

Amperometric Glucose Sensor Based on The Entrapment of Glucose Oxidase in Electrochemically Synthesized Pyrrole/N,N-Dimethylaminopyrrole (Py/DMAPy) Copolymer Film

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Glucose oxidase (GOx) was entrapped on the films obtained by electrochemical copolymerization of pyrrole (Py) and N,N-dimethylaminopyrrole (DMAPy), to fabricate GOx-immobilized electrodes for amperometric sensing of glucose. Polymeric film was characterized with cyclic voltammetry (CV) and Fourier transform infrared (FTIR) spectroscopy. The amperometric response of the enzyme electrode was measured at +0.70V vs. Ag/AgCl, which was due to the electrooxidation of enzymatically, produced H₂O₂. The effects of the composition of copolymer, pH, temperature and substrate concentration on the response of the enzyme electrode were investigated. The optimum pH was found to be 7.4 at 25 °C and response time of enzyme electrode was determined as 45 s. The upper limit of the linear working portion was obtained as 10 mM glucose concentration with a detection limit of (5.0 × 10⁻⁶ M) with the calculated value of apparent Michaelis-Menten constant (KM_{app}) was 3.70 mM. The activation energy of this immobilized enzyme system were found to be 30.25 kJ mol⁻¹.

Key Words: Pyrrole, N,N-Dimethylaminopyrrole, Copolymerization, Glucose oxidase, Sensor.

INTRODUCTION

A biosensor is an analytical device for the detection of an analyte that combines a biological or biologically derived material with a physicochemical transducer. The goal is to generate an electronic response in suitable proportion with the concentration of analyte. Immobilizing the appropriate enzyme into the polymer matrix provides long life time to enzyme molecules. However, the usefulness of immobilized enzyme electrodes depends on some factors such as the immobilization method, the chemical and physical conditions (pH and temperature) and the stability of the transducer membrane used to attach the enzyme. Immobilization of enzyme in several matrices has been used for the fabrication of biosensors for detection of glucose¹, urea², dopamine³ cholesterol⁴, etc.

Conducting polymers are promising candidates for the components of the enzyme-electrodes because they are synthesized readily in the form of thin films by electrochemical polymerization of heterocyclic compounds such as azulenes, furans, pyrroles and thiophenes. 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 of enzymes to conducting polymer films and their adsorption on conducting polymer films^{5,6}. It has been established, that it has no influence, what kind of immobilization technique is adopted, the morphology of these polymer films should have a great influence on the performance of the enzyme electrodes fabricated with them. The preparation of conducting polymer films with ordered porous structure has attracted an enormous interest in recent years owing to their novel three-dimensional structure favourable for fabrication of enzyme electrodes. Such a porous structure has been realized in various conducting polymers, including polyaniline, polypyrrole, polythiophene and their derivatives, by means of chemical^{7,8} and electrochemical polymerization⁹⁻¹³. Copolymerization is very useful for tailoring diverse properties of coatings¹⁴ and authors have reported that the glucose oxidase (GOx) immobilized glucose sensor prepared by electropolymerization and electrocopolymerization of pyrrole derivatives that have carboxyl and hydroxyl groups, 3-(1-pyrrolyl) propionic acid (PPA) and 3-(1-pyrrolyl) propanol, respectively, can afford higher electrode stability and reproducibility than compared with that of unsubstituted pyrrole^{15,16}. Structural modification of the polymer backbone has been utilized to control the resulting properties, e.g., incorporation of various functional groups can induce porosity¹⁷.

It is well known that an increase in the 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 entrapment of enzymes in conducting copolymer matrix during electrochemical polymerization is a simple process to construct biosensors¹⁸⁻²¹.

The current interest is to develop enzyme electrodes suitable for biosensors, 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. The copolymer of pyrrole (Py) and N,N-dimethylaminopyrrole (DMAPy), whose structure is shown in Fig. 1, was employed in this work as a novel support-material for fabricating GOx-immobilized electrodes. In our earlier studies, the Py/DMAPy copolymer was synthesized chemically and we have examined the electrorheological properties of chemically synthesized copolymer²². To the best of our knowledge, the present study is the first report about electrochemical synthesis of Py/DMAPy copolymer. The goal of this study is to the preparation of a new enzyme electrode to determine glucose using with the Py/DMAPy copolymer modified Pt electrode. Firstly, Py/DMAPy copolymer was synthesized in presence of glucose oxidase in phosphate buffer solution at pH 7.4²³ and a homogeneous black colour film can be observed on the electrode, with good mechanical stability. The GOx immobilized electrodes

were prepared using this technique with the copolymers of various compositions and applied to amperometric sensing of glucose. Their sensing ability was investigated in relation between the effect of copolymer composition and the response of enzyme electrode to the glucose. In order to confirm the formation of Py/DMAPy copolymer, cyclic voltammetry (CV) and Fourier transform infrared spectrometry (FTIR) were also carried out. The changes in the response of the enzyme electrode with composition of copolymer, substrate, pH and temperature were measured to optimize the working conditions. The results obtained with enzyme electrode were compared with that of free enzymes in solution using uncoated platinum electrode.

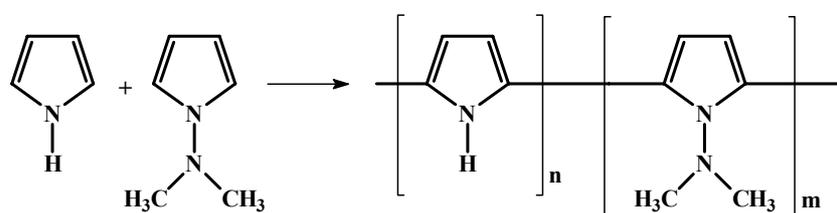


Fig. 1. Structure of the Py/DMAPy copolymer

EXPERIMENTAL

Pyrrole (Merck) was purified by distillation at reduced pressure prior to use. N,N-Dimethylaminopyrrole was purchased from Sigma, Aldrich. Glucose oxidase (GOx, EC 1.1.3.4, 179,000 units/g, type VII-S from *Aspergillus niger*, Sigma) and D-(+)-glucose anhydrous (Fluka) were used to perform the fabricated biosensor. The buffer solution was prepared using $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (Riedel De Haen) and NaOH (Riedel De Haen). Alumina polishing suspension agglomerate (0.05 μm) (Baikowski) was used as electrode polisher. Double-distilled water was used for preparation of the buffer solution. All other compounds were of analytical reagent grade.

FTIR spectra of the polymers were recorded between 4000–400 cm^{-1} with a resolution of 4 cm^{-1} on a Perkin-Elmer spectrometer (Beaconsfield, HP91QA). Electrochemical copolymerization of monomers and biosensor measurements were carried out in the 3-electrode cell equipped with a Gamry potentiostat (Gamry Instruments, US). The platinum bare and Py/DMAPy copolymer modified platinum electrodes were used as working electrodes, platinum wire as counter and Ag/AgCl as a reference electrodes were used.

Electrode fabrication: The Pt working electrode was polished with agglomerate-free alumina polishing suspension and washed with phosphate buffer solution. Electrochemical copolymerization of pyrrole and N,N-dimethylaminopyrrole (DMAPy) was performed in 0.1 M phosphate buffer (pH 7.4), containing 5 mg/mL GOx, Py and DMAPy monomers (total concentration was 0.5 M) and was carried out by cyclic voltametric method in the range of -0.20–1.50 V with 10 cycle at room temperature.

After electropolymerization, the electrode was washed several times with buffer solution, than distilled water to remove any loosely bound enzyme and any remaining monomer. It was then stored at 4 °C in the phosphate buffer (pH 7.4). To optimize the synthetic conditions, several molar ratios of the Py and DMAPy monomers (in the range 0.1/0.4-0.4/0.1) were assayed with respect to the efficiency of enzyme entrapment. Different ratios of monomers were used to find the best glucose sensing. The highest sensitivity and enzyme stability was provided at 0.3/0.2 molar ratio and this Py/DMAPy system were selected for biosensor fabrication. For comparison, the same procedure was applied to enzyme electrodes which are consists of polypyrrole (PPy) and poly N,N-dimethyl amino pyrrole (PDMAP) homopolymers.

Electrochemical measurements were carried out in a 3-electrode cell with separate compartments for the reference electrode and for the counter electrode (Pt wire). Oxygen was introduced into the solution in this cell at a constant flow rate to obtain a solution saturated with oxygen. Oxygen flow was continued above the solution to keep it saturated with oxygen during the measurements. In order to determine the steady-state background current of the enzyme electrode, a potential of +0.70 V *versus* SCE was applied to the enzyme electrode. After the steady-state current value had been determined, previously known amounts of the glucose were added to the cell from a stock glucose solution and the solution was stirred for 5 s. The glucose response of the enzyme electrode was measured at constant potential of +0.70V *vs.* SCE amperometrically due to the electrooxidation of H₂O₂ produced enzymatically.

RESULTS AND DISCUSSION

Cyclic voltammetry studies of Py/DMAPy copolymer: Cyclic voltammetry (CV) experiments were carried out in unstirred solutions at a scan rate of 100 mV s⁻¹. A low proportion of Py in copolymer (Py/DMAPy (molar ratio): (0/0.5; 0.1/0.4) resulted in an inefficient electrode coverage and enzyme entrapment, while higher concentrations resulted in a decreased sensitivity due to an increased resistivity of the polymer formed onto the surface of the electrode. Electrochemical characterization of Py-DMAPy copolymer was carried out by cyclic voltammetry (CV) with a Pt as working electrode. Fig. 2(a-c) shows the CVs of PPy, PDMAPy and Py/DMAPy copolymer modified Pt electrodes, respectively. CVs were performed in 0.1 M NaH₂PO₄ containing 5 mg/mL GOx enzyme. Fig. 2d shows the CV of 0.1 M NaH₂PO₄ containing 5 mg/mL GOx enzyme without monomers.

Fig. 2a shows the cyclic voltammogram of PPy. There is a broad peak in range of 0.60-1.30 V which has a maximum at 0.7 V can be observed. Peak intensity was quite low according to the PDMAPy and copolymer (Fig. 2b,c). An oxidation peak, which is irreversible and intense can be seen from the CV of PDMAPy (Fig. 2b). The CV of Py/DMAPy copolymer in 0.1 M phosphate buffer solution containing GOx at pH 7.4 is shown in Fig. 2c. There are two irreversible oxidation peaks corresponding to the polymerization of pyrrole and N,N-dimethylaminopyrrole were observed at

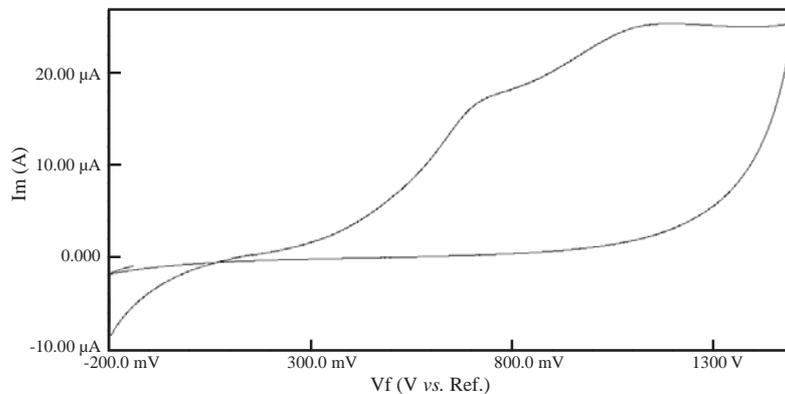


Fig. 2a. Cyclic voltammogram of PPy modified Pt electrode in 0.1 M NaH_2PO_4 including 5 mg/mL glucose oxidase

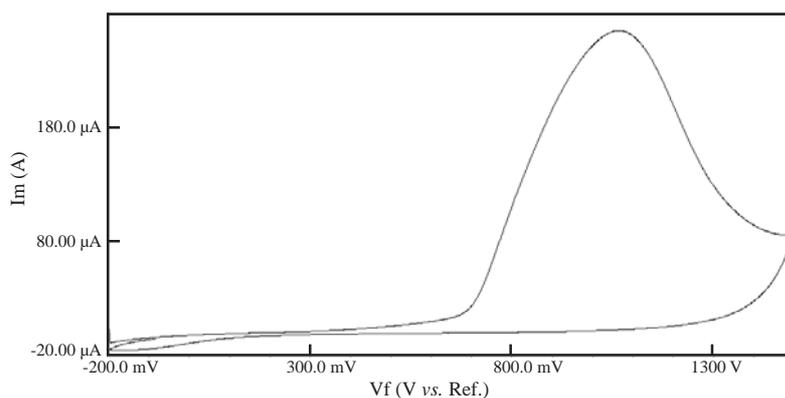


Fig. 2b. Cyclic voltammogram of PDMAPy modified Pt electrode in 0.1 M NaH_2PO_4 including 5 mg/mL glucose oxidase

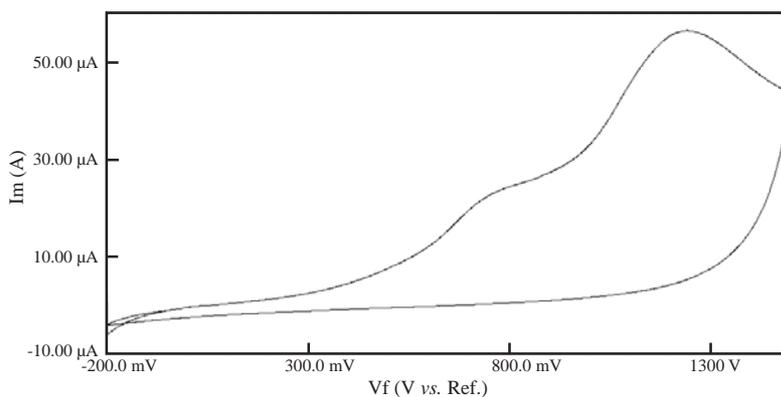


Fig. 2c. Cyclic voltammogram of Py/DMAPy copolymer modified Pt electrode in 0.1 M NaH_2PO_4 including 5 mg/mL glucose oxidase

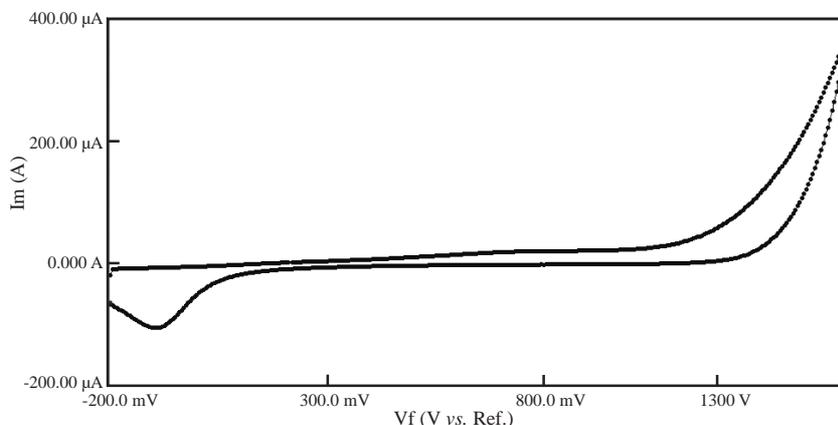


Fig. 2d. Cyclic voltammogram of Pt electrode in 0.1 M NaH_2PO_4 solution including 5 mg/mL glucose oxidase at a scan rate of 100 mV s^{-1}

ca. 0.75 and 1.25 V, respectively. These values are in agreement with previous studies²⁴⁻²⁶. Oxidation peak values of homopolymers are shifted to higher values in the CV of Py/DMAPy copolymer. Furthermore, intensity of oxidation peaks of copolymer was decreased according to homopolymers. This electrochemical behaviour is attributed to the formation of copolymer film onto Pt electrode.

FTIR studies of Py/DMAPy copolymer: The FTIR spectra of PPy, PDMAPy homopolymers and Py-DMAPy (0.3/0.2) copolymer are shown in Fig. 3(a-c). Transmission peaks at 3439, 3425 and 3435 cm^{-1} corresponds to N-H stretching in the spectra of PPy, PDMAPy homopolymers and Py/DMAPy copolymer, respectively. For the polymers synthesized in presence of GOx, it displays the mainly characteristic peaks at 1640 and 1540 cm^{-1} due to the secondary amide C=O stretching. Additionally, in the FTIR spectra of the polymers, a shoulder at the vicinity of 3290 cm^{-1} , which is characteristic of GOx, was observed²⁷. The peaks observed under the value of 700 cm^{-1} attributed to GOx enzyme²⁸ in the spectra of polymers. These results suggest existence of GOx on the polymer films.

The bands at 1454 and 1457 cm^{-1} in the spectra of polymers corresponds to C-N stretching vibration in the aromatic ring while the band at around of 1266 cm^{-1} in the spectrum of homopolymers corresponds to C-H or C-N in-plane deformation modes. The band of C-H out of plane deformation vibrations of the aromatic ring has a maximum at 772, 783 and 763 cm^{-1} for PPy, PDMAPy and copolymer, respectively. These bands correspond to the characteristic bands for PPy are in good agreement with earlier reported work²⁹. The FTIR spectra support the polymerization of PPy. There is a broad doublet peak in the range of 1400-900 cm^{-1} in the spectra of PPy (Fig. 3a). In the spectrum of copolymer (Fig. 3c), the peak shape of band at in this range resembles to the band at 1079 cm^{-1} situated in the spectrum of PDMAPy (Fig. 3b). Moreover, the new peak at 1106 cm^{-1} appears in spectrum of Py/DMAPy copolymer is the collective peak of homopolymers in this region. The spectrum of results confirms the formation of copolymer onto platinum surface.

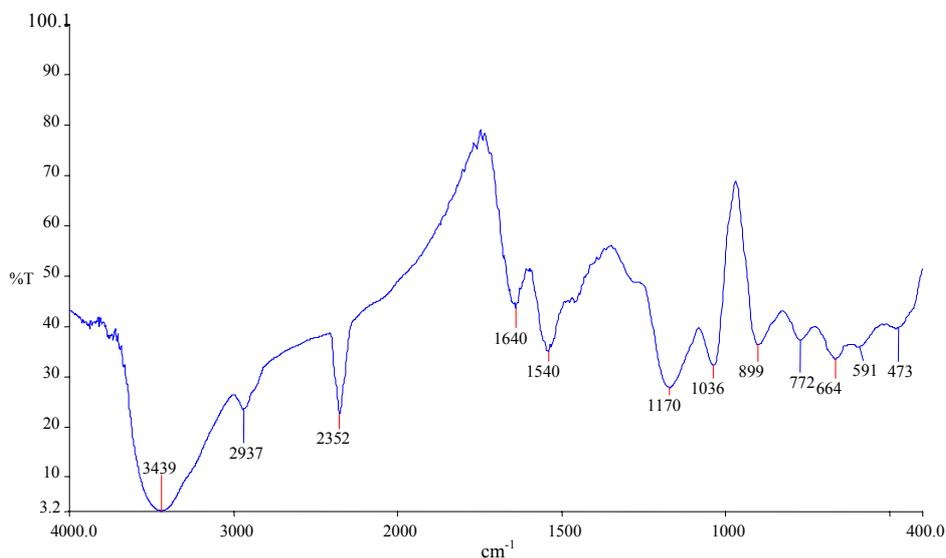


Fig. 3a. FTIR spectrum of PPy synthesized on to Pt electrode in 0.1 M NaH₂PO₄ including 5 mg/mL glucose oxidase

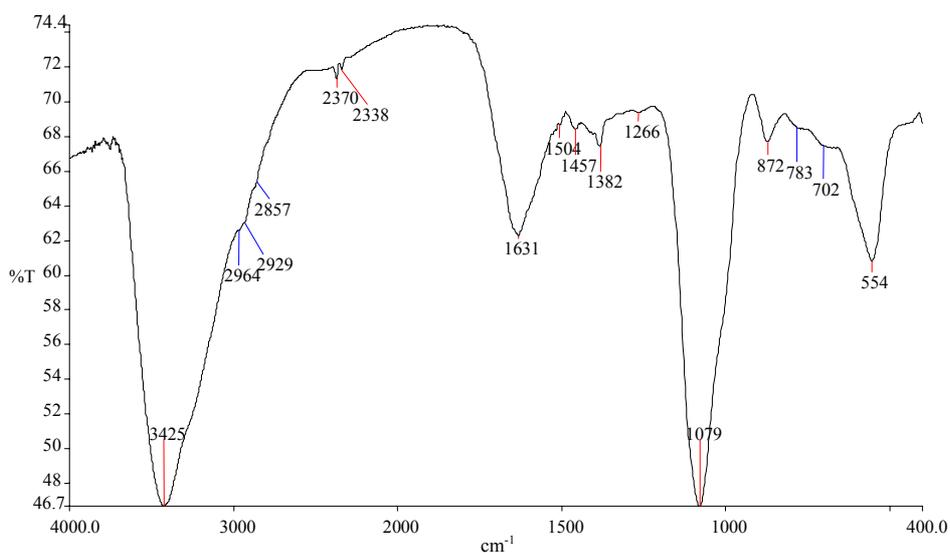


Fig. 3b. FTIR spectrum of PDMAPy synthesized on to Pt electrode in 0.1 M NaH₂PO₄ including 5 mg/mL glucose oxidase

Glucose sensing: Fig. 4 shows the proposed mechanism for the Py/DMAPy copolymer modified enzyme electrode, polarized at +0.70V *versus* Ag/AgCl. The added glucose transferred fast into the copolymer film, to oxidized it by the entrapped GOx in the presence of O₂.

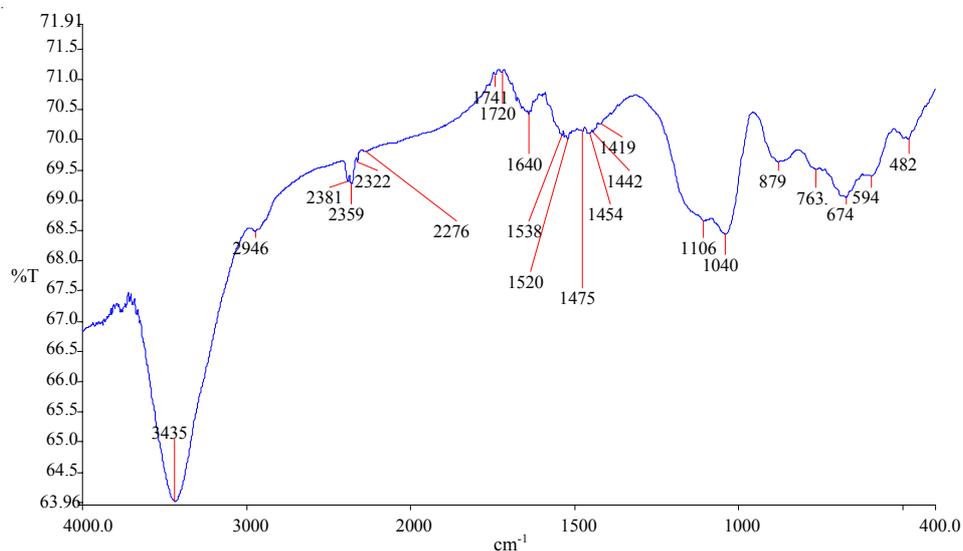


Fig. 3c. FTIR spectrum of Py/DMAPy copolymer synthesized on to Pt electrode in 0.1 M NaH_2PO_4 including 5 mg/mL glucose oxidase

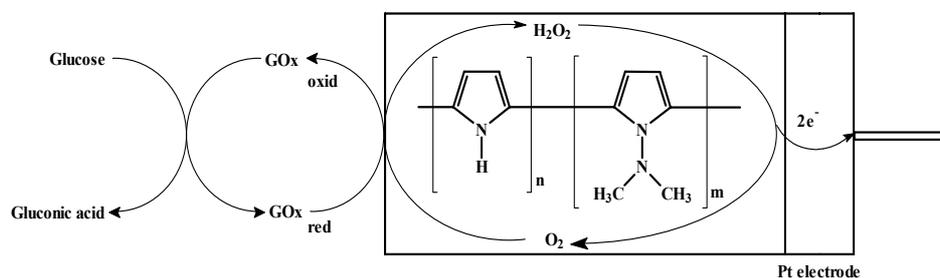
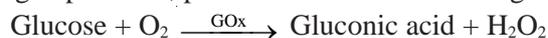


Fig. 4. Proposed mechanism for the Py/DMAPy copolymer modified enzyme electrode, polarized at +0.70V versus Ag/AgCl.

The amount of glucose can be determined by measuring the anodic current of oxidation of hydrogen peroxide, produced in the reaction as given below:



The formation of hydrogen peroxide is detected by the amperometric current technique during electrode oxidation:



In order to construct the amperometric enzyme sensor, GOx is used as an example of a redox protein. The enzyme catalyze in the presence of molecular oxygen, leading to the oxidation of glucose into gluconic acid and hydrogen peroxide. The conversion of glucose into gluconic acid involves the transfer of 2H^+ and 2e^- from the substrate to the flavin moiety of the enzyme²⁵.

Influence of the composition of copolymer: With a view to determine the optimum composition of copolymer for the detection of glucose, the molar ratio of Py in the copolymer was varied in the range of 0.1 to 0.4 M. For comparison purpose, homopolymers were tried to be accumulated on Pt electrode surface in the presence of GOx. Although, Py was polymerized on the Pt electrode by cyclic voltametric method in the range of -0.20-1.50 V at room temperature, but DMAPy was not polymerized under the same conditions. After preparation of polymer modified enzyme electrodes, response of electrodes on the glucose were examined. Fig. 5 shows the effect of copolymer composition on the response of glucose. Glucose response on the current with the molar ratio of Py in the copolymer was measured for the polymer modified electrode by applying to constant potential of +0.70 V vs. Ag/AgCl. It has been observed that (Py/DMAPy) (0.3/0.2) molar ratio gives the best response to glucose concentration.

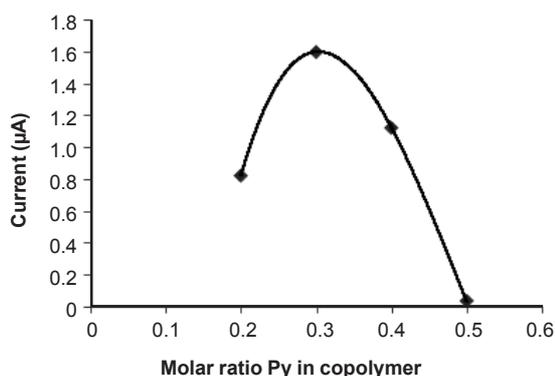


Fig. 5. Effect of the molar ratio of Py in copolymer on the response of the enzyme electrode in phosphate buffer solution at 25 °C

Influence of the pH: The pH value is another variable parameter that affects the current response of the enzyme electrodes. The pH dependence of the response of the Py/DMAPy copolymer modified enzyme electrode was investigated by using 0.1 M NaH₂PO₄ (Fig. 6). Fresh enzyme electrode was prepared for each pH value to eliminate the errors that might appear from re-using. The current increases in moving from pH 6.0-7.4, while it decreases sharply above pH 7.4. The maximum current of the enzyme electrode were obtained at pH 7.4 where it is in agreement with the biosensor which was developed for glucose sensing by using with a printable biosensor prepared with polypyrrole coated latex particles³⁰.

Influence of the temperature: The working temperature is also an important factor for the activity of enzyme electrode. The response of the enzyme electrode was measured in the temperature range of 15-70 °C as shown in Fig. 7. The current response increases linearly with temperature from 15 to 50 °C, but decreases significantly from 50 to 70 °C because of the deactivation of GOx. Thus, the highest amperometric response of enzyme electrode was obtained at 50 °C. This value is

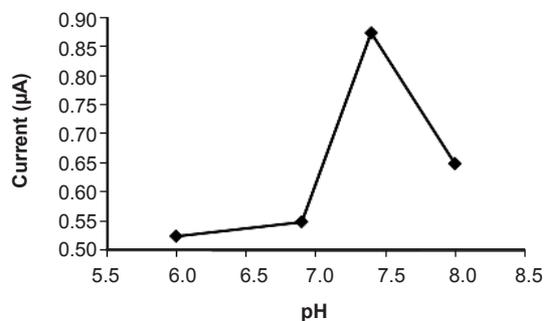


Fig. 6. Influences of pH on the responses of the enzyme electrode in phosphate buffer solution at 25 °C

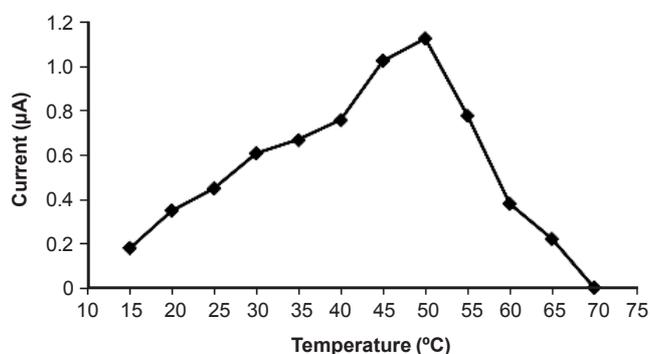


Fig. 7. Effects of the temperature on the activity of the enzyme electrode in phosphate buffer solution (pH 7.4)

better than the enzyme electrode which was developed before with PPy for glucose determination³¹. However, it is known that the free glucose oxidase lost its activity at higher temperatures above 30 °C³². As a result, copolymer matrix provides higher stability against high temperatures than the free enzyme.

The activation energy of the immobilized GOx can be calculated based on the Arrhenius Formula as given below:

$$\ln k = \ln A - (E_a/RT)$$

where k is the rate constant and E_a is the apparent activation energy. Since the electrode surface areas, the quantity of the enzyme and concentration of substrate are constant, the response current is proportional to the rate constant k ³³. $\ln I$ can be used instead of $\ln k$ in the formula. The relationship between $\ln I$ and $1/T$ was plotted (not shown here) and straight line was obtained. The activation energy of the entrapped GOx was calculated as 30.25 kJ/mol which is in concurrence with previous reports^{34,35}. Accordingly, we can conclude that the Py/DMAPy copolymer can offer a good environment for GOx, which make the sensor more stable at high temperature. Considering the convenience of the practical application, 25 °C was selected in this experiment.

Influence of the substrate concentration: Fig. 8 shows the response of the enzyme electrodes to glucose. Constant potential of +0.70V *versus* Ag/AgCl was applied to the enzyme electrode to determine the amperometric response due to the electrooxidation of H₂O₂, which is propagated enzymatically. When the steady-state background current value was provided, known amounts of substrate (glucose) solution were added and the currents for each added to concentration of glucose were listed. A current rise is observed after each glucose addition. The currents reached a steady-state value after the initial stirring was stopped in about 45 s following the addition of the substrate. These steady-state current values were used to form the calibration plots. The response time of the glucose enzyme electrode could be compared with the literatures^{24,35-37}. Response time values of enzyme electrode developed in this study are in congruity with those studies.

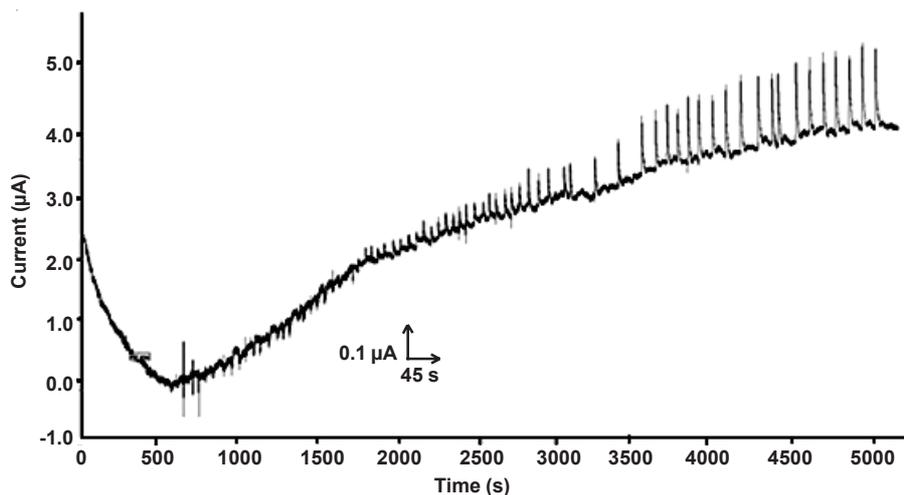


Fig. 8. Current response to glucose addition of glucose enzyme electrode (Py/DMAPy: 0.3/0.2) applying a potential step to +0.70V *vs.* Ag/AgCl in 0.10 M phosphate buffer solution (pH 7.4) at 25 °C

Fig. 9 illustrates the response of the enzyme electrode based on Py/DMAPy copolymer as a function of glucose concentration. The calibration plot was prepared at the optimum working conditions [pH 7.4, 25 °C, with molar ratio of (0.3/0.2)]. As seen from Fig. 9, linear working range of enzyme electrode is up to a value of 10 mM of glucose concentration. The detection limit of Py/DMAPy copolymer modified enzyme electrode were 5.0×10^{-6} M. According to the Lineweaver-Burk form of the Michaelis-Menten equation³⁸, the relation between the reciprocal of the response current (i^{-1}) and the reciprocal of glucose concentration (C^{-1}) was obtained. The apparent Michaelis-Menten constant (K_{Mapp}) for glucose was calculated from the slope and intercept ($K_{Mapp} = 3.70$ mM). The free GOx enzyme in solution which produced a current response on an uncoated Pt electrode gave a K_M value of 3.19

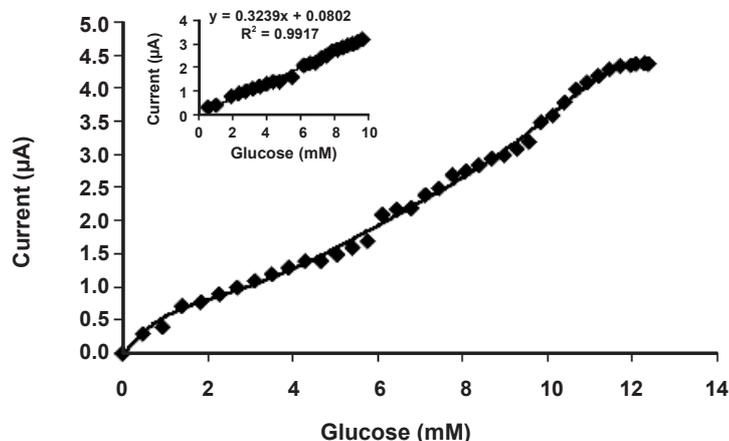


Fig. 9. Relation between the response of the glucose enzyme electrode with the substrate concentration in 0.10 M phosphate buffer solution (pH 7.4) at 25 °C

mM glucose. KM values obtained from both the systems indicate no diffusional limitations in the entrapped state and the non-denaturing character of the procedure of enzyme anchoring³⁹. According to the character of Michaelis-Menten constant (KM), the smaller the value of KM, the stronger will be the affinity between enzyme and substrate. The KMapp value of enzyme electrode obtained in this work was compared with literature and Table-1 exhibit the low value of KMapp.

TABLE-1
CHARACTERISTICS OF SOME CONDUCTING POLYMER
BASED GLUCOSE BIOSENSORS

Matrix	Method of immobilization	Linear range	KMapp (mM)	Ref.
Pyrrole/N,N-dimethylaminopyrrole (Py/DMAPy) copolymer	Entrapment	Up to 10 mM	3.70	This work
Polypyrrole	Entrapment	–	19.96	40
Polypyrrole	Entrapment	0.005-20.0 mM	23.3 0	34
Polypyrrole nanotube	Physical adsorption	500μM-13mM	7.01	1
Polypyrrole–polyvinyl sulphonate (Ppy–PVS) composite film	Cross-linking	1-50 mM	6.25	41
Polypyrrole/polyacrylamide microparticles	Entrapment	Up to < 10 mM	5.50	37
Polypyrrole-coated glucose oxidase nanoparticles	Self-encapsulation	Up to 25 mM	4.11	42
Poly(<i>o</i> -aminophenol) film on polypyrrole-Pt nanocomposite	Entrapment	0.0015-13 mM	23.90	35

Conclusion

This study demonstrates that the new glucose sensor can be successfully prepared by entrapment of GOx into Py/DMAPy copolymer matrix. Formation of copolymer film was confirmed by CV and FTIR measurements. For glucose sensing studies, it was determined that the copolymer matrix provides higher stability against high temperatures (50 °C) than the free enzyme. The calculated E_a value of the entrapped GOx in copolymer film is 30.25 kJ/mol which is in agreement with literature. KMapp values of immobilized (3.70 mM) and free enzyme (3.19 mM) systems show that there was no denaturation at enzyme structure when enzyme electrode was prepared. While the detection limit of Py/DMAPy copolymer modified enzyme electrode was determined as 5.0×10^{-6} M, the linear working range of enzyme electrode was found up to a value of 10 mM of glucose concentration. This study shows that the synthesized Py/DMAPy copolymer can be used to construct stable and sensitive biosensors and can also be exploited for the fabrication of other biological sensors. The biosensors based on Py/DMAPy copolymer possess several advantages with respect to temperature stability, simplicity, response time, high relation between enzyme and substrate and low cost that will allow the rapid detection of glucose. The cost effectiveness and simple method of fabrication of Py/DMAPy copolymer modified enzyme electrode is an additional advantage when compared with conventional electrodes.

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