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Synthesis of Poly(*o*-anisidine)/H_2SO_4 Film for the Development of Glucose Biosensor

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Abstract The poly(*o*-anisidine)–sulfuric acid–glucose oxidase (POA–H_2SO_4–GOx) electrode has been investigated in the present work. Platinum electrode was used for the synthesis of poly (*o*-anisidine)–sulfuric acid (POA–H_2SO_4) film using galvanostatic method with 0.2 M *o*-anisidine, 1.0 M H_2SO_4 solution, 1.0 pH and 2 mA/cm^2 applied current density. The synthesized film was characterized using electrochemical technique, conductivity measurement, UV–visible spectroscopy, Fourier transform infrared spectroscopy, and scanning electron microscopy. GO_X was immobilized on synthesized POA–H_2SO_4 film by cross-linking via glutaraldehyde in phosphate and acetate buffer. The Michaelis–Menten constant ($K_m^{'}$) was determined for the immobilized enzyme. The glucose oxidase electrode shows the maximum current response at pH 5.5 and potential 0.6 V. The sensitivity of POA–H_2SO_4–GO_X electrode in phosphate and acetate buffer has been recorded. The results of this study reveal that the phosphate buffer gives fast response as compared to acetate buffer in amperometric measurements.

Keywords Galvanostatic · Poly(*o*-anisidine) · Glucose oxidase · Cross-linking · Biosensor

Introduction

Since the first demonstration by Clark and Lyons that an enzyme could be integrated into an electrode to form a biosensor, the developments of such electrochemical devices have made considerable progress [1–3]. The advantages associated with these devices are their high

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selectivity and simple use in complex media as well as the possibility of developing compact and portable analyzers. Several conducting polymers such as polypyrole, poly(*N*-methylpyrole), polyaniline, poly(*o*-anisidine), poly(*o*-toluidine), etc. have been used to immobilize desired enzymes (glucose oxidase, urease, cholesterol oxidase, etc.) using physical adsorption and entrapment techniques [4–9]. Polyaniline and its derivatives are considered to be an attractive class of polymer because this conducting material exhibits two redox couples in the right potential range to facilitate an enzyme–polymer charge transfer. Besides, polyaniline has shown variety of applications such as in rechargeable batteries, electrocatalysis, electrochromic displays, gas separation, and biosensors. However, the role of structural and mechanical behavior of polyaniline and its derivatives for their applications to biosensor is yet to be explored.

Electrochemically synthesized conducting polymers have received considerable attention. 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.

The synthesized film should have uniform, porous surface morphology and high conductivity. The conducting polymers fulfill both requirements. Sensor systems based on conducting polymers also rely on sensible changes in the optical and electrical features of this kind of materials [10–14]. Conducting polymers have considerable flexibilities in modifying their chemical structures. By chemical modeling and synthesis, it is possible to modulate their electrical and mechanical properties [15]. The polymer itself can be modified to bind with protein molecules [16]. Conducting polymers are also known for their ability to be compatible with biological molecules in neutral aqueous solutions. Additionally, conducting polymers have the ability to efficiently transfer the electric charges produced by biochemical reactions to electronic circuits [17–19]. Polyaniline is a technologically important conducting polymer because of its unique electrical, electrochemical, and optical properties. The combination of oxidoreductases and amperometric electrodes is a most commonly studied biosensor concept. Through various strategies, the enzyme reaction can be easily followed and sensitively measured by electrochemical techniques [20–30].

The various types of biosensors and methods of enzyme immobilization on electrodes have been summarized in compressive reviews [31–34]. The immobilization of enzymes or proteins in such polymers can be achieved by several techniques like physical entrapment, chemical cross-linking, covalent coupling, etc. The physical entrapment, covalent coupling, and other methods suffer from leaching problems of the enzyme. However, this problem can be significantly overcome using chemical cross-linking method of immobilization via glutaraldehyde. The polyelectrolyte characteristic of the polymer is also useful for sensor applications. The surface morphology and conductivity of the synthesized films are two key factors for sensor applications. Conducting polymer-based biosensors have found promising applications in various fields such as biotechnology, food and agriculture product processing, health care, medicine, and pollution monitoring [35–37].

In the present investigation, we have reported the electrochemical synthesis of conducting poly(*o*-anisidine) (POA) film and immobilization of GO_X on synthesized POA–H_2SO_4 film by cross-linking via glutaraldehyde in phosphate and acetate buffer. We have studied influence of these buffers on GO_X-immobilized POA–H_2SO_4 film by cross-linking via glutaraldehyde on platinum electrode and its response to glucose measurement as an approach towards the development of sensitive amperometric biosensor.

Experimental

Synthesis of POA–H_2SO_4 Film

The monomer *o*-anisidine was distilled twice before use. The electrolyte solution was prepared in distilled water. The electropolymerization of *o*-anisidine was carried out by galvanostatic technique in one compartment electrochemical cell. Platinum rectangular sheet (20×10×0.25 mm) was used as a counter electrode, and another platinum rectangular sheet (20×5×0.25 mm) was used as a working electrode. The reference electrode was Ag/AgCl. All three electrodes were placed vertically in the cell. The reference electrode was kept in close proximity to the working electrode to minimize the electrolytic ohmic drop. The pH of the electrolyte was measured by a calibrated ELICO LI120 pH meter.

The POA–H_2SO_4 film was synthesized from an aqueous solution of containing 0.2 M *o*-anisidine and 1.0 M of sulfuric acid using electrochemical deposition method carried out by galvanostatic technique at 29 °C. The applied current density 2 mA/cm^2 and the 1.0 pH were kept constant during synthesis of POA film. The pH was adjusted by adding nitric acid (HNO_3) or sodium hydroxide (NaOH). All of the above reagents were obtained from Rankem Ranbaxy New Delhi (India). After synthesis, polymer-coated electrodes were rinsed thoroughly in distilled water and dried in cold air and then used for subsequent characterization.

Immobilization of GO_X on POA–H_2SO_4 Film

The stock solution of glucose oxidase (GO_X; EC 1.1.3.4, Type VII; 200 U/ml; Aldrich) was prepared in 0.1 M phosphate and/or acetate buffer (pH 5.5). The enzyme GO_X was immobilized by cross-linking via (1%) glutaraldehyde (Loba Chemie) on POA–H_2SO_4 films and left 30 min and then washed with phosphate and acetate buffer to remove any loosely bound enzyme. The enzymatic incorporation was done in glutaraldehyde media. This kind of immobilization results in greater physical and chemical stability of the catalytic material because of the cross-linking formed with the glutaraldehyde and enzyme. In this case, the active site of the enzyme could be more accessible for the enzyme reaction. An adequate concentration of GO_X was chosen so that it ensures higher enzyme loading and provide 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 reaction given below

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

The formation of hydrogen peroxide is detected by the amperometric method during electrode oxidation.

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

The enzyme electrode formed by glucose oxidase with glutaraldehyde is used for amperometric measurement of glucose [38–39].

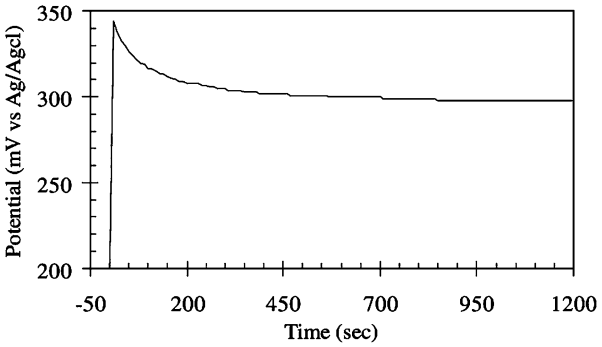

Fig. 1 The chronopotentiogram recorded during the synthesis of POA film with optimized electrochemical process parameters

To construct the amperometric enzyme sensors, GO_X is used as a redox protein. The enzyme catalyses, in the presence of molecular oxygen, leading 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 [40]. 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 POA–H_2SO_4 Film

The chronopotentiogram of synthesized POA–H_2SO_4 film is shown in Fig. 1. The POA–H_2SO_4 film was synthesized on platinum substrate from 0.2 M concentration of *o*-anisidine and 1.0 M of H_2SO_4 with 2 mA/cm^2 applied current density at 1.0 pH. This has resulted in high conductivity, with uniform and porous surface morphology of synthesized POA–H_2SO_4 film. The behavior of the galvanostatic synthesis overshoot during the first few seconds probably indicates difficult formation of dimmers and oligomers. After this, the potential remain constant, suggesting that building up of the films proceeds in accordance to the same reaction along the full thickness of the polymer. It was found that green polymeric film on platinum electrode was deposited with very good uniformity. The electrical conductivity of synthesized POA–H_2SO_4 was measured by four probe technique (Model DRF-02 Owen 1038-Optochem International, New Delhi), and it was found to be 1.91 S/cm.

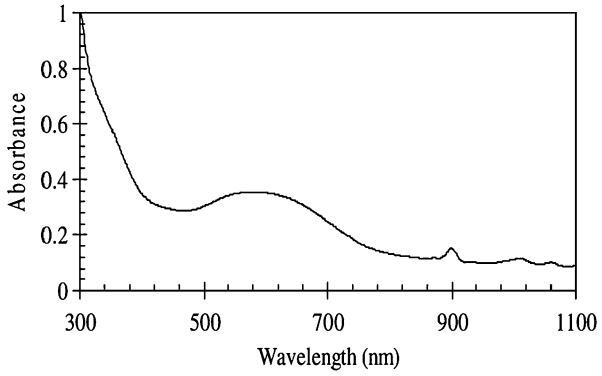

Fig. 2 The optical absorption spectrum of synthesized POA film with optimized electrochemical process parameters

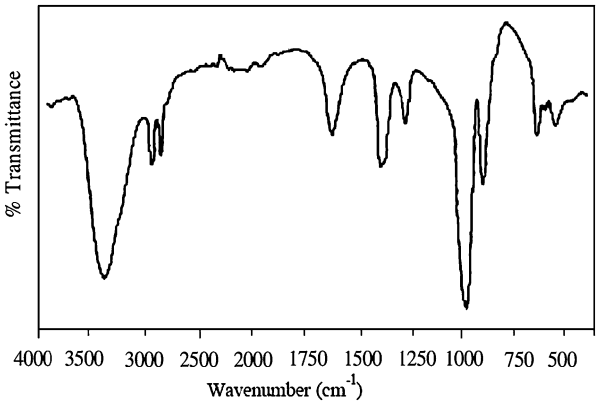

Fig. 3 The FTIR spectrum of synthesized POA film with optimized electrochemical process parameters

UV–Visible Studies of POA–H_2SO_4 Film

The optical absorption spectrum of synthesized POA film with optimized process parameters is as shown in Fig. 2. The UV–visible spectrum was recorded using UV–visible spectrophotometer Shimadzu 1601 in the wavelength range 300–1,100 nm. The peak appearing at 300 nm is assigned to a $\pi \rightarrow \pi^*$ electronic transition between the valance and conduction bands of the polymer, plus a second strong peak appearing at 600 nm attributed to an intermolecular charge transfer excitation associated with quinoide ring, whereas a small hump at 894 nm indicates that the formation of emeraldine salt with higher conductivity. It shows the formation of conducting POA film. It shows very good resemblance with earlier reported work [41, 42].

FTIR Studies of POA–H_2SO_4 Film

The Fourier transform infrared spectroscopy (FTIR) spectra were recorded (Testscan Shimadzu FTIR-8400 series) in the region 4,000–400 cm^{-1}. The FTIR spectrum of synthesized POA film with optimized process parameters is as shown in Fig. 3. The peak at 3,444.6 cm^{-1} corresponds to the N–H stretching vibrations. The strong bands at 1,660.6 cm^{-1} represents the presence C=N bonding. The presence of duplex peaks at 1,411.8 cm^{-1} corresponds to C–O group. The bands at 954.7 cm^{-1} represents the O–C=O

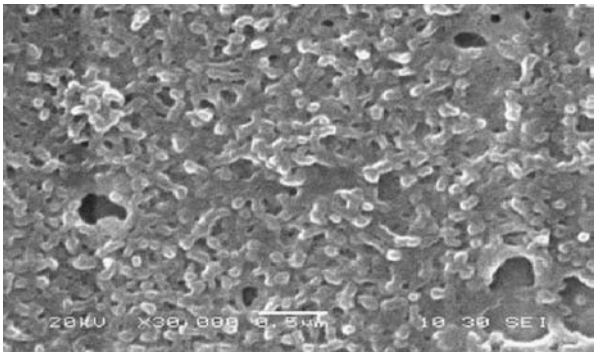

Fig. 4 The scanning electron micrograph of synthesized POA film with optimized electrochemical process parameters

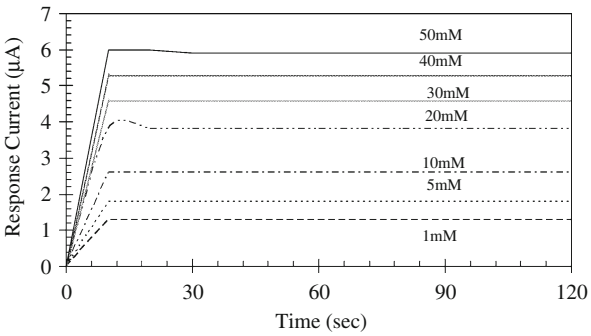

Fig. 5 Current–time curve for the glucose oxidase electrode of POA at 0.6 V. Glucose solution 1, 5, 10, 20, 30, 40, and 50 mM in 0.1 M phosphate buffer with pH 5.5

in the plane deformation. The peak at 1,311.5 cm^{-1} corresponds to the C–H stretching vibrations. The strong bands at 1,660.6 cm^{-1} indicates the presence of stretching vibrations in quinoid ring, whereas the band at 1,411.8 cm^{-1} represents the stretching vibrations of benzoid ring. This characteristic band confirms the structure of POA film [42].

SEM Studies of POA–H_2SO_4 Film

The scanning electron micrograph (SEM) was recorded using JEOL, JSM-6360A SEM machine. The scanning electron micrograph of synthesized POA film with optimized process parameters is as shown in Fig. 4. The synthesized POA film with H_2SO_4 shows isolated granules, giving a sponge-like structure with excellent surface morphology with very good porosity which is suitable for immobilization of biocomponent. It shows very good resemblance with earlier reported work [42].

Amperometric Response of POA–H_2SO_4–GO_X Electrode

The change in response current of the active device results with the change in the concentration of glucose. The response current of the device depends on several factors such as (1) the contact resistance between the metal electrode 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 and enzyme loading, the diffusion layer thickness, and the diffusion coefficients of reactants and products in the polymer film. As we have developed the sensor for the measurement of glucose concentration, glucose oxidase is the parameter of

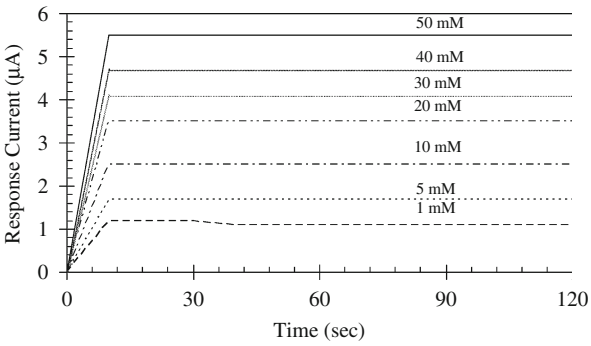

Fig. 6 Current–time curve for the glucose oxidase electrode of POA at 0.6 V. Glucose solution 1, 5, 10, 20, 30, 40, and 50 mM in 0.1 M acetate buffer with pH 5.5

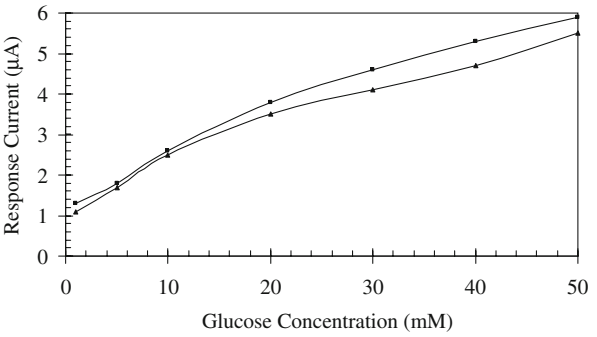

Fig. 7 The relationship between response current and glucose concentration for GO_X electrode of POA in 0.1 M phosphate buffer (*filled square*), and 0.1 M acetate buffer (*filled triangle*), pH 5.5

interest for sensor applications. The current–time relationship of POA–H_2SO_4–GO_X electrode when the applied potential was set at 0.6 V in phosphate and acetate buffer is as shown in Figs. 5 and 6, respectively.

It was found that the response current of the enzyme electrode easily reached to steady state. The sensor was tested for different glucose solution in the presence of phosphate and/or acetate buffer at pH 5.5. It was observed that the response times of glucose solution (1 to 50 mM) in phosphate and acetate buffers are slightly different. The relationship between response current and glucose concentration in 0.1 M phosphate buffer and acetate buffer at 5.5 pH is shown in Fig. 7. It was observed that the response current increases with increasing glucose concentration in the range 1 to 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 [43, 44].

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 [45]. The relationship between reciprocal of current against reciprocal of glucose concentration in 0.1 M phosphate buffer and 0.1 M acetate buffer is shown in Fig. 8.

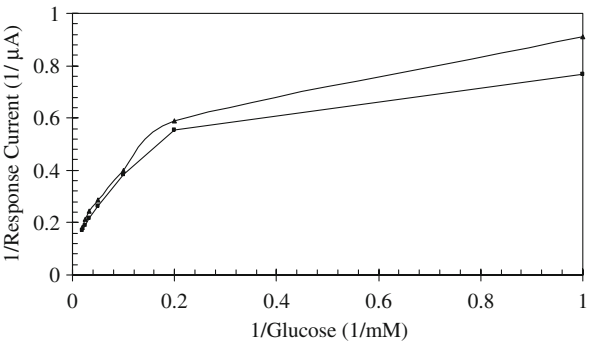

Fig. 8 Determination of apparent Michaelis–Menten constant ($K_m^{'}$) for the GO_X electrode of POA in 0.1 M phosphate buffer (*filled square*), and 0.1 M acetate buffer (*filled triangle*), pH 5.5

Table 1 Comparison of the analytical performance of POA–H_2SO_4–GO_X electrode for phosphate buffer and acetate buffer at pH 5.5.

Sr. No.	Parameters	Buffers	
		Phosphate	Acetate
1	I_{max} (μA)	5.9	5.2
2	$K_m^{'}$ (mM)	13	10
3	Linearity (mM)	1–5	1–5
4	Sensitivity (μA/mM)	6.92	5.55

The maximum current (I_{max}) and apparent Michaelis–Menten constant ($K_m^{'}$) can be calculated from the intercepts. The I_{max} and $K_m^{'}$ values were calculated for POA–H_2SO_4–GO_X films in phosphate buffer and acetate buffer. In the phosphate buffer, the I_{max} was 5.9 μA with $K_m^{'}$ 13 mM, and in acetate buffer, it was 5.2 μA and $K_m^{'}$ 10 mM, respectively. The value of $K_m^{'}$ depends on the immobilization of enzyme, and the lesser $K_m^{'}$ gives faster response of the electrode to glucose. The POA–H_2SO_4–GO_X electrode had shown excellent sensitivity in phosphate buffer as compared to acetate buffer (Table 1).

Effect of Potential

The current–potential relationship of the enzyme electrodes in 0.1 M phosphate buffer and 0.1 M acetate buffer solution containing 10 mM glucose at pH 5.5 is shown in Fig. 9. 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 the electrode reaction is related to the concentration of electroactive species, the pH value of solution, and the applied potential [46]. In the potential 0.6 V onwards, the response was almost steady, which could be explained by the rate-limiting process of enzyme kinetics, diffusion control of H_2O_2, and substrate [47]. Considering the decrease in response of the POA–H_2SO_4–GO_X electrode at higher potential, which also has affected the electrochemical response of the enzyme electrode, we preferred to set the potential at 0.6 V for the further studies of POA–H_2SO_4–GO_X electrode.

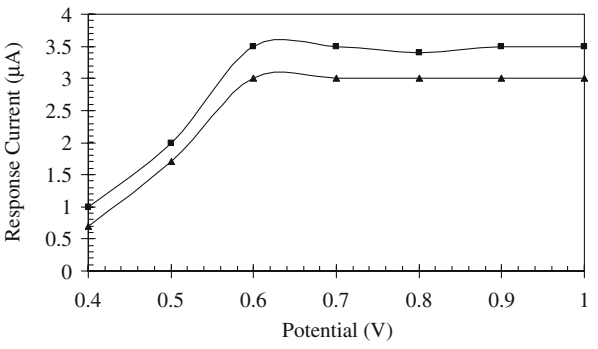

Fig. 9 Current–potential curves for the GO_X electrode of POA in 0.1 M phosphate buffer (*filled square*), and 0.1 M acetate buffer (*filled triangle*), pH 5.5

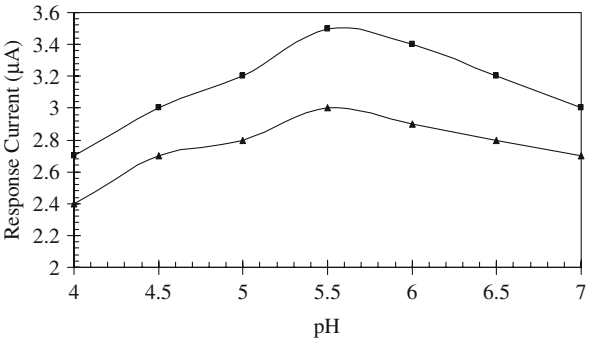

Fig. 10 Effect of pH on GO_X electrode response of POA. Steady state currents measured at 0.6 V in 10 mM glucose solution in 0.1 M phosphate buffer (*filled square*), and 0.1 M acetate buffer (*filled triangle*)

Effect of pH

In an optimized polymerization, the value of pH of reaction medium allows an efficient entrapment of the enzyme, which prevents the loss of the enzyme activity under polymerization conditions [48]. The effect of pH on the behavior of the enzyme electrode was studied with 0.1 M phosphate buffer and 0.1 M acetate buffer solution with 10 mM glucose. The steady-state current at 0.6 V as a function of pH values is shown in Fig. 10. The electrochemical response is quite good at pH ranging from 4 to 7, and the maximum current occurred at pH 5.5.

Stability and Lifetime of the POA–H_2SO_4–GO_X Electrode

Stability and lifetime of the POA–H_2SO_4–GO_X electrode have been studied. It shows excellent stability and response for 3–4 weeks (Fig. 11). At the beginning of the test of stability, current response decreased rapidly. After a few days the sensor shows stable response. The current response of the POA–H_2SO_4–GO_X electrode in the acetate buffer decreased much more as compared to that of the phosphate buffer. The test was carried out for 30 days for both the buffers.

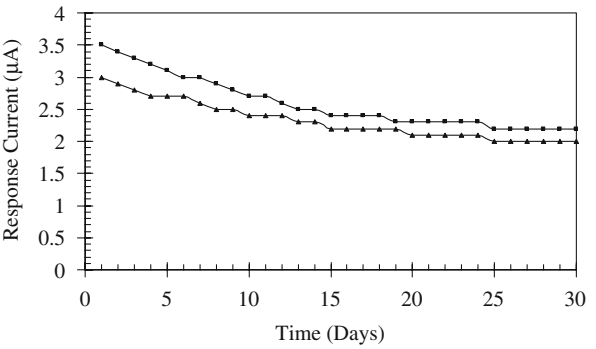

Fig. 11 Stability of the POA electrode on storage in 0.1 M phosphate buffer (*filled square*), and 0.1 M acetate buffer (*filled triangle*), pH 5.5 at room temperature

Conclusions

We have successfully developed POA–H_2SO_4–GO_X biosensor for the determination of glucose. It was found that the conducting POA–H_2SO_4 film can be utilized as a suitable matrix for immobilization of GO_X by cross-linking via glutaraldehyde. This efficient cross-linking via glutaraldehyde of the functionalized porous H_2SO_4-doped POA film lead to the enzyme electrode to exhibit a good performance in terms of dynamic range of detection and short response time. The cost effectiveness and simple method of development of POA–H_2SO_4–GO_X electrode is an additional advantage as compared to conventional electrodes. The POA–H_2SO_4–GO_X electrode in phosphate buffer gives fast response as compared to acetate buffer in amperometric measurement.

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