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Covalent immobilization of glucose oxidase on poly[1-(2-carboxyethyl)pyrrole] film for glucose sensing

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Poly[1-(2-carboxyethyl)pyrrole] film (PPy-COOH film) was electrochemically prepared and glucose oxidase (GOD) was covalently immobilized on the PPy-COOH film by the condensation reaction with carboxyl groups of the PPy-COOH. The apparent activity of the GOD immobilized on the PPy-COOH film was 152 mU cm^{-2}. The GOD-immobilized PPy-COOH film (PPy-GOD film) was applied to amperometric glucose sensing. Plots of response current *versus* glucose concentration gave a straight line with a slope of 1.7 $\mu A\ cm^{-2}$ per 1 mM glucose up to 80 mM. The glucose oxidation with the GOD on the PPy-GOD film was considered to proceed through a Michaelis–Menten mechanism.

(Keywords: poly[1-(2-carboxyethyl)pyrrole]; glucose oxidase; glucose sensing)

INTRODUCTION

Immobilization of enzymes in conducting polymers by electropolymerization is an attractive technique for fabricating micro enzyme-electrodes[1,2]. Biosensors based on the micro enzyme-electrodes are expected to be applied to *in vivo* and *in vitro* measurements in clinical analyses[3–5]. Although conventional immobilization of enzymes in conducting polymers has been achieved by physical entrapment during electropolymerization in aqueous solution containing enzymes and monomers[6,7], leakage of the entrapped enzymes from conducting polymers is a significant problem.

In order to overcome this problem, covalent attaching of enzymes to conducting polymers has been attempted and applied to fabrication of biosensors[8,9]. Considering that an enzyme reaction occurs at the interface of a biosensor/bulk solution, the sensitivity of the sensor will mainly attributed to the enzyme molecules immobilized on the surface of conducting polymer layer. However, covalent immobilization of enzymes on the surface of conducting polymers has been hardly reported. In the present study, covalent immobilization of glucose oxidase (GOD) on the poly[1-(2-carboxyethyl)pyrrole] film (PPy-COOH film) prepared by electropolymerization was attempted by the condensation reaction of GOD with the carboxyl groups on the PPy-COOH film. The GOD-immobilized PPy-COOH film (PPy-GOD film) was applied to glucose sensing.

EXPERIMENTAL

The 1-(2-cyanoethyl)pyrrole, obtained from Aldrich, was distilled under 15 mmHg at 140°C and stored at 4°C in the dark under nitrogen gas. GOD (EC 1.1.3.4, grade 2, from *Aspergillus* species), which was supplied by Toyobo, had an activity of 164 units mg^{-1}. Peroxidase (POD) (EC 1.11.1.7, type 1, from horseradish), which was supplied by Sigma, had an activity of 87 units mg^{-1}. *p*-Benzoquinone was obtained from Nacalai Tesque, and was sublimed before use. 1-Cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMC), tetra-*n*-butylammonium tetrafluoroborate ($(C_4H_9)_4NBF_4$), propylene carbonate, D-(+)-glucose, 4-aminoantipyrine and phenol were commercial materials and used without further purification.

1-(2-Carboxyethyl)pyrrole was synthesized as described in the previous literature[10]: a mixture of 25 g of 1-(2-cyanoethyl)pyrrole and 100 ml of 5% potassium hydroxide was stirred at 50°C for 30 h. The reaction mixture was cooled to room temperature and acidified with hydrochloric acid. After ether extraction, the product was obtained by removal of ether using a rotary evaporator. The product was confirmed to be 1-(2-carboxyethyl)pyrrole by melting point measurement and infrared (i.r.) spectroscopy.

PPy-COOH film was prepared on gold-deposited alumina plate (0.25 cm^2) by electropolymerization in 25 ml of propylene carbonate containing 0.2 M 1-(2-carboxyethyl)-pyrrole and 0.1 M $(C_4H_9)_4NBF_4$ at +1.2 V *versus* saturated calomel electrode (SCE). A potentiostat/galvanostat (Hokuto Denko HA-301) and a coulomb/amperehour meter (Hokuto Denko HF-201) were used. The amount of passed charge was 0.40 C cm^{-2}, and the thickness of obtained polymer film was determined to be 2.3 μm by using a surface roughness meter (Tokyo Seimitsu SURFCOM200B). When the passed charge was less than 0.35 C cm^{-2}, the obtained PPy-COOH films lacked uniformity. On the contrary, the passed charge exceeding 0.45 C cm^{-2} gave poor reproducibility of the film thickness.

Immobilization of GOD on PPy-COOH film was carried out by using CMC as a condensing agent as follows: 30 mg

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of GOD and 60 mg of CMC were dissolved in 1 ml of 0.1 M phosphate buffer (pH 5.0) and the solution was stirred. The solution (20 μl) was spread on the PPy-COOH film. The immobilization reaction was carried out at 4°C for 18 h, and the GOD-immobilized PPy-COOH film (PPy-GOD film) was rinsed with distilled water.

The apparent activity of GOD immobilized on PPy-COOH film was measured by colorimetric method based on POD catalysed reaction of hydrogen peroxide, produced in glucose oxidation reaction, with 4-aminoantipyrine and phenol[11]. In 4.0 ml of 0.1 M phosphate buffer, 32.0 mg of 4-aminoantipyrine and 2.5 mg of POD were dissolved: Solution 1. A solution of 4.2 mg of phenol in 4.0 ml of distilled water was prepared: Solution 2. In a dark bottle containing 0.1 ml of Solution 1 and 0.1 ml of Solution 2, 4.8 ml of 0.1 M phosphate buffer, 0.5 ml of 1 M glucose solution and PPy-GOD film were added. The mixture was incubated at 30°C for 1 h, and cooled to 0°C. The PPy-GOD film was removed from the mixture and the residual solution was subjected to measurement of absorbance at 505 nm using a spectrophotometer (Shimadzu UV-3100PC). The apparent activity was measured over the pH range 4.5 to 9.0.

On the basis of a preceding study[12], the amperometric response of the PPy-GOD film to glucose was measured by applying a constant potential of + 0.35 V *versus* SCE in 25 ml of 0.1 M phosphate buffer containing 1 mM *p*-benzoquinone used as a mediator. After the background current was allowed to be constant, a given amount of 1 M glucose was injected into the solution and then the current change was monitored by a pen recorder (Chino Works EB2P00).

RESULTS AND DISCUSSION

In order to confirm the immobilization of GOD, the i.r. spectrum and apparent activity of PPy-GOD was measured. *Figure 1* shows the i.r. spectra of the PPy-GOD film, the PPy-COOH film and GOD. Absorption peaks at around 1700 cm^{-1} in the i.r. spectra of the PPy-GOD film and the

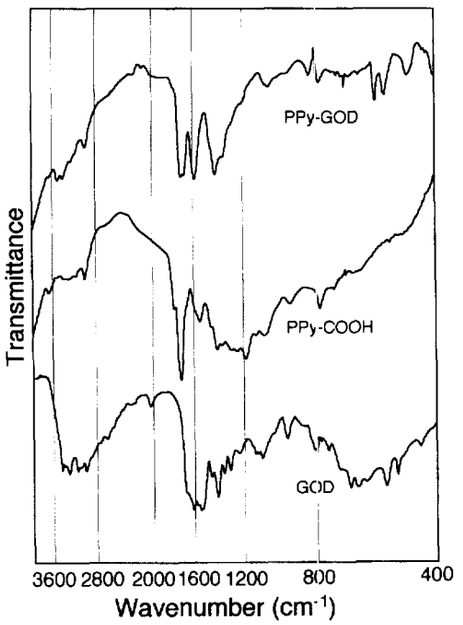

Figure 1 I.r. spectra of PPy-GOD film, PPy-COOH film and GOD

Table 1 Amount and apparent activity of immobilized GOD

Support film	Condensing agent	GOD immobilized (μg)	Apparent activity (mU cm^{-2})
PPy-COOH	CMC	6	150
PPy-COOH	–	0	31
PPy	CMC	0	11

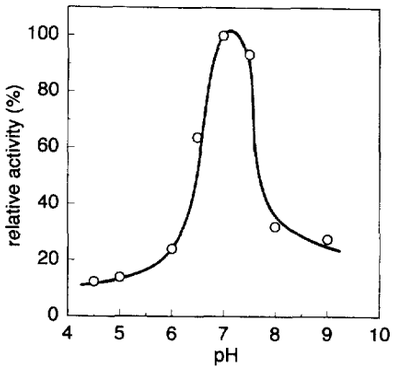

Figure 2 Effect of pH on apparent activity of the GOD immobilized on the PPy-COOH film

PPy-COOH film correspond to C = O stretching. In addition, in the i.r. spectrum of the PPy-GOD, an absorption in the vicinity of 1570 cm^{-1}, which is characteristic of GOD, was observed. This result suggests existence of GOD on the PPy-GOD film.

Table 1 shows the amount and the apparent activity of immobilized GOD. The amount of GOD immobilized on the PPy-COOH film in the presence of CMC was estimated to be 6 μg by Lowry *et al.*'s method[13], while the GOD immobilized in the absence of CMC could not be detected. The apparent activity of the GOD immobilized in the presence of CMC was five times higher than that of the GOD immobilized in the absence of CMC. These results suggest that GOD was covalently attached to carboxyl groups on the PPy-COOH film by condensation reaction in the presence of CMC. However, the specific activity of the immobilized GOD estimated from the data in *Table 1* is 4% of native GOD's. On the other hand, the apparent activity of GOD immobilized on polypyrrole film having no carboxyl groups(PPy) was less than a tenth of that of the GOD immobilized on the PPy-COOH film, which can be attributed to adsorption of GOD on the PPy film.

Figure 2 shows the effect of pH on the apparent activity of the GOD immobilized on the PPy-COOH film. The maximum activity of the immobilized GOD was observed at pH 7.0 though it was reported elsewhere that the maximum activity of native GOD was observed at around pH 6.0[14,15]. The shift of optimum pH by immobilization is considered to be due to residual carboxyl groups on the PPy-GOD film. It is conceivable that the shift of optimum pH is equivalent to cancelling the acidity around the immobilized GOD due to the residual carboxyl groups.

Taking the pH effect into account, the amperometric response of PPy-GOD film to glucose was measured at the optimum pH 7.0. The measurement system contains following reactions:

$$\text{GOD(FAD)} + \beta - \text{D} - \text{glucose} \rightarrow \text{GOD(FADH}_2\text{)} + \text{D} - \text{glucono} - \delta - \text{lactone} \quad (1)$$

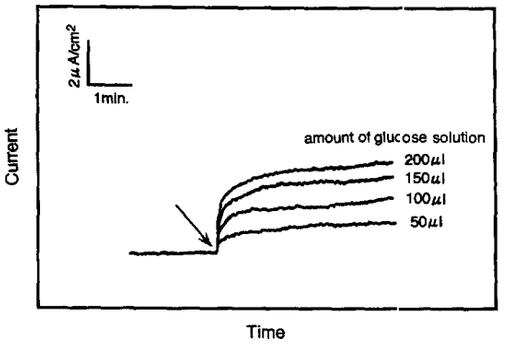

Figure 3 Amperometric response of the PPy-GOD film to 1 M glucose injected in phosphate buffer containing 1 mM *p*-benzoquinone at + 0.35 V versus SCE

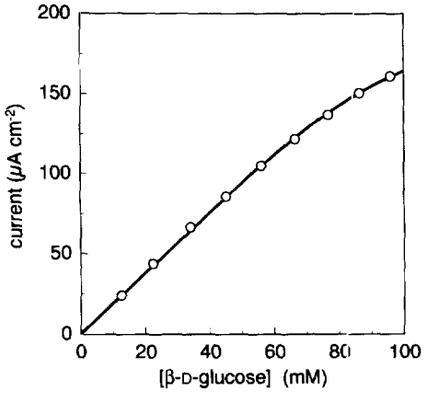

Figure 4 Relation between β-D-glucose concentration and response current

$$GOD(FADH_2) + Q \rightarrow GOD(FAD) + H_2Q \quad (2)$$

$$H_2Q \rightarrow Q + 2H^+ + 2e^- \quad (3)$$

where Q and H_2Q are *p*-benzoquinone and hydroquinone, respectively. It is not still confirmed whether the direct electron transfer occurs between oxidoreductase and conducting polymer or not. Numerous biosensors composed of oxidoreductase and conducting polymers, therefore, adopt mediators such as *p*-benzoquinone or ferrocene derivatives[16–18] for detection of substrates. *Figure 3* shows the amperometric response of the PPy-GOD film to 1 M glucose solution injected in phosphate buffer containing 1 mM *p*-benzoquinone. The arrow in *Figure 3* indicates a point at which 1 M glucose solution was injected. The current became almost constant within 2 or 3 min after injection of the glucose solution. An increase of the amperometric response with increasing amount of the glucose solution injected suggests that the PPy-GOD film could detect the electrons generated in the above reactions. Furthermore, the amperometric response of the PPy-GOD film was about three times as large as that of the polypyrrole film containing physically entrapped GOD[12]. The GOD attached to the PPy-COOH film should be located mainly on the surface of the film. Therefore, it is considered that the above reactions efficiently occurred at the interface of the PPy-GOD film/bulk solution.

Figure 4 shows the relation between glucose concentration and response current of the PPy-GOD film at 5 min after injection of the glucose solution. The relationship was approximately linear up to 80 mM with the slope of 1.7 μA cm^{-2} per 1 mM glucose. Assuming that glucose is oxidized with the GOD on the PPy-GOD film, as well as native GOD, the following equation can be derived[19]:

$$1/I = (K_{m,app}/I_{max})/[S] + 1/I_{max} \quad (4)$$

where I is the current, [S] is the glucose concentration and I_{max} and $K_{m,app}$ are called the maximum current and apparent Michaelis constant, respectively. Equation (4) means that plots of $1/I$ against 1/[S] (Lineweaver–Burk plots) give a straight line with the $1/I$-axis intercept, $1/I_{max}$ and 1/[S]-axis intercept, $-1/K_{m,app}$. *Figure 5* shows the Lineweaver–Burk plots based on the data in *Figure 4*. The plots gave a straight line which was of typical Michaelis–Menten form. The I_{max} and the $K_{m,app}$ values determined from *Figure 5* were 690 μA cm^{-2} and 340 mM, respectively. Considering that the Michaelis constant of native GOD was reported to be 9.6 mM[20], the calculated value of $K_{m,app}$ for the GOD on the PPy-GOD film was significantly high. As described earlier, the specific activity of immobilized GOD was lower than that of native GOD. Therefore, the high $K_{m,app}$ value suggests that the covalent binding of GOD to the PPy-COOH film led to suppression of GOD-glucose complex formation.

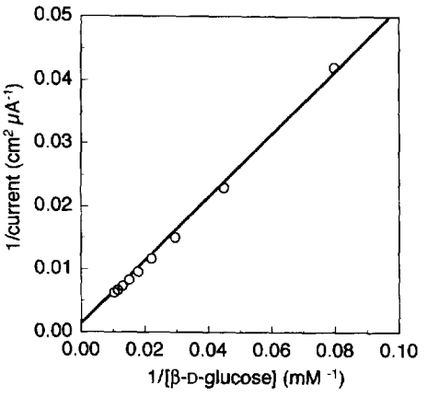

Figure 5 Lineweaver–Burk plots based on the data in *Figure 4*

CONCLUSIONS

The film of polypyrrole having carboxyl groups (PPy-COOH) was prepared by electrochemical polymerization of 1-(2-carboxyl)pyrrole. GOD-immobilized PPy-COOH film (PPy-GOD film) was successfully prepared by the condensation reaction of GOD with carboxyl groups of the PPy-COOH film. Amperometric glucose sensing with the PPy-GOD film gave the result that response current increased linearly with glucose concentration up to 80 mM. The Lineweaver–Burk plots based on the relation between glucose concentration and response current gave a straight line and, therefore, the glucose oxidation with the GOD on the PPy-GOD film was considered to proceed through a Michaelis–Menten mechanism, as well as native GOD. The covalent enzyme-immobilization technique described in the present paper can be employed as a useful method of fabricating micro enzyme-electrodes for *in vivo* and *in vitro* measurements in clinical analyses.

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