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Immobilization of glucose oxidase and electron-mediating groups on the film of 3-methylthiophene/thiophene-3-acetic acid copolymer and its application to reagentless sensing of glucose

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Abstract

Enzyme electrodes were prepared by covalent immobilization of glucose oxidase (GOx) on the films of conducting copolymer obtained by electrochemical copolymerization of 3-methylthiophene and thiophene-3-acetic acid. Onto the enzyme electrodes, 2,5-dihydroxyphenyl (DHP) groups were introduced as redox mediators by the reaction of 2,5-dihydroxybenzaldehyde using alkylenediamines as linker molecules. The mediator-carrying enzyme electrodes were applied to reagentless glucose sensing system. It was found that response current to glucose was increased by introducing DHP groups, and the longer the alkylene chain of used alkylenediamine was, the higher the response current became. For a comparison, poly-L-lysine was linked onto the films of the conducting copolymer in advance, and immobilization of GOx and then introduction of DHP groups were carried out. The mediator-carrying electrode prepared thus gave higher amperometric response than those prepared with alkylenediamines, suggesting that amino groups of poly-L-lysine functioned effectively as the sites for binding GOx and DHP groups.

Keywords: Conducting polymer; Enzyme electrode; Reagentless sensing

1. Introduction

In fundamental research to apply enzymes to biosensors, redox enzymes have been immobilized on electrodes for fabrication of amperometric sensors [1–14]. One of the crucial steps in the sensing with such enzyme electrodes is electron transfer from enzyme molecules to electrode surface. Although this step can be promoted if an electron mediator, such as *p*-benzoquinone/hydroquinone, is coexisting with analyte [15–20], addition of the mediator makes the sensing procedure troublesome and diffusion of the mediator limits application of the enzyme electrode. Therefore, it will be a key technique to bind the mediator onto the enzyme electrode. There has been a method using redox polymers as the mediators, which carry redox species such as ferrocene on their side chains [21–23]. In this method, the redox polymers play an important role in electron transfer from enzyme to electrodes as well as in immobilization of the enzyme on the electrodes. In addition, covalent binding of the mediator is a highly effective method to prevent disclosure of the mediator from the electrode to analyte solution [24–29]. Thus, covalent combination of the enzyme electrode and the mediator will give promise of making a simple sensing system and performing easy reagentless operation.

Enzyme electrodes carrying electron transfer mediators have been prepared with conducting polymers. Such electrodes can be obtained readily, for example, by the electrochemical polymerization in the presence of enzymes and mediators [3,15,30,31]. However, the enzymes thus buried within the conducting polymer films are unfavorable for the reaction with substrates. If a conducting polymer has functional groups available as covalent binding sites, enzymes and mediators can be immobilized firmly on the surface of its film. By using the enzyme electrode prepared in this manner, amperometric sensing has been carried out successfully [29,32]. On the other hand, it is the convenience of using conducting polymers that their films

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can be obtained quite easily and quickly by electrochemical synthesis on the surface of various conducting materials (not only Au but also inexpensive stainless steel).

In a previous study, the authors have fabricated the enzyme electrode composed of a conducting copolymer film (Fig. 1) and glucose oxidase (GOx) and applied it to amperometric sensing of glucose. The copolymer was electrochemically synthesized from 3-methylthiophene (3MT) and thiophene-3-acetic acid (T3A). The copolymer has great advantage for chemical modification, such as covalent immobilization of enzyme, because it has carboxyl groups in its T3A units, which can be used as the sites for binding various organic groups. Furthermore, the carboxyl groups can be also used for binding the mediator. Thus, a high content of T3A units in the copolymer seemed preferable for the modification, but an increased content of T3A caused a decrease in conductivity of the copolymer to result in a significant decrease in sensitivity of the enzyme electrode to glucose. The optimum content of T3A has been found to be ca. 10% [33].

Succeeding to the previous study, binding of electron-mediating groups onto the enzyme electrode was attempted for reagentless amperometric sensing of glucose. 2,5-Dihydroxyphenyl (DHP) groups, derived from 2,5-dihydroxybenzaldehyde (HBA), were introduced as electron mediators onto the GOx-immobilized electrode based on 3MT/T3A copolymer of the optimum composition. HBA has an aldehyde group on its hydroquinone structure and, therefore, can react with amino groups to form shiff-base linkage. In the present paper, two kinds of effective methods for fabricating the enzyme electrodes carrying DHP groups are proposed, the methods involving use of different types of linker molecules: Alkylenediamines (C2–C12) and poly-L-lysine.

2. Experimental

2.1. Materials

All the chemicals used were commercial products: 3MT, tetraethylammonium perchlorate, *N*-hydroxysuccinimide (NHS), hexamethylenediamine and dodecamethylenediamine from Nacalai Tesque, Inc.; T3A from Tokyo Kasei Kogyo Co. Ltd; GOx (EC 1.1.3.4., 147 U/mg) from Toyobo Co. Ltd; ethylenediamine, 25% glutaraldehyde solution and D-glucose from Wako Pure Chemical Industries, Ltd; HBA and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMC) from Aldrich Chem. Co. Inc.; poly-L-lysine (M_w 4000–15,000) from Sigma-Aldrich Co. They were used without further purification.

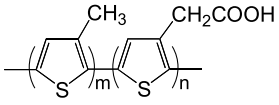


Fig. 1. Copolymer of 3-methylthiophene and thiophene-3-acetic acid (3MT/T3A copolymer).

2.2. Apparatus

Electrochemical copolymerization of 3MT and T3A, cyclic voltammetry and glucose sensing were carried out in the tree-electrode cell shown in Fig. 2, equipped with a potentiostat/galvanostat (Hokuto Denko Corp. HA-150G), a bipolar coulomb/amperehour meter (Hokuto Denko Corp. HF-203D) and an arbitrary function generator (Holuto Denko Corp. HB-105A). The working electrodes (Au, 0.25 cm^2) were used for preparation of 3MT/T3A copolymer film and for cyclic voltammetry. A Pt plate and saturated calomel electrode (SCE) were used as the counter and reference electrodes, respectively.

2.3. Preparation of 3MT/T3A copolymer film on Au electrode

A solution of 3MT (0.45 M), T3A (0.05 M) and tetraethylammonium perchlorate (0.10 M) in acetnitrile (20 ml) was placed in the electrochemical cell, and nitrogen gas was passed through the solution for 20 min. Then a potential at +2.2 V vs. SCE was applied on the Au working electrode until the amount of passed charge was reached to 0.80 C/cm^2. The 3MT/T3A copolymer was obtained as a deep brown film on the working electrode.

2.4. Preparation of enzyme electrode carrying DHP groups

2.4.1. Enzyme electrodes carrying DHP groups bound with alkylenediamines (Samples **2**–**4**)

The copolymer films were immersed stepwise in the following aqueous solutions (5.0 ml) at 4 °C: (1) 6.0 mg/ml NHS and 24 mg/ml CMC, (2) 5.0 mg/ml GOx, (3) 0.5% glutaraldehyde, (4) 50 mM ethylenediamine and (5) 50 mM HBA. The immersing time for each was 24 h. The film was rinsed with distilled water after each treatment. Variants of the treatment are listed in Table 1.

For a comparison, the enzyme electrode carrying no DHP groups was prepared by following the treatments (1) and (2) (Sample **1**).

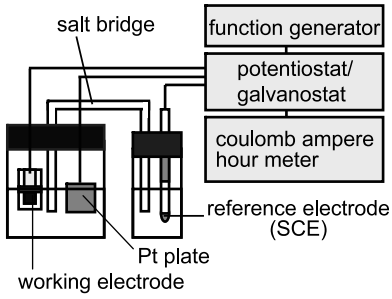


Fig. 2. Apparatus for electrochemical experiments.

Table 1
Reagents used for preparation of the enzyme electrode carrying DHP groups with alkylenediamines

Sample no.	Reagent for electrode modification		
	Glutaraldehyde	Alkylenediamine	HBA
1	nu	nu	nu
2	u	C2	u
3	u	C6	u
4	u	C12	u

u, used; nu, not used; C2, ethylenediamine; C6, hexamethylenediamine; C12, dodecamethylenediamine.

*2.4.2. Enzyme electrodes carrying DHP groups bound with poly-L-lysine (Samples **5–7**)*

The copolymer films were immersed stepwise in the following aqueous solutions (5.0 ml) at 4 °C: (1) 6.0 mg/ml NHS and 24 mg/ml CMC, (2) 2.0 mg/ml poly-L-lysine, (3) 5.0 mg/ml GOx and 24 mg/ml CMC and (4) 50 mM HBA. The immersing time for each is shown in Table 2. The films were rinsed with distilled water after each treatment.

2.5. Glucose sensing

The enzyme electrode carrying DHP groups was placed in 20 ml of 0.01 M phosphate buffer (pH 7.0), and a constant potential of +0.40 V vs. SCE was applied. After background current was allowed to become constant, amperometric response to incremental addition of a glucose solution was monitored. An increase in current was measured on the basis of the back ground current and referred to as response current.

3. Results and discussion

3.1. Characteristics of 3MT/T3A copolymer

It was confirmed by IR spectroscopy that electrochemical polymerization of 3MT and T3A (3MT:T3A = 9:1) gave the copolymer consisting of 90% 3MT and 10% T3A. This copolymer had sufficient amount of carboxyl groups for immobilization of GOx and, therefore, T3A content larger than 10% did not increase the amount of immobilized GOx [34]. The 3MT/T3A copolymer with 10% T3A units had a conductivity of 1.9×10^{-3} S/cm, and afforded high sensitivity when the copolymer film was applied to amperometric glucose sensing by immobilization of GOx on it. Hence the copolymer with 10% T3A was used throughout the present study.

Table 2
Treating time for preparation of the enzyme electrode carrying DHP groups with poly-L-lysine

Sample no.	Reaction time (h)			
	NHS and CMC	Poly-L-lysine	GOx and CMC	HBA
5	24	1	1	24
6	24	3	1	24
7	24	24	1	24

3.2. Nature of HBA as a redox mediator

In order to examine the redox characteristics of HBA, cyclic voltammetry was carried out with 1 mM solution of HBA in 0.1 M phosphate buffer (pH 7.0). Prior to the cyclic voltammetry, the HBA solution was bubbled with nitrogen gas for 20 min or more to remove oxygen. In the cyclic voltammogram shown in Fig. 3, the oxidation and reduction peaks are observed clearly at +0.27 and −0.02 V vs. SCE, respectively.

Succeedingly, by use of the GOx-immobilized film (Sample **1**) as an enzyme electrode, amperometric response to glucose was measured at a constant potential of +0.40 V vs. SCE in the presence of 1 mM HBA. As shown in Fig. 4, response current was obtained depending on the concentration of glucose. The result demonstrates that HBA acts as an effective redox mediator by applying +0.40 V vs. SCE to the enzyme electrode.

3.3. Glucose sensing using enzyme electrodes carrying DHP groups bound with alkylenediamine

Scheme 1(a) illustrates the preparation of enzyme electrode carrying DHP groups with alkylenediamine. According to the scheme, GOx was immobilized on the 3MT/T3A copolymer film, and DHP groups were bound to both the film and the immobilized GOx through C2–C12 alkylenediamines. The preparation was carried out quite readily as follows: 3MT/T3A copolymer film was prepared by electrochemical polymerization for ca. 1.5 min, and then the copolymer film was simply immersed in the solutions for the surface modification with GOx and DHP groups. The mechanism of amperometric glucose sensing is expected as shown in Scheme 1(b), where the response current is generated via the transfer of electrons to the 3MT/T3A

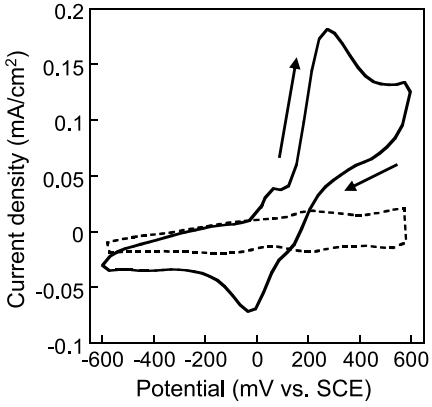


Fig. 3. Cyclic voltammograms measured with Au electrode in the presence (solid line) and the absence (dotted line) of HBA at a potential scan rate of 50 mV/s.

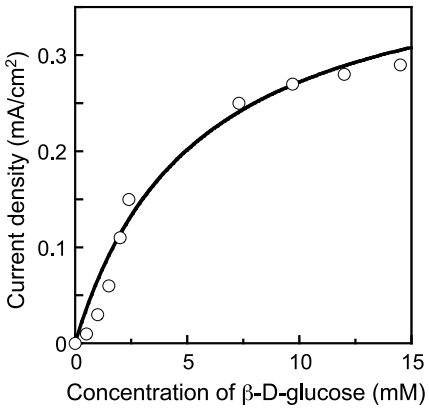
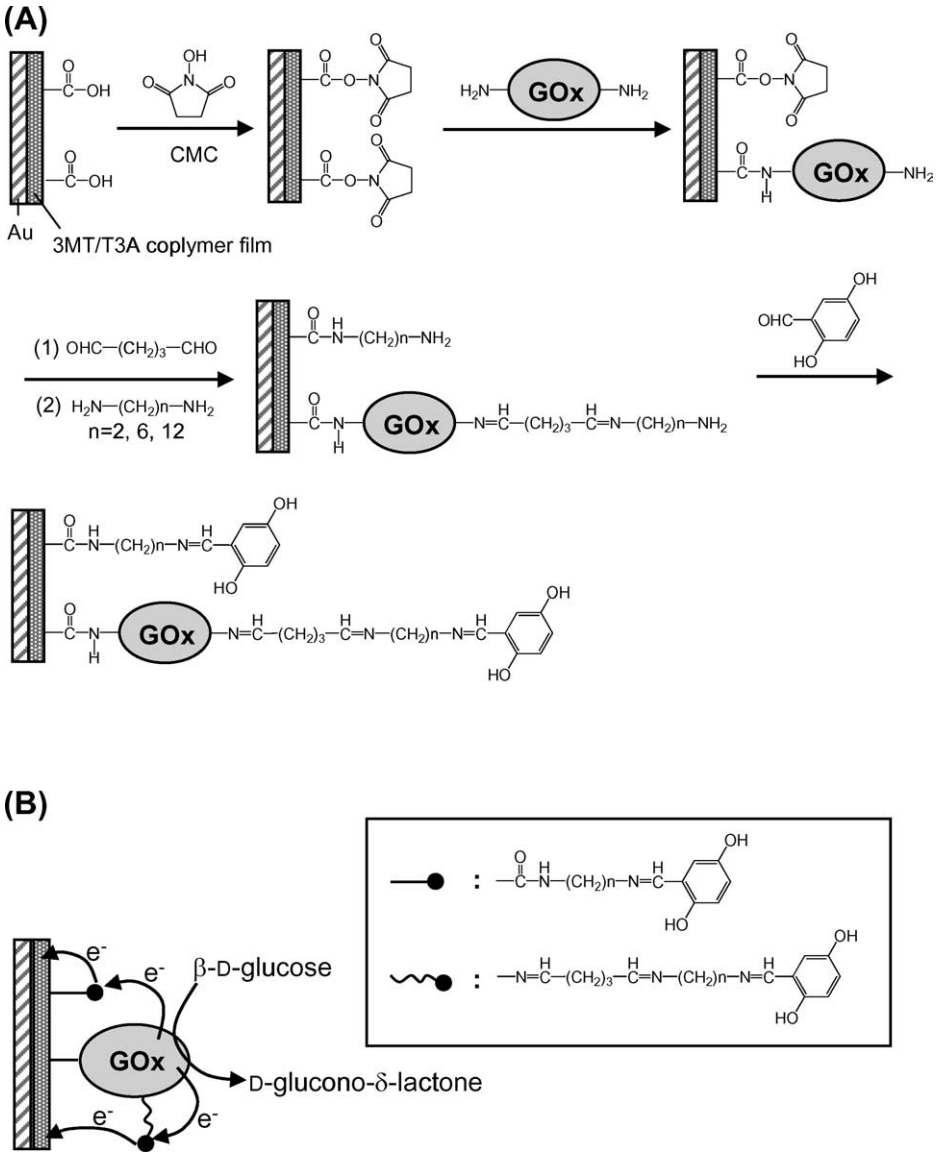


Fig. 4. Amperometric response of the enzyme electrode to glucose.

copolymer film accompanying to the glucose oxidation by GOx.

Electrochemical properties of the enzyme electrodes carrying DHP groups were examined by cyclic voltammetry in 0.1 M phosphate buffer (pH 7.0) free from oxygen, and compared with those of the enzyme electrodes carrying no DHP groups (prepared through the step (1)–(4) in Section 2. 4.1). Fig. 5 shows, as a typical example, the cyclic voltammogram measured with Sample **4**, where the oxidation peak was observed similarly to the voltammograms for dissolved HBA (Fig. 3). This peak was observed in the voltammograms measured with Sample **2**, **3**, and can be attributed to the introduced DHP groups. From the area of the oxidation peak, the amounts of DHP groups on Samples **2**, **3** and **4** were estimated to be 28, 26 and 25 nmol, respectively. Thus, the amount of introduced DHP groups



Scheme 1. Preparation of the enzyme electrode carrying HBA with alkylenediamine (A) and the mechanism of amperometric sensing of glucose (B).

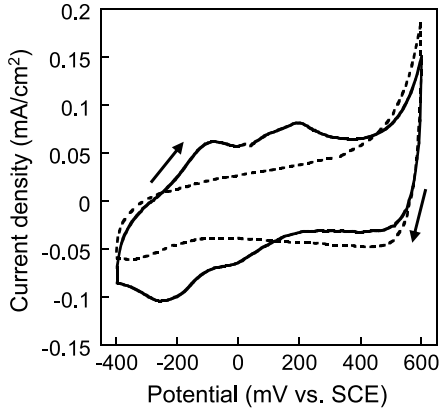


Fig. 5. Cyclic voltammograms measured with Sample **4** (solid line) and the enzyme electrode carrying no DHP groups but C12-alkylenediamine (dotted line) at a potential scan rate of 5 mV/s.

little depended on the length of the alkylenediamines used as linker molecules.

Fig. 6 shows the amperometric response obtained by applying the enzyme electrodes carrying DHP groups to glucose sensing. For each of the electrodes, the response current was found to increase linearly with an increase in glucose concentration up to 2.5 mM. The sensitivity of the enzyme electrodes carrying DHP groups (Samples **2**–**4**) was higher than that of the electrode carrying no DHP groups (Sample **1**). Moreover, it is a point of interest that higher response current was observed for the enzyme electrode carrying DHP groups prepared with alkylenediamine having a longer alkylene chain.

GOx has a flavin adenine dinucleotide (FAD) as a cofactor [34,35]. The glucose oxidation reaction by GOx proceeds through the reduction of FAD. In the glucose sensing system designed in the present study, DHP groups have an electron mediating function like *p*-benzoquinone/hydroquinone system [14,15] to accept electrons from reduced FAD and to transport them to the electrode, i.e. 3MT/T3A copolymer film. To facilitate the electron transfer, it must be required for a redox mediator to access

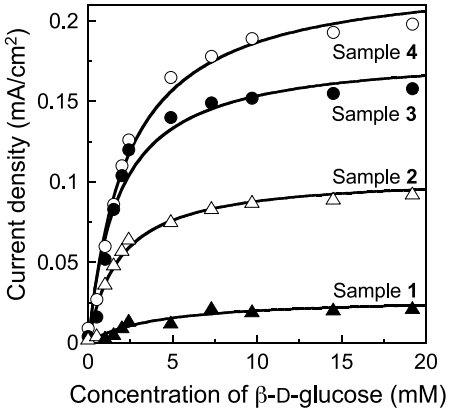


Fig. 6. Influence of the length of linkers on amperometric response to glucose.

to FAD which is covered by shell of protein [36,37]. If the mediator is dissolved in analyte solution, the redox reaction between the mediator and FAD will occur quite easily. However, although DHP groups are considered to have capability of accelerating the electron transfer as shown by the effect of HBA in Fig. 4, their mobility should be limited by the length of alkylenediamine used as a linker. Considering the result that Sample **2**–**4** had almost same amounts of DHP groups on their surface, the length of the linker should be taken into account as a significant factor to control the amperometric response. Thus, the DHP groups bound with alkylenediamine with a longer alkylene chain, probably because of their higher mobility, could access considerably to FAD and the 3MT/T3A copolymer film to give larger response current to glucose as shown in Fig. 6.

3.4. Glucose sensing using enzyme electrodes carrying DHP groups bound with poly-L-lysine

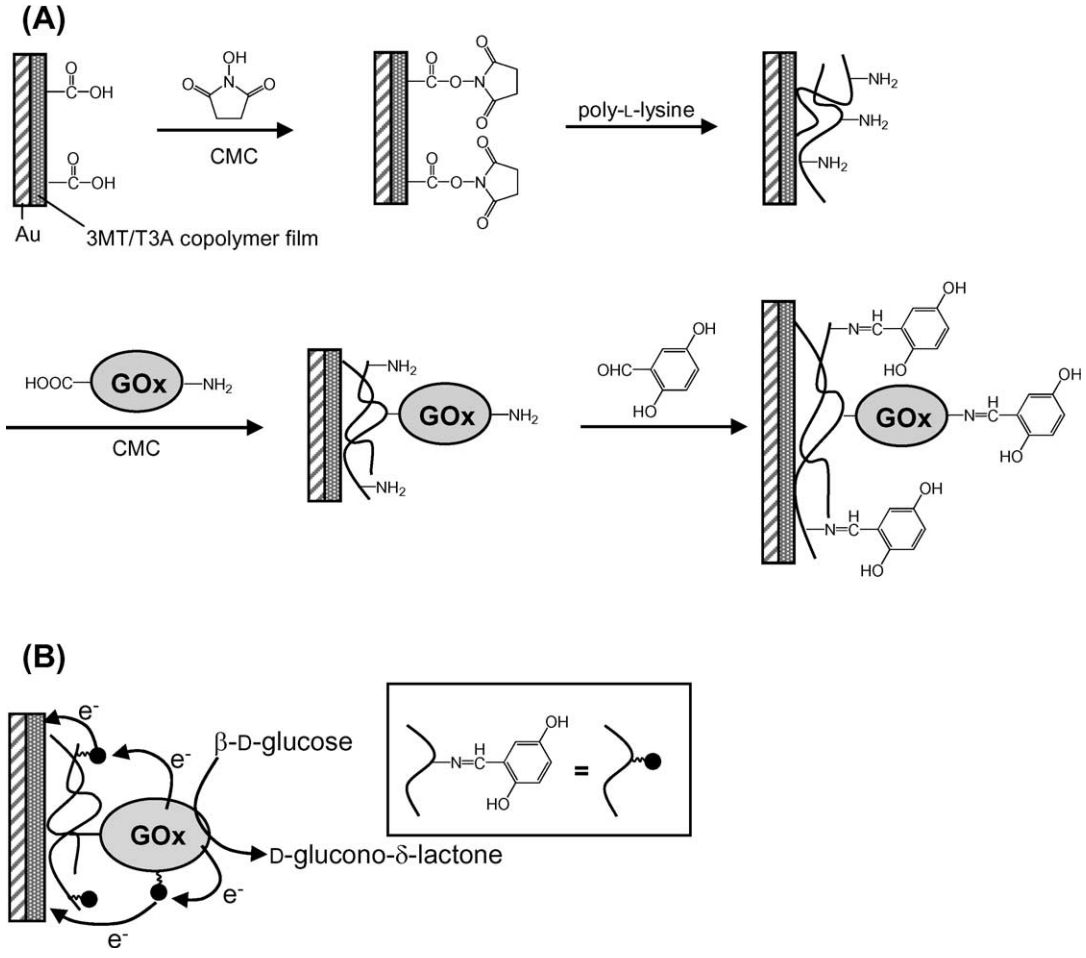
The amount of DHP groups bound to the enzyme electrode may be increased by increasing the content of T3A units in 3MT/T3A copolymer film but, as reported previously [33], a large content of T3A units causes a decrease in conductivity of the copolymer film. Therefore, by use of poly-L-lysine as a linker, DHP groups were introduced onto the enzyme electrode because poly-L-lysine was expected to provide not only multiple site for binding DHP groups but also mobility of the DHP groups bound to the enzyme electrode. Scheme 2 illustrates preparation of the enzyme electrode carrying DHP groups with poly-L-lysine (a) and the mechanism of amperometric glucose sensing with the enzyme electrode (b).

Fig. 7 shows comparison of amperometric response to glucose between the enzyme electrode carrying DHP groups prepared with poly-L-lysine (Sample **5**) and that prepared with dodecamethylenediamine (Sample **4**). It was found that Sample **5** had higher sensitivity than Sample **4** whereas both electrodes gave response current depending on the concentration of glucose. The enhanced response observed for Sample **5** can be attributed to increased sites for binding of GOx and DHP groups, and the result demonstrates that it is effective in preparation of mediator-carrying enzyme electrode to employ poly-L-lysine as a linker.

However, glucose sensitivity of the enzyme electrode was influenced by the time of treatment of the 3MT/T3A copolymer film with poly-L-lysine. As shown in Table 3, the

Table 3
Influence of the time of treatment with poly-L-lysine and air on amperometric response to glucose

Sample no.	Current response to 10 mM glucose (mA/cm^2)	
	Under nitrogen	Under air
4	0.19	0.18
5	0.25	0.23
6	0.16	–
7	0.14	–



Scheme 2. Preparation of the enzyme electrode carrying HBA with poly-L-lysine (A) and the mechanism of amperometric sensing of glucose (B).

treatment for a longer time than 3 h resulted in a decrease in the sensitivity (Samples **6** and **7**). Although the reason for this result is still under consideration, it can be speculated as a possible explanation that the long treatment time makes the surface of the copolymer film crowed with a large amount of poly-L-lysine to keep the immobilized GOx away from the surface. Furthermore, the mobility of bound GOx and DHP groups may be limited by a high density of poly-L-lysine. Such dense layer of poly-L-lysine is considered to be unfavorable for the electron transfer from GOx (FAD) to the copolymer film via DHP groups.

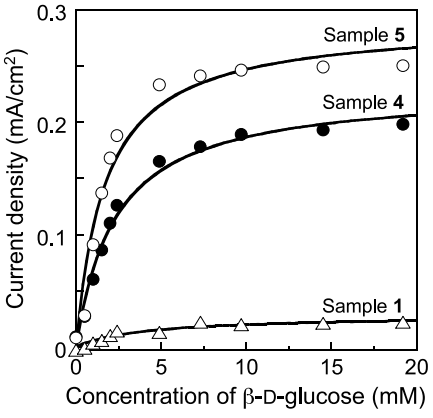


Fig. 7. Comparison between the effects of poly-L-lysine and dodecamethylenediamine as linkers on amperometric response to glucose.

Table 3, in addition, shows the results of glucose sensing with Sample **4** and **5** under air. Slightly lower response was observed under air than under nitrogen for both the electrodes prepared with C12-alkylenediamine (Sample **4**) and with poly-L-lysine (Sample **5**). These results may be interpreted by the consideration that dissolved oxygen oxidizes $FADH_2$ of immobilized GOx to become reduced to hydrogen peroxide, which cannot be oxidized on the electrodes at 0.40 V vs. SCE because of its high oxidation potential (0.80 V vs. SCE) [31].

4. Conclusions

Electron-mediating DHP groups were introduced onto the GOx-immobilized electrode based on 3MT/T3A copolymer by two kinds of methods involving the use of alkylenediamine (C2–C12) and poly-L-lysine as linker molecules, respectively. The amperometric sensing of

glucose with the enzyme electrodes carrying DHP groups gave the result that the sensitivity was increased remarkably by introducing DHP groups onto the electrode. With respect to the enzyme electrode carrying DHP groups prepared with alkylenediamines, the longer the alkylene chain of used alkylenediamine was, the larger the response current became, reflecting facilitated electron transfer due to high mobility of bound DHP groups. On the other hand, the enzyme electrode carrying DHP groups prepared with poly-L-lysine gave enhanced response current, which suggested that poly-L-lysine functioned as an effective linker to provide a sufficient amount of the sites for binding of GOx and DHP groups. The methods for introducing the electron-mediating groups in the present study will be available for practical fabrication of the enzyme electrodes consisting of 3MT/T3A copolymer and various redox enzymes. These enzyme electrodes are worth investigating intensively as promising devices for reagentless operation of amperometric biosensing systems.

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