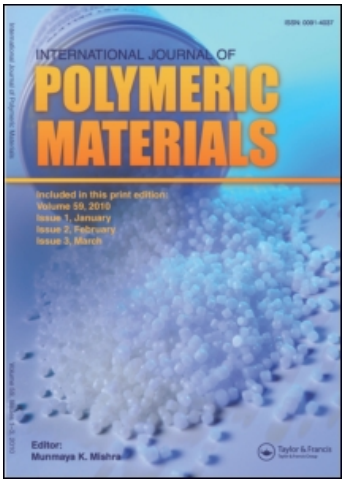


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# Development of a POA/DBS/GOx Biosensor for the Determination of Glucose

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# Development of a POA/DBS/GOx Biosensor for the Determination of Glucose

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*In the present work, a simple technique is described for constructing a poly(o-anisidine) (POA)-dodecylbenzene sulphonic acid (DBS)-glucose oxidase (GOx) (POA-DBS-GOx) electrode. The enzyme glucose oxidase (GOx) was immobilized by crosslinking via glutaraldehyde on the POA-DBS film. The POA-DBS films were synthesized electrochemically on platinum substrate. The synthesized films were characterized by using electrochemical technique, conductivity measurement, UV-visible spectroscopy, Fourier transform infrared spectroscopy (FTIR) and scanning electron microscopy (SEM). The conductivity of the polymer films was found to be about* $7.61 \times 10^{-2} S/cm$. *The crosslinking of enzyme and the porous morphology of the polymer film lead to good stability and good response time of the enzyme electrode. The stability and lifetime of the POA-DBS-GOx electrode have been studied. It shows very good stability and response for 3–4 weeks at* $4^{\circ}C$. *The results of this study reveal that a phosphate buffer gives better response than acetate buffer in amperometric measurements.*

**Keywords:** biosensor, glucoseoxidase, immobilization by cross-linking, poly(o-anisidine)

## INTRODUCTION

Biosensors are devices that combine the selectivity and specificity of a biologically active compound with a signal transducer and an

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electronic amplifier. The transducer converts the biochemical signal to an electronic signal. The biosensor signal is proportional to the concentration of a measured analyte or a group of analytes. Amperometric biosensors measure the current on an indicator electrode caused by direct oxidation of the products of the biochemical reaction. The amperometric biosensors' potential at the electrode is held constant while the current flow is measured. The amperometric biosensors are reliable, relatively cheap and highly sensitive for clinical, industrial and environmental purposes [1–4]. The determination of glucose is one of the most well-known and popular biosensor applications. The glucose is of special importance because of its involvement in human metabolic process. In the past 20 years numerous efforts have been devoted to develop a glucose biosensor with a fast and accurate response [5–11]. The immobilization technique for localizing enzymes at the surface of various electrodes plays a very important role in the research of glucose biosensors [12–17].



Today, the conducting polymer films polypyrrol [18,19], polyphenols [20,21], polyaniline [22,23] and its derivatives poly(o-anisidine) and poly(o-toluidine) [24] have drawn the attention of many researchers and scientists; the major advantages being the miniaturization, which is useful in the developments of biosensor devices. These materials have challenged the reign of the inorganic semiconductors. The simplicity in preparation, cost effectiveness, and ability to tailor properties of these organic molecules, mean they have potential applications in various fields [25–29]. The conducting polymers are being widely used in biosensor applications because they provide a stable and porous matrix for the immobilization of the biocomponent and also facilitate the electron transfer process [30–32].

The electronic conductivity of polymers exhibits a strong dependence on the redox and protonation state of the polymer. This most important and unique feature has been utilized to fabricate and develop the biosensor concept. This concept involves immobilization of the appropriate enzyme in the conducting polymer matrix. The enzyme-catalyzed reaction of the biomolecule causes a change in redox potential and pH of the microenvironment and triggers a change in the electronic conductivity. The sensor action is a consequence of the enzyme-catalyzed reaction specific to glucose. The change in the pH of the microenvironment of an enzyme-immobilized polymer film is caused by the formation of gluconic acid. A more important possibility of response is due to the effect of $H_2O_2$ produced by the enzymatic reaction. The porosity is an important factor for the facile immobilization of the enzyme [33,34].

The development of an enzyme-based biosensor can be carried out by immobilization of the biorecognition element using electrochemical technique [35,36]. This method is simple and can be used to localize the biocomponent, which is randomly oriented within the polymer matrix and often inaccessible to the target analyte [37,38]. The advantage of using the composite POA-DBS film lies in the electrostatic rejection of anions. Sulfonate ions of the POA-DBS composite film provide a charged surface for electrostatic interaction between the enzyme and the surface [39]. Crosslinking via glutaraldehyde is likely to lead to greater stability of the enzyme in POA-DBS film.

In the present investigation, we describe the results of our systematic studies relating to the electrochemical synthesis and characterization of the POA-DBS film and the development of POA-DBS-GOx electrode. We have studied the influence of phosphate and acetate buffer on GOx immobilized on POA-DBS film, by crosslinking via glutaraldehyde, and its response for glucose measurement as an approach for development of a sensitive amperometric biosensor.



## EXPERIMENTAL

### Synthesis of POA-DBS 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 a one-compartment electrochemical cell. A platinum rectangular sheet ($20\,mm \times 10\,mm \times 0.25\,mm$) was used as a counter electrode and another platinum rectangular sheet ($20\,mm \times 5\,mm \times 0.25\,mm$) 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-DBS film was synthesized from an aqueous solution containing 0.2 M o-anisidine and 0.5 M of dodecylbenzene sulphonic acid (DBS) using electrochemical deposition method. It was carried out by galvanostatic technique at 27°C. The applied current density of $1\,mA/cm^2$ 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 reagents were obtained from Rankhem Ranbaxy New Delhi (India). After synthesis, the polymer-coated electrodes were rinsed thoroughly in distilled water and dried in cold air and then used for subsequent characterization.

## Immobilization of GOx on POA-DBS Film

A stock solution of glucose oxidase (GOx) (EC 1.1.3.4, Type VII) (200 U/ml) (Aldrich) was prepared in 0.1 M phosphate or acetate buffer (pH 5.5) solution containing 2 mg/ml GOx and 100 ml distilled water. The dried films were dipped 24 h at room temperature in a 0.1 M phosphate or acetate buffer (pH 5.5) solution before use. These films were dipped for 30 min in the GOx solution. The enzyme GOx was immobilized by crosslinking via (1%) glutaraldehyde (Loba Chemic) on the POA-DBS films for 30 min. It was then washed with a phosphate and acetate buffer, in order to remove any loosely bound enzyme, thus restricting the leaching of the enzyme in the film.

The enzymatic incorporation was done in glutaraldehyde media. This kind of immobilization results in greater physical and chemical stability of the catalytic material due to the crosslinking between 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 GOx was chosen to ensure 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

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

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

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

The enzyme electrode formed by attaching glucose oxidase by means of glutaraldehyde is used for amperometric measurement of glucose. The glutaraldehyde plays a significant role in current response [40,41].

In order to construct the amperometric enzyme sensor, GOx is used as an example of a redox protein. The enzyme catalyses, in the presence of molecular oxygen, 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 [42]. The electron transfer from the redox cofactor to the sensing electrode is also facilitated by the presence of a polymeric conducting material.

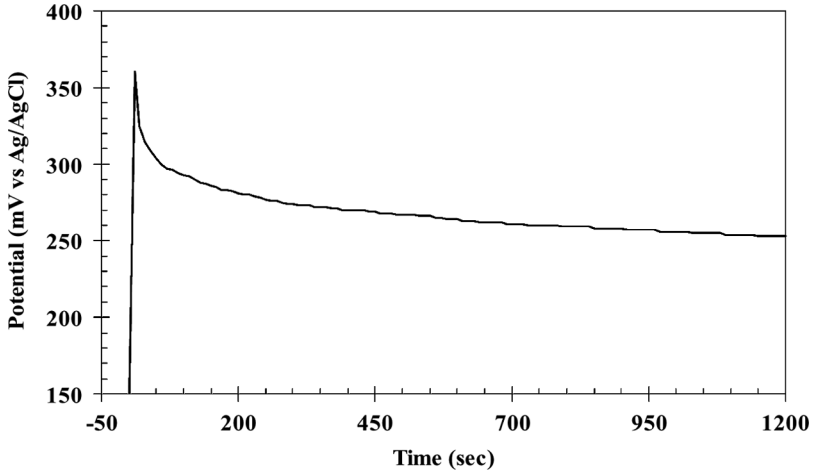


**FIGURE 1** The chronopotentiogram recorded during the synthesis of POA film with optimized electrochemical process parameters.



## Galvanostatic Studies of POA-DBS Film

The chronopotentiogram of the synthesized POA-DBS film is shown in Figure 1. The POA-DBS film was synthesized on platinum substrate from 0.2 M concentration of o-anisidine and 0.5 M of DBS with $1\,mA/cm^2$ applied current density at 1.0 pH. This has resulted in high conductivity, with uniform and porous surface morphology of the resulting POA-DBS film. The behavior of the galvanostatic synthesis overshoot during the first few seconds probably indicates difficult formation of dimers and oligomers.

After, the potential remains constant, suggesting that build-up of the films proceeds according to the same reaction along the full thickness of the polymer. It was found that green polymeric film on the platinum electrode was deposited with high uniformity. The electrical conductivity of the synthesized POA-DBS was measured by four-probe technique (Model DRF-02 Owen 1038-Optochem International, New Delhi) and was $7.61 \times 10^{-2}$ S/cm [43,44].

## UV-visible Studies of POA-DBS Film

The optical absorption spectrum of the synthesized POA film with optimized process parameters is shown in Figure 2. The UV-visible spectrum was recorded using UV-visible spectrophotometer Shimadzu 1601 in the wavelength range 300–900 nm. A green-colored film shows

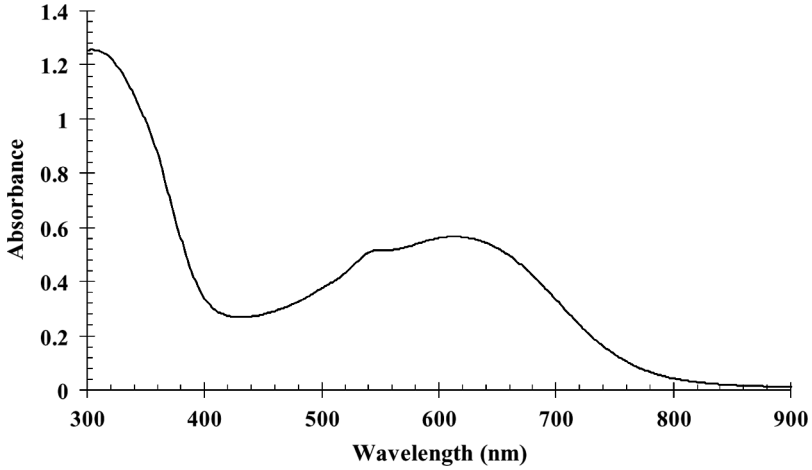


**FIGURE 2** The optical absorption spectrum of synthesized POA film with optimized electrochemical process parameters.



a peak appearing at 300 nm, which is assigned to a $\Pi \rightarrow \Pi^*$ electronic transition between the valence and conduction bands of the polymer, plus a strong peak at 600 nm attributed to an intramolecular charge transfer excitation associated with quinoide ring. A tail at 800 nm indicates the formation of emeraldine salt (ES), which shows a strong resemblance to earlier reported work [45,46].

## FTIR Studies of POA-DBS Film

The FTIR spectra were recorded (Testscan Shimadzu FTIR-8400 series) in the region 4000–400 $cm^{-1}$. The FTIR spectrum of the POA film with optimized process parameters is shown in Figure 3. The characteristic band at 3435.0 $cm^{-1}$ arises mainly from N−H stretching and the bands at 1656.7 $cm^{-1}$ arises from C=N group, while the bands at 952.8 $cm^{-1}$ arise from O−C=O. The characteristic band at 1423.4 $cm^{-1}$ arises mainly from C−O group whereas the band at 1315.4 $cm^{-1}$ arises from C−H stretching. Thus, FTIR spectral results confirm the structure of POA film. It shows a strong resemblance to earlier reported work [45].

## SEM Studies of POA-DBS Film

The scanning electron micrograph was recorded using JEOL, JSM-6360A SEM machine. The scanning electron micrograph of the

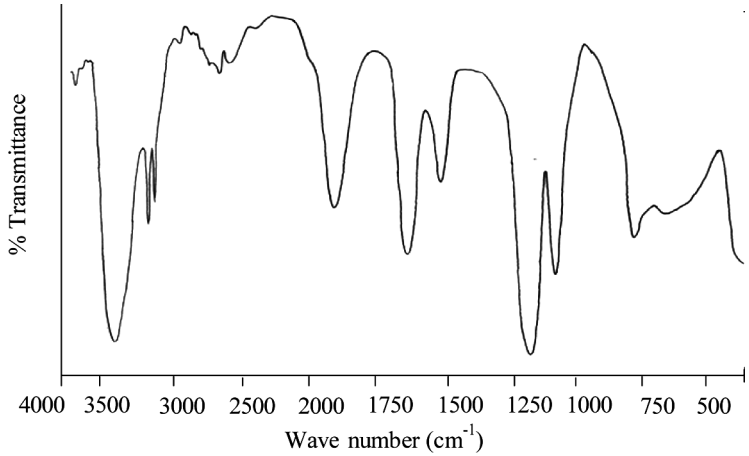


**FIGURE 3** The FTIR spectrum of synthesized POA film with optimized electrochemical process parameters.



synthesized POA film with optimized process parameters is shown in Figure 4. The synthesized POA-DBS film shows a sponge-like structure morphology, uniform and porous. This excellent surface morphology with very good porosity is suitable for immobilization of the biocomponent.

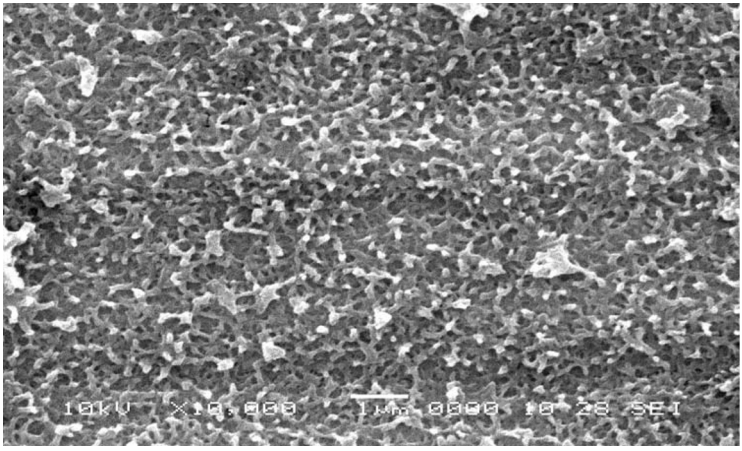


**FIGURE 4** The scanning electron micrograph of synthesized POA film with optimized electrochemical process parameters.

## Amperometric Response of the POA-DBS-GOx Electrode

The change in response current of the active device glucose oxidase is the parameter of great interest for sensor applications. The response current of the device depends on several factors, such as the contact resistance between the metal electrode and the polymer film, the geometric factor of the film and 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. The current-time relationship of POA-DBS-GOx electrode when the potential of the enzyme was set at 0.6 V in a phosphate and acetate buffer is shown in Figures 5 and 6, respectively.

It was found that the response current of the enzyme electrode easily reached a steady state. The steady state was attained within 10 sec for all concentrations. The glucose solutions for current measurements were contained in a phosphate or acetate buffer with pH 5.5. It was observed that the response times of the glucose solution (1 mM to 50 mM), in phosphate and acetate buffers were little 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 Figure 7 (the linear regression equation is $y = 0.0813x + 3.4539$ and



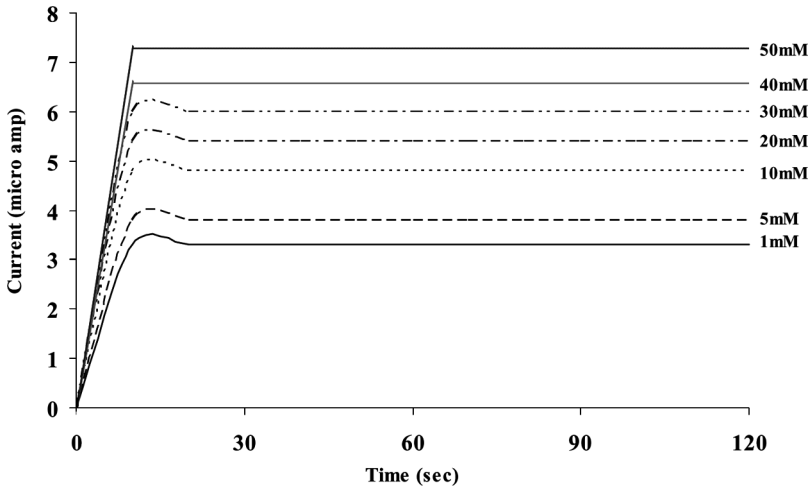


**FIGURE 5** Current-time curve for the glucose oxidase electrode of POA at 0.6 V. Glucose solution 1 mM, 5 mM, 10 mM, 20 mM, 30 mM, 40 mM, and 50 mM in 0.1 M phosphate buffer, pH 5.5.

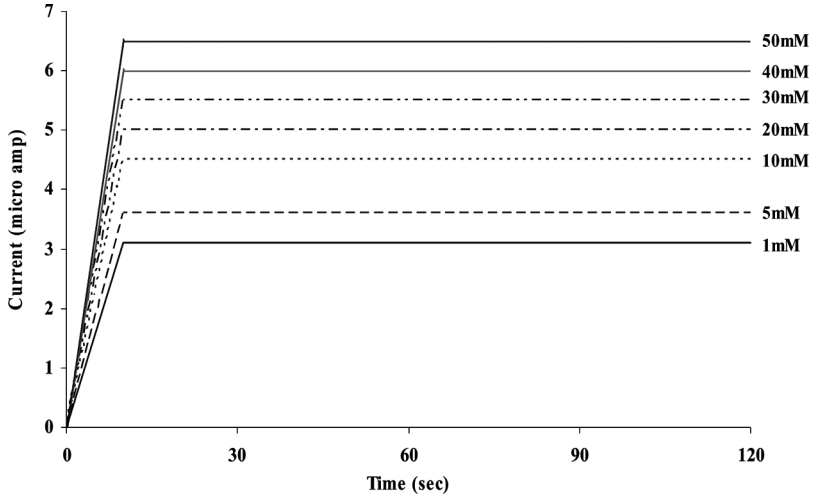


**FIGURE 6** Current-time curve for the glucose oxidase electrode of POA at 0.6 V. Glucose solution 1 mM, 5 mM, 10 mM, 20 mM, 30 mM, 40 mM, and 50 mM in 0.1 M acetate buffer, pH 5.5.



the linear regression coefficient is $R^2 = 0.967$), and Figure 8 (the linear regression equation is $y = 0.0688x + 3.3064$ and the linear regression coefficient is $R^2 = 0.9542$), respectively.

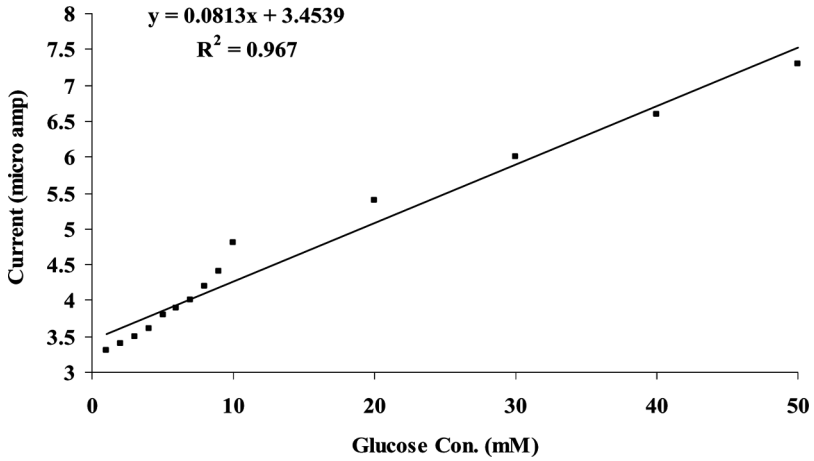


**FIGURE 7** The relationship between response current and glucose concentration for the GOx electrode of POA in 0.1 M phosphate buffer (■), pH 5.5.

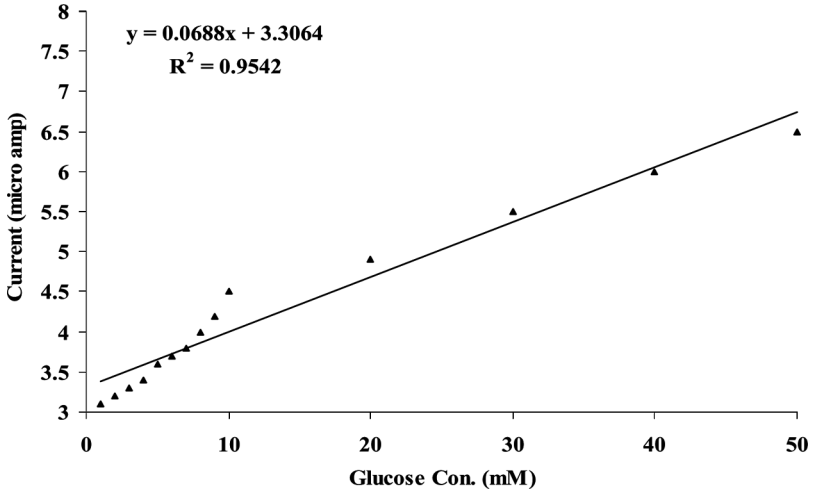


**FIGURE 8** The relationship between response current and glucose concentration for the GOx electrode of POA in 0.1 M acetate buffer (▲), pH 5.5.



Again, it was found that the response current increases with increasing glucose concentration in the range 1 mM 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, slowing the attainment of a steady state value [47,48] of the response current.

## 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 [49]. 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 9. 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-DBS-GOx films: In the phosphate buffer, the $I_{max}$ was 7.3 μA with $K'_m$-5 mM, and in acetate buffer it was 6.5 μA and $K'_m$-3 mM respectively. The value of $K'_m$ depends on immobilization of the enzyme and a lesser $K'_m$ gives a faster response of the electrode to glucose. It was found

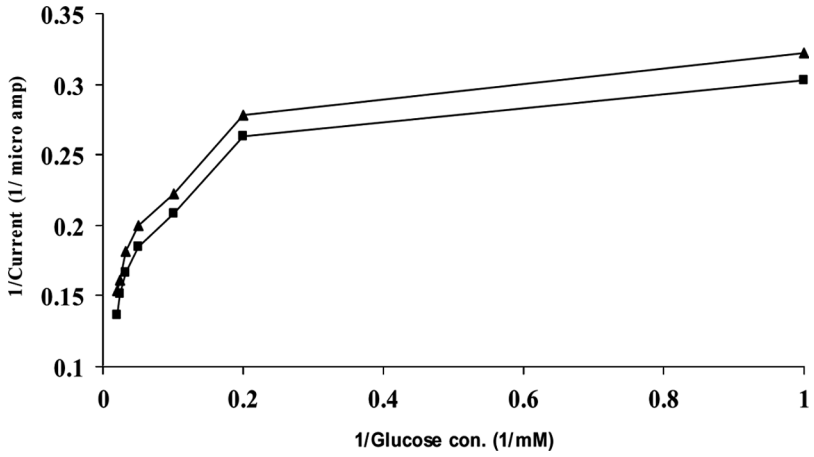


**FIGURE 9** Determination of apparent Michaelis-Menten constant ($K'_m$) for the GOx electrode of POA in 0.1 M phosphate buffer (■), and 0.1 M acetate buffer (▲), pH 5.5.



that a more sensitive POA-DBS-GOx electrode had developed in the phosphate buffer than in the 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 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 the electrode reaction is related to the concentration of electroactive species, the pH value of

**TABLE 1** Comparison of the Analytical Performance of the POA-DBS-GOx Electrode for Phosphate Buffer and Acetate Buffer at pH 5.5

| Sr. No. | Parameters | Buffers | |
|---|---|---|---|
| | | Phosphate | Acetate |
| 1 | $I_{max}$ (μA) | 7.3 | 6.5 |
| 2 | $K'_m$ (mM) | 5 | 3 |
| 3 | Linearity (mM) | 0–9 | 0–9 |
| 4 | Sensitivity (μA/mM) | 16.66 | 20 |

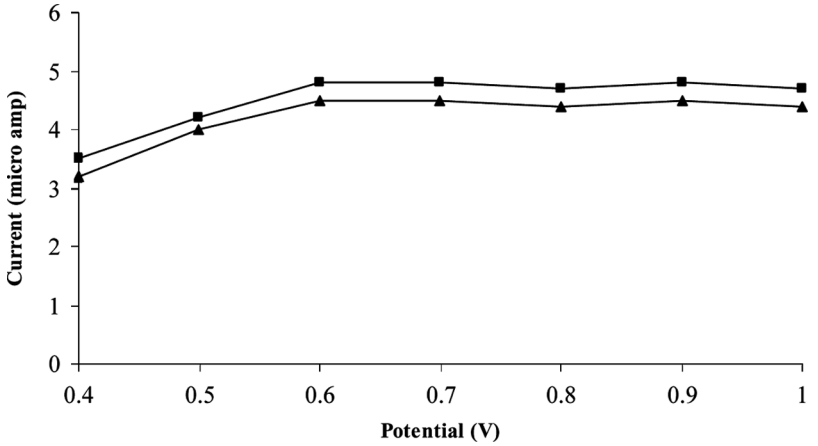


**FIGURE 10** Current-potential curves for the GOx electrode of POA in 0.1 M phosphate buffer (■), and 0.1 M acetate buffer (▲), pH 5.5.



solution and the applied potential [50]. Above the potential of 0.6 V the response was almost steady, which could be explained by the rate-limiting process of enzyme kinetics, the diffusion control of $H_2O_2$ and the substrate [51]. Considering the decrease in response of the POA-DBS-GOx 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 further studies of the POA-DBS-GOx electrode.

## Effect of pH

In an optimized polymerization the value of the pH of the reaction medium allows an efficient entrapment of the enzyme. It also prevents the loss of the enzyme activity under polymerization conditions [52]. Thus, enzyme sensor response depends on the pH of the sampling solution. 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 Figure 11. 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-DBS-Gox Electrode

The stability and lifetime of the POA-DBS-GOx electrode have been studied. It shows very good stability and excellent response for

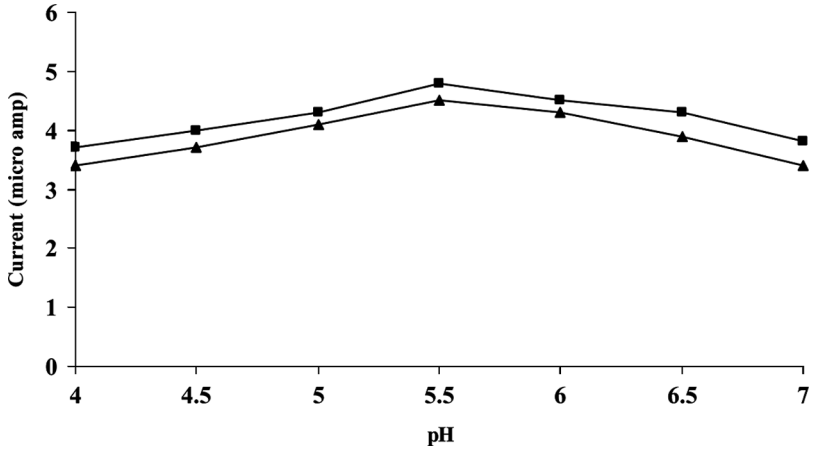


**FIGURE 11** Effect of pH on the GOx electrode response of POA. Steady-state currents measured at 0.6 V in 10 mM glucose solution in 0.1 M phosphate buffer (■), and 0.1 M acetate buffer (▲).



3–4 weeks (Figure 12). At the beginning of the test of stability, the current response decreased rapidly and then slowly stabilized. The current response of the POA-DBS-GOx electrode in the acetate buffer decreased much more than that of the phosphate buffer. The test was carried out for 30 days for both buffers.

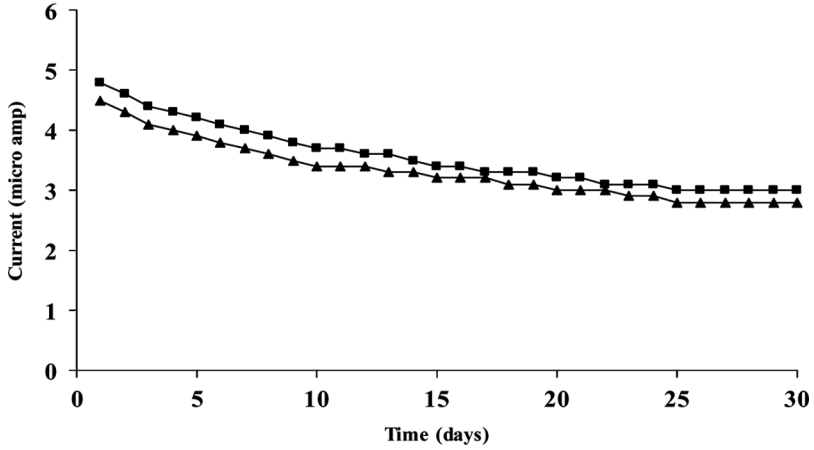


**FIGURE 12** Stability of the POA electrode on storage in 0.1 M phosphate buffer (■), and 0.1 M acetate buffer (▲), pH 5.5 at room temperature.

## CONCLUSIONS

We have successfully developed a POA-DBS-GOx biosensor for the determination of glucose. It was found that the conducting POA-DBS film can be utilized as a suitable matrix for immobilization of GOx by crosslinking via glutaraldehyde. The electrochemical response is quite good at pH 5.5. The value of $K'_m$ depends on immobilization of the enzyme, and lower $K'_m$ gives faster response of the electrode to glucose. The biosensor shows a very good stability and excellent response for 3–4 weeks. The POA-DBS-GOx electrode in a phosphate buffer gives a faster response than an acetate buffer in amperometric measurement.

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