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Covalent immobilization of glucose oxidase on the film prepared by electrochemical polymerization of *N*-phenylglycine for amperometric glucose sensing

Toshimasa Homma, Toshifumi Ichimura, Mizuki Kondo, Takashi Kuwahara, Masato Shimomura *

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

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ABSTRACT

N-Phenylglycine, an aniline-derived amino acid, was polymerized electrochemically. The resulting poly(*N*-phenylglycine) (PPG) was in the form of a thin film having the conductivity of 2.5×10^{-3} S cm^{-1}. The surface of the PPG film had a fibrous structure, which made the actual area of the surface larger than the apparent one. Glucose oxidase (GOx) was immobilized covalently on the PPG film by the condensation reaction of amino groups of GOx with the carboxyl groups present on the film. The quantity and activity of the immobilized GOx were determined to be 66.4 μg cm^{-2} and 121 mU cm^{-2}, respectively. The GOx-immobilized PPG film was applied to amperometric glucose sensing in the presence of *p*-benzoquinone employed as an electron mediator. It was found that the current response increased with an increase in glucose concentration up to 7.5 mM. The sensitivity was 21.0 μA mM^{-1} cm^{-2} with the relative standard deviation of 6.2% (n = 5).

1. Introduction

During the last three decades, conducting polymers have attracted much attention as important materials for fabricating enzyme electrodes which are applied to bioelectrochemical devices, such as biosensors and biocatalytic fuel cells [1–3]. Conventionally, enzyme electrodes have been fabricated by entrapping enzyme molecules physically within conducting polymer films during electrochemical polymerization [4–8]. However, the enzyme reaction is considered to occur predominantly on the surface of the enzyme electrode and, therefore, the enzyme molecules entrapped deep into the conducting polymer film will little contribute to sensing ability of the enzyme electrode. In addition, leakage may be inevitable for the enzyme molecules entrapped in close vicinity to the electrode surface. From such a viewpoint, many researchers have attempted to immobilize enzymes covalently by using the surface functional groups of conducting polymer films as binding reaction sites [9–14].

Conducting polymers substituted with carboxyl groups can be subjected to covalent immobilization of enzymes because amide linkages are formed by the condensation reaction of the carboxyl groups with amino groups of the enzymes. In general, however, the conductivity of the polymers is considerably lowered by such substituents as carboxyl groups [15,16]. For example, the conductivity of poly(*o*-amino benzoic acid) is 3.0×10^{-8} S cm^{-1}, which is 8 orders of magnitude lower than that of polyaniline [17]. In a previous study, it has been shown that the conductivity of the polymers affects the amperometric biosensing performance of the enzyme electrodes fabricated with them [18,19].

Recently, chemically oxidative polymerization of *N*-phenylglycine, an aniline-derivative amino acid, has been carried out. In this manner, poly(*N*-phenylglycine) (PPG), whose structure is shown in Fig. 1, has been obtained in the form of powder having a relatively high conductivity of 1.7×10^{-4} S cm^{-1} [20]. PPG has carboxyl groups and,

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

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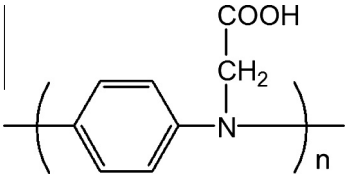

Fig. 1. Chemical structure of PPG.

therefore, is a promising candidate for the conducting material of enzyme electrodes. In the present study, PPG was synthesized electrochemically in the form of a thin film, and glucose oxidase (GOx) was immobilized covalently on the PPG film by the condensation reaction of amino groups of GOx with the carboxyl groups present on the film. The GOx-immobilized PPG film (PPG-GOx film) was applied to amperometric glucose sensing. To our knowledge, no previous attempts have been made to employ PPG as a conducting component of the enzyme electrode for biosensing.

2. Experimental

2.1. Materials

N-Phenylglycine of higher purity than 97.0% was obtained from Tokyo Chemical Ind. Co. and used as received. GOx (EC 1.1.3.4, from *Aspergillus* species) was supplied from Toyobo Co., whose activity was 162 U mg^{-1}. D-Glucose of guaranteed reagent grade from Wako Pure Chemical Ind. was used without further purification. *N*-Cyclohexyl-*N*-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate (CMC, used as a condensing reagent) and *N*-hydroxysuccinimide (NHS, used for activation of carboxyl groups) were from Sigma–Aldrich, Inc. and Nacalai Tesque, Inc., respectively. Other chemicals were of guaranteed reagent grade or analytical grade, which were used without further purification. All aqueous solutions were prepared with distilled water. A glucose solution was allowed to stand for at least 24 h to reach anomeric equilibrium.

2.2. Preparation of the PPG film

The PPG film was prepared by electrochemical polymerization in a 1.0 M HNO_3 solution containing 0.1 M *N*-phenylglycine. Prior to the polymerization, for removal of dissolved O_2, the solution was saturated with N_2 by bubbling via an external source. The polymerization was carried out with a conventional three-electrode cell equipped with a potentiostat/galvanostat (μAutolab Type III, Eco Chemie). A gold film deposited on an alumina plate was used as a working electrode (0.25 cm^2). In advance of 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 applying a constant potential of 0.75 V vs. SCE to the working electrode and continued until the amount of passed charge reached 4.0 C cm^{-2}. The PPG film obtained in this manner was washed thoroughly with distilled water.

2.3. Characterization of the PPG film

Prior to immobilization of GOx, the PPG film was characterized as follows: The IR spectrum of the film surface was measured with a diamond ATR crystal on a Nicolet iS10 FT-IR spectrometer. Surface morphology of the film was observed by scanning electron microscopy (SEM) on a JEOL JSM-6301F microscope. The sample for SEM was coated with gold (10 nm thick) by ion sputtering. The conductivity of the PPG film was measured on a MCP-T610 resistivity meter (Mitsubishi Chemical Analytech Co.) by a four-point probe method. The sample for the conductivity measurement was prepared in the same manner as described in a previous publication [21].

2.4. Immobilization of GOx on the PPG film

GOx was immobilized on the PPG film by the reaction shown in Scheme 1. The PPG film was immersed in 2 mL of an aqueous solution of 100 mg CMC and 15 mg NHS for 20 min to activate the carboxyl groups on the film by esterification with NHS. After washing with distilled water, 15 μL of a 5 mg mL^{-1} aqueous solution of GOx was dropped onto the PPG film, and the film was left at room temperature for 30 min being covered with a Petri dish to avoid water evaporation. Then the PPG film treated in this manner (PPG-GOx film) was washed with 3.0 mL of distilled water. The water containing unbound GOx was recovered, and the amount of immobilized GOx was estimated from the difference in GOx content between the solution recovered and that prepared for the immobilization. The GOx content was determined by use of the QuantiPro BCA Assay Kit (Sigma–Aldrich, Inc.).

2.5. Biochemical measurement

The activity of the GOx immobilized on PPG film was measured at 25 °C by a colorimetric method according to the procedure of Trinder [22]. This method includes the reaction of hydrogen peroxide, which is produced in glucose oxidation by GOx, with phenol and 4-aminoantipyrine in the presence of peroxidase to yield a colored product. In advance, a standard curve was drawn on the basis of the absorbance at 505 nm due to the colored product. The activity was determined from the standard curve, in which the absorbance was measured on a Shimadzu UV-3100 PC spectrometer.

2.6. Glucose sensing with the PPG-GOx film

Taking preceding studies [18,19] into account, amperometric response of PPG-GOx film to glucose was measured by applying a constant potential of 0.4 V vs. SCE in 20 mL of a phosphate buffer solution (0.1 M, pH 7.0) containing 1.0 mM *p*-benzoquinone. The solution was stirred continuously with a magnetic bar at a speed of 400 rpm to provide convective transport. After the background current was allowed to be constant, a given concentration of glucose