Transactions of the Materials Research Society of Japan 29 [3] 771-774 (2004)

Electrochemical Biosensor Based on Thin Conducting Polymer Film for Phenol

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An electrochemical biosensor has been developed for the quantitative estimation of phenol in aqueous solution. The enzyme, tyrosinase (PPO), was covalently immobilized to an electrochemically prepared novel copolymer poly (N-3-aminopropyl pyrrole-co-pyrrole) film onto an indium-tin-oxide (ITO) coated glass plate support. The biosensor has been characterized by UV-visible and infrared spectroscopy. Electrochemical studies have been conducted on these enzyme immobilized polymer films and amperometric response was measured as a function of concentration of phenol, at fixed bias voltage of 0.1V vs. Ag/AgCl in a phosphate buffer solution containing 0.01M potassium hexacyanoferrate (II) as redox mediator. The linearity and sensitivity of the enzyme electrode towards phenol is 2.8-140.2μM and 42.4 μA/mM, respectively. The biosensor is stable for about 4 months at 4°C.

Key words: biosensor, tyrosinase, copolymer, covalent bonding, and peptide linkage

1. INTRODUCTION

Phenolic compounds are released into the environment by a large number of industrial wastes [1]. These phenolic compounds have toxic effects on animal, plants and humans, resulting in an acute environmental problem. Phenol is easily adsorbed in humans, regardless of the type of exposure. Prolonged oral or subcutaneous exposure causes damage to the lungs, liver, kidney and genito-urinary tract. A number of phenol compounds are listed in the European and US-EPA list of priority pollutants due to their toxicity and persistence in the environment [2,3]. According to US-EPA the existence of 4.0 mg l^{-1} of phenol in domestic drinking water is not expected to cause any adverse noncarcinogenic effects in a 70 Kg adult over a lifetime of exposure, with a margin of safety [4]. In view of major concern regarding toxicity and chemical importance of these compounds, there is a growing need for innovative devices to monitor phenols in complex environments, food, pharmaceutical and industrial matrices. Many methods are available for the determination of phenolic compounds, including gas chromatography and spectrophotometric analysis [5,6]. However, these methods suffer from complicated sample pretreatment and are unsuitable for on-site monitoring. Therefore, a large number of efforts have been made for the simple and effective determination of phenolic compounds. Amperometric biosensors that combine the high specificity of biological catalysts such as enzymes with the sensitivity and accuracy of electrochemical indicator reactions have been proven to be promising for this purpose. Electrochemically polymerized conducting polymers have received considerable attention over the last two decades. The poly conjugated conducting polymers have been recently proposed for biosensing applications because of a number of favorable characteristics, such as (1) direct and easy deposition on sensor electrode by electrochemical oxidation of monomer, (2) control of thickness by deposition charge, and (3) redox conductivity of the polymer useful for sensor application. These requirements are met with polypyrrole (PPY), which is the most commonly used polymer because of easy oxidation, low cost of the monomer and chemical stability of the polymer [7].

The aim of this paper is to present our work on the fabrication of an amine functional group derived pyrrole copolymer electrode and the subsequent covalent attachment of tyrosinase, also known as Poly phenol oxidase (PPO), via carbodiimide coupling reaction. This work illustrates the versatility of an amine-based conducting poly (aminopropyl pyrrole-co-pyrrole) (PAPCP) as an immobilization platform for the fabrication of biosensor. The application of the resulting biosensor for the amperometric detection of phenol in aqueous medium is described.

2. EXPERIMENTAL

2.1 *Material and methods*

Mushroom tyrosinase (EC 1.14.18.1, 2578 U/mg from mushroom) and L-tyrosine were obtained from sigma. 1-Cyanoethylpyrrole monomer was obtained from Aldrich. Pyrrole monomer was distilled thrice and the p- toluene sulphonic acid solution was freshly prepared before use. 1-ethyl-3-(3-dimethylaminopropyl) car bodiimide hydrochloride (EDC), and N-hydroxy-succinimide (NHS) were obtained from Sigma

Chem. Co. All other chemicals were of analytical grade and used without further purification.

2.2 *preparation of monomer N-(3-aminopropyl)-pyrrole*

A solution of 1-cyanoethyl pyrrole (0.041 mol) in anhydrous ether was added to a suspension of $LiAlH_4$ (0.1 mol) in anhydrous ether (300ml) and the mixture was refluxed for 20h. After cooling, the excess hydride was destroyed by the successive addition of water (3.4 ml), a solution of 15% NaOH (3.4ml) and water (10.2 ml). The solution was heated to 40^0C for 2h and was filtered before evaporating to dryness. Yellow oil was obtained as a final product with a yield of about 80%. ^{1}H NMR δ ($CDCl_3$): 1.92 (m,2H, CH_2-2); 2.70 (t,2H,CH_2-3); 4.0 (t,2H, CH_2-1); 6.15(d, 2H, CH-β); 6.65(d, 2H, CH-α)

2.3 *Preparation of a copolymer poly (N-3-aminopropyl pyrrole-co-pyrrole)(PAPCP)*

The copolymer film of N-(3-aminopropyl)-pyrrole and pyrrole (0.25 cm^2 area) was electrochemically prepared from an aqueous solution containing 0.05M pyrrole, 0.05M N-(3-aminopropyl) pyrrole and 1.0M p-toluene sulphonic acid sodium salt on an ITO glass plate, at a fixed voltage of 0.8V Vs SCE. The thickness of the film obtained was about 1.4 μm as calculated from the injected charge.

2.4 *Fabrication of covalent bonded enzyme electrode (PPO/PAPCP/ITO)*

The enzyme, tyrosinase, was covalently attached to the above said copolymer electrode by using the exposed surface free amine groups of said copolymer. The copolymer electrode (PAPCP) was immersed in a phosphate buffer solution (0.1M, pH 7.2) containing 0.015M EDC and 0.03M NHS for 1.5 h, and immediately placed in an enzyme solution of 3.6mg ml^{-1} PPO in the same buffer solution for another 1.5h. The enzyme electrode was rinsed with phosphate buffer solution (pH 7.2) to remove the excess unbound enzyme and dried at room temperature. The enzyme electrode was stored under dry condition at 4^0C in a refrigerator when not in used.

2.5 *Instrumentation*

Ultraviolet-visible (UV-vis) absorbance data were collected with a JASCO (model V-570) spectrophotometer. Fourier transform infrared (FTIR) spectra of the films were recorded on JASCO, (model 230). Scanning electron micrographs were obtained with a Shimadzu; super scan (model SS-550) at an acceleration voltage of 12.0 KV. All electrochemical studies were performed on cyclic voltammetry instrument Hokuto Denko (model HSV-100).

2.6 *Enzyme activity measurements*

Tyrosinase (PPO) oxidizes L-tyrosine to dihydroxyphenylalanine, which in turn is oxidized to o-quinone. The later is accompanied by an increase in absorbance at 280 nm. The rate of increase is proportional to enzyme concentration and linear during a period of 5-10 minutes after an initial lag. One unit causes a change in absorbance at 280 nm (A_{280}) of 0.001 per minute at 25°C at pH 6.5. 1.0 mL of 0.001M L-tyrosine, 1.0 mL of 0.5M phosphate buffer, pH 6.5 and 0.9 mL of reagent grade water were mixed and taken into cuvettes. The above said reaction mixture was oxygenated by bubbling oxygen into cuvettes through a capillary tube for 4-5 min. After that cuvettes were transferred to the spectrophotometer and A_{280} was recorded for 10-15 minutes to achieve temperature equilibrium and to establish blank rate. PPO/ PAPCP/ITO electrode was immersed for 20 minutes in phosphate buffer solution. The electrode was taken out from the buffer solution and 0.1mL of the said buffer solution was added to the above reaction mixture in cuvettes and recorded A_{280} for 10-15 minutes to determine the possible leaching of enzyme from the (PPO/PAPCP/ITO) electrode. ΔA_{280} was determined from the linear portion of the curve and the enzyme activity was calculated by using a formula [8]:

Units/ mL = ΔA_{280} x 1000/ mL of reaction mixture (buffer solution)

However, no leaching (disorption) of enzyme (PPO) from the immobilization copolymer film matrix was observed. This may be attributed to an efficient covalent entrapment of enzyme, PPO, through peptide linkage to the exposed amino functional group at the surface of copolymer film.

3. RESULTS AND DISCUSSION

3.1 *Characterization of enzyme electrode (PPO/PAPCP/ITO)*

The steps involved in the fabrication of the covalent immobilization of enzyme, PPO, to a copolymer poly (N-3-aminopropylpyrrole-co-pyrrole) electrode surface are shown below in Fig.1

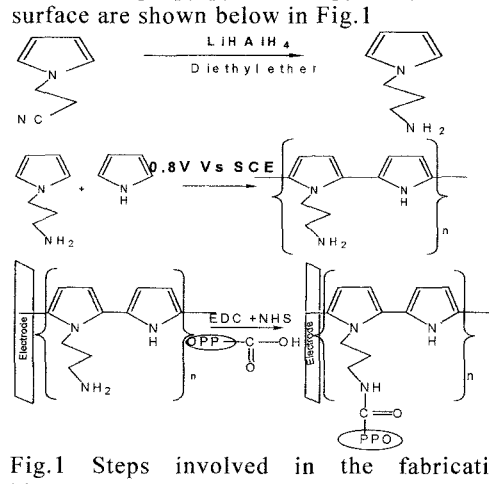

Fig.1 Steps involved in the fabrication of biosensor strip

The free amine groups present at the surface of the copolymer film have been utilized for the covalent attachment of enzyme, PPO, through peptide linkage with a carboxylic acid group using the linkage reagents EDC and NHS [9,10]. Fig. 2 shows the FT- IR spectra of PAPCP (·····) and

PPO/PAPCP (—). Sharp peaks seen at 1540 cm-1 and 1000-1100 cm-1 in native copolymer PAPCP film have been attributed to C=C stretching mode and C-C stretching respectively [11]. However, in the spectra of enzyme immobilized polymer film (PPO/PAPCP) the peaks seen in the spectra of native film (PAPCP) at 1000-1100 cm^{-1} have been merged into a broad peak at 950-1200 cm^{-1} and a new sharp peak is obtained at 1645 cm^{-1}. This new peak at 1645 cm^{-1} has been attributed to C=O stretching, also called as amide I band and peak appearing at 3390 cm-1 is assigned to NH deformation, also called as amide II band [12].

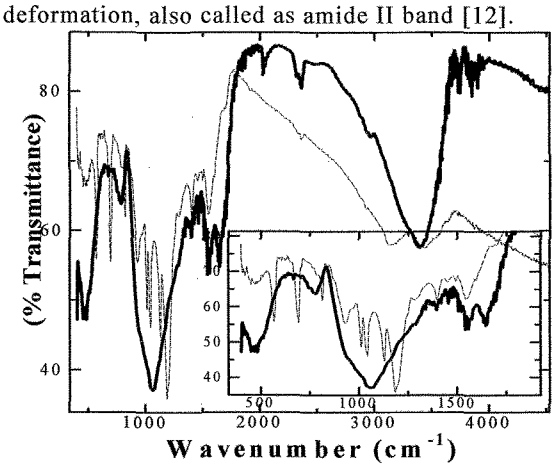

Fig.2. FTIR spectra of PAPCP (....) and PPO/PAPCP (—)

When the PPO/PAPCP/ITO electrode was immersed in phosphate buffer containing L-tyrosine, the observed increase in absorbance at 280 nm is characteristic of L 3,4-dihydroxyphenyl alanine (DOPA) produced by enzymatic reaction. The results further indicate that the enzyme (PPO) has been incorporated in the copolymer, PAPCP, film. The PPO/PAPCP/ITO electrodes were also studied for the enzyme stability at both room temperature and 4^0C. The PPO/PAPCP/ITO electrode was found with no significant decay in stability for about 4 months when stored at 4^0C. However, at room temperature the film was stable only for 4 weeks. The physical morphology of the immobilizing matrix has a great effect on the performance of the enzyme electrode. The morphologies of poly (N-3-aminopropylpyrrole-co-pyrrole)(PAPCP/ITO) and enzyme immobilized electrode (PPO/PAPCP/ITO) were characterized by scanning electron microscopy (SEM). A typical SEM picture of PAPCP/ITO (Fig.3A) at 2.5x 20000 magnification displays a three- dimensional porous open structure. This porous structure has contributed a significant role towards the high enzyme stability within the polymer matrix and good reproducibility of the enzyme electrode (PPO/PAPCP/ITO). When enzyme was immobilized in the PAPCP/ITO matrix, many rectangular shape particles were distributed uniformly through out the surface of the polymer matrix, as can be seen in the SEM micrograph (Fig 3B) at 2.0 x 20000 magnification, which are assigned to protein molecular chain.

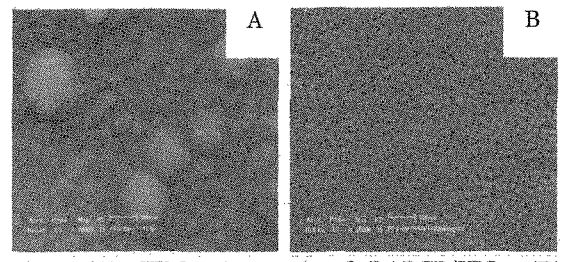

Fig.3. (A) SEM micrograph of PAPCP/ITO; (B) (PPO/PAPCP/ITO).

3.2 *Cyclic voltammetric studies*

The biosensor tends to be more stable as compared to direct electrochemical oxidation of phenol to quinone. The enzyme electrode has an advantage over direct electrochemical oxidation of phenol because of is its low potential detection (0 to -0.2V vs. Ag/AgCl) as compared to (+0.80 to +0.95V vs. Ag/AgCl), since at higher voltage the enzymatically produced quinone get polymerized and is responsible for the fouling of electrode [13]. Potassium hexacyanoferrate (II) has been used as a reducing agent for the quinone species aqueous phenol sensors [14] and it is suggested that the reaction between the quinone and hexacyanoferrate (II) reduces the possibility of enzyme inactivation by quinone. Keeping this in view we have used potassium hexacyanoferrate (II), as redox mediator, in solution during electrochemical detection of phenol in aqueous medium.

A three-electrode cell was used for the electrochemical studies using PPO/PAPCP/ITO electrode as the working electrode, platinum foil as the counter electrode and Ag/AgCl as the reference electrode. The cyclic voltammetric studies have been done with increasing phenol aliquots using PPO/PAPCP/ITO electrode in 0.1M-phosphate buffer (pH 7.2). Before experiment oxygen was passed through the sample solution for about 15 minutes.

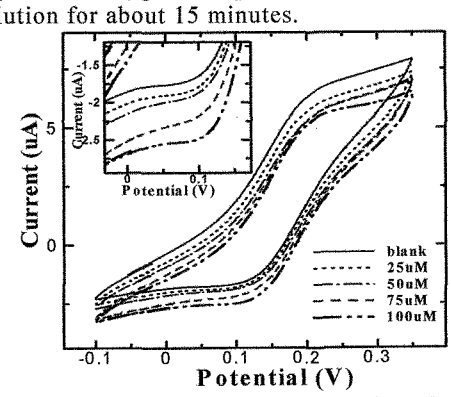

Fig.4. Cyclic voltammograms of PPO/PAPCP/ITO electrode in 0.1M-phosphate buffer (pH 7.2) containing 0.01 M potassium hexacyanoferrate (II) (mediator) with increasing concentration of Phenol; Scan rate 5mV/Sec.

Fig.4 shows a reduction peak at 0.1V, which keeps on increasing in cathodic current with increase in concentration of phenol in phosphate buffer. This is attributed to the direct reduction of Fe^{3+} to Fe^{2+} ion by the enzymatically-produced

quinone at enzyme electrode surface. However, a decrease in the oxidation peak current at 0.25V with increasing concentration of phenol, further indicates a fast enzymatic reaction between the enzymatically produced quinone and $[Fe^{2+}(CN)_6]^{4-}$ species at the electrode surface.

3.3 *Amperometric studies*

A three-electrode cell configuration similar to the one used in cyclic voltammetric experiments has been used for the amperometric response studies of biosensor in oxygen purged phosphate buffer (pH 7.2). Chronoamperometric response monitoring was obtained by carrying the measurements at 1-minute time intervals in accordance to the chronoamperometric protocol and the results are shown in Fig.5.

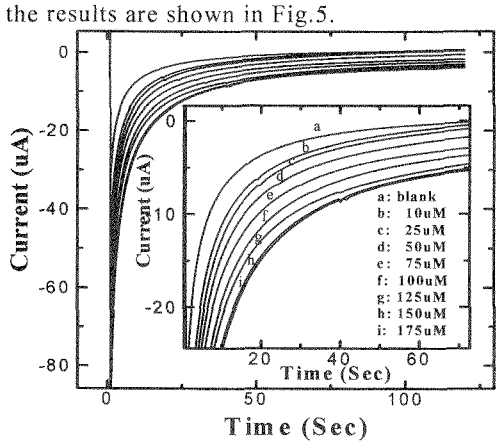

Fig.5. Steady state current response of the biosensor to an increasing concentration of Phenol

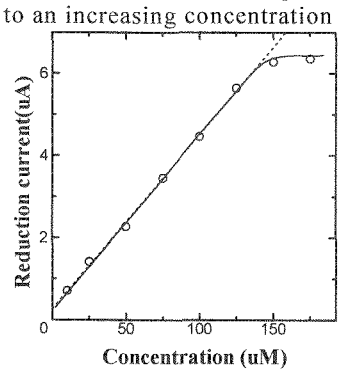

Fig.6. Calibration curve of biosensor (PPO/PAPCP/ITO) for phenol in 0.1M-phospahate buffer (pH7.2) at 25^0C; applied potential 0.1V.

The amperometric response was measured at PPO/PAPCP/ITO after the 95 % decay of transient current for phenol in a phosphate buffer solution containing 0.01M hexacyanoferrate (II) as redox mediator, at a fix bias voltage of 0.1V vs. Ag/AgCl under slow stirring, at room temperature. Figure 6 illustrates the steady state current dependence calibration curve on the concentration of phenolic solution. The enzyme electrode responses rapidly to the substrates and 95% of the transient current decay were obtained at 40 second. This short response of PPO/PAPCP/ITO electrode reveals that the faster electronic exchange occurs in between the enzyme (PPO) produced quinone and hexacyanoferrate (II). Linearity was observed within a range of 2.8-140.2μM (r = 0.999). The sensitivity of the enzyme electrode obtained towards phenol was 42.4 μA/mM. The detection limit was calculated according to the formula $3\sigma_b/m$ [15] criteria, where m is the slop of the calibration graph and σ_b is the standard deviation of the blank signal. The lowest detection limit observed was 1.4μM. A lose in linearity at higher concentration of phenol is attributed to slow surface fouling by the reaction product [16]. However, no significant decrease in response was observed after at least 10 uses in testing and displayed a good reproducibility. The relative standard deviation determined by 10 successive analyses of a 50μM phenol standard using a single PPO/PAPCP/ITO electrode was found to be about 6%. In a series of 10 PPO/Fe^{2+}-PPY/ITO electrode sensors, a relative standard deviation of about 9 % was obtained for the individual current response for the same sample (50 μM phenol).The biosensor is safe and easy to handle for onsite monitoring of phenol in aqueous solution.

4. ACKNOWLDGEMENT

One of the authors (Rajesh) Scientist, IPMD, CSIR, New Delhi, India is thankful to JSPS, Japan, for providing a postdoctoral fellowship.

5. REFERENCES

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(Received October 10, 2003; Accepted December 3, 2003)