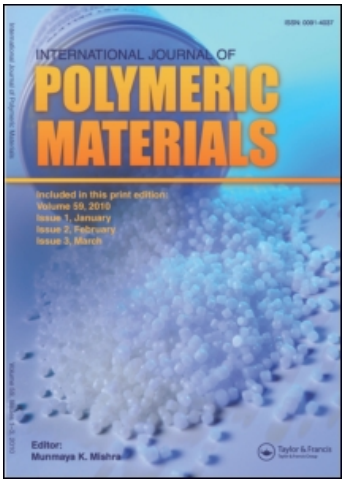

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Immobilization of GOD on Ppy-PVS Composite Film for Determination of Glucose: A Comparative Study of Phosphate and Acetate Buffers

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Immobilization of GOD on Ppy–PVS Composite Film for Determination of Glucose: A Comparative Study of Phosphate and Acetate Buffers

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The Polypyrrole-polyvinylsulphonate-glucose oxidase (Ppy–PVS–GOD) biosensor for determination of glucose has been described in the present investigation. The enzyme, glucose oxidase (GOD) was immobilized by crosslinking via glutaraldehyde on a polypyrrole–polyvinyl sulphonate (Ppy–PVS) composite film. The Ppy–PVS film was electrochemically synthesized on indium-tin-oxide (ITO)–coated glass plate. The synthesized composite films were characterized using galvanostatic electrochemical technique, electrical conductivity, UV-Visible spectroscopy, Fourier transform infrared spectroscopy (FTIR), and scanning electron microscopy (SEM). The crosslinking 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. Characterization of resulting amperometric biosensor for the estimation of glucose has been experimentally determined in terms of linear response range, optimum pH, applied potential, and shelf-life. These Ppy–PVS–GOD electrodes can be used for glucose estimation from 1 to 50 mM and have a shelf-life of about 5–6 weeks at 4°C. The sensitivity of Ppy–PVS–GOD electrode in phosphate and acetate buffer has been studied. It was found that the phosphate buffer gives fast response as compared to acetate buffer in amperometric measurements.

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Keywords: biosensor, composite film, crosslinking, glucose-oxidase, immobilization

INTRODUCTION

There has been a significant increase in demand for the development of a fast and sensitive method for the determination of various analytes present in the blood serum. Biosensors have been considered one of the most suitable devices due to selectivity, fast response, miniature size, reliable and reproducible results. Biosensors using immobilized enzymes as a bio-recognition element are among the most widely investigated devices for both fundamental and applications-oriented research [1–4]. In this context, design and study of new amperometric biosensors for the estimation of concentration of various analytes of clinical interest such as cholesterol, lactate, urea, and glucose have received considerable attention [5–11].

Organic conducting polymers have recently emerged as a new class of electroactive materials and are interesting subjects for research and development [11–14]. The remarkable switching capability of these electroactive materials between the conducting 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. Various conducting polymers have been extensively considered as the material for immobilization of enzymes, such as polyacetylene, polythiophene, polypyrrole (Ppy), polyindole, and polyaniline. Among these conducting polymers, Ppy is well characterized and is probably one of the most suitable polymers for biosensor applications because it has good environmental stability and biocompatibility. Its low oxidation potential enables a conducting polymer film to be grown from aqueous solutions that is compatible with most of the biological elements. Moreover, its easy polymerization, high electrical conductivity, chemical stability, and ability to form freestanding films are added advantages for its application to biosensors [15–16].

Various methods to hybridize Ppy with other materials have been attempted in order to increase mechanical strength of Ppy film for various applications such as functional electrodes, electrochromic devices, sensors, and so on [17–18]. The materials, which can be doped with Ppy to increase stability and mechanical strength, are Nafion,

polyvinyl alcohol, poly(methylmethacrylate), polystyrene sulphonate, polyvinyl sulphonate, dodecylbenzene sulphonate, p-toluene sulphonate, and so on [19–20]. It has been reported that use of polyelectrolyte in polymerization solution with pyrrole causes an increased, growth rate, higher compactness of the synthesized film, and improved environmental stability [19,21]. The ion exchange properties and stability of polypyrrole (Ppy)–polyvinylpyrrolidone (PVP), polypyrrole (Ppy)–polyvinyl alcohol (PVA), and polypyrrole (Ppy)–polystyrene sulphonate (PSS) composites have been studied by some authors [22]. However, it is still important to find high quality polymers and effective dopants showing their desirable properties. The stability of polymer matrix depends on anions. The incorporation of a large size dopant anion, such as polyvinyl sulphonate (PVS), p-toluene sulphonate (pTS), and dodecylbenzene sulphonate (DBS) into Ppy films during electropolymerization makes the Ppy film more stable and porous [23]. The porosity is an important factor for the facile immobilization of enzyme. The authors have immobilized enzyme on large anion-doped porous Ppy–PVS film by crosslinking technique for biosensor application.

The stability of the enzyme over the matrix is the vital factor in the development of biosensor. This is beneficial to biosensor transport as well as reducing measurement cost. The major cause of poor stability is the disorption (leaching out) of enzyme from immobilization materials. Therefore, crosslinking method via glutaraldehyde has been chosen for the immobilization of enzyme in the present investigation. A method of electrochemical entrapment of enzyme has been described that 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, the authors have initially electrochemically synthesized Ppy–PVS composite film and then GOD was immobilized. This article describes the results of the systematic studies related to the electrochemical preparation and characterization of the Ppy–PVS–GOD electrode for determination of glucose. The advantage of using the composite Ppy–PVS films lies in the electrostatic rejection of anions [27]. Sulphonate ions of the Ppy–PVS composite films provide a charged surface for electrostatic interaction between the enzyme and the surface [28]. The authors have immobilized enzyme on large anion (PVS)-doped porous Ppy film by crosslinking via glutaraldehyde for development of glucose biosensor. Crosslinking via glutaraldehyde has led to greater stability of the

enzyme in the Ppy–PVS films. An attempt has been made to investigate the effect of pH, potential, phosphate, and acetate buffers on the activity of the Ppy–PVS–GOD electrode.

EXPERIMENTAL

Preparation of Polypyrrole–Polyvinyl Sulphonate (Ppy–PVS) Composite Films

Ppy–PVS films were synthesized from an aqueous solution of distilled 0.1 M pyrrole (Spectrochem) and 0.025 M sodium salt of polyvinyl sulphonate (25% by weight) (Aldrich) using electrochemical deposition method. It was carried out by galvanostatic technique at room temperature (27°C) in a one-compartment, three-electrode glass cell. The ITO coated glass plate was used as a working electrode, platinum foil 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 $1\,mA \cdot cm^{-2}$ and the pH 3.0 were kept constant during the synthesis of composite films. After synthesis the polymer-coated electrodes were rinsed thoroughly in deionized water, dried in cold air, and then used for subsequent characterization.

Immobilization of GOD on Polypyrrole–Polyvinyl Sulphonate (Ppy–PVS) Composite Films

The enzyme GOD (SISCO) was immobilized by crosslinking via glutaraldehyde (Loba Chemie) on composite Ppy–PVS 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 and/or acetate buffer. The stock solution of GOD ($2\,mg.ml^{-1}$) prepared in 0.1 M phosphate buffer and/or 0.1 M acetate buffer (pH 7.0) was adsorbed onto the surface of Ppy–PVS 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 crosslinking 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°C) in phosphate buffer and acetate buffer. An adequate concentration of GOD and glutaraldehyde in the crosslinking mixture were chosen so that it ensures higher enzyme loading and provides excellent amperometric response with an efficient retention of the enzyme.

RESULTS AND DISCUSSION

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

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

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

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

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 and leads 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 Ppy–PVS Composite Films

The potential time curve of the galvanostatically synthesized Ppy–PVS composite film is shown in Figure 1. The Ppy–PVS film was synthesized on ITO coated glass plate from 0.1 M concentration of pyrrole and 0.025 M of PVS with $1\,\text{mA}\cdot\text{cm}^{-2}$ current density at pH 3.0.

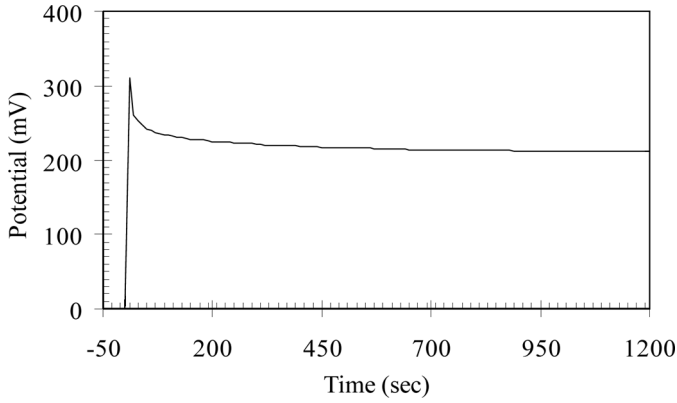

FIGURE 1 Potential–time curve during the synthesis of Ppy–PVS film in aqueous solution at $1\,\text{mA}\cdot\text{cm}^{-2}$ and pH 3.0.

This has resulted in high conductivity, with very good uniformity and porous surface morphology. The behavior of the galvanostatic synthesis overshoot during the first few seconds probably indicates difficult formation of dimers and oligomers. After this, the potential remains constant suggesting that building up of the film proceeds according to the same reaction along the full thickness of the polymer. A blackish polymeric film on ITO coated glass electrode was deposited with very good uniformity. The electrical conductivity of the synthesized composite Ppy–PVS films was measured by Keithley 6514 Electrometer and it is 2.412×10^{-3} S · cm^{-1}.

UV-Vis. Studies of Ppy–PVS Composite Films

UV-Vis. spectrum of synthesized Ppy–PVS film is shown in Figure 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 of bipolaron charge transfer bands, which indicate high conductivity, and absorption at around 460 nm, while indicates π-π^*. This indicates that higher sulphonate group introduction to the polymer system may induce more doping of the Ppy backbone because the sulphonate group can be anionically charged so that it can stabilize the doped state of Ppy more effectively. This shows very good resemblance with the polymerization potential. The absorption spectra observed for synthesized composite films are in good agreement with the earlier reported work [30–31].

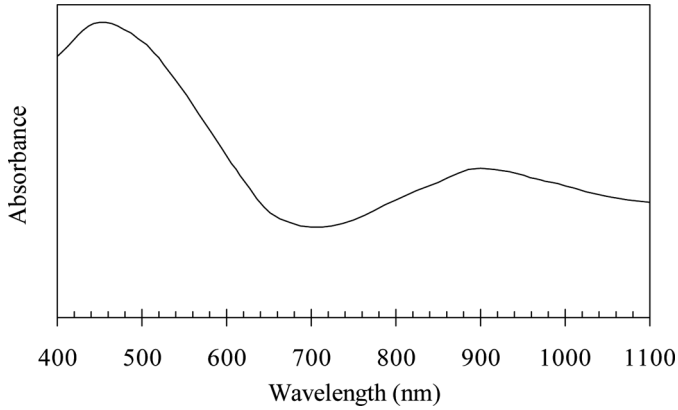

FIGURE 2 UV–Vis spectra of Ppy–PVS film in aqueous solution at current density 1 mA.cm^{-2} and pH 3.0.

FTIR Studies of Ppy–PVS Composite Films

The FTIR spectrum of the synthesized composite Ppy–PVS film is shown in Figure 3. The FTIR spectrum was recorded using Shimadzu FTIR-8400 series, using KBr pellets in the region 350–4,000 cm^{-1}. Spectrum shows broad peak at 3423 cm^{-1} corresponds 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} (C=O), 1527 cm^{-1} (N–H bending). These bands correspond to the characteristic bands for Ppy; it shows very good agreement with earlier reported work [32–33]. Thus, the FTIR spectral results confirm the formation of Ppy.

SEM Studies of Ppy-PVS Composite Films

The scanning electron micrograph of synthesized Ppy–PVS composite film is shown in Figure 4. The scanning electron micrograph was recorded using a JEOL JSM-6360A SEM machine. It can be seen that the surface morphology is porous, uniform with globular or cauliflower-like structure, which is suitable for immobilization of biocomponent. It shows very good agreement with earlier reported work [34–35].

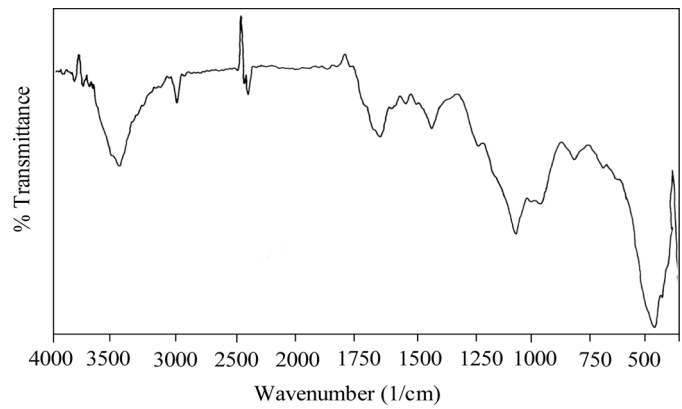

FIGURE 3 FTIR spectra of Ppy–PVS film in aqueous solution at current density 1 $mA \cdot cm^{-2}$ and pH 3.0.

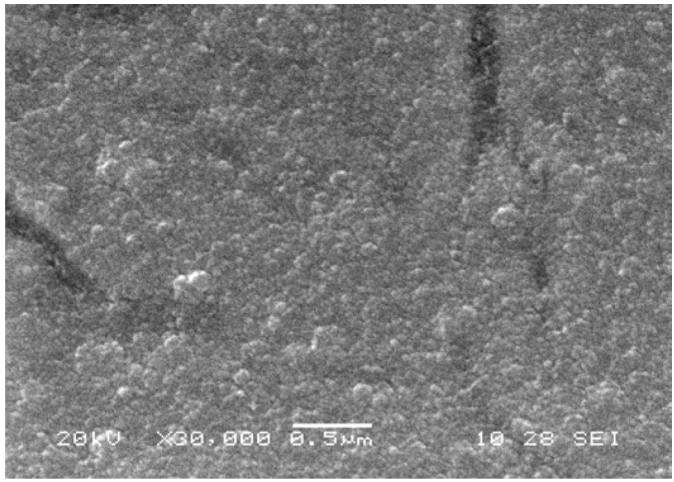

FIGURE 4 SEM of Ppy–PVS film in aqueous solution at current density $1\,mA \cdot cm^{-2}$ and pH 3.0.

Current Response of Ppy–PVS–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 depends on several factors, such as analyte pH, temperature, polymer film potential, substrate concentration, enzyme loading, and so on.

The current-time relationship when the potential of the enzyme electrode was set at 0.7 V is as shown in Figures 5 and 6 for phosphate and acetate buffers, respectively. It was found that the response current of the enzyme electrode easily reaches a steady state. The relationship between response current and glucose concentration in 0.1 M phosphate buffer and/or 0.1 M acetate buffer at pH 7.0 is shown in Figure 7. It was found that, current increases with increasing glucose concentration in the range 1 mM–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 a steady

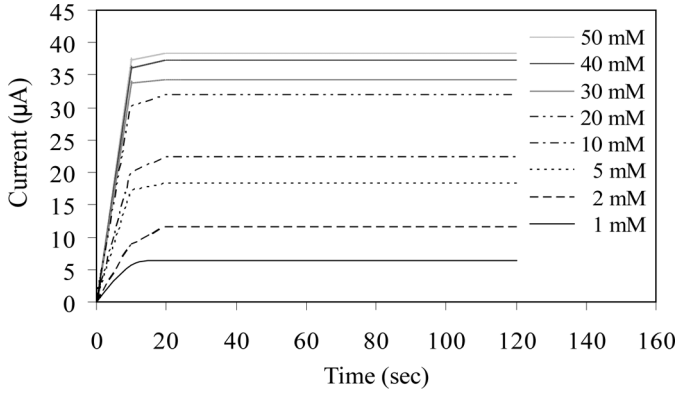

FIGURE 5 Current–time curve during the Ppy–PVS–GOD electrode at 0.7 V and pH 7.0 in 0.1 M phosphate buffer for different glucose solution of 1–50 mM.

state value. The response time of glucose solution in phosphate and acetate buffers is little different.

Determination of Michaelis-Menten Constant (K_m)

The apparent Michaelis-Menten constant (K_m) was calculated for the immobilized enzyme by an amperometric method as suggested by Shu and Wilson [36]. The relationship between 1/current against 1/Glucose concentration in 0.1 M phosphate buffer and 0.1 M acetate buffer is shown in Figure 8. The maximum current (I_{max}) and apparent

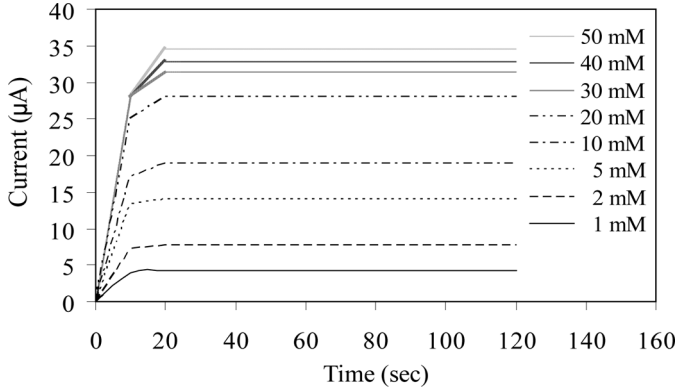

FIGURE 6 Current–time curve during the Ppy–PVS–GOD electrode at 0.7 V and pH 7.0 in 0.1 M acetate buffer for different glucose solution of 1–50 mM.

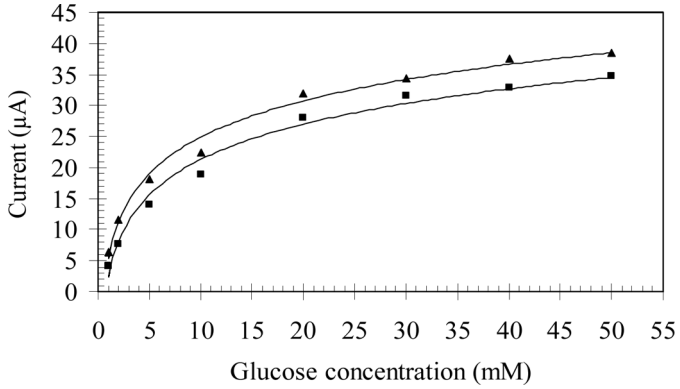

FIGURE 7 The relationship between response current and glucose concentration for the Ppy–PVS–GOD electrode in 0.1 M phosphate buffer (▲) and 0.1 M acetate buffer (■), pH 7.0.

Michaelis-Menten constant (K_m) can be calculated from the intercepts. The I_{max} and K_m values were calculated for Ppy–PVS–GOD films in phosphate buffer and acetate buffer with pH 7.0. In the phosphate buffer, the maximum current was 40 μA with K_m 6.25 mM and in the acetate buffer it was 42 μA with K_m 8.33 mM. The value of K_m depends on immobilization of enzyme: a lesser K_m gives a faster response of the electrode to glucose. An excellent response of Ppy–PVS–GOD electrode

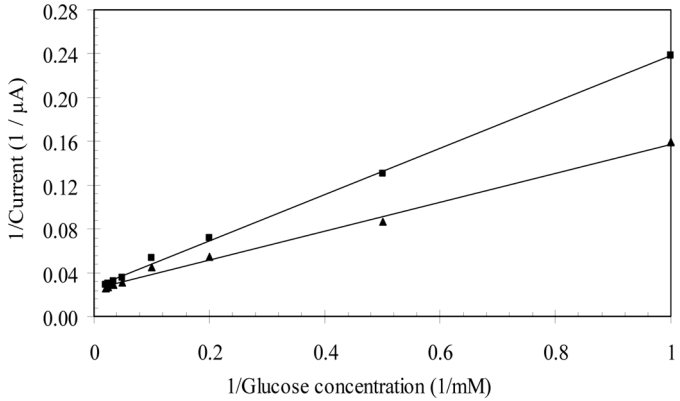

FIGURE 8 The determination of apparent Michaelis-Menten constant (K_m) for the Ppy–PVS–GOD electrode in 0.1 M phosphate buffer (▲) and 0.1 M acetate buffer (■), pH 7.0.

TABLE 1 Comparison of the Analytical Performance of Ppy–PVS–GOD Electrode for Phosphate and Acetate Buffer at pH 7.0

Sr. No.	Parameters	Buffers	
		Phosphate	Acetate
1	I_{max} (μA)	40	42
2	K_m (mM)	6.25	8.33
3	Linearity (mM)	1–5	1–5
4	Sensitivity ($\mu A \cdot mM^{-1}$)	2.2	2.1
5	Lifetime (days)	40	38

was observed when it was developed in phosphate buffer as compared to acetate buffer (Table 1).

Effect of pH

In an optimized polymerization the value of pH of reaction medium allows an efficient entrapment of the enzyme. It also prevents the loss of the enzyme activity under polymerization conditions [37]. Therefore enzyme sensor response depends on the working pH of the sampling solution. The effect of pH on the behaviour of the enzyme electrode was studied with 0.1 M phosphate and/or 0.1 M acetate buffers solution with 5 mM glucose. The steady state currents at 0.7 V as a function of pH values are shown in Figure 9. The electrochemical response

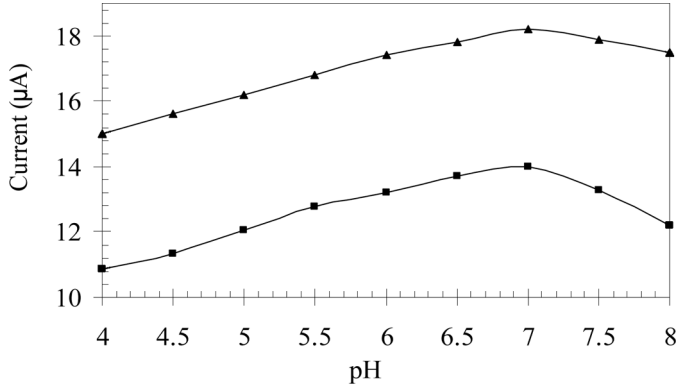

FIGURE 9 Effect of pH on the GOD electrode response of Ppy–PVS. Steady currents measure at 0.7 V in 5 mM glucose solution in 0.1 M phosphate buffer (▲) and 0.1 M acetate buffer (■).

is quite good at pH ranging from 4.0 to 8.0 and the maximum current occurred at pH 7.0 [38–39].

Effect of Potential

The current-potential relationships of the enzyme electrodes in 0.1 M phosphate buffer and 0.1 M acetate buffer solution containing 5 mM glucose at pH 7.0 are shown in Figure 10. The response current increases rapidly with increase in potential, which indicates that the response of the enzyme electrode was controlled by the electrochemical methods. It is well known that the velocity of an electrode reaction is related to the concentration of electroactive species, the pH value of solution and applied potential [40–41]. It can be seen that, above 0.7 V, the response is almost steady, which could be explained by the rate-limiting process of enzyme kinetics, diffusion-control of H_2O_2 and substrate [42–43]. Considering the decrease in response of the Ppy–PVS–GOD electrode at higher potential, which also has affected the electrochemical response of the enzyme electrode, the authors preferred to set the potential at 0.7 V for the analysis of Ppy–PVS–GOD electrode.

The stability and lifetime of the Ppy–PVS–GOD electrode have been studied. It shows very good stability and excellent response for 5–6 weeks (Figure 11). In the beginning current response decreased rapidly and then stabilized. It was observed that the current response of the Ppy–PVS–GOD electrode in the acetate buffer decreases much more than that of phosphate buffer.

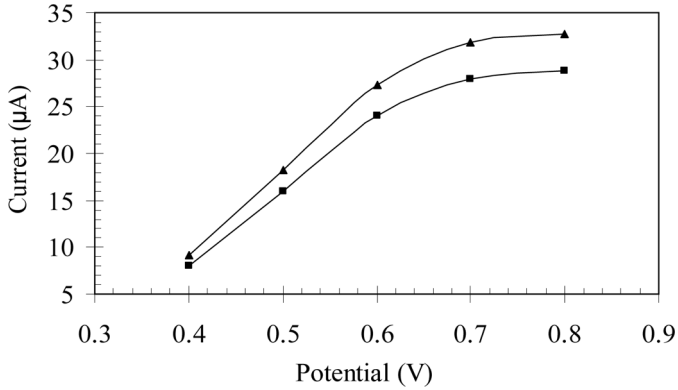

FIGURE 10 Current–potential curves for the Ppy–PVS–GOD electrode in 0.1 M phosphate buffer (▲) and 0.1 M acetate buffer (■), pH 7.0.

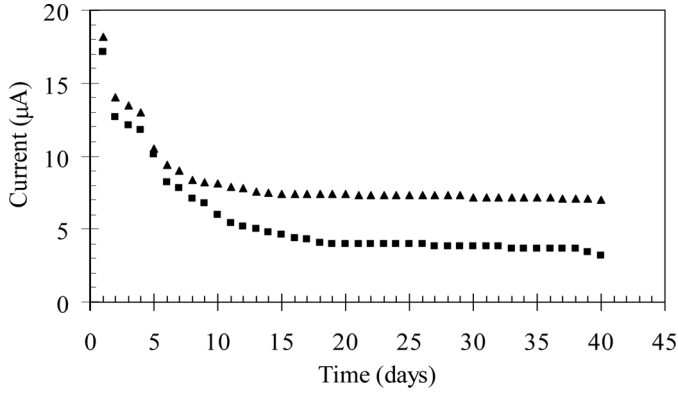

FIGURE 11 Stability of the Ppy–PVS–GOD electrode on storage in 0.1 M phosphate buffer (▲) and 0.1 M acetate buffer (■), pH 7.0.

CONCLUSION

A rapid and convenient method for the development of an amperometric biosensor for glucose estimation has been presented. The conducting Ppy–PVS composite film was found as a suitable matrix for the crosslinking via glutaraldehyde for entrapment of enzyme (GOD). This efficient crosslinking of the enzyme with amine functionalized porous PVS doped Ppy film leads 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 Ppy–PVS–GOD biosensor works efficiently at 0.7 V and exhibits linearity with concentration in the range of 1–5 mM with pH 7.0. The sensitivity of Ppy–PVS–GOD electrode in phosphate buffer is $2.2\,\mu A \cdot mM^{-1}$ and in acetate buffer it is $2.1\,\mu A \cdot mM^{-1}$ and the apparent K_m values for phosphate and acetate buffer are 6.25 mM and 8.4 mM, respectively. The Ppy–PVS–GOD electrode shows excellent response in phosphate buffer as compared with acetate buffer.

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