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Immobilization of Glucose oxidase on PANI-2 Amino Pyridine composite film by cross-linking via glutaraldehyde for determination of glucose

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Abstract: *The PANI-2AP-GOD biosensor for determination of glucose has been described. The enzyme, glucose oxidase (GOD) was immobilized by cross-linking via glutaraldehyde on a polyaniline–2amino pyridine (PANI-2AP) composite film. The PANI-2AP film was electrochemically synthesized on Indium–tin-oxide (ITO) coated glass plate. We have synthesized PANI-2AP film with 0.1 M aniline, 0.025 M 2-AP at 3.0 pH and 1 mA/cm^2 current density. The immobilization was done by cross-linking 2 mg/ml GOD via 0.1% glutaraldehyde at 0.1 M phosphate buffer with 7.0 pH. The synthesized composite films were characterized using galvanostatic electrochemical technique, electrical conductivity, UV–Vis spectroscopy, Fourier transform infrared spectroscopy (FT-IR), and scanning electron microscopy (SEM). The cross-linking of enzyme and porous morphology of the polymer film leads to high enzyme loading and an increase in lifetime, stability and fast response time of the enzyme electrode. Amperometric response was measured as a function of different concentration of glucose. It was observed that current increases with increasing glucose concentration in the range 1–50 mm.*

Keywords*: Immobilization; Composite film; Glucose-oxidase; Cross-linking; Biosensor*

I. Introduction

Glucose is a bio-molecule that is known to play a variety of roles in the welfare of mankind. The development of amperometric glucose biosensors is extensively investigated research area because of its importance in the treatment of diabetes mellitus health care, food and environmental monitoring and process industries, etc. [1–3]. In the last decade various types of biosensors have been reported with many applications [4,5].The fabrication of enzyme-based biosensors can be carried out by immobilization of enzymes as a bio recognition element during electrochemical transduction [6,7]. This is one of the best methods for technical development of biosensor based on oxido reductase enzymes because of its simplicity and low cost. Conducting polymers have recently been used as electroactive materials in chemical structure and redox characteristics [8,9].Electrochemically polymerized conducting polymers had received considerable attention over the last two decades. The remarkable switching capability of these electroactive materials between the con-ducting oxidized (doped) and the insulating-reduced (undoped) state is the basis of many applications. Among others, 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; and (3) redox conductivity. The polyelectrolyte characteristic of the polymer is also useful for sensor application. Aniline fulfills above requirements together with having the characteristics of easy oxidation and high chemical stability [10–15]. Aniline molecular configuration is responsible for increase in electrical conductivity. The ion exchange process in Aniline leads to morphological changes; one can therefore utilize this process as a tool for controlling the morphology [16].Various methods to hybridize Aniline with other materials have been attempted to increase mechanical strength of Aniline film for various applications such as functional electrodes, electrochromic devices, sensors, etc. [17,18]. Doping other materials can increase the stability and mechanical strength of Aniline matrix. The materials, which can be doped with Aniline to increase stability and mechanical strength are Nafion, polyvinyl alcohol, poly (methylmethacrylate), polystyrene sulphonate, polyvinyl sulpho-nate, dodecylbenzenesulphonate and p-toluene sulphonate, etc. [19,20]. It has been reported that use of polyelectrolyte in polymerization solution with aniline causes to increase growth rate, higher compactness of the synthesized film and improved environmental stability [19,21]. The ion exchange properties and stability of polyaniline (Aniline), polyvinyl pyrrolidone (PVP), polyaniline (Aniline)-polyvinyl alcohol (PVA), polyaniline (Aniline) polystyrene sulphonate (PSS) composites have been studied by some authors [22]. However, it is still important to find high quality polymers and active dopants showing their desirable properties. The stability of polymer matrix depends on anions. The incorporation of a large size dopant anion, such as 2 amino pyridine (2-AP), p-toluene sulphonate (pTS), and

dodecylbenzenesulphonate (DBS) into Aniline films during electropolymerization makes Aniline film more porous [23]. The porosity is an important factor for the facile immobilization of enzyme. We have immobilized enzyme on large anion-doped porous PANI-2AP film by cross-linking technique for biosensor application.Long lifetime stability of the enzyme over the matrix is the vital factor in the development of bio-sensor. This is beneficial to biosensor transport as well as reduces measurement cost. The major cause of poor stability is the desorption (leaching out) of enzyme from immobilization materials. Therefore cross-linking method via glutaraldehyde has been chosen for the immobilization of enzyme in the present investigation. The method of electrochemical entrapment of enzyme has been described which is induced by polymerization of the monomer in the presence of the bioactive moiety [24]. This method is simple and can be used to localize the bioactive component. However, as the biological component is randomly oriented within the polymer matrix it is often inaccessible to the target analyte [25,26].Therefore in the present investigation, we have initially electrochemically synthesized PANI-2AP composite film and then GOD was immobilized. We have described the results of our systematic studies related to the electrochemical preparation and characterization of the PANI-2AP-GOD electrode for determination of glucose. The advantage of using the composite PANI-2AP films lies in the electrostatic rejection of anions [27]. The PANI-2AP composite films provide a charged surface for electrostatic interaction between the enzyme and the surface [28]. We have immobilized enzyme on large anion (2-AP) doped porous Aniline film by cross-linking via glutaraldehyde for development of glucose biosensor. Cross-linking via glutaraldehyde has lead to greater stability of the enzyme in the PANI-2AP films. An attempt has been made to investigate the effect of pH and potential on the activity of the PANI-2AP-GOD electrode.

II. Experimental

Preparation Of Poly Aniline/ 2 Amino Pyridine (Pani-2ap) Composite Films

PANI-2AP films were synthesized from an aqueous solution of distilled 0.1 M aniline (Spectrochem) and 0.025 M Two amino pyridine (25% by weight) (Aldrich) using electro-chemical deposition method. It was carried out by galvanostatic technique at room temperature in a one-compartment three-electrode glass cell. The ITO coated glass plate was used as a work-ing electrode, graphite as a counter electrode and Ag/AgCl was used as a reference electrode. The electrolyte solution was prepared in deionized water. The applied current density 1 mA/cm^2 and the 3.0 pH were kept constant during synthesis of composite films. After synthesis the polymer coated electrodes were rinsed thoroughly in deionized water dried in cold air and then use for subsequent characterization.

Immobilization Of God On Polyaniline 2 Amino Pyridine (Pani-2ap) Composite Films

The enzyme GOD (SISCO) was immobilized by cross-linking via glutaraldehyde (LobaChemie) on composite PANI-2AP films, thus restricting the leaching of the enzyme from the film. These films were subsequently dipped in 0.1% glutaraldehyde solution, left for 30 min and washed 2–3 times with phosphate buffer. The stock solution of GOD (2 mg/ml) prepared in 0.1 M phosphate buffer (pH 7.0) was adsorbed onto the surface of PANI-2AP films. The enzymatic incorporation was done in glutaraldehyde media. This kind of immobilization results in a greater physical and chemical stability of the catalytic material due to the cross-linking formed with the glutaraldehyde and enzyme. In this case, the active sites of the enzyme could be more accessible for the enzymatic reaction. The lifetime of the biosensor was studied when it was kept at (4 ^{0}C) in phosphate buffer. An adequate concentration of GOD and glutaraldehyde in cross-linking mixture were chosen so that it will ensure higher enzyme loading and provide excellent amperometric response with an ancient retention of the enzyme.

III. Results And Discussion

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

$$\text{Glucose} + O_2 \xrightarrow{\text{GOD}} \text{Gluconic acid} + H_2O_2$$

And formation of hydrogen peroxide is detected by the amperometric current method during electrode potential

$$H_2O_2 \longrightarrow O_2 + 2H + 2e^-$$

In order to construct the amperometric enzyme sensor, GOD is used as an example of a redox protein. The enzyme catalyses in the presence of molecular oxygen, lead to the oxidation of glucose into gluconic acid and hydrogen peroxide. The conversion of glucose to gluconic acid involves the transfer of two protons and two

electrons from the substrate to the flavin moiety of the enzyme [29]. The electron transfer from the redox cofactor to the sensing electrode is also facilitated by the presence of a polymeric conducting material.

Galvanostatic Studies Of Pani-2ap Composite Films

The potential time curves of the galvanostatically synthesized PANI-2AP a composite film is shown in Fig. 1.The PANI-2AP film was synthesized on ITOcoated glass plate from 0.1 M concentration of polyaniline and 0.025 M of 2-AP with 1 mA/cm^2 current density at 3.0 pH. This has resulted in high conductivity, with very good and porous surface morphology. The behaviour of the galvanostatic synthesis overshoot during first few seconds probably indicates difficult formation of dimmers and oligomers. After this, potential remains constant suggesting that building up of the film proceeds according to the same reaction along the full thickness of the polymer. It was found that a blackish polymeric film on ITO coated glass electrode was deposited with very good uniformity. The electrical conductivity of synthesized composite PANI-2AP films was measured by Keithley 6514 Electrometer and it is $2.412 \cdot 10^{3}$ S/cm.

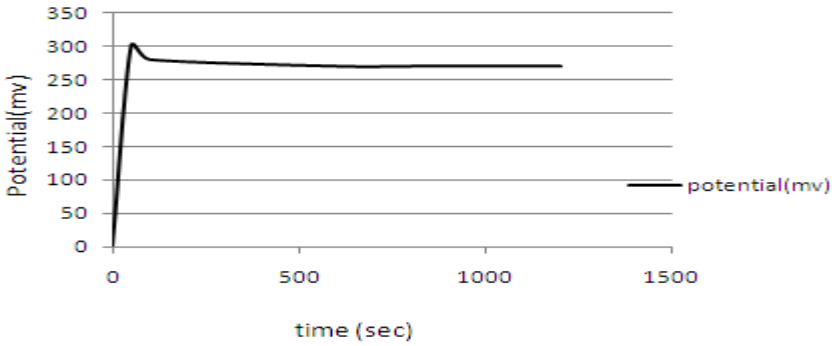

Fig.1 Current–time curve during the synthesis of PANI-2AP film in aqueous solution At current density 1 mA/cm^2 and pH 3.0

Uv–Visible Studies Of Pani-2ap Composite Films

UV–Vis spectrum of synthesized PANI-2AP film is shown in Fig. 2. UV spectrum was recorded using UV Visible 1601 spectrophotometer, Shimadzu in the range of 400–1100 nm. It showed absorption at around 900 nm as bipolaron charge transfer bands indicate high conductivity and absorption at around 460 nm indicates p–p*. This indicates that higher sulphonate group introduction to the polymer system leads to the stabilized doped state of the Aniline. More doped Aniline has resulted in to stronger absorption at 900 nm, which is responsible for bipolaron state [30]. This shows very good resemblance with the polymerization potential. The absorption spectra observed for synthesized composite films gives good agreement with the earlier reported work [30,31].

Fig 2 UV–Vis spectra of PANI-2AP film in aqueous solution at current density 1 mA/cm^2 and pH 3.0.

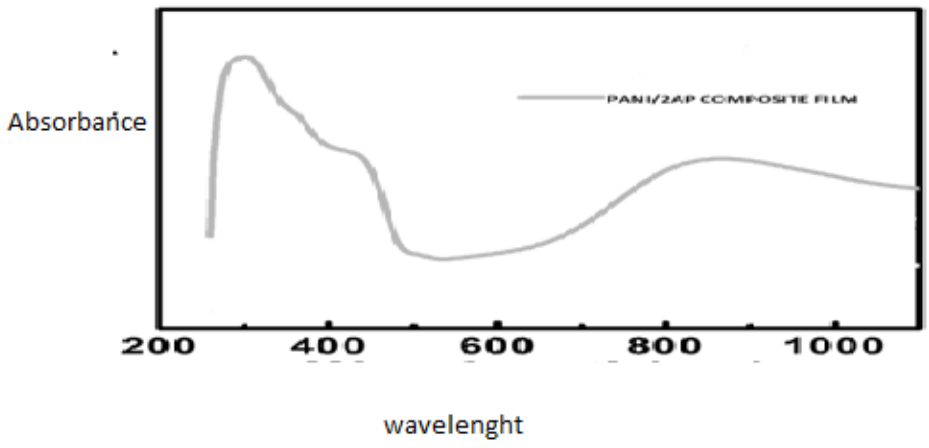

Fig 2 UV–Vis spectra of PANI-2AP film in aqueous solution at current density 1 mA/cm^2 and pH 3.0.

Ft-Ir Studies Of Pani/2ap Composite Films

The FT-IR spectrum of synthesized composite PANI-2AP is shown in Fig. 3. The FT-IR spectrum was recorded using Shimadzu FT-IR-8400 series, using KBr pellets in the region 350–4000 cm ^{1}. Spectrum show broad peak at 3423 cm ^{1}corre-sponds to N–H stretching. The incorporation of the counter anion in the polymer is evidenced by the peaks at 2924 and 2854 cm ^{1} assigned to aliphatic $–CH_3$ and $–CH_2$, related to the sulphonate anion. Further evidence of the presence of this anion in the polymer film is revealed by peaks at 1382 and 1635 cm ^{1} which may be assigned to SO_2 stretch in sulphonates. The vibration bands are observed at 1728 cm ^{1} (CO), 1527 cm ^{1} (N–H bending). These bands correspond to the characteristic bands for Aniline; it shows very good agreement.

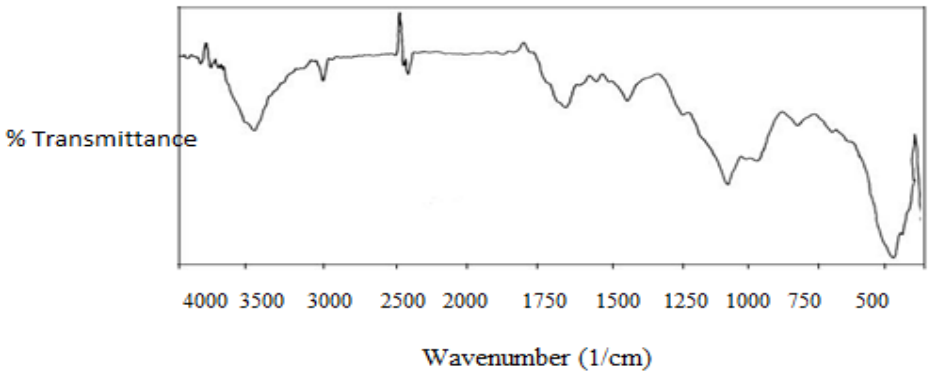

Fig 3 . FT-IR spectra of PANI-2AP film in aqueous solution at current density 1 mA/cm^2 and pH 3.0.

Sem Studies Of Pani-2ap Composite Films

The scanning electron micrograph of synthesized PANI-2AP composite film is shown in Fig. 4. The scanning electron micrograph was recorded, using JEOL JSM-6360A SEM machine. It can be seen that the surface morphology is more porous, uni-form with globular or cauliflower like structure [34].It shows very good agreement with earlierreported work [35,36].

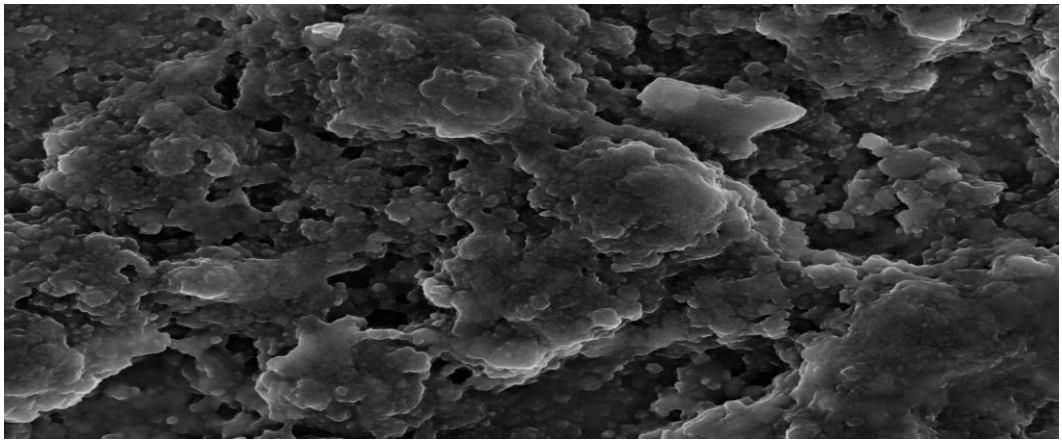

Fig 4 SEM image of PANI/2AP composite film

Current Response Of Pani-2ap-God Electrodes

The change in response current of the active device glucose oxidase is the parameter of interest for sensor applications. The response current of the device depends on several factors; such as: (1) the contact resistance between the metal electrodes and the polymer film; (2) the geometric factor of the film; and (3) the film conductivity. The film conductivity is dependent on several factors such as analyte pH, temperature, polymer film potential, substrate concentration and enzyme loading.The current–time relationship when the potential of the enzyme electrode was set at 0.7 V is as shown in Fig. 5. It was found that the response current of the enzyme electrode easily reaches to steady state. The relationship between response current and glucose concentration in 0.1 M phosphate buffer, at 7.0 pH, is shown in Fig. 6. It was found that, current increases with increasing glucose concentration in the range 1–50 mM. In the present case, assuming that the enzyme is uniformly distributed throughout the film, the reaction takes place predominantly on the surface of the film in the lower concentration. However, the reaction on the surface of the film and the diffusion occurring

simultaneously at higher concentrations delays the response time. With increasing concentrations of glucose, the response current also increased and finally reached to steady state value.

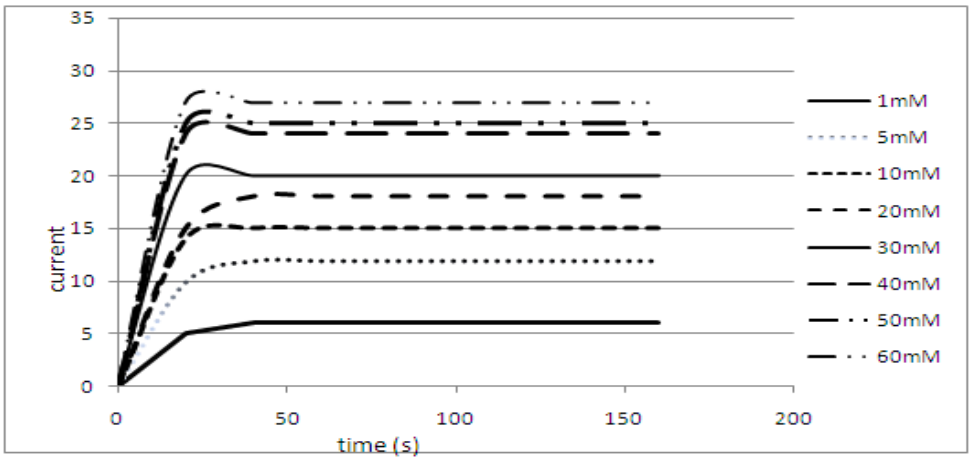

Fig 5. Current–time curve during the PANI-2AP-GOD electrode at 0.7 V and pH 7.0 in 0.1 M phosphate buffer for different glucose solution of 1–50 mM.

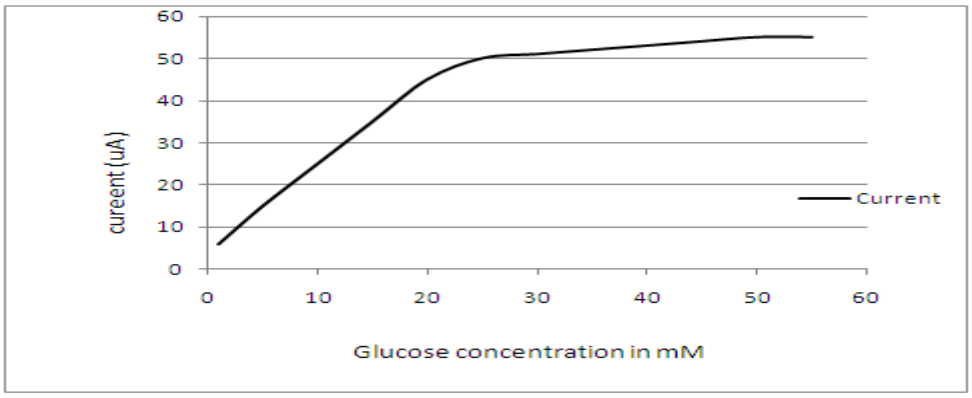

Fig 6 The relationship between response current and glucose concentration for the PANI-2AP-GOD electrode in 0.1 M phosphate buffer, pH 7.0

Michaelis–Menten Constant (K^0_m)

The apparent Michaelis Menten constant (K^0_m) was calculated for the immobilized enzyme by an amperometric method as suggested by Shu and Wilson [37]. The relationship between 1/current against 1/Glucose concentration in 0.1 M phosphate buffer is shown in Fig. 7. The maximum current (I_{max}) = 42 lA and Michaelis Menten constant ðK^0_mÞ ¼ 6:25 were calculated from intercepts. The value of K^0_m depends on immobilization of enzyme and lesser the K^0_m gives the faster response of the electrode to glucose.

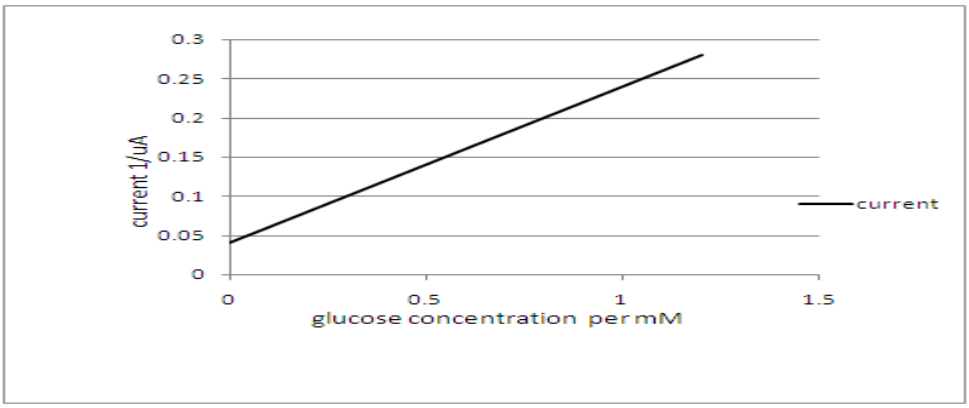

Fig 7 . The determination of apparent Michaelis–Menten con-stant (K^0_m) for the PANI-2AP-GOD electrode in 0.1 M phosphate buer, pH 7.0.

Effect Of Ph

In an optimized polymerization the value of pH of reaction medium allow an efficient entrapment of the enzyme. It also prevents the loss of the enzyme activity under polymerization conditions [38].Therefore enzyme sensor response depends on the working pH of the sampling solution. The effect of pH on the behavior of the enzyme electrode was studied with 0.1 M phosphate buffer solution with 5 mM glucose. The steady state currents at 0.7 V as a function of pH values is shown in Fig. 8. The electrochemical response is quite good at pH ranging from 4.0 to 8.0 and the maximum current occurred at pH 7.0 [39,40].

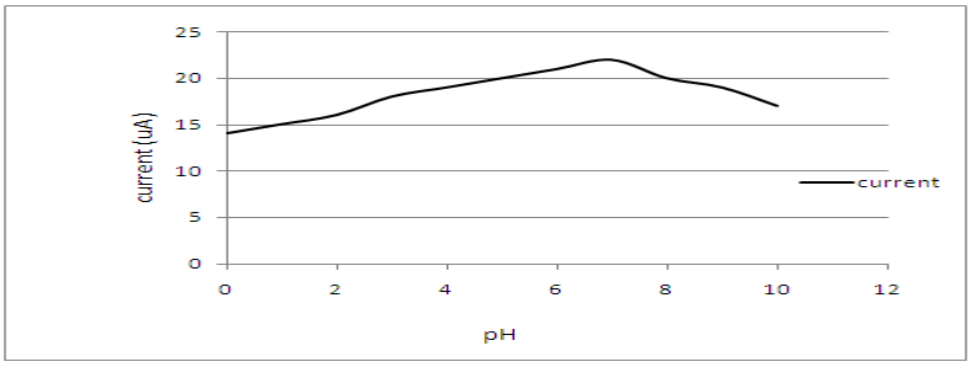

Fig 8 . Effect of pH on the GOD electrode response of PANI-2AP. Steady currents measure at 0.7 V in 5 mM glucose solution in 0.1 M phosphate buffer

Effect Of Potential

The response current increases rapidly with increase in potential, which indicates that the response of the enzyme electrode was controlled by the electrochemical methods (Fig. 9). It is well known that the velocity of an electrode reaction is related to the concentration of electro active species, the pH value of solution and applied potential [41,42].Above the potential 0.7 V, the response was almost steady, which could be explained by the rate-limiting process of enzyme kinetics, diusion control of H_2O_2 and substrate [43]. Considering the decrease in response of the PANI-2AP-GOD electrode at higher potential, which also has acted the electrochemical response of the enzyme electrode, we preferred to set the potential at 0.7 V for the further studies of PANI-2AP-GOD electrode. The stability and lifetime of the PANI-2AP-GOD electrode have been studied. It shows very good stability and excellent response for 38–40 days (Fig. 10)

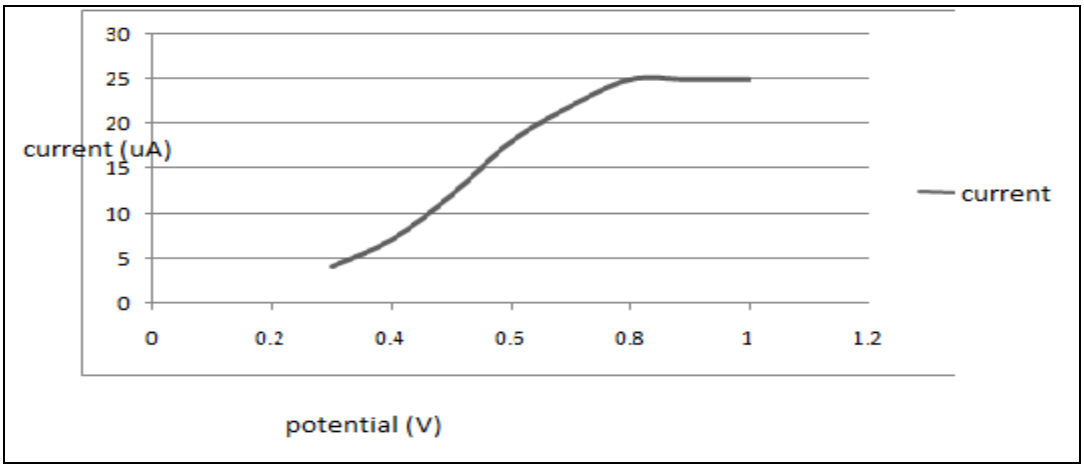

Fig 9 Current–potential curves for the PANI-2AP-GOD electrode in 0.1 M phosphate buffer, pH 7.0.

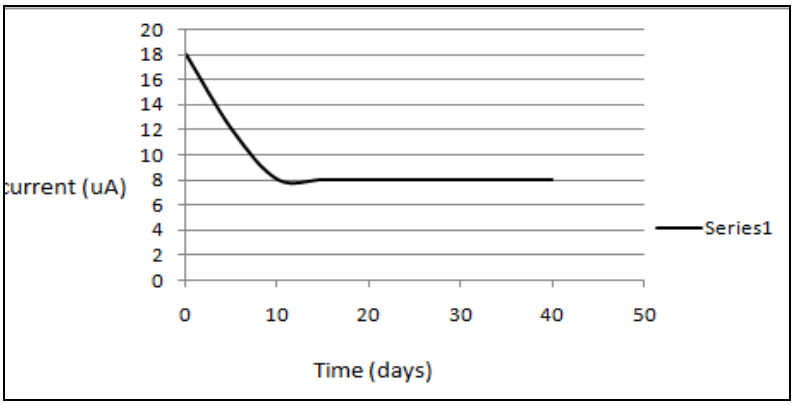

Fig 10 Stability of the PANI-2AP-GOD electrode on storage in 0.1 M phosphate buffer, pH 7.0

IV. Conclusion

This study has demonstrated the feasibility of development of a conducting polyaniline based bio-sensor for monitoring glucose. The conducting Aniline– 2-AP having amine functional groups can be utilized as a suitable matrix for the cross-linking via glutaraldehyde entrapment of enzyme (GOD). This e-cient cross-linking of the amine functionalized porous 2-AP doped Aniline film; lead to the enzyme electrode to exhibit a good performance in terms of dynamic range of detection, short response time and long lifetime and stability. The cost effectiveness and simple method of fabrication of PANI-2AP-GOD electrode is an additional advantage as com-pared with conventional electrodes.

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