or counterions are covalently attached to the polymer backbone, was employed to entrap HRP molecules. The concept of self-doped conducting polymers was first introduced in 1987.⁸ The covalent binding of sulfonate groups to polypyrrole, resulting in either water-soluble *N*-alkylsulfonate or 3-alkylsulfonate polypyrroles, has been reported.⁹⁻¹⁵ Owing to the very poor conductivity of the *N*-substituted polypyrrole, polypyrrole derivatized in the 3-position appears to be more preferable for this purpose.

In this paper, we report for the first time on the use of a poly{pyrrole-co-[4-(3-pyrrolyl)butanesulfonate]} (Py-PS) copolymer for fabricating hydrogen peroxide biosensors with high-performance sensibility. This is carried out through the electrochemical copolymerization of pyrrole and 4-(3-pyrrolyl)butanesulfonate, *i.e.* 3-alkylsulfonate pyrrole, in the presence of HRP. An overoxidized copolymer of pyrrole and 3-(pyrrol-1-yl)propanesulfonate (*N*-alkylsulfonate pyrrole) was previously used for the selective detection of catecholamine neurotransmitters, dopamine and norepinephrine.¹³ Here, we demonstrate the utility of Py-PS copolymer as an enzyme immobilization matrix for the determination of hydrogen peroxide. Simultaneously, a comparison between the novel HRP/Py-PS electrode and the conventional HRP/PPy electrode with regard to the sensor response and lifetime is discussed. The effect of the pH of the electrodeposition solution employed during enzyme electrode preparation on the response of the resulting electrode is demonstrated. The entrapment of HRP in the poly{pyrrole-co-[4-(3-pyrrolyl)butanesulfonate]} copolymer matrix is a very promising method for constructing a hydrogen peroxide biosensor with an extended linear range, improved stability, and good reproducibility.

Fig. 1 Structures of 4-(3-pyrrolyl)butanesulfonic acid sodium salt (PS) and poly{pyrrole-co-[4-(3-pyrrolyl)butanesulfonate]} (Py-PS).

Experimental

Materials

Tin oxide (9000 Å thick, F-doped) coated glass plates as a base electrode were obtained from Nippon Sheet Glass (Japan). Horseradish peroxidase (grade II) from Boehringer (FRG) and pyrrole (Py) from Kanto Chemicals were used without further purification. 4-(3-Pyrrolyl)butanesulfonic acid sodium salt (hereafter abbreviated as "pyrrole sulfonate, PS") was synthesized following a procedure described in the literature.¹⁵ Pyrrole was first protected as an *N*-(phenylsulfonyl) derivative with benzenesulfonyl chloride under phase-transfer conditions, followed by acylation in the 3-position of pyrrole with 4-chlorobutyl chloride under Friedel-Crafts reaction conditions; then, a Clemmensen reduction to give the protected 3-(4-chlorobutyl)pyrrole, which was subsequently converted to the protected sulfonated butylpyrrole, and finally deprotection with base to give the required sulfonate butylpyrrole. The thus-obtained 4-(3-pyrrolyl)butanesulfonic acid sodium salt (PS) was characterized by NMR (^1H NMR (D_2O): δ 1.4–1.7 (m, 4H), 2.3 (t, 2H), 2.8 (t, 2H), 5.9 (m, 1H), 6.45 (br s, 1H), 6.6 (m, 1H)) and elemental analysis (calculated: C, 39.50; H, 5.80; N, 5.76; found: C, 39.39; H, 5.60; N, 5.30). The molecular structure of PS is shown in Fig. 1.

Diluted hydrogen peroxide standard solutions were freshly prepared from a 31% hydrogen peroxide aqueous solution directly prior to use. All other reagents were of analytical grade or the best grade available.

Fabrication of enzyme electrodes

An SnO_2 -coated glass plate (1.0 cm $\times$ 1.0 cm) was pretreated with sulfuric acid that had been heated by dilution with water (1:1) for about 30 min and used as a working electrode. HRP/PPy electrodes were fabricated by electropolymerization in a 6-mL aqueous solution containing 0.06 M KCl, 0.05 M pyrrole, and 0.6 g L^{-1} HRP at room temperature. Ag/AgCl and a platinum black wire were used as reference and counter electrodes, respectively. HRP/Py-PS electrodes were prepared in a similar way in an aqueous solution (6 mL) containing 0.04 M pyrrole, 0.01 M sodium 4-(3-pyrrolyl)butanesulphonate (PS), and 0.6 g L^{-1} HRP, unless otherwise noted. Electrochemical polymerization was done in a galvanostatic mode at an electropolymerization charge of 5 mC cm^{-2} (current density, 0.1 mA cm^{-2}).

Measurement of sensor performance

Electrochemical measurements were carried out in a conventional three-electrode system using Ag/AgCl/KCl (saturated) as a reference electrode, a platinum-black wire as a

Fig. 2 Calibration curves for H_2O_2 obtained with HRP/Py-PS (PS 20%) and HRP/PPy electrodes.

counter electrode, and an enzyme catalytic working electrode. The experiments were performed in a 1/15 M phosphate-buffered solution (pH 6.4) at 30°C. The enzyme electrodes were polarized at +150 mV *versus* Ag/AgCl to obtain background currents. Aliquots of H_2O_2 standard solution were then added to the phosphate buffer solution, followed by temporary magnetic stirring; the generated cathodic currents in the quiescent solution were recorded. The current responses toward H_2O_2 were determined by subtracting the background currents from the obtained current values. Calibration plots were obtained by successive injections of H_2O_2 standard solutions.

Preparation of Py-PS copolymer films for elemental analysis

An SnO_2 -coated glass plate (2.5 cm $\times$ 4.0 cm) was pretreated and used as a working electrode. Py-PS copolymer films were formed by electropolymerization in a 36-mL aqueous solution containing pyrrole and 4-(3-pyrrolyl)butanesulphonate (PS). The concentrations of PS in the electrogenerated solution were 10, 20 and 80% of the total monomer concentration (Py+PS) of 0.05 M. Electropolymerization was carried out in a galvanostatic mode at a current density of 0.1 mA cm^{-2} for 2 h. After the thus-obtained Py-PS electrodes were dried in a desiccator overnight, the Py-PS copolymer films were peeled off from the electrode surface and dried in a desiccator again.

Results and Discussion

Sensor response to H_2O_2

To investigate the sensor sensitivity and reproducibility, an HRP/Py-PS electrode was first constructed by immobilizing HRP in a Py-PS copolymer film, deposited onto an SnO_2 electrode by electropolymerization in an aqueous solution containing PS 20% (0.01 M PS and 0.04 M Py) and 0.6 g L^{-1} HRP. Three independent HRP/Py-PS electrodes and one HRP/PPy electrode for a comparison were fabricated, and the corresponding calibration curves for hydrogen peroxide were measured (Fig. 2). Optimization of the ratio of the two monomers, Py and PS, during Py-PS copolymer formation at a constant HRP concentration is discussed later.

It must be pointed out that *no* additional conducting salt was

Fig. 3 Calibration curves for HRP/Py-PS and HRP/PPy electrodes prepared in electropolymerization solutions containing PS at 0 (●), 20 (○), 40 (△), 60 (□), 80 (◇) % of total monomer concentration (Py+PS = 0.05 M). The inset shows the sensor responses obtained in a high H_2O_2 concentration region.

added for the electrochemical polymerization process, because the PS monomer, itself, also acts as a conducting salt. As expected, it appears that the sensor sensitivity and the linear range toward H_2O_2 were similar with or without using KCl as a supporting electrolyte during Py-PS copolymer growth. Therefore, the HRP/Py-PS electrode can be prepared without adding an extraneous conducting salt.

The sensor response measurement of the enzyme electrodes is based on the cathodic current generated at a potential of +150 mV vs. Ag/AgCl by the addition of a substrate. It appears that the sensitivity of the HRP/Py-PS electrode is comparable to that of the HRP/PPy electrode, except for an enhanced upper sensing limit observed at the HRP/Py-PS electrode. While the HRP/PPy electrode response is linear over a range of 10^{-7} – 10^{-4} M H_2O_2 , the HRP/Py-PS electrode gives a broader working concentration range of 10^{-7} – 10^{-3} M H_2O_2 with high electrode-to-electrode reproducibility, as can be seen in Fig. 2. This may have resulted from: (i) the capability of entrapping a larger amount of enzyme molecules in the negatively charged Py-PS copolymer films than in the positively charged polypyrrole films and/or (ii) the promotion of the internal diffusion of H_2O_2 within the hydrophilic Py-PS copolymer films. A slightly rapid current response following hydrogen peroxide addition was observed at the HRP/Py-PS electrode. Thus, in a quiescent electrolyte, the output current reached a steady state within 2 – 5 min for the HRP/Py-PS electrode, while it took 3 – 6 min for the HRP/PPy electrode. Though it was difficult to directly quantify the actual amount of enzyme entrapped in these ultra-thin polymer films, the increase in the amount of entrapped enzyme within the Py-PS copolymer film can be derived from the electrostatic interactions between HRP and the sulfonate groups of the copolymer during fabrication. The pH of the electropolymerization solution used for the HRP/Py-PS electrode preparations is *ca.* 6.9, and since horseradish peroxidase has an isoelectric point of 7.2, the enzyme is positively charged at pH 6.9, and hence could be incorporated into the Py-PS copolymer *via* electrostatic attractions with the sulfonate groups of the polymer during electropolymerization.

In order to optimize construction of the HRP/Py-PS electrode, the effect of varying concentrations of two monomers, Py and

Table 1 Elemental analysis of Py-PS copolymers and estimated Py and PS mole fractions

Compound	C (%)	H (%)	N (%)	S (%)	Py (m)	PS (n)
PS = 10%	53.22	4.69	12.43	6.23	0.78	0.22
PS = 20%	53.06	4.94	12.53	10.00	0.65	0.35
PS = 80%	46.50	4.53	9.18	12.69	0.40	0.60

PS, in the electropolymerization solution was examined. The total monomer concentration (Py + PS) was kept constant at 0.05 M and the concentrations of Py and PS were varied from PS 20% to 100% relative to the total monomer concentration with a constant amount of HRP (0.6 g L⁻¹). Figure 3 shows the influence of the concentration of PS in the electrodeposition solution on the sensor performance. It was expected that for a greater amount PS used, a greater amount of the enzyme would be immobilized and, consequently, a higher response would be observed. However, the sensor signal was not observed from an enzyme electrode based on HRP entrapped within a PS homopolymer film. Although all of the HRP/Py-PS electrodes prepared in an electrodeposition solution containing PS 20 – 80% exhibited the same sensitivity and linear response in the range of 10^{-7} – 10^{-3} M toward H_2O_2 , the sensor signal decreased in the high-concentration region (10^{-4} – 4×10^{-3} M) of H_2O_2 as the amount of PS present in the monomer solution increased. These results can be attributed to the following two possibilities: (i) the composition of the electropolymerization solutions has not been reflected in the resulting copolymer films; or (ii) the water solubility of the Py-PS copolymer films increases at high concentrations of PS, leading to poor adhesion onto the electrode surface.

To test which factor contributes to these observed results, the mole-fraction ratios of Py and PS in the Py-PS copolymers electrosynthesized from the monomer feed solutions of different composition were estimated by elemental analysis. The copolymer Py-PS consisting of repeated units of pyrrole and 4-(3-pyrrolyl)butanesulfonate may be generically represented as in Fig. 1. The values for *m* and *n*, the mole fractions of Py and PS, respectively, were obtained from the sulfur-to-nitrogen ratio, as determined by elemental analyses. The results obtained from Py-PS copolymers prepared from an electropolymerization solution containing PS 10, 20 and 80% are given in Table 1. Apparently, the composition of the monomer feed solution regarding to the Py and PS monomers is reflected in the composition of the resulting copolymer films. This result demonstrates that the decrease in the sensor response with increasing the concentration of PS present in the electrodeposition solution can be reasonably ascribed to an increase in the water solubility of the Py-PS copolymers, thus causing the films to have a poor adherent characteristic.

From the results mentioned above, a ratio of 0.01 M of PS for 0.04 M of pyrrole (PS 20%) corresponding to the highest sensor response to H_2O_2 in the high concentration region was chosen as the optimum ratio, and was used in all of the experiments described below.

As mentioned above, the optimized HRP/Py-PS electrode can sense H_2O_2 up to 10^{-3} M with a detection limit of 10^{-7} M. In a quiescent electrolyte, the output current following hydrogen peroxide addition took 2 – 5 min to reach a steady state. The response times were longer when larger amounts of the substrate were added.

The reproducibility mentioned as RSD for 10 repeated measurements of the sensor response to 10^{-5} M H_2O_2 was 5.1%.

Fig. 4 Effect of the electropolymerization solution pH on the cathodic current response to 10^{-5} M H_2O_2 observed with HRP/PPy and HRP/Py-PS electrodes.

The reproducibility between the electrodes was 4.8% for 10^{-5} M H_2O_2 and 7.1% for 10^{-4} M H_2O_2 (three HRP/Py-PS electrodes).

The effect of ascorbic acid as a common physiological interferent on the sensor response was also examined. At an operating potential of +150 mV vs. Ag/AgCl, the anodic currents for ascorbic acid oxidation on a bare SnO_2 electrode were 12 and 114 nA cm^{-2} at 10^{-5} and 10^{-4} M, respectively. These are only 2% of the output cathodic currents of the HRP/Py-PS electrode at the corresponding concentrations of the substrate, H_2O_2 . This demonstrates the advantage of using a mild potential for biosensor operation.

Effect of the electropolymerization solution pH on the sensor response

To verify that an electrostatic interaction plays a major role in enzyme incorporation in the present system, the effect of an electrodeposition solution on the sensor response of the HRP/PPy and HRP/Py-PS electrodes was investigated. HCl and KOH were used to adjust the pH of the monomer solution for fabricating HRP/PPy electrodes, and NaOH was used instead of KOH for preparing HRP/Py-PS electrodes. The pH was varied from 4 to 9, and the sensor response was investigated for a 10^{-5} M H_2O_2 solution. Two independent HRP/Py-PS and HRP/PPy electrodes were prepared from the corresponding Py-PS and PPy monomer solutions with a given pH value; the symbols and error bars in Fig. 4 represent the mean and standard deviation, respectively. As is evident in Fig. 4, the response of the HRP/Py-PS electrodes is comparable to that of the HRP/PPy electrodes when electrosynthesized from neutral or mildly acidic solutions, while the response decreases remarkably when prepared from an alkaline electropolymerization solution. Similar results were obtained with two other sets of HRP/Py-PS electrodes. This indicates that enzyme molecules negatively charged at the polymerization pH could be inhibited from incorporating into the polyanion Py-PS copolymer during electropolymerization. This result demonstrates that electrostatic interaction is, at least to a certain extent, responsible for enzyme incorporation into the Py-PS copolymer films.

It has been demonstrated that in a self-doped electroactive polymer having anionic dopants covalently bound to the

Fig. 5 Operational stability of HRP/Py-PS and HRP/PPy electrodes. About 80 measurements were performed over a 3-week period for each electrode. The relative responses to 10^{-5} M H_2O_2 are plotted as a function of time. The inset shows the relative responses over an 8-h period operation after preparation.

polymer chain, the charge compensation on electrochemical doping derives from the ejection of cations instead of the injection of anions. This phenomenon suggests that the covalently attached anions that are localized within the polymer matrix repel anions while allowing cations to permeate through the film.

In the case of the HRP/PPy electrodes, the observed response is not significantly affected by the pH variation during electropolymerization. The electrostatic attraction seems not to be the key factor in determining the enzyme loading in polypyrrole films in the present experimental system.

It should be noted that between pH 4 and 5, the current responses tend to decrease for both kinds of enzyme electrodes; this may result partly from the denaturation of HRP under acidic conditions.

Sensor lifetime

The operational stability, tested with HRP/Py-PS and HRP/PPy electrodes, was examined by measuring the response to 10^{-5} M H_2O_2 . Firstly, the measurements were repeated approximately 18 times over an 8-h period after fabricating enzyme electrodes. The electrodes were then tested periodically to ascertain the stability of the response to hydrogen peroxide (approximately 5 measurements each day). The electrodes were stored in a phosphate buffer solution (pH 6.4) at 4°C between runs. About 80 measurements were performed with each type of electrode over a 3-week period. From Fig. 5, which shows the operational stability test results, it is apparent that the HRP/Py-PS electrode retained more than 80% of its initial activity after 8 h, while the response of the HRP/PPy electrode decayed to 50% of the initial current response. After a period of 21 days, the HRP/PPy electrode lost its activity almost completely, while the HRP/Py-PS electrode response was maintained at approximately 60% of its initial value. These results indicate that the effect of repeated measurements of the sensor response on the stability of the HRP/PPy electrode is very significant, while there was almost no influence on the stability of the HRP/Py-PS electrode. The higher rate of loss in activity for the HRP/PPy electrode, as compared with that for the HRP/Py-PS electrode, is probably due to the higher rate of leaching of the enzyme out of the PPy film, caused by agitation during sensor response measurements. For the HRP/Py-PS electrode, the enzyme molecules were entrapped tightly in the Py-PS copolymer matrix, retained by electrostatic interactions with the

sulfonate groups of polymer and, thus, were suppressed from leaching out.

To study the storage stability of the HRP/Py-PS and HRP/PPy electrodes, both of the enzyme electrodes were prepared, and after measurements of the sensor response to 10^{-5} M H_2O_2 they were stored in a phosphate buffer solution (pH 6.4) at *ca.* 4°C in a refrigerator. After 14 days, they were tested again, showing that the HRP/Py-PS electrode retained up to 83% of its activity, while the HRP/PPy electrode response decreases down to 70% of its initial value.

Another experiment was conducted to study the long-term stability by preparing HRP/Py-PS and HRP/PPy electrodes, and periodically testing their responses to 10^{-5} M H_2O_2 over a course of 5 weeks. After 35 days and 14 measurements, the HRP/Py-PS electrode was shown to have retained 50% of its initial response, while the HRP/PPy electrode response decayed to less than 25% of its initial value.

These results strongly suggest that the stability of the enzyme electrode is improved through an electrostatic binding of the positively charged peroxidase molecules with the negatively charged sulfonate groups attached to the polymer chain. To reach a final conclusion, however, it is still necessary to quantitate the temporal variation of the amount of enzyme molecules during the course of storage, and to assess the enzyme activity for the two types of HRP-carrying conductive polymer electrodes.

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