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ENZYME IMMOBILIZATION ON ULTRAMICROELECTRODES THROUGH ELECTROPOLYMERIZATION: EFFECT OF POLYMERIC FILM THICKNESS ON THE AMPEROMETRIC RESPONSE OF THE ELECTRODE

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The rapid growth in the science and technology of the biosensors demands improved methods of enzyme immobilization and miniaturization of sensor devices. The formation, on electrode surfaces, of enzyme-entrapped polymer films through electropolymerization from monomer solutions containing the enzyme offers an elegant and reliable approach for enzyme immobilization, especially on ultramicroelectrodes. Platinum ultramicrodiscelectrodes (UMDE) were fabricated in-house and immobilized with glucose oxidase (GOx) enzyme through electropolymerized films of polypyrrole and poly(1,3- diaminobenzene). The UMDE, modified with GOx-trapped polymer film was tested for its amperometric response to glucose in solution. The polymer film thickness was varied from about 10 to 466 nm by controlling the charge required for the electropolymerization and the influence of the polymer film thickness on the amperometric response to glucose was investigated. Moreover, the effect of oxygenation and de-oxygenation of the test solutions on the amperometric response was also examined. The results of the above studies are presented and discussed in this communication.

Keywords: Pt ultramicrodisc-electrode, electropolymerization, polymer film thickness, glucose oxidase, amperometry, glucose sensing

INTRODUCTION

'Biosensors' is an important area of research [1,2] currently pursued worldover. A biosensor is an analytical device made up of a sensitive and selective biological element (such as, an enzyme, a microbe, etc.) placed in close proximity of a transducer [3-5]. The transducer can be electrochemical, optical, thermal or piezo- electric [6]. Amperometric biosensors [7-9], belonging to the broad category of electrochemical sensors, measure the current produced during the oxidation/reduction of the reactant/product of the bio-catalyzed reaction, at a constant potential. The efficacy of the amperometric enzyme-based biosensors has been demonstrated in many analytical applications [3,10]. The success of the biosensor depends on many important factors like, nature of the electrode, enzyme immobilization, stability and selectivity of the enzyme, screening of interferents, etc [11]. Of late, the miniaturization [12] of electrochemical biosensors has assumed importance for a variety of reasons: i) potential uses in physiological and neurological studies [13,14], ii) improved biocompatibility by minimizing immunological responses, iii) measurement in very small volumes [14], iv) determination of spatial concentration profile [15], v) fabrication of multisensor arrays and probes [16,17] and vi) design of biochips [18]. The advent of ultramicroelectrodes(UMEs) having μm dimensions [19] has paved the way for the development of miniaturized electrochemical biosensors [20-22]. Enzyme immobilization on UMEs calls for precise control of the process to achieve uniform film thickness and enzyme distribution on the electrode surface. Physical methods like dipping and solvent-casting do not prove successful for this purpose. On the other hand, electropolymerization, an all-chemical approach, offers immobilization of the enzyme uniformly on UMEs [12,23]. Electropolymerization, initiated generally by the oxidation of the monomer leads to the deposition of a thin, insoluble, conducting or insulating film, which could be made to trap enzyme moities by taking the enzyme in the monomer solution. Depending on the experimental conditions, like monomer concentration, voltage/current imposed and the conductivity of the deposited film, films of increasing thickness can be obtained by carrying out electropolymerization for longer durations. Enzyme immobilization in a wide variety of electropolymerized polymer films on electrode surfaces has been reported [23-30]. It will be interesting to study how the film thickness will influence the sensing activity of the immobilized enzyme. In the present investigations, glucose oxidase (GOx)

enzyme was immobilized on platinum ultramicrodiscelectrodes (UMDEs), fabricated *in-house*, using electropolymerized films of polypyrrole(PPy) and poly(1,3- diaminobenzene) (PDAB). The PDAB is obtained as a very thin, self-regulated film due to its insulating characteristics. The thickness of the PPy film was varied by choosing appropriate experimental conditions and the sensing activity of the immobilized GOx was analyzed as a function of the polymer film thickness. Also, the influence of dissolved oxygen on the amperometric response of the electrode to glucose has been discussed.

EXPERIMENTAL

Chemicals and reagents:

1,3-Diaminobenzene(DAB), 99%, (M/s Aldrich, USA) was purified by high vacuum sublimation. Pyrrole was purified by distillation. GOx (Type II, Sigma, USA) was used for immobilization. All other reagents and chemicals were of AnalaR grade. Distilled, deionized water was used making solutions.

Apparatus:

Voltammetric set-up (PAR model 264A) was used for carrying out cyclic voltammetric experiments and electropolymerization of DAB. Potentiostatic polymerization of pyrrole was performed using a Potentiostat (BAS Model LC-4B) and the quantity of charge for deposition was calculated from i - t curves. Amperometric measurements with the enzyme-immobilized UMDEs were carried out in a Faraday cage using Voltammograph (BAS Model CV-27) coupled with a Pre-amplifier (Model BAS PA1) and an X-t recorder. An all glass three-electrode cell was employed for the electrochemical studies. A Pt wire and an Ag/AgCl, 3 *M* NaCl electrode served as the auxiliary and the reference electrodes respectively. All potentials reported in this study are versus the above reference electrode.

Preparation of Pt UMDE

Approximately 1 cm length of Pt wire of 25 μm dia. (M/s AESAR Johnson Matthey, USA) was placed into a soft glass tube μ4 mm OD, 10 cm length). A miniature welding torch (Smith Equipments, USA) was used to flame-seal one end of the glass tube with the Pt wire inside, such that wire protrudes out 1 or 2 mm on either side of the seal. The wire protruding on the outer side is then cut and the sealed end is polished to yield a flat glass surface presenting a Pt disc (dia 25 μm) at its centre.(cf. Fig. 1). The Pt wire inside the glass tube is joined to a copper lead using Woods alloy for making electrical connections. The pretreatment of UMDE was carried out as follows: 1) UMDE was successively polished with 1200 emery, 5.0, 0.3 and finally 0.05 μm size alumina using a mechanical polishing machine (M/s Struers, Denmark). 2) Sonicated in distilled, deionized water for about 10 minutes. 3) Cycled at 50 mV/s for 30 minutes in 1*M* sulfuric acid between - 0.8 and 1.2 V and 4) Again sonicated in distilled, deionized water for 5 minutes.

RESULTS AND DISCUSSION

The Pt UMDEs, fabricated *in-house*, were tested with ferro/ferricyanide redox system to ascertain their spherical diffusion characteristics. CVs were recorded at 50 mV/s in 0.1 *M* KCl containing 5 mM each of ferro- and ferri-cyanide. A representative CV showing the sigmoidal behaviour is presented in Fig.2.

Enzyme immobilization on UMDEs was done by trapping the enzyme in the polymer film obtained by electropolymerization from monomer solutions. The PDAB film entrapping GOx was obtained by cycling the UMDE

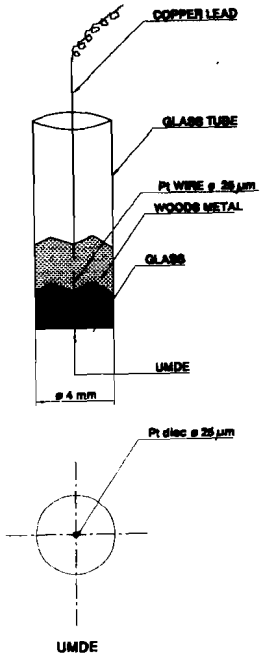

Fig.1: Schematic diagram of Pt ultramicrodiscelectrode, fabricated in-house

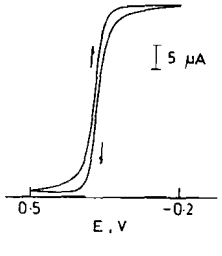

Fig.2:Cyclic voltammogram of ferro/ferricyanide (5 mM each) in 0.1 M KCl at 50 mV/s on Pt UMDE

between 0 and 0.82 V at 50 mV/s in 0.1 *M* phosphate buffer (*p*H = 7.4) containing 3 m*M* 1,3-DAB and 600 U/ml GOx, for 15.5 hours. The GOx-containing PPy film on UMDE was prepared by polymerizing pyrrole potentiostatically at 0.75 V from a solution containing 200 mM pyrrole, 100 mM KCl and 600 U/ml GOx. Time of deposition was varied to get films of different thickness. The thickness of PPy-GOx film was calculated using the earlier report [29] that a charge of 45 mC/cm^2 corresponds to 0.1 μm thickness. PPy-GOx films of three thickness, viz., 26, 88 and 466 nm (approximately) were prepared and studied in this investigation. The electrodes were washed thoroughly with buffer prior to and after the experiments and then preserved in buffer at 277 K.

Amperometric measurements:

The amperometric response of the polymer-GOx electrodes to glucose was monitored by following the electrolytic decomposition of H_2O_2 produced by the reaction between the reduced GOx and the dissolved O_2 in the buffer medium. The current measurements were made at 0.7 V, in 0.1 *M* phosphate buffer (*p*H = 7.0). Glucose concentration was increased by successive additions and the solution was stirred by continuous bubbling of compressed air. The current versus glucose concentration for the electrodes is given in Fig. 3. A linear response to glucose results in 0 - 6 mM range (Fig. 4). It may be noticed that the response is the highest for the PDAB-GOx electrode and it decreases with the increase of film thickness. It may be expected that thicker films could trap increasing amounts of the enzyme and thereby show enhanced enzyme activity. However, the observed decrease in amperometric response with the increase in film thickness suggests that the enzyme moities get trapped deeper and deeper into the film as the film grows, thereby constraining the access of the glucose molecules to trapped enzyme moities. It is also seen that the decrease in enzyme activity (as measured by the amperometric response) is not linear with increase in film thickness. The relatively high (current/thickness) value obtained for the PDAB-GOx electrode could be understood as follows. The thickness of 1,2-PDAB film is less than 10 nm [31,32] and that of 1,3-PDAB will also be same. The average diameter of the GOx molecule is about 8 nm [33] and its entrapment in the PDAB film offers much easier access for glucose molecules from solution to reach enzyme sites than in the other cases where film thickness is higher. Also, these results are in good agreement with the earlier observation [34] that the ideal biosensor configuration, based on a digital simulation, would use the thinnest possible biochemical layer and associated membranes and that the biochemical layer should have the highest possible activity.

Effect of dissolved O_2 :

The influence of dissolved O_2 on the amperometric response of the electrode to glucose was investigated. For this, PPy - GOx electrode (thickness ≈ 88 nm) was used and the buffer medium was continuously bubbled with pure oxygen during the amperometric evaluation of the electrode to glucose. Later, the same electrode was subjected to evaluation in presence of continuous bubbling with compressed air and then with pure nitrogen. The current versus glucose concentration obtained under the three different conditions is depicted in Fig. 5. It could be seen that in the presence of N_2 bubbling, the response is very low even from the initial

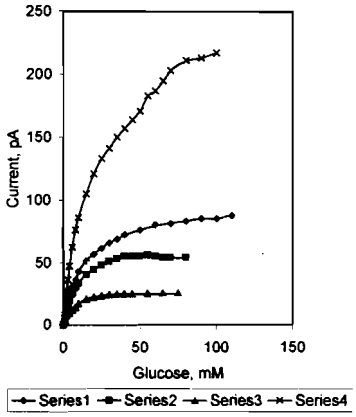

Fig.3: Amperometric response of the GOx-immobilized Pt UMDEs to glucose at 0.7 V in 0.1 M phosphate (pH = 7.0) buffer. Series 1: PPy-GOx film, thickness ≈ 26 nm Series 2: PPy-GOx film, thickness ≈ 88 nm Series 3: PPy-GOx film, thickness ≈ 466 nm

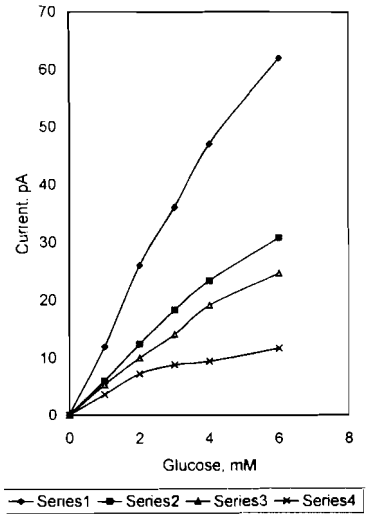

Fig.4: Amperometric linear response of the GOx-immobilized Pt UMDEs to glucose at 0.7 V in 0.1 M phosphate (pH = 7.0) buffer: Series 1: PDAB-GOx film, thickness ≈ 10 nm Series 2: PPy-GOx film, thickness ≈ 26 nm Series 3: PPy-GOx film, thickness ≈ 88 nm

stage itself. On the other hand, the response is markedly high in both the other cases. Moreover, the response in the presence of O_2 is almost similar to that observed in the presence of air in the initial stages (upto 6 mM); but at high glucose concentrations, the response is higher in the case of O_2 than in air. This could be explained as follows. The glucose probe reaction proceeds as follows.

$$\text{Glucose} + GOx_{ox} \text{ -------- } \text{Gluconolactone} + GOx_{red}$$

$$GOx_{red} + O_2 \text{ -------- } GOx_{ox} + H_2O_2$$

The O_2 available at the enzyme site (inside the polymer film) helps to restore the reduced enzyme back to its oxidized state and thereby sustains the catalytic cycle to continue further. The availability of O_2 at the enzyme site within the polymer film will depend on the concentration of dissolved O_2 in the buffer medium. Thus the variation of the concentration of dissolved O_2 in buffer medium as a result of bubbling the medium with O_2/air/N_2 would account for the above observed results.

CONCLUSIONS

1. Platinum UMDEs exhibiting spherical diffusion were successfully fabricated *in-house*. They could be immobilized with GOx using electropolymerized films of PDAB and PPy. The enzyme- immobilized electrodes respond amperometrically to glucose and exhibit a linear response in 0 - 6 mM range.
2. Sensitivity to glucose increases with the decrease in the thickness of the polymer film.
3. The amperometric response of the electrode to glucose depends on the concentration of the dissolved O_2 and it decreases with the depletion of O_2 in the buffer medium.

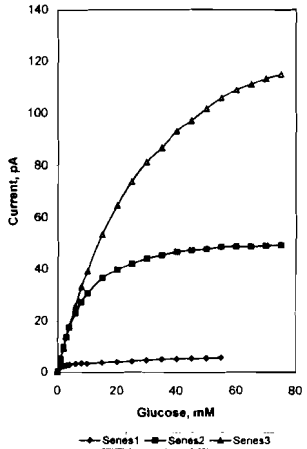

Fig 5: Effect of dissolved O_2 : Amperometric response of the Pt/PPy-GOx electrode (film thickness ≈ 88 nm) to glucose at 0.7 V in 0.1 M phosphate (pH = 7.0) buffer in the presence of continuous bubbling of :- Series 1: N_2, Series 2: compressed air, Series 3: O_2

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