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Amperometric glucose-responding property of enzyme electrodes fabricated by covalent immobilization of glucose oxidase on conducting polymer films with macroporous structure

Chuanjun Liu, Hokuto Ohta, Takashi Kuwahara, Masato Shimomura *

Department of Bioengineering, Faculty of Engineering, Nagaoka University of Technology, 1603-1 Kamitomioka-machi, Nagaoka, Niigata 940-2188, Japan

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Abstract

Macroporous conducting polymer films were prepared by the electrochemical copolymerization of 3-methylthiophene and thiophene-3-acetic acid on the ITO-coated glass plates bearing different sizes of polystyrene template particles, and enzyme electrodes were fabricated by covalent immobilization of glucose oxidase on the macroporous copolymer films. It was found that the doping level and conductivity of the copolymer films was significantly affected by the treatment with solvent to remove the polystyrene particles, which was considered to result in deterioration in amperometric glucose-responding property of the enzyme electrodes fabricated with the copolymer films. Three-dimensionally ordered macroporous structure on the copolymer films led to enhancement of amperometric response of the enzyme electrodes, and this effect was attributed to the geometry of the interconnected channel structure formed by the linkage of macropores. It was suggested that the amperometric response of the enzyme electrodes was determined by whether the interconnected channel structure on the copolymer films had long distance regularity and a proper size to allow the enzyme and electron-mediator molecules to penetrate into the interior pores of the copolymer film. In particular, the interconnected channel structure seemed to play an important role in the electron-transfer reaction between the mediator molecules and the surface of electrodes.

Keywords: 3-Methylthiophene; Thiophene-3-acetic acid; Conducting polymer; Glucose oxidase; Enzyme electrode

1. Introduction

During last two decades, the immobilization of enzymes on conducting polymer films has been exploited as an excellent technique to fabricate enzyme electrodes that can be applied to biosensors [1–3] and biofuel cells [4–6]. Generally, the immobilization of enzymes is achieved either by in situ entrapment during the electrochemical polymerization in enzyme-containing solution, or by post immobilization, such as covalent binding and adsorption of enzymes, on conducting polymer

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[*] Corresponding author. Tel./fax: +81 258 47 9404.
E-mail address: smasato@vos.nagaokaut.ac.jp (M. Shimomura).

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films [7,8]. No matter what kind of approach is adopted, the morphology of the conducting polymer films should have a great influence on the performance of the enzyme electrodes fabricated with them. It is well known that an increase in surface area or porosity of conducting polymer films will facilitate enzyme immobilization and mass diffusion at solid/liquid interface, and thus improve the performance of the enzyme electrodes.

The preparation of conducting polymer films with ordered macroporous structure has attracted a great interest in recent years owing to the novel three-dimensional structure favorable for their application to enzyme electrodes. Such macroporous structure has been realized for various conducting polymers including polyaniline, polypyrrole, polythiophene and their derivatives, which can be synthesized by chemical [9,10] and electrochemical [11–14] polymerization in the presence of self-assembled template particles. As for the application of such conducting polymer films, an optical-affinity biosensor and a potentiometric biosensor have been fabricated with the highly ordered macroporous film of polythiophene-*co*-(3-thiophenemethanol) [15] and with that of creatinine deiminase doped polypyrrole [16], respectively. Neural prosthetic probe devices were developed by use of macroporous poly(3,4-ethylenedioxythiophene) and polypyrrole films [17]. A nanocomposite of porous polyaniline was applied to fabrication of a nitrite sensor though it was not an example of a biosensor [18]. Glucose oxidase (GOx) and glucose dehydrogenase were immobilized on highly ordered macroporous gold electrodes with precisely controlled pore size and number of pore layers, and the bioelectrocatalytic activity of these enzyme electrodes was investigated in detail [19,20].

Our current interest is to develop enzyme electrodes suitable for biosensors and biofuel cells, in which a large amount of loaded enzyme as well as effective mass diffusion is critically required for achieving high performance of the enzyme electrodes. In the present study, in view of the novel structure and properties of macroporous materials, enzyme electrodes were fabricated by covalent immobilization of GOx on conducting polymer films with macroporous structure. The films were prepared by electrochemical copolymerization of 3-methylthiophene (3MT) and thiophene-3-acetic acid (T3A) on the electrodes bearing polystyrene (PS) particles as the template of the macroporous structure. The PS template was removed by dissolution in tetrahydrofuran (THF) and, in this manner, the macroporous structure was obtained. Fig. 1 shows the structural formula of the copolymer. GOx was immobilized on the surface of the copolymer films through amide linkages by the condensation reaction with COOH groups on the surface. Throughout the present study, the films obtained by the copolymerization of 3MT and T3A with the ratio of 5/1 was employed because they had both considerably high conductivity and a sufficient amount of COOH groups for immobilizing GOx [21]. Amperometric glucose-sensing characteristics of thus fabricated enzyme electrode (GOx-electrode) were compared with those of the one fabricated with the conducting polymer film prepared in the absence of the PS template. The effect of the THF treatment, as well as the size of PS template particles, on the glucose-sensing characteristics of the GOx-electrode was also investigated.

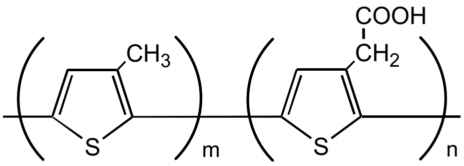


Fig. 1. Structure of 3MT/T3A copolymer.

2. Experimental

2.1. Materials

3MT, tetraethylammonium perchlorate (TEAP) and *p*-benzoquinone (BQ) were purchased from Nacalai Tesque, Inc., and T3A was purchase from Tokyo Kasei Kogyo Co.: all these chemicals were of guaranteed-reagent grade. GOx (EC 1.1.3.4, from *Aspergillus* species) was supplied by Toyobo Co., which had an activity of 165 U/mg. Two kinds of sulfonated PS latex (10 wt% dispersion in H_2O) were obtained from Aldrich Chemical Co., whose average particle diameters were 0.46 and 0.80 μm. 1-Cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate (CMC) from Aldrich Chemical Co. was used as a condensing agent. Other chemicals and solvents were of guaranteed-reagent or analytical grade and used without further purification.

2.2. Apparatus

The electrochemical copolymerization of 3MT and T3A, cyclic voltammetry and measurement of amperometric response to glucose were carried out in a three-electrode cell 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 (Hokuto Denko Corp. HB-105A). The working electrode for the copolymerization was an ITO-coated glass plate. A platinum wire and a saturated calomel electrode (SCE) were used as the counter and reference electrodes, respectively (Fig. 2).

Scanning electron microscopy (SEM) imaging was carried out on a JEOL JSM-6301F microscope by applying an acceleration voltage of 10 kV on sputtered samples. UV/vis absorption spectra of the copolymer film were measured on a Shimadzu UV-3100PC spectrometer.

2.3. Preparation of PS template on ITO-coated glass

The PS template was prepared on an ITO-coated glass plate by means of gravitational sedimentation. A cleaned surface of the ITO-coated glass plate was masked, but a circular area (0.283 cm^2) of the surface being left unmasked. Diluted PS latex (30 μl, 1.0 wt%) was cast on the surface of the ITO-coated glass plate. Subsequently, the plate was sealed in a glass container and then kept in an incubator at 25 °C, in which water was allowed to evaporate slowly over a period of 2–3 weeks.

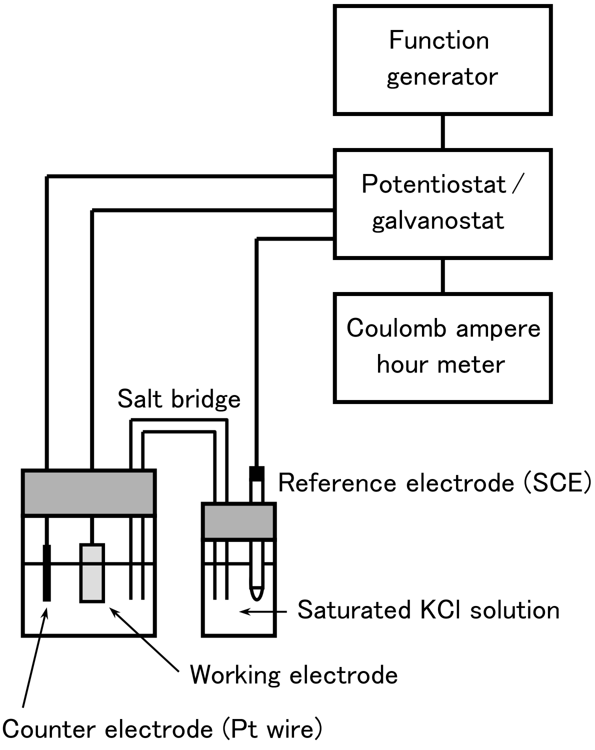


Fig. 2. Apparatus for electrochemical experiments.

The drying process is very important for the preparation of PS template with well-arranged structure. Especially for the gravitational method adopted in the present study, the sedimentation of the PS particles with small sizes is extremely slow. Fast drying at an elevated temperature will reduce the self-assembling ability of the PS particles and result in the formation of non-uniform template.

2.4. Electrochemical copolymerization of 3MT and T3A

The macroporous conducting polymer films were prepared by the electrochemical copolymerization of 3MT and T3A using the ITO-coated glass plate bearing the PS template as a working electrode, which was carried out in 10 ml of acetonitrile solution containing 0.1 M of TEAP and 0.6 M of the monomers of 3MT and T3A with a ratio of 5/1. This monomer ratio has been proved by a previous study [21] to be optimum for the application of 3MT/T3A copolymer to the GOx-electrode. Before the polymerization, the solution was purged with nitrogen gas for 20 min to remove the oxygen. The polymerization was conducted at a potential of 2.2 V vs. SCE until a charge of 20 mC was passed through. After the polymerization, the obtained 3MT/T3A copolymer film was dried in air for 24 h and then dipped into THF for 24 h to remove the PS template particles. For the purpose of making a comparison, the electrochemical copolymerization was also carried out using a bare ITO-coated glass plate as a working electrode under the same conditions except that the amount of passed charge was 100 mC.

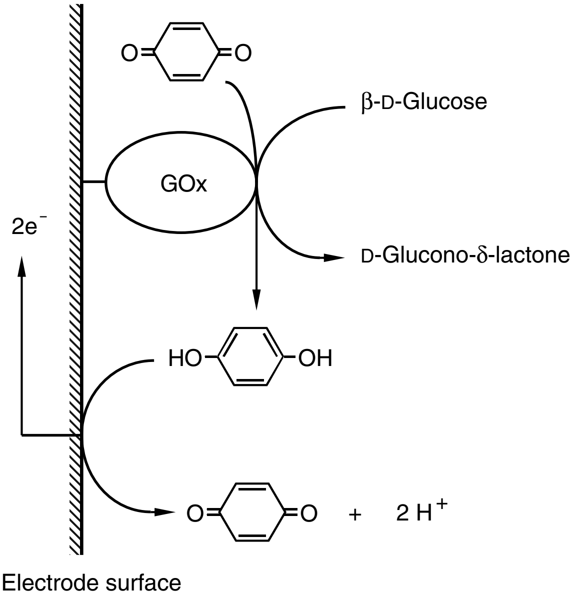
It was difficult to equalize the amounts of passed charge for the preparation of templated and non-templated films. The templated film lost its macroporous structure when the amount of passed charge was larger than 20 mC. On the contrary, it was hard to obtain the non-templated film uniformly covering the surface of the ITO-coated glass plate when the amount of passed charge was smaller than 100 mC. Therefore, the different amounts of charge were chosen. As a result, the average thicknesses of the templated and non-templated films were 7 and 15 μm, respectively.

2.5. Immobilization of GOx on 3MT/T3A copolymer films

Both the templated and non-templated 3MT/T3A copolymer films were immersed in 2.5 ml of deionized water containing 60 mg CMC and 25 mg of GOx for 4 h at 4 °C. The copolymer films treated thus were then rinsed with deionized water and kept in 0.1 M phosphate buffer (pH 7.0) at 4 °C. The amount of immobilized GOx was determined by the analysis with Folin–Ciocalteu phenol reagent after alkaline copper treatment according to the method of Lowry [22]. It was reported elsewhere [23] that the activity of immobilized GOx was 80 mU/cm^2, which was hardly dependent on the composition of the 3MT/T3A copolymer.

2.6. Measurement of amperometric response to glucose with GOx-electrode

The amperometric response to glucose was measured for the GOx-immobilized films of 3MT/T3A copolymer (GOx-electrodes) in the same manner as described in a previous publication [24]. As shown in Scheme 1, glucose was oxidized with the immobilized GOx in the presence of BQ which was employed as an electron mediator. The produced hydroquinone was oxidized electrochemically on the electrode surface and the accompanying electron-generation was detected as current change. The apparatus shown in Fig. 2 was used for the measurement of the amperometric response. The GOx-electrode was placed in 20 ml of 0.1 M phosphate buffer (pH 7.0) containing 1 mM of BQ. A constant potential of +400 mV vs. SCE was applied and the solution was stirred with a magnetic agitator at a rate of 400 rpm. After the background current was allowed to be constant, 100 μl of 0.2 M β-D-glucose solution was injected into the measurement system with a time interval of ca. 200 s, and the real-time current response to the addition of glucose was recorded. After the measurement, the surface morphology of the macroporous GOx-electrode was investigated by SEM observation.



Scheme 1. Glucose oxidation with immobilized GOx and role of *p*-benzoquinone as a mediator.

3. Results and discussion

3.1. Influence of THF treatment on the properties of 3MT/T3A copolymer film

It is an essential process to remove the particles used as the template for the preparation of macroporous conducting polymer films. THF and toluene are favorable solvents for removal of PS particles from the 3MT/T3A copolymer films prepared with the PS template whereas HF or NaOH solution is used to remove inorganic templates such as Al_2O_3 and SiO_2 particles. It has been demonstrated that the doping state is influenced by the HF or NaOH treatment for removal of the inorganic particles used as the templates for the preparation of conducting polymer nanotubes and nanowires [25–27]. In addition, a change in transition between the valence band and the bipolaron state of conduction polymers has been indicated to be probably caused by dedoping upon exposure to THF [15]. However, details are unknown with respect to the influence of the THF treatment on the performance of the enzyme electrodes fabricated with the conducting polymers. As discussed in a previous publication [24], the amperometric characteristics of enzyme electrodes are significantly affected by the electrochemical properties of the conducting polymer films used. Therefore, prior to the preparation of the templated copolymer film, the non-templated film was treated with THF and the electrochemical properties of the THF-treated film were compared with those of untreated one.

As seen in the SEM image of Fig. 3, the 3MT/T3A copolymer film prepared on the ITO-coated glass plate without the PS template gave the surface

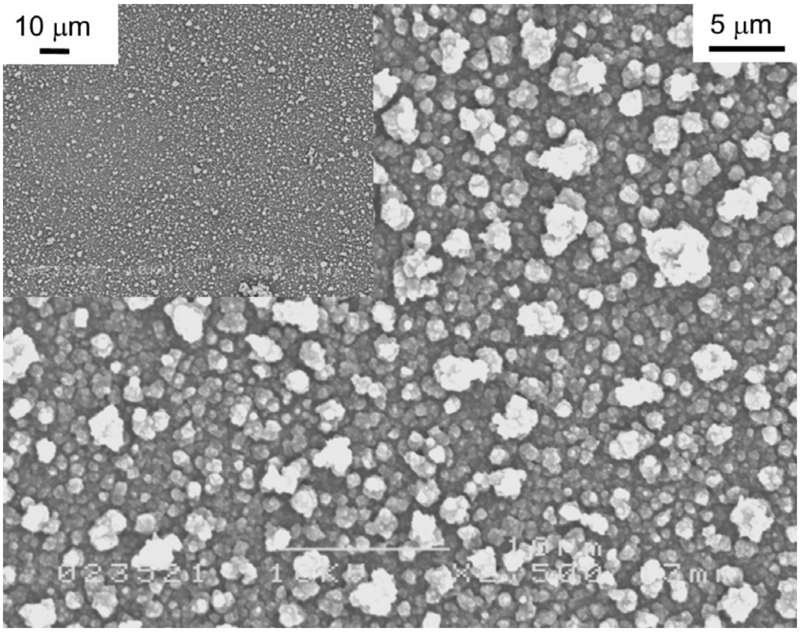


Fig. 3. SEM images of the non-templated 3MT/T3A copolymer film. The inset shows the image at lower magnification.

morphology typical for a thin film of the conducting polymer prepared with a small amount of passed charge (100 mC in the present case). Fig. 4 shows the UV/vis absorption spectra of the copolymer film before and after the THF treatment together with the spectrum after the toluene treatment. According to the description in a literature [27], the peaks at 2.97 and 1.70 eV are attributed to the $\pi-\pi^*$ transition of the conjugated backbone and the absorption by the bipolarons, respectively. It should be noted that, after the THF treatment, the absorbance of the bipolaron decreased while the intensity of the $\pi-\pi^*$ transition increased in reverse. In addition, the both peaks were shifted to a low energy side by the THF treatment. These results demonstrate that the THF treatment has a dedoping effect on the conducting polymer. Furthermore, the cyclic voltammetry for the copolymer film in the presence of BQ gave the result that the redox currents of BQ was decreased by the THF treatment, suggesting a lowered electrochemical activity of the THF-treated copolymer film. On the other hand, it was confirmed by SEM that the THF treatment did not cause any perceptible change in the morphology of the copolymer film. Fig. 5 shows the amperometric response to glucose measured for the GOx-electrodes fabricated with THF-treated and as-prepared 3MT/T3A copolymer films (non-templated). The response of the GOx-electrode fabricated with the THF-treated film was only one third of that fabricated with the as-prepared one. Thus, the conductivity of the copolymer film was decreased by the dedoping during the THF treatment and, as a result, the GOx-electrode fabricated with the THF-treated film showed such small response to glucose.

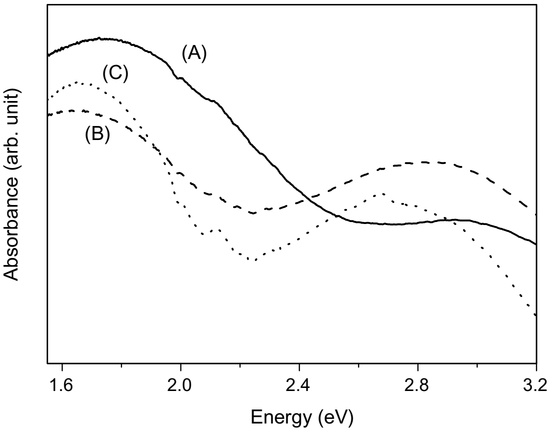


Fig. 4. UV/vis absorption spectra of the non-templated 3MT/T3A copolymer film: as-prepared (A), THF-treated (B) and toluene-treated (C).

It should be mentioned that toluene is also a common solvent used for removal of the PS template. The films of conducting polymers, such as

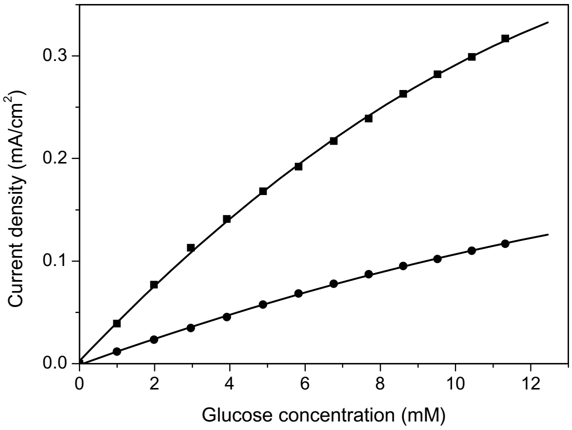


Fig. 5. Amperometric response to glucose measured for the GO*x*-electrodes fabricated with THF-treated (●) and as-prepared (■) 3MT/T3A copolymer films (non-templated).

polyaniline, polypyrrole and polybithiophene, with ordered macroporous structure has been prepared by use of toluene to dissolve the PS template. It was reported that the removal of PS particles by dissolution in toluene had no adverse effect on the conductivity of the polymers [12]. This seems to be contrary to our results that the change in UV/vis spectra of 3MT/T3A copolymer by the toluene treatment was similar to that by the THF treatment as shown in Fig. 4, and decreased response to glucose was observed for the GOx-electrode fabricated with the toluene-treated film. The difference in polymerization conditions, such as monomer, electrolyte solution and dopant, may be a possible reason for the divergent results.

3.2. Surface structure of the macroporous 3MT/T3A copolymer films

Two kinds of latex containing PS particles with average diameters of 0.46 and 0.80 μm were used in the present study to prepare the templates for macroporous 3MT/T3A copolymer films. Fig. 6 shows SEM images of the 3MT/T3A copolymer films obtained by use of the PS templates. Both films had macroporous structures on their surfaces, which were significantly different from the films obtained with no template (see Fig. 1). The size of the pores on the surface was found to reflect the particle size of the PS template. In addition, these pores were linked together by interconnected channels. Therefore, a certain degree of three-dimensional architecture can be expected to be formed within the macroporous films though the detailed structure in the interior is not clear. It should be mentioned, however, that the pores of the film surface were not arranged in such ordered hexagonal closest packing as reported elsewhere [12]. This might be relevant to the irregular arrangement of PS particles in the templates shown in the insets of Fig. 6.

The PS template preparation by gravitational sedimentation has the advantage that its process is simple and does not need any complicated equipment, but the disadvantage is that it is difficult to control the uniformity in thickness and density of the template layer over the whole casting area, especially in the case of thick template due to the reduced self-assemble ability of PS particles in the latex. Such non-uniformity of macroporous structure is more obvious for polymer films than for

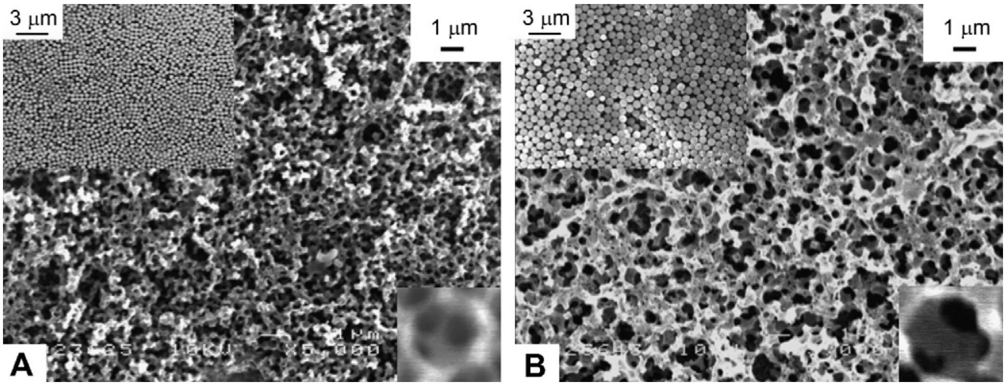


Fig. 6. SEM images of the macroporous 3MT/T3A copolymer films prepared by use of PS templates of 0.46 μm (A) and 0.80 μm (B). The insets in the top left show the images of the PS templates. The insets in the bottom right show the images of interconnected channel structure of each film.

metal films [12]. It is likely that the irregularity of the macroporous 3MT/T3A copolymer films observed in the present study is closely related to the kinetics of film growth in the course of the electrochemical polymerization 3MT and T3A. The strict regularity of macroporous structure will be less critical for the application of conducting films to enzyme electrodes than for the preparation of optoelectronic materials such as photonic crystals. Therefore, the poor regularity of the macroporous 3MT/T3A copolymer films is not considered to prevent them from bioelectrochemical applications. Nevertheless, the investigation of morphology should be essential to eliminate possible experimental errors caused by the irregularity of the macroporous 3MT/T3A copolymer films.

3.3. Glucose-responding property of GOx-electrodes fabricated with the macroporous 3MT/T3A copolymer films

Fig. 7 shows the amperometric response to glucose measured for the GOx-electrodes fabricated with the macroporous 3MT/T3A copolymer films. The plots represent the average values obtained from five measurements for each of the GOx-electrodes fabricated by use of the PS templates of 0.46 and 0.80 μm. The maximum values of relative standard deviation were 14.6% and 12.2% for 0.46 and 0.80 μm samples, respectively. Compared with the result for the GOx-electrode fabricated with the non-templated film (THF-treated) shown in Fig. 5, an improved response was observed for both of the macroporous GOx-electrodes. This result showed that the macroporous structure of the GOx-electrode was effective for enhancement of the amperometric response. If the dedoping were not caused by the THF treatment, this enhancing effect should be more significant. It is a point of interest that a larger response to glucose was achieved by use of the PS template of 0.46 μm than 0.80 μm. This seems to disagree with the common sense that the macroporous film with a smaller pore size has a larger surface area and thus brings higher performance to the enzyme electrode. As a matter of fact, it has been demonstrated for a three-dimensionally ordered macroporous gold electrode that the electrode with smaller pores shows higher electrochemical activity than that with larger ones [19]. With regard to the unusual result mentioned above, the amount of immobilized GOx was determined for the templated (macroporous) and non-templated GOx-electrodes (Table 1).

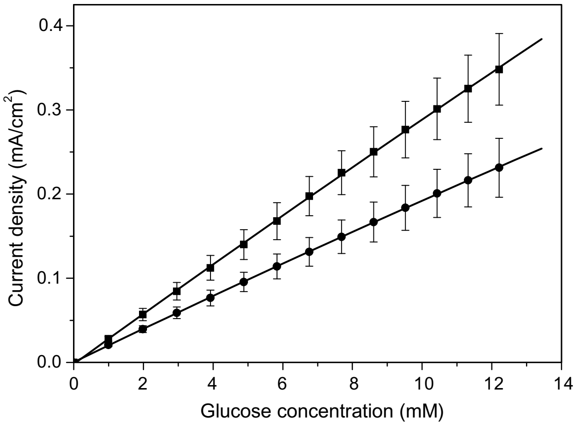


Fig. 7. Amperometric response to glucose measured for the GOx-electrodes fabricated with the macroporous 3MT/T3A copolymer films prepared by use of PS templates of 0.46 μm (●) and 0.80 μm (■).

As for the enzyme electrodes fabricated by enzyme immobilization on the surface of conducting films, the relation between the surface porosity of the electrodes and the amount of immobilized enzyme has been seldom given in the literatures, though it has been well accepted that the amount of immobilized enzyme increases with an increasing porosity of the surface. The possible reason is that common methods of protein determination, including the well-established method of Lowry, are not always suitable for precise evaluation of immobi-

Table 1
Amounts of GOx immobilized on various 3MT/T3A copolymer films

3MT/T3A copolymer film	Amount of immobilized GOx (μg/cm^2)[a]	RSD (%)[b]
Non-templated (as-prepared)	59	10.4
Non-templated (THF-treated)	76	10.2
Templated with PS particles (0.46 μm)	61	6.3
Templated with PS particles (0.80 μm)	69	9.2

[a] Determined by the method of Lowry [22].

[b] Relative standard deviation for three samples of each film.

lized enzymes such as the GOx on the conducting polymer films. In the present study, therefore, plural samples of GOx-electrode were prepared in one batch experiment in order to eliminate a relative error as possible as it can be. The result in Table 1 seems to demonstrate that the amount of immobilized GOx lay on the almost same level, whereas the GOx-electrodes showed a difference in amperometric response to glucose. In particular, both the two kinds of macroporous films did not bear any marked amount of immobilized GOx compared with the non-templated ones.

With respect to a three-dimensionally ordered macroporous gold electrode, it has been demonstrated that an enhanced active surface area of the bulk film can be obtained by use of template particles with smaller diameter or an increased number of pore layers, owing to formation of interconnected channel structure between neighboring pores, though its geometric area is independent of the pore size [28]. Thus the structure (the regularity and mouth open size) of interconnected channels should significantly affect the property of the ordered macroporous film. However, the formation mechanism of the interconnected channel structure for conducting polymers is more complicated than that for metals. Particularly, it has been proved for conducting polymer films that the channel size is affected by various factors, such as the diameter of template particles, polymerization potential, the nature of monomer, shrinkage of the films in solvent, and so on [11–13]. As mentioned in Section 3.2, the interconnected channel structure was observed for two kinds of templated 3MT/T3A copolymer films. The poor regularity of the two- and three-dimensional macroporous structure was a marked shortcoming of both films. In view of the irregular arrangement of pores seen in Fig. 6, it is likely that the probability to form the interconnected channels was more readily increased by use of the PS template of 0.80 μm than 0.46 μm because the PS template of larger particle size is considered to give larger open mouths of the interconnected channels. Therefore, a larger active surface area can be expected for the macroporous 3MT/T3A copolymer film obtained by use of the PS template of 0.80 μm than 0.46 μm. This is indirectly evidenced by the cyclic voltammograms shown in Fig. 8, which demonstrates that a higher redox current was observed for the 3MT/T3A copolymer film obtained by use of the 0.80 μm template, suggesting the facilitated penetration of BQ molecules into interior pores of the copolymer film. It should be mentioned that, in the present study, enzyme immobilization was carried out by static immersion of the 3MT/T3A copolymer films into GOx solution and, accordingly, large GOx molecules could hardly enter the hollow pores in the interior of the copolymer films, especially for the ones with smaller size of the interconnected channels. Thus, it is suggested that, in spite of the films having the macroporous structure, most GOx molecules were located just on the outermost surface of the films and, as a result, almost same amount of GOx was immobilized on the both films prepared by use of the PS templates of 0.46 and 0.80 μm as shown in Table 1. Therefore, the larger response to glucose observed for the GOx-electrode fabricated by use of the 0.80 μm template, which was independent of the amount of immobilized GOx, was attributed to the higher rate of the electron-mediating reaction between BQ molecules and the electrode surface due to its larger active area.

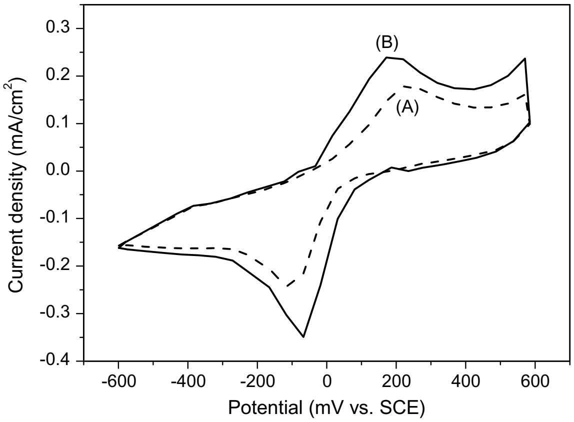


Fig. 8. Cyclic voltammograms measured with the macroporous 3MT/T3A copolymer films prepared by use of PS templates of 0.46 μm (A) and 0.80 μm (B) in the presence of 0.2 mM of BQ in 0.1 M phosphate buffer (pH 7.0) at a scan rate of 50 mV/s.

It should be pointed out that the unusual result of amperometric response to glucose observed for the two kinds of GOx-electrodes is probably limited just in the present study, for the 3MT/T3A copolymer films had irregular macroporous structure on their surfaces. If the three-dimensional ordering of the macroporous structure were improved, it would not strange at all to meet with a completely contrary result. The result in the present study demonstrated in only one aspect that the amperometric glucose-responding property of the GOx-electrode was determined by whether the interconnected channel structure on the 3MT/T3A copolymer films had long distance regularity and a proper size to allow GOx and BQ molecules to penetrate into the interior pores of the copolymer film. Optimization of these parameters will be an essential work for the fabrication of the enzyme electrodes based on macroporous conducting polymer films.

4. Conclusions

The macroporous conducting polymer films were prepared by the electrochemical copolymerization of 3MT and T3A on the ITO-coated glass plates bearing different sizes of PS template particles, and GOx-electrodes were fabricated by covalent immobilization of GOx on the macroporous 3MT/T3A copolymer films. Two major conclusions were drawn: (1) the doping level and conductivity of the copolymer films were significantly affected by the treatment with solvent to remove the PS particles, which was considered to result in deterioration in amperometric glucose-responding property of the GOx-electrodes fabricated with the copolymer films; (2) the copolymer films prepared by use of the PS template had three-dimensionally ordered macroporous structure of the copolymer films which led to enhancement of amperometric response of the GOx-electrodes, and this effect could be relevant to the geometry of the interconnected channel structure formed by the linkage of macropores.

With respect to enzyme electrodes based on macroporous conducting polymer films, the interconnected channel structure on the films seemed to play an important role in the electron-transfer reaction between mediator molecules and the electrode surface. In addition, many complicated problems, such as the long distance ordering and open mouth size of the interconnected channels, should be taken into account for the fabrication of enzyme electrodes. These geometric factors should have significant effects on both the immobilization of enzyme and the electron-transfer reaction of mediator molecules on the electrode surface though the macroporous structure of the 3MT/T3A copolymer films prepared in the present study did not bring an increase in the amount of immobilized enzyme.

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